MAK HILL Publications Research Journal of Medical Sciences

Research Article
doi: 10.36478/makrjms.2024.11.341.348

# Detection of *Mycobacterium tuberculosis* by Polymerase Chain Reaction: A one-year Cross Sectional Study in the Rural Population of Belagavi District

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**ABSTRACT**
Tuberculosis (TB) has long been a significant threat to humanity, affecting health, society and the economy. Recently, the Belagavi district experienced a decline in TB notifications amid the COVID-19 pandemic, potentially leading to increased morbidity, mortality and household transmission. New sputum samples with prior consent and detailed patient histories were collected from the Respiratory OPD of Dr Prabhakar Kore Hospital and MRC. Petroff's decontamination was performed on the samples in a BSL-2 cabinet, followed by ZN-stain and Auramine O' stain screening under compound and fluorescent microscopes, respectively. PCR targeting IS6110 was conducted on the remaining samples after storage at (-80 ), following DNA extraction using Qiagen Kit. Standardization included preparation of Master mix (Takara) and PCR procedure with specific primers. Gel Electrophoresis identified amplified products showing a band at 123 bp as MTB. Data analysis was employed. The study identified a majority (38%) of positive MTB cases in the 31- 40 years age group using conventional PCR, with lower rates observed in younger age groups. Syndemic effects of coexisting DM and HIV were evident in 5.13% of patients. PCR demonstrates considerable potential for *Mycobacterium tuberculosis* detection from sputum samples, offering a safe and effective diagnostic alternative to the traditional staining techniques, despite limitations in throughput and cost-effectiveness in rural populations.

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**Key Words**
PCR, IS6110, 123bp, mycobacterium tuberculosis, HIV

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**Received:** 20 August 2024
**Accepted:** 26 October 2024
**Published:** 30 October 2024

**Citation:** Sneha Roy Choudhury, 2024. Detection of Mycobacterium tuberculosis by Polymerase Chain Reaction: A one-year Cross Sectional Study in the Rural Population of Belagavi District. Res. J. Med. Sci., 18: 280-289, doi: 10.36478/ makrjms.2024.11.341.348

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## INTRODUCTION

Tuberculosis (TB) has been a persistent threat to humanity, impacting health, society and the economy, particularly in less developed areas. Mycobacterium tuberculosis, the causative agent, primarily spreads through respiratory droplets and historically, through contaminated bovine milk. In India, since the milk is generally boiled, chances of spread of infection with *Mycobacterium* bovis are comparatively rare. Our country faces a significant TB burden, exacerbated by recent epidemiological shifts[1]. In order to curb transmission and TB mortality rates, the WHO strategized to eliminate TB by 2035 under the banner of WHO's END TB programme[2].

TB diagnosis traditionally relied on direct smear examination and culture methods hindered by sensitivity, mass screening and speed limitations. PCR technology has revolutionized TB diagnostics by enabling rapid, specific pathogen detection directly from clinical specimens. In this study, conventional PCR targeting IS6110 from newly diagnosed sputum samples was found to be at par compared to the traditional staining methods.

Effective TB management relies on accurate diagnosis and prompt treatment initiation, critical for curbing transmission and reducing mortality rates. Despite advancements like CB-NAAT and LPA, accessibility remains a challenge in remote, underserved areas, highlighting the urgent need for cost-effective, reliable diagnostic tools[3].

This study aimed to address these loopholes by leveraging conventional PCR for *Mycobacterium tuberculosis* detection in sputum samples, alongside demographic profiling of TB patients in Belagavi district. By enhancing diagnostic accuracy and accessibility, initiatives like this contribute to global TB eradication efforts.

## MATERIALS AND METHODS

**Sputum Specimens:** New sputum specimens submitted in the respiratory department of Dr Prabhakar Kore Hospital and MRC were included in the study.

**Processing of Specimens and Detection in PCR:**

**Conventional Staining Techniques:**

Ziehl-Neelsen (ZN) Technique STAIN/Hot method-Principle:

- Acid fastness is due to the high content of lipids, fatty acids and higher alcohols which constitute almost 40% of the dry weight of tubercle bacilli.
- Mycolic acid (a wax), acid – fast in the Free State is found in all acid-fast bacteria.
- The integrity of the cell wall is also important for acid-fastness of bacteria.

**Smear Preparation:** Smear measuring 2 x 3 cm in size of oval shape is prepared in the centre of a new clean grease free, scratch free slide from the yellow purulent portion of the sputum. The smear should be translucent (i.e. neither too thick nor too thin, the print beneath should be readable through the smear. Smear preparation should be done near a flame, as six inches around the flame is considered sterile zone (as heat coagulates the aerosols which are raised during the smear preparation).

**Heat Fixation:** The smear is air dried for 15-30 minutes and then heat fixed by passing over the flame 3-5 times for 3-4 seconds each time. Coagulation of the proteinaceous material in the sputum will facilitate fixing of the smear.

**Procedure:**

**Step 1 (Primary Stain):** Smear is poured with strong carbol fuchsin (1%) for 5 minutes. Intermittent heating is done by flaming the underneath of the slide until the vapor rises. Heating helps in better penetration of the stain. Hence heat here is also referred to as a mordant. To ensure that the smear does not dry out, to counteract drying more solution of stain is added to the slide and the slide is reheated. Rinse the slide with tap water, until all free carbol fuchsin stain is washed away. At this time the smear on the slide looks red in colour.

**Step 2 (Decolorization):** It is done by pouring 25% sulfuric acid over the slide and allowing it to stand for 2-4 minutes. The slide is gently rinsed with tap water and tilted to drain of the water. A properly decolourized slide appears light pink. If the slide still looks red, sulfuric acid is reapplied for 1-3 minutes and then rinsed gently with tap water.

**Step 3 (Counter staining):** 0.1% methylene blue is poured onto the slide and left for 30 seconds. Then the slide is rinsed gently with tap water and allowed to dry. The slide is examined under the binocular microscope using low power objective (10 X ) to select a suitable area and then examined under oil immersion field (100 X ), Contaminated materials / slide should be discarded in a jar containing 5 % phenol.

**Fluorescence Staining Technique / Auramine-phenol Solution:**

**Principle:** Fluorescent materials emit visible light when they are irradiated with ultra-violet or violet blue visible rays.

**Smear Preparation:** Smear measuring 2 x 3 cm in size of oval shape is prepared in the centre of a new clean

grease free, scratch free slide from the yellow purulent portion of the sputum. The smear should be translucent (i.e. neither too thick nor too thin, the print beneath should be readable through the smear).

**Air Drying and Heat Fixation:** The slide is allowed to air dry for 15-30 minutes to clear air bubbles which would spurt while heating to fix the smear.

**Step 1 (Primary Stain):** The smear containing slide is flood with filtered 0.1% Auramine solution. Do not heat. Keep the stain as it is for atleast 20 minutes, make sure that the smear area is continuously covered with auramine by adding more, if needed. Rinse with water and drain.

**Step 2 (Decolourization):** Apply 0.5% acid alcohol as decolourising solution for 3 minutes. Gently rinse with water until the macroscopically visible stain has been washed away and drained.

**Step 3 (Counter staining):** The smear is flooded with 0.5% potassium permanganate solution for 1 minute. Here 1 minute time is very critical because counter staining for longer time may quench the acid-fast bacilli fluorescence. Gently rinse with water and drain. Air dry on a slide rack away from sunlight. The slide is then examined under the fluorescent LED (light-emitting diode) microscope. The smears are screened by using 20 X 25 objective, hence can be screened faster (2 minutes) for 100 fields.

**Molecular Method:**

**Standardization:** PCR-reaction was made by adding 1µL of each specific forward and reverse primers (20 mM) and 5 µL of template DNA into the 10 µL of master mix (Takara) and volume adjusted to 25 µL by adding 8 µL of NFW.

**Lysis of Organisms and Preparation of DNA from Clinical Specimens:**

**Decontamination by Petroff's Technique:**

**Principle:**

**Modified Petroff's Method (4% NaOH):** Here sputum is mixed with 4% sodium hydroxide, centrifuged and sediment is neutralised with phosphate buffer saline. Since this method is mostly recommended for LJ-culture, we utilised the other method namely NALC-NaOH method.

**NALC (N-acetyl-L-Cysteine) +2% NaOH:**

- This method is more compatible with automated culture system and is superior to Petroff's method for isolation of *Mycobacteria*.
- NALC liquifies the thick purulent sputum.
- NaOH kills the normal flora present in the sputum other than *Mycobacterium tuberculosis*.

**DNA Extraction as Per Qiagen Kit:**

We took 30 bulk sputum samples which were kept at -80 prior to DNA extraction.

- Remove Tris HCl from Eppendorf tube.
- To the pellet added 180 µl ATL buffer + 20 µl proteinase K.
- Mix by vortexing and then incubate at 56 until completely lysed for 10 minutes. (vortex occasionally during incubation)
- Add 200 µl buffer AL then mix by vortexing for 15 seconds.
- Incubate at 70 for 10 minutes. Briefly centrifuge the tube to remove drops from the lid.
- Add 200 µl ethanol (96-100%). Vortex for 15 seconds. Briefly centrifuge the tube to remove drops from the lid.
- Pipet the mixture onto the QIAamp Mini spin column (in a 2 ml collection tube). Centrifuge at 6000 x g (8000 rpm) for 1 minute. Discard the flow-through and collection tube.
- Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl buffer AW1. Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard the flow through and collection tube.
- Place the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW2. Centrifuge a full speed (20,000 x g; 14,000 rpm) for 3 minutes. Discard the flow-through and collection tube.
- As recommended, we placed the QIAamp Mini spin column in a new 2ml collection tube and centrifuge at full speed for 1 minute. This eliminates the chance of possible Buffer AW2 carry over.
- Place the QIAamp Mini spin column in a new 1.5 ml microcentrifuge tube and then we added 200 µl Buffer AE or distilled water and incubate at room temperature for 1 minute. Centrifuge at 6000 x g (8000 rpm) for 1 minute to elute the DNA.

**Oligonucleotides:** The primers used in the PCR were targeted to detect *Mycobacterium tuberculosis* based on the amplification of the repetitive DNA sequences of 123 bp from IS6110. The Oligonucleotide sequences used in the amplification were as follows: (Table 1)

Table1: PCR Primers for Mycobacterium Tuberculosis

| | |
|---|---|
| Forward primer: | 5′ CCTGCGAGCGTAGGCGTCGG 3′ |
| Reverse Primer: | 5′ CTCGTCCAGCGCCGCTTCGG 3′ |

**Preparation and Amplification of PCR:** The PCR amplification reactions were performed in a total volume of 25 µl. The reaction mixture consisting of 5 µl of PCR buffer, 0.2µM of each forward and reverse primer 0.2mM of dNTP mixture, 2 mM MgCl2, 0.425 U of Taq Polymerase, and 5.0 µl of template DNA. The PCR reactions were performed at 94 for 5 minutes (followed by 32 cycles at 94 for 45s, at 68 for 45s, at

72 for 2 min and a final extension at 72 for 5 minutes, using Genetix (Gradient) thermocycler at Molecular microbiology section of the department.

**Gel Electrophoresis:**

**Preparation of 1 % Agarose Gel:**

1 g Agarose powder dissolved in 100 ml TE buffer was boiled till it got dissolved completely, after cooling down to (40-45) , 3-5 µl (EtBr) Ethidium bromide was mixed and the entire mixture was poured slowly in the entire tank and allowed to cool. The comb which was placed was removed slowly after hardening of the agarose gel. 10 µl of the samples were loaded in along with positive and negative controls and the ladder. A current of 90 V for 50 minutes was passed over the gel. Post 50 minutes the gel was removed and placed in the UV -chamber. The results were visualised in Syngene. Fluorescent dyes such as Ethidium bromide or Sybergreen I bind specifically to DNA and are used to visualise the sample preparation. Here we used Ethidium bromide as the fluorescent dye. The Ethidium bromide dye (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide [EtBr]) is one of these agents and was the most widely dye in early DNA and RNA analyses. Because EtBr is carcinogenic, precautions are of utmost important to limit exposure. Under excitation with UV light at 300 nm, EtBr in DNA emits visible light at 590 nm. Therefore, DNA separated in Agarose and exposed to EtBr emits orange light when illuminated at 300 nm.

**Quality Control:** The quality of the amplification was monitored by the simultaneous testing of a negative and a positive control sample in each run. The negative control samples consisted of distilled water and the positive control were partly obtained from patient's positive samples and partly by the known *Mycobacterial* strain, M. *tuberculosis*, H37RV.

## RESULTS AND DISCUSSIONS

The study was conducted at the K-FIST-II, VGST laboratory, Government of Karnataka, Department of Science and technology, at the Molecular section of the Microbiology department, KAHER Jawaharlal Nehru Medical College, Belagavi, using BSL – II safety precautions.

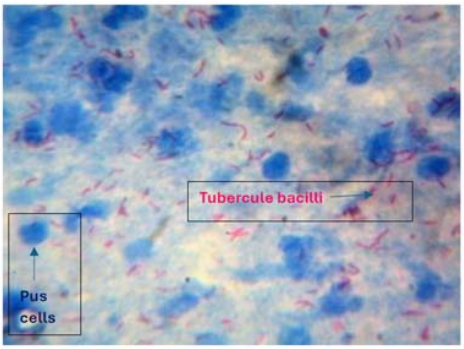


Fig. 1: In ZN-Stain appearance

Fig. 1: In ZN-Stain appearance the tubercle bacilli manifested as lean, straight, beaded and barred forms, occasionally slightly curved entities, measuring 2-4 µm in length and 0.2 -0.8 µm in breadth, appearing individually, in pairs and in tiny clusters. Pus cells are marked in arrow.

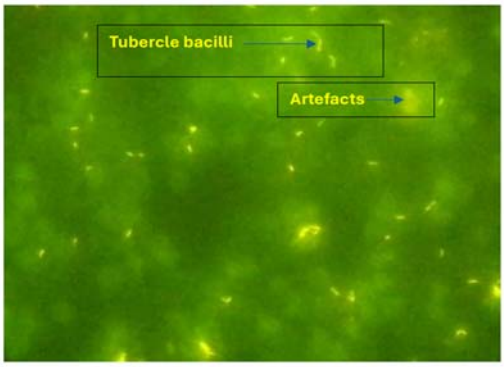


Fig. 2: Auramine O'Stain appearance,

Fig. 2: Auramine O'Stain appearance, the *Mycobacterium tuberculosis* bacilli ( arrow mark) appeared as bright yellow fluorescence, with a dark background, very short and slightly curved organisms and Artefacts ( arrow mark ) in the same suspected PTB patient.


Fig.3 MTB detected

Fig.3 Lane 1 shows positive control H37Rv strain, lanes 2,3,4,7 are negative samples, lane 5 shows 100 bp ladder, lane 6 and 8 shows MTB detected at 123 bp, lane 9 is the negative control with water.

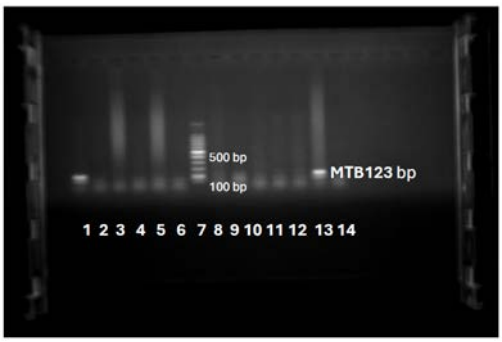


Fig.4: PTB samples

Fig 4 Lanes 1 and 14 are the positive and negative controls for MTB respectively. Lane 7 is the 100 bp ladder, lane 13 is positive for PTB and lanes 2,3,4,5,6,8,9,10,11,12 are negative PTB samples.

A total no. of 161 sputum samples were received in the molecular section of the Microbiology department, J.N Medical College. Out of these 99 (61%) were from male patients and the rest 62 (39%) were from female patients. Thick, purulent, yellowish samples of 3-5 ml were collected in a wide mouthed sterile container.

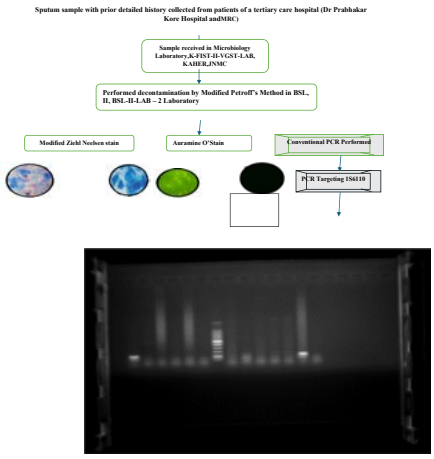


Fig. 5: MTB sputum sample

Fig.5: Lane 1 shows the positive control, lane 7 shows a 100 bp DNA ladder, lane 14 is the negative control, Lane 13 shows positive MTB sputum sample, whereas lanes 2,3,4,5,6,8,9,10,11,12 and 14 are negative for MTB sputum Samples. Total 11 sputum samples of 11 suspected PTB patients were screened at once in one of the bulks of patient samples.

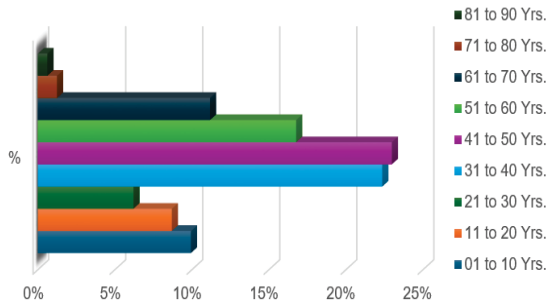


Fig. 6: Age Wise Distribution of Cases ( n= 161)

Among the participants of various age groups, maximum patients were belonging to the age group of 41yrs -50yrs (n = 37, 23%), followed by 31yrs-40 yrs (n= 36, 22% and 51- 60 yrs ( n=27, 17% ). Least number of participants were from the age group of 81yrs-90 yrs (n=1, 1%), followed by 71yrs-80 yrs (n=2, 1%). (Fig 6).

Table 2: MTB Positive Patients with Comorbities

| Parameter | Results | | | |
|---|---|---|---|---|
| | Negative | Positive | Cochran's Q | p-value |
| Zn Stain | 126 (78.26%) | 35 (21.74%) | 2.167 | 0.338 |
| Auramine-o stain | 123 (76.4%) | 38 (23.6%) | | |
| PCR | 122 (75.78%) | 39 (24.22%) | | |

**The Approximate Time Taken for Each Method are as Follows:** ZN-Stain-Total time required for the preparation of smear till the result interpretation step is 60 minutes per sample approximately.

**Auramine O' Stain:** Total time required for the preparation of smear till the result interpretation step is 57 mins per sample approximately.

**Conventional PCR:** Total turnaround Time for 30 samples-4-5 hours approx. till results. Hence maximum of 96 samples can be used in case of conventional PCR with a total duration of only 2 hours approximately (Only PCR step requires 2 hours-the duration of the rest of the initial processing steps would depend on the efficiency of the technician) . This can be used in case of a high patient load healthcare setting where numerous samples needs to be screened at once.

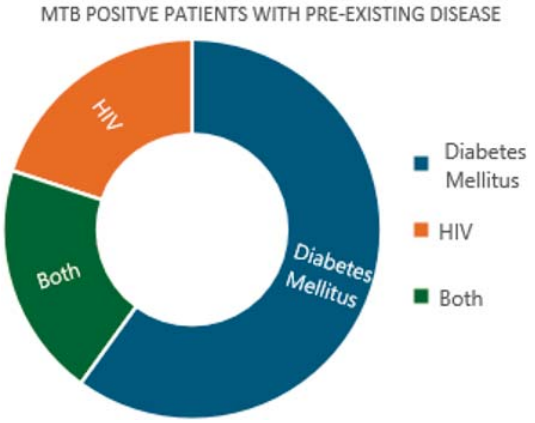


Fig.7: MTB Positive with Pre-Existing Disease

Out of 161 patient samples, MTB positive patients detected by PCR with pre-existing non-communicable disease as Diabetes Mellitus constitute 6 (n=6, 15.38%), this is followed by MTB positive patients with pre-existing communicable disease i.e. HIV constitute around (n=2, 5.13%) and patients presenting with both DM and HIV along with pre-existing Pulmonary Tuberculosis constitute (n=2, 5.13%). Fig (7).

**Comparison of Diagnostic Methods for *Mycobacterium tuberculosis* Detection**:

**P-Value is Obtained by Cochran's Q Test:**

**The Table Compares Three Diagnostic Methods for Detecting *Mycobacterium tuberculosis:*** Zn Stain,

Auramine-o stain, and PCR. The results show that Zn Stain detected 21.74% positive cases, Auramine-o stain detected 23.6% positive cases and PCR detected 24.22% positive cases. Cochran's Q test resulted in a value of 2.167 with a p-value of 0.338, indicating that there is no statistically significant difference in the detection rates among these three procedures. Therefore, the effectiveness of these three diagnostic methods appears to be similar. Table 2

Our discussion on the current study would be incomplete without throwing some light on the essence and cultural integrity of Belagavi, a charming city nestled at the foothills of the Sahyadri mountain range, approximately 779 meters above sea level and located 100 kilometres from the Arabian Sea. Known also as Kundanagari, Belagavi has assimilated cultural influences from neighbouring Maharashtra and Goa, blending them with local Kannada traditions to create a uniquely rich heritage[4].

Covering a total area of 13,433 square kilometres as per the 2011 census, the Belagavi district is home to an inhabitants of 4,779,661, with approximately 75.97% residing in rural areas spread across 1,275 villages. The demographics predominantly include migrant labourers, slum dwellers and residents of underdeveloped regions and tribal communities, who are most vulnerable to the impact of certain diseases. Factors such as illiteracy, poor living conditions, malnutrition, inadequate housing, and overcrowding contribute significantly to the spread of these illnesses. With regard to the progression of tuberculosis infection into active disease, HIV is a particularly significant risk factor that should be taken into consideration[5-8].

In our study, we collected 161 samples from suspected patients, all of which consisted entirely of sputum. The sputum appeared thick, purulent and yellowish, with approximately 2% showing a brick-red color (indicating haemoptysis). This differs from a study conducted by Pallavi Sinha at BHU, India, where they received a total of 721 clinical samples from patients suspected of pulmonary tuberculosis. Their specimens included 656 pulmonary samples (636 sputum and 20 bronchoalveolar lavage [BAL] ) and 65 extra-pulmonary samples (20 urine, 15 pus, 15 fine-needle aspirations [FNAs], 9 cerebrospinal fluid [CSF], 1 bone marrow, and 5 pleural fluid). Our study did not include comparison with the 'gold standard culture' method due to its longer turnaround time for detecting Mycobacterial growth. Therefore, our focus was solely on determining the prevalence of positivity using Zn-stain, Auramine O' stain, and conventional PCR methods. Subsequently, we analyzed distributions related to gender, age, family members, and comorbidities [9-10].

Our study focused on the IS6110 gene, which has a gene product size of 123 bp. The H37Rv strain served as the positive control, whereas H2O functioned as the negative control. Numerous targets have been identified for the detection of Mycobacterium tuberculosis, including genes that encode the 32-kDa, 38-kDa, and 65-kDa antigens (groEl, mtb-4, and dnaJ, respectively), insertion sequences such as IS986 and IS6110, the 16S-23S spacer region, the heat shock protein (hsp) 65 genes, and 16S rRNA. IS986 and IS6110 are among the most common repeated elements in M. tuberculosis strains, usually found in 10-16 copies. A study by de Lassence and Walker DA revealed that IS6110 had greater sensitivity and specificity compared to IS986. Consequently, in our research, we focused on the IS6110 gene in uncultured samples from probable MTB patients, as it serves as a significant marker for MTB identification. The presence of this target in Mycobacterium bovis may result in false positive outcomes, as demonstrated in a study by Bauer J, Cowan[9-11-12].

In another investigation conducted by Farzam and Imami they utilized PCR to target the cyp 141 gene for detecting MTB in clinical sputum specimens (n=123). Their findings indicated higher sensitivity compared to targeting the commonly used IS6110 gene. Specifically, the cyp 141 gene showed a sensitivity of 85.7% for direct specimens, whereas IS6110 exhibited lower sensitivity of 42.9% for direct specimens. Both genes had equal specificity at 100%. The cyp 141 gene encodes a metabolic protein in M. tuberculosis that serves as a crucial virulence component. In contrast, a study by McLean KJ, Dunford AJ *et al.* reported conflicting results regarding the genomic location of the gene encoding this Cytochrome P450 protein.

In our study, out of the 161 newly detected sputum samples, 99 (61%) sputum samples were from male patients and the rest 62 (39%) sputum samples were from female patients. In our study, males exhibited higher positivity rates across all the three diagnostic methods: ZN-stain (62.86%), Auramine-O Stain (63.16%) and conventional PCR (58.97%), whereas females showed lower positivity rates in Zn-stain (37.14%), Auramine-O Stain (36.84%) and conventional PCR (41.03%). This disparity in positivity between genders can be attributed to the social dynamics in rural areas. Males often have greater exposure to external environments and higher prevalence of risk factors such as alcohol consumption, tobacco chewing, cigarette smoking and chewing paan and betel nut, which are detrimental to respiratory health. In contrast, females of rural areas, primarily the

homemakers, tend to stay indoors more and may hesitate to seek healthcare due to stigma and cultural norms, resulting in lower detection rates of MTB among females. Many studies have shown that tuberculosis disproportionately affects the female counterpart, particularly in the procreative age group, and is a significant cause of female infertility in India. For instance, in Bengaluru rural area, there has been a steady trend in tuberculosis incidence, with a decline in smear-positive cases from approximately 65-23 per 100,000 over a certain period, as reported by A K Chakraborty *et al.* This decline correlates with improvements in acute respiratory infection (ARI) rates [13-18].

The global incidence of tuberculosis exhibits considerable regional variation, with lower rates in Europe (55 per 100,000) and the USA (8 per 100,000), contrasted by higher rates in Africa (317 per 100,000) and South-East Asia (536 per 100,000), as reported by the World Health Organization's Global TB Control Report 2006. Reducing tuberculosis (TB) fatalities by 95% and TB incidence by 90% from 2015 levels, with a target of 90% coverage in treatment and prevention, is part of the World Health Organization's (WHO) End TB plan, which seeks to eradicate TB by 2035. India's NTEP intensified TB elimination efforts after COVID-19, achieving significant milestones in 2023 towards eradicating TB by 2025, including a 16% decline in TB incidence and an 18% reduction in mortality since 2015, along with enhanced case notifications, comprehensive care packages and innovations like scaling up BCG vaccinations and integrating AI. The Government of Karnataka's unwavering commitment to eradicate TB by 2025 through the "Kashaya Muktha Karnataka (KMK)" initiative, aligned with the National Strategic Plan 2017-2025, exemplifies commendable dedication. Based on the number of TB cases that were reported in 2019, the Belagavi district in North Karnataka was classified as the fourth highest TB burden district in the state of Karnataka. The use of laboratory detection tests that are both quick and sensitive is absolutely necessary in order to curb the spread of tuberculosis and bring down the death rate. Through the use of amplification of the cyp141 gene, Darban-Sarokhalil and colleagues established the first fast diagnostic approach for M. tuberculosis. Within the scope of our investigation, we focused on the IS6110 gene, which has a product size of 123 base pairs. The positive control was the standard H37Rv strain, and the negative control was water[19-21]].

A research by Chakraborty *et al.* in Wardha district, Maharashtra, correlating TB prevalence with socio-economic determinants, showed disparities influenced by literacy, employment and housing conditions. The frequency of tuberculosis was minimal among graduates and maximal among illiterates. Professionals exhibited the highest incidence of tuberculosis cases, succeeded by farmers and agricultural labourers. Individuals residing in 'kutcha' houses exhibited a greater frequency of tuberculosis than those inhabiting 'pucca' houses. Among females, 48% of tuberculosis cases were attributed to the unemployed, including homemakers. Chakravarty *et al.* conducted another study revealing that tuberculosis rates were consistently lower in females than in males across all demographic characteristics. Dholakia *et al.* conducted a study revealing that around 52% of workers in India estimated to have tuberculosis were aged 15-44 years. Approximately 40% of workers in urban areas were women, while the percentage of women workers with tuberculosis in rural regions was at 17.9%. The Wardha survey indicated that urban professionals and rural service workers exhibited a higher frequency of tuberculosis, although the proportion of females was low, leading to a reduced overall count of female TB cases. Numerous studies demonstrate that tuberculosis morbidity is a substantial public health concern in India, especially among males in the economically productive age bracket and females in the reproductive age bracket [22,23].

Diabetes mellitus is the predominant co-morbidity in pulmonary tuberculosis, succeeded by malignancy, and is correlated with an increased probability of cavitary nodules, as indicated by a study conducted by Wang JI and Lee In our investigation, approximately 137 (85%) of patients exhibited a negative result for diabetes mellitus. Among these, only 6% were positive for diabetes mellitus regardless of their tuberculosis status and the diabetes mellitus status of 14 (9%) patients was unknown. In our study, 6 (15.38%) patients had co-existing diabetes mellitus (DM) with TB, 2 (5.13%) had TB-HIV co-infection and another 2 (5.13%) exhibited a syndemic presentation of DM (a non-communicable disease) and HIV (a communicable disease) along with tuberculosis. Table 3

Table3: Studies Depicting Tuberculosis and Diabetes Mellitus Comorbidity

| Region/Country | Percentage of TB patients with Diabetes mellitus | Year Published |
|---|---|---|
| Karnataka, India | 32% | 2011 |
| Kerala, India | 44% | 2012 |
| Tamil Nadu, India | 25% | 2012 |
| Texas U.S.A | 39% | 2011 |
| Mexico | 36% | 2011 |
| Tanzania | 17% | 2011 |
| Pakistan | 16% | 2012 |
| South Pacific | 40-45% | 2013 |

A recent study by Stevenson, Dye C factor for new cases of pulmonary tuberculosis, using India as a case study. Diabetes mellitus constituted 14.8% of pulmonary tuberculosis and 20.2% of sputum smear-positive tuberculosis patients, with the frequency of smear-positive cases approximately 15%

elevated in urban regions relative to rural areas. These observations indicate that diabetes mellitus considerably contributes to the burden of tuberculosis patients in India, particularly the infectious variety. A study by Faruqi suggested that the prevalence of diabetes mellitus in India is expected to rise, underscoring the necessity for rigorous tuberculosis control measures in both urban and rural regions. Al Webel *et al.* proposed that in individuals with diabetes mellitus, tuberculosis primarily manifests in the lower lobes, often presenting with cavitary lesions. Bashar conducted a study revealing a notable rise in multidrug-resistant tuberculosis among patients with diabetes mellitus (36% vs. 10%) relative to those without diabetes mellitus. A study by Kameda demonstrated no correlation between glycaemic management and relapse rate, suggesting that tuberculosis patients with diabetes mellitus exhibit comparable responses and long-term recurrence rates to those without diabetes mellitus, while having a worse prognosis upon relapse. The extensive engagement of endocrine glands in clinical tuberculosis is a significant factor for physicians treating the condition. Additional research is required to definitively investigate the observed susceptibility to certain endocrine problems, particularly the heightened incidence of diabetes mellitus in tuberculosis patients. Multiple explanations have been suggested to elucidate the heightened incidence of diabetic mellitus (DM) in tuberculosis (TB). Research by Nichols indicates that a reciprocal association is anticipated when two diseases are correlated. Bloom JD et al. suggested that latent glucose intolerance may increase susceptibility to tuberculosis infection. Tripathi, Das associated tuberculosis with malnutrition, suggesting that malnutrition may influence the aetiology of diabetes mellitus[24,25].

Roychowdhury and Sen proposed that tuberculosis of the pancreas may be a potential cause of glucose intolerance, notwithstanding the rarity of pancreatic involvement in tuberculosis. Mugusi Swai propose that stress-induced diabetes mellitus (DM) may arise during significant illnesses such as tuberculosis (TB). Glucose tolerance abnormalities observed in tuberculosis patients generally improve following the commencement of anti-tuberculosis therapy. Atkin SL, Masson EA, reported early phase hyperglycaemia in some patients induced by Rifampicin, possibly due to increased intestinal absorption of glucose, as intravenous glucose tolerance was found to be normal in these patients. Furthermore, evidence linking mycobacteria to DM has been rapidly increasing, as noted by Goswami, Mishra and Kochupillai[9-11].

In numerous developing nations, such as India, the prevalence of Human Immunodeficiency Virus (HIV) infection and tuberculosis (TB) presents considerable health obstacles. In this investigation, 132 (82%) patients were found to be HIV-negative, 15 (9%) were identified as HIV-positive, while the HIV status of 14 (9%) patients remained undetermined. Our findings indicate that 2 patients (5.13%) were diagnosed with TB-HIV co-infection, while an additional 2 patients (5.13%) presented with a syndemic infection involving HIV, diabetes mellitus (DM) and TB. Tuberculosis is the most prevalent opportunistic infection among individuals infected with HIV in India, which ranks third in the world for the number of people living with HIV. India represents approximately 9% of the global burden of HIV-associated TB, positioning it as the second highest in the world. The co-infection of HIV and TB presents a significant mortality risk, accounting for approximately 25% of global HIV/AIDS-related deaths annually, as highlighted in research conducted by Lawn et al. The majority of individuals identified with TB-HIV co-infection present with advanced stages of HIV disease, which is marked by diminished CD4+ T-cell counts, elevated viral loads, or classification within WHO clinical stages 3 and 4. In regions where HIV prevalence is significant, tuberculosis programs must focus on the identification of infectious sputum smear-positive cases using microscopy techniques. Diagnosing tuberculosis in patients with advanced HIV infection presents significant challenges, as individuals who are HIV-positive and have pulmonary tuberculosis frequently exhibit a higher incidence of negative sputum smears, making sputum culture essential for accurate confirmation. The tuberculin skin test often produces false negative or invalid results because of immune system dysfunction and chest radiography may be less effective in HIV patients since they show fewer cavitations. Furthermore, extra-pulmonary tuberculosis occurs more frequently in cases of TB-HIV co-infection. All patients with HIV and TB should be offered antiretroviral therapy (ART), irrespective of their CD4 cell count. Initiate anti-tuberculosis treatment first, and subsequently commence ART once TB treatment is tolerated, usually within a timeframe of 2 weeks to 2 months. The application of Highly Active Anti-Retroviral Therapy (HAART) in tuberculosis patients can result in a persistent decrease in HIV viral load. The comparison of diagnostic methods for detecting Mycobacterium tuberculosis is crucial for improving clinical outcomes and resource allocation. In this study, ZN- Stain, Auramine-O' stain, and PCR were evaluated based on their respective detection rates. The following results indicated that conventional PCR had the highest detection rate at 24.22%, followed closely by Auramine-O' stain at 23.6%, and ZN Stain at 21.74%. Despite these slight variations, Cochran's Q test revealed a non-significant Q value of 2.167 ($p = 0.338$), suggesting no statistically significant differences among the methods. This finding underscores the

comparable efficacy of these diagnostic techniques in identifying Mycobacterium tuberculosis infections, aligning with previous studies that have also reported similar diagnostic performance across these modalities[14-21].

## CONCLUSION

To mitigate delays in M. tuberculosis detection and initiate timely treatment, selecting appropriate diagnostics tailored to diverse settings and patient profiles is paramount. While newer molecular methodologies hold promise, revitalizing existing technologies like Conventional PCR can extend diagnostic reach, particularly in rural areas lacking sophisticated infrastructure. The Conventional PCR, once instrumental in COVID-19 diagnostics, can be repurposed for MTB detection, leveraging existing equipment and expertise.

In conclusion, this study provides evidence supporting the equivalency of ZN Stain, Auramine-O' stain, and PCR in detecting Mycobacterium tuberculosis. The lack of statistical significance in detection rates, as indicated by Cochran's Q test, indicates that healthcare providers can choose any of these methods based on availability, cost-effectiveness and laboratory infrastructure without compromising diagnostic accuracy. Further research could focus on assessing these methods in diverse patient populations or settings to validate their robustness and reliability across different epidemiological contexts. Overall, these findings contribute to optimizing tuberculosis diagnostics, thereby enhancing patient care and public health interventions aimed at controlling tuberculosis transmission.

Moving forward, research should focus on affordable, portable diagnostic solutions suited for resource-constrained settings. Innovations such as Whole Genome Sequencing (WGS) and Computer-Aided Detection (CAD) for radiographs offer promising avenues for enhancing TB diagnostics.

To conclude, integrating technological advancements with practical considerations is essential for improving TB diagnosis and management. By optimizing existing resources and embracing new innovations, the fight against TB can achieve broader and more effective public health outcomes.

**Summary:** Effective management of *Mycobacterium tuberculosis* (MTB) pivots on timely diagnosis and treatment, underscoring the critical need for tailored diagnostic approaches. While newer molecular techniques are invaluable, revitalizing conventional technologies such as PCR can significantly broaden diagnostic capabilities, especially in rural areas. Through this study we aimed to compare the efficacy of three diagnostic methods-ZN - Stain, Auramine-O' stain and PCR-for detecting *Mycobacterium tuberculosis*. The detection rates were found to be 21.74%, 23.6% and 24.22% respectively for ZN- Stain, Auramine-O' Stain and PCR. Statistical analysis using Cochran's Q test yielded a Q value of 2.167 with a corresponding p-value of 0.338. These results suggest no significant difference in detection rates among the three methods, implying similar effectiveness in diagnosing Mycobacterium tuberculosis among the three methods. Hence Conventional PCR can prove to be an effective initial diagnostic screening weapon to fight against this ancient disease and make our country **TB MUKHT BHARAT** by **2025**.

**Recommendations:** Research Focus: Future studies should prioritize the development of affordable and portable diagnostic solutions tailored for rural settings, addressing challenges such as cost, sensitivity, 24x7 power supply and accessibility.

**Technological Advancements:** Innovations like Whole Genome Sequencing (WGS) and Computer-Aided Detection (CAD) for radiographs present promising avenues for enhancing TB diagnostics, offering improved accuracy and efficiency.

**Limitations:** The study recognises limitations, including restraints in sample size and a singular focus on MTB, indicating the need for a broader perspective in future research efforts.

The limitations of conventional PCR are numerous, including the necessity for individual assays and the careful optimisation of reagents and amplification conditions for each PCR run.

Primer design is crucial for good PCR amplification, as cross-reaction with non-target DNA might lead to non-specific products. Laboratories conducting these assays must invest significant resources in specialised "DNA-free" laboratory space and equipment. DNA-free laboratory space and equipment are crucial to reduce contamination of following specimens by PCR amplicons, which can result in false positive outcomes. Despite these limitations, traditional PCR assays will remain relevant in smaller, rural, regional diagnostic laboratories.

**Acknowledgements:**

- Esteemed Principal ma'am, Dr (Mrs) N S Mahantashetti, I.E.C committee, J N Medical College
- Respected HOD and Professors of Microbiology Department, Technical Officers, Technicians, Staffs, J.N Medical College.
- Respected HOD and Professors of Microbiology Department, Technical staff, BIMS
- Dignitaries, Consultants, Staffs, Technicians of

KLES Dr Prabhakar Kore Hospital and MRC.
- Dignitaries, Consultants, Staffs, Technicians of Civil Hospital
- Government of India.
- Government of Karnataka
- Konark Enterprises for kits
- Parents and In-laws, Spouse Er. Sovan Mukherjee (MBA, IIM I), Kid Miss Sohanshi Mukherjee for constant support.

Presented paper in part at the 3rd Bienniel Conference of the Assam Chapter of Indian Association of Medical Microbiologists, DMC. Presented poster in part at the 46thAnnual conference, IAMM, KGMU, Lucknow (U.P), Abstract Selected in 3rd International Conference on Clinical and applied Microbiology, Jakarta, Indonesia.

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