" DETECTION OF *Mycobacterium tuberculosis* BY POLYMERASE CHAIN REACTION – A ONE YEAR CROSS - SECTIONAL STUDY IN THE RURAL POPULATION OF BELAGAVI DISTRICT."

BY

REG NO: BIO121002

DISSERTATION

Submitted to the KLE Deemed University, Belagavi,

Karnataka,

In partial fulfilment of the requirements

for the degree of

MD (DOCTOR OF MEDICINE)

IN

MICROBIOLOGY

Under the Guidance of

PROFESSOR

DEPARTMENT OF MICROBIOLOGY
JAWAHARLAL NEHRU MEDICAL COLLEGE BELAGAVI, KARNATAKA

,AUGUST - 2024

Declaration by the Candidate

I hereby declare that this dissertation entitled **"DETECTION OF *Mycobacterium tuberculosis* BY POLYMERASE CHAIN REACTION: A ONE YEAR CROSS-SECTIONAL STUDY IN THE RURAL POPULATION OF BELAGAVI DISTRICT."** is a bonafide and genuine research work carried out by me under the guidance of Professor, Department of Microbiology, Jawaharlal Nehru Medical College, Nehru Nagar, Belagavi- 590010.

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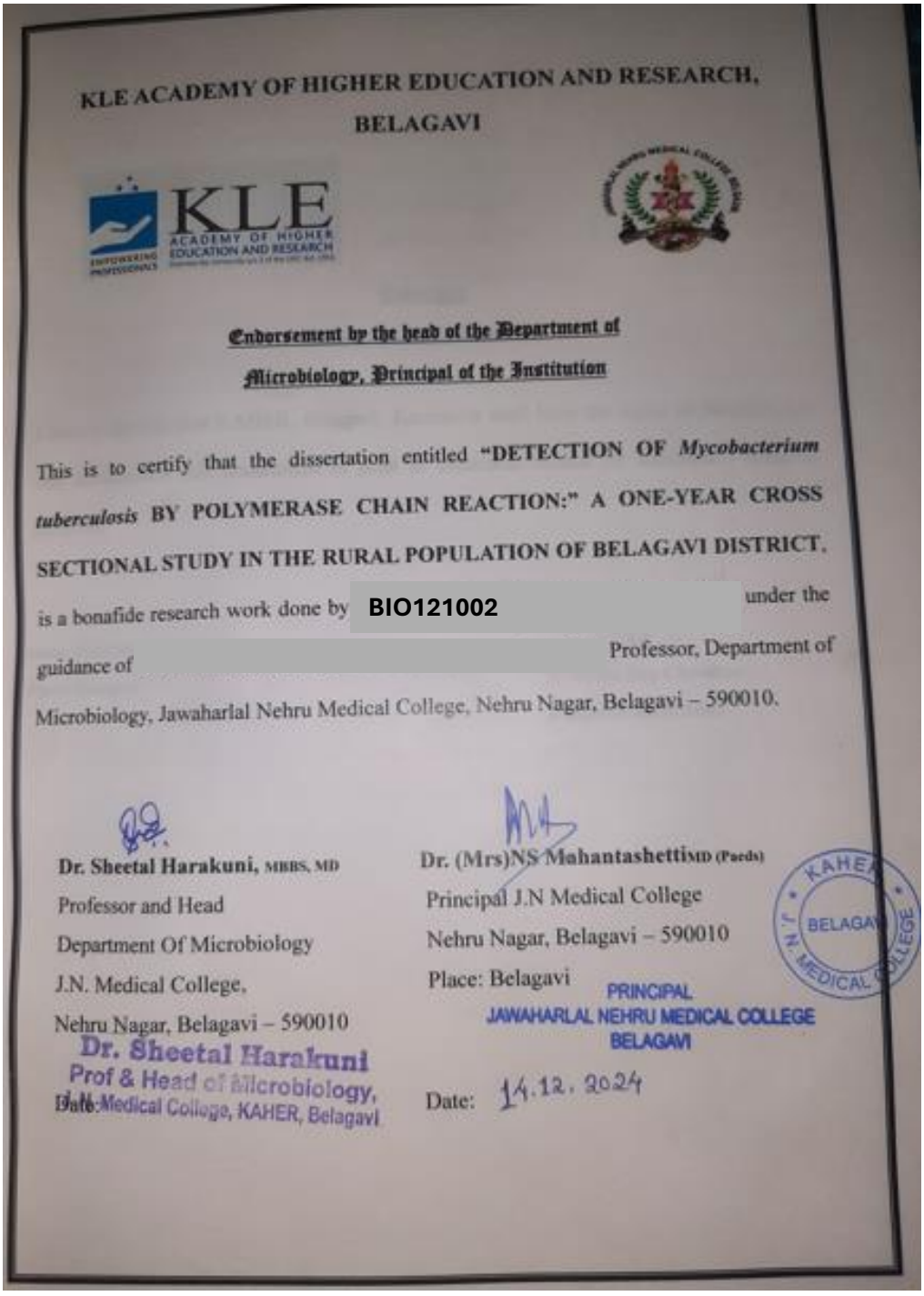

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To,
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J. N. Medical College, Belagavi.

LISTS OF WELL-KNOWN LEGENDS FROM VARIOUS FIELDS WHO SUCCUMBED TO TUBERCULOSIS:

FIELD	WELL KNOWN VICTIMS OF TB	IMAGE
MATHEMATICIAN	Srinivasa Ramanujan	
DOCTORS	Rene Theophile Hyacinthe Laennec	
	Edward Livingston Trudeau	
STATESMEN/STATES WOMEN	Kamala Nehru	
	Eleanor Roosevelt	
	Mohammed Ali Jinnah	

	Nelson Mandela	
MUSICIANS	**Frederic Francois Chopin**	
	Niccolo Paganini	
	Carl Maria Von Weber	
PERFORMING ARTISTS	**Vivian Leigh**	
	Elisa Rachel Felix	
WRITERS AND POETS	**Munshi Prem Chand**	
	Sir Walter Scott	

		
	John Keats	
	Elizabeth Barret Browning	
	Charlotte Bronte	
	Emily Bronte	
	Anne Bronte	
	Alexander Pope	
	Robert Louis Stevenson	

ABSTRACT

STUDY BACKGROUND: 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.

OBJECTIVES: The main objectives of this discourse were to detect Mycobacterium tuberculosis in suspected Pulmonary tuberculosis patients using conventional PCR on sputum samples and to examine the demographic profile of TB patients attending hospitals in and around Belagavi.

METHODOLOGY: 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°C), 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.

Forward primer: 5' CCTGCGAGCGTAGGCGTCGG 3'
Reverse Primer: 5' CTCGTCCAGCGCCGCTTCGG 3'.

RESULTS: 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.

CONCLUSION: 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.

KEYWORDS: PCR, IS6110, 123bp, *Mycobacterium tuberculosis,* HIV

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 is 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.


OBJECTIVES

OBJECTIVES OF THE STUDY

1. Detection of *Mycobacterium tuberculosis* in suspected Pulmonary tuberculosis patients from sputum samples using Conventional Polymerase Chain Reaction.

2. To evaluate the demographic profile of Tuberculosis patients attending hospitals in and around Belagavi.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less **- Maria Salomea Sklodowska- Curie**[4]

Tuberculosis continues to create a havoc and stigma among the human race since aeons ago. It is very aptly known as 'the barometer of social welfare' because of its higher prevalence in under privileged areas. It causes both pulmonary and extrapulmonary infections sparing only the hair and nails[5]. The disease has been referred to as 'yaksma' in the oldest of the Rig Veda in 1500 BC[6,7].

Lehman and Neumann coined the generic name *Mycobacterium* in 1896. This was named so because of the resemblance of the bacilli being mold like [myco: fungus; bacterium: bacteria] pellicular growth of these organisms in liquid medium[8]. This genus named as Mycobacterium is the only genus in the family Mycobacteriaceae belonging to the order Actinomycetales. The mycobacteria has got somewhat similar nucleic acid patterns as that observed in Nocardia, with the resemblance of 62 to 70 mol per cent of the guanine + cytosine [G+C] ratio in the deoxyribonucleic acid [DNA] of mycobacteria[9].

Since in this study we have detected Mycobacterium TB in sputum samples from patients presenting with suspected Pulmonary TB, our knowledge would be incomplete, if we do not delve deep about this Disease and the causative pathogen.

TABLE 1:

CLASSIFICATION OF MYCOBACTERIA ON THE BASIS OF PATHOGENICITY
GROUP 1 – OBLIGATE PATHOGENS **e.g : 1) Mycobacterium tuberculosis, 2) Mycobacterium leprae, 3) Mycobacterium bovis**
GROUP 2 – SKIN PATHOGENS **e.g : 1) Mycobacterium marinum, 2) Mycobacterium ulcerans**
GROUP 3 – OPPORTUNISTIC PATHOGENS **e.g : 1) Mycobacterium kansasii, 2) Mycobacterium avium intracellulare [MAIC]**
GROUP 4 – NON-OR RARELY PATHOGENIC **e.g : 1) Mycobacterium gordonae, 2) Mycobacterium smegmatis**
GROUP 5 – ANIMAL PATHOGENS **e.g. : 1) Mycobacterium paratuberculosis, 2) Mycobacterium lepraemurium**

Reference table. Rajesh Bhatia, Chapter-6,the Mycobacteria, page. 102, Alladi mohan,2nd edition, Jaypee bros.

TABLE 2:

MAJOR GENERA AND SPECIES TO BE CONSIDERED

Mycobacterium tuberculosis complex	Nontuberculous Mycobacteria
1. M. tuberculosis 2. M. bovis 3. M. bovis bacillus Calmette-Guerin (BCG) 4. M. africanum 5. M. caprae 6. M. canettii 7. M. microti 8. M. mungi 9. M. orygis 10. M. pinnipedi	**A] Slow-Growing Pathogens** 1. M. avium complex (10 species, not listed here) 2. M.gordonae 3. M.haemophilum 4. M.kansasii 5. M.malmoense 6. M.marinum 7. M.ulcerans 8. M.xenopi **B] Rapid -Growing Opportunistic Pathogens** 9. M. fortuitum group (10 species) 10. M. chelonae/ abscessus group (6 species and 3 subspecies) 11. M. mucogenicum group (3 species) 12. M. smegmatis group (2 species) **C] Early Pigmented Rapid-Growing Opportunistic Pathogens** 13. M. bacteremicum 14. M.canariasense 15. M. cosmeticum 16. M. mageritense/ M. wolinskyi group(2 species) 17. M. monacense 18. M. neoaurum **D] Nonculturable Nontuberculous Mycobacteria** 19. M. leprae

Reference table: chapter 42, Mycobacteria, section 14, Mycobacteria and other bacteria with unusual growth requirements.,pg.. 530, Bailey and Scott's Diagnostic Microbiology,15th edition, Elsevier

MYCOBACTERIUM TUBERCULOSIS

Morphology

The tubercle bacilli are morphologically slender, straight or slightly curved rod-shaped organisms which measures around 2-4 µm in length and 0.2 to 0.8µ in breadth, sometimes occurring singly, in pairs or in small groups. The bacilli are Gram positive organisms though they do not readily take up the stain. These organisms are called acid and alcohol fast as they resist decolorization. *Mycobacterium tuberculosis* are frequently seen in beaded or barred forms while *Mycobacterium bovis* stains more uniformly. In some younger cultures granules like, and in some, non-acid-fast rods have been reported**.** [10].

CELL WALL: The cell wall of Mycobacteria has a high lipid content and made up of several distinct layers. The lipid content of the cell wall accounts for 60% of the cell wall weight. The cell wall constituting the inner layer, overlying the cell membrane is composed of peptidoglycan [murein]. External to the murein is a layer of Arabinogalactan, which is being covalently linked to a group of long chain fatty acids termed *mycolic acid* [11]

STRUCTURE OF MYCOBACTERIUM TUBERCULOSIS: OUTER AND INNER LAYER OF THE CELL WALL.

- The below diagram depicts a schematic representation of Mycobacterium, showing the main components of the outer and inner layer of the cell wall. The presence of mycolic acid in the complex of covalent – linked MA-AG-PG is a unique character for the identification of the genus Mycobacterium and plays an important role as a permeability barrier. 1. Cell wall (insoluble) antigens: The cell wall consists of several distinct layers as depicted in the below figure:
- Peptidoglycan layer: It maintains the shape and rigidity of the cell.
- Arabinogalactan layer: It is a major structural component of the mycobacterial cell wall.
- Mycolic acid layer: It forms the principal constituent, and this layer is made up of long chain of fatty acids which is attached to arabinogalactan. As it confers very low permeability to the cell wall hence it reduces the entry of most of the antibiotics, also being responsible for acid fastness.

- Outermost layer: This layer consists of lipids (dimycocerosates and acylglycerols), glycolipids and mycosides(phenolic glycolipids)
- Proteins (e.g. porins, transport proteins): These proteins are found throughout the various layers.
- Plasma membrane: This layer is present beneath the cell wall, into which various proteins, phosphatidylinositol mannosides and lipoarabinomannan (LAM) are inserted. LAM is an important antigen, which facilitates the survival of tubercle bacilli within the macrophages and is also used as a target Ag for the TB diagnosis.
- Cytoplasmic(soluble) antigens: These include Ag 5, Ag 6, Ag 60 and are used in the serodiagnosis of tuberculosis.[12]

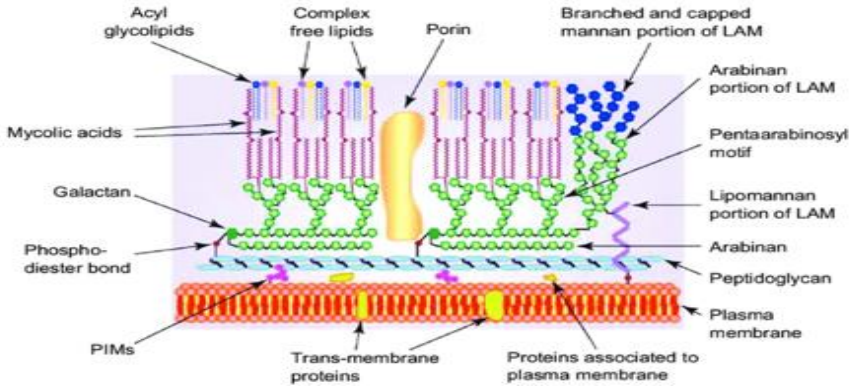


Fig. 1 Mycobacterium tuberculosis structure showing outer and inner layers of the cell wall. Source: Internet, slide share, a scribed company, Structure of Mycobacterium phcl 413, Jun 6, 2017, College of medical Sciences, Gombe State university, department of pharmacology.

GROWTH: The Mycobacteria, which are obligate aerobes, derive energy from oxidation of many simple carbon compounds. The growth rate of this bacilli is much slower than that of most other bacteria and the biochemical activities are as well not characteristic. The generation time is about 18 hours in vitro. The earliest growth occurs in about 2 weeks but may be delayed as late as 6-8 weeks. The optimum temperature for growth of this bacilli is around 37°C whereas growth does not occur below 25°C and above 40°C. Optimum pH required for growth of Mycobacterium tuberculosis is around 6.4 to 7.0. Increased CO2 tension [5% to 10%] enhances growth. Human strains grow more luxuriantly in culture [eugonic] than do

bovine strains [dysgonic]. The addition of 0.2 % of glycerol to the medium encourages the growth of human strains but the growth of the bovine strains may in fact be inhibited [13]

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS: The tubercle bacilli are inactivated by heat. The bacilli takes around 15 to 20 minutes at 60℃ to perish which is known as the thermal death time. The survival of these organisms can be traced to the following percentages of chemicals such as 5% phenol, 15 % sulphuric acid, 3% Nitric acid, 5% oxalic acid and 4% sodium hydroxide. Tincture Iodine can destroy the bacilli in 5 minutes whereas 80 % ethanol does so in 2-10 minutes. It takes 5 minutes for tincture iodine to destroy it whereas 80 % ethanol does so in 2-10 minutes. The bacilli can survive for 20 -30 hours in sputum whereas in droplets it can survive for 8-10 days. If the cultures of the bacilli are exposed to direct sunlight for continuous three hours, the chances are that the tubercle bacilli might get killed. Cultures of Mycobacterium tuberculosis can be stored continuously for 2 years in a deep freezer at around -20℃ temperature. These bacilli can survive for 20-30 hours in dried sputum and are highly resistant to drying. Even the ordinary daylight is seen to be lethal to these mycobacteria. [14]

Other than *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis* can also cause pulmonary TB. Usually smear-positive patients with cavitary lesions on the Chest Skiagram are an important source of infection as their one bout of cough which produces around 3000 tiny infectious droplet nuclei each measuring around 1-5 µm in diameter and inhabiting 1-10 TB bacilli are potent enough to transmit infection. Seldom tubercle bacilli may lodge in the tonsils or in the wall of the intestine due to the ingestion of raw contaminated milk or milk products. These bacilli can stay in the air for a sufficiently long period, in the dark for several hours, direct exposure to sunlight kills these bacilli within few minutes. The two important factors determining an individual's risk of exposure to the contaminated air include concentration of droplet nuclei in contaminated air and the length of time that impure air is breathed.[15]

DEMOGRAPHIC FACTORS INFLUENCING THE INCIDENCE OF TUBERCULOSIS:

1. **Population factors**- The jeopardy of acquiring tuberculosis is imminent during the extremes of ages due to compromised and incompetent immunity and exposure to newer antigens .[16] Unlike industrialized countries where older population fall prey to TB, this disease infects individuals, economically in their most productive life years of 15 -59 years of age in developing nations.
2. **Gender**- At all age groups, the prevalence of this dreaded disease was estimated to be imminently higher in male genders compared to their female counterparts. It has been documented that Tuberculosis kills more women in their reproductive age groups than does any other pathologies causing maternal mortality combined, hence creating more bereaves.(orphans). Approximately one-third of female infertility in India is caused by Tuberculosis.[17]
3. **Alcoholism and tobacco use**- In a study by Pai M et.al, it is has been evidenced that tobacco smokers are more prone to develop TB than the non-smokers. The smoke causes inhibitory effect on macrophages, resulting in chronic cough, dyspnea, chest skiagram appearance of upper zone involvement of the lung, cavity and miliary appearance and positive sputum culture. Smoking has been found to be gravely associated with both TB relapse and mortality. [18]
4. **Socio-economic factors**- These are the non-medical factors which contribute to the spread of the disease, that is why it is aptly also known as 'the barometer of social welfare'. HIV is also the most compelling risk factor for the progression of TB infection to disease. The disease spreads by coughing, sneezing, aerosol, unhealthy practices.

5. **Malnutrition**- In a recent experimental study on Guineapigs by McMurray DN, Bartow RA et.al it was established that protein calorie malnutrition could be a causative factor for TB. It is presumed that the function of the T-lymphocyte and cell mediated immunity are drastically impaired. More recent studies attempt to identify specific diets and micro-nutrient deficiencies that may increase TB risk. Two studies examining Hindu vegetarian versus non-Hindu omnivore of South Asian immigrants in Britain found vegetarianism correlated with a three-to four-fold increase in

the relative risk of Active TB. Moreover, the latter study by Strachan et al demonstrated a dose response relationship, whereby consuming a lower intake of fish and meat correlated with an increased risk of Tuberculosis. In one cohort study, it was found that low levels of vitamins A and C increase TB risk, while in another study by Hamila H. et al found vitamin C to be unassociated. Finally, by encouraging increased protein intake and general micro-nutrient supplementation, as well as aggressively treating intestinal parasites and anemia , all treating clinicians can address malnutrition in all their patients as a mode of TB anticipation. [19,20,21,22]

5. **Occupation-** There is an increased risk of Tuberculosis among workers with silica, mines, healthcare workers. These respirable silica elements directly damage the function of macrophages, thereby hindering their ability to defend mycobacteria.
6. **Corticosteroids** and other immunosuppressive drug/ agents- Patients with Long-term kidney injury or lymphoproliferative disorders receiving steroids or immunosuppressant drugs have repressed immunity and an increased risk of mounting clinical TB.
7. **Human Immunodeficiency Virus Infection**- The frequency of TB in HIV infected patients is about a 100-times greater than that in the general inhabitants. In HIV infected persons, active TB seems to advance rapidly after infection and progresses swiftly, resulting in demise. Hence a prompt initiation of ART is strictly recommended for TB patients at very extreme risk for HIV disease progression and mortality. For all those patients with a CD4+ count less than 200 cells/mm3, ART is endorsed as soon as the ATT is tolerated, usually between two weeks and two months. TB Patients with CD4+ counts in the 200 to 350 cells/mm3 range, ART should be commenced after the first two months of ATT, because the toxicity of TB treatment is utmost during this period. In patients with CD4+ count superior than 350 cells/ mm3, the ART should be deferred and the patient is monitored closely.[23]

8. **Other diseases**- Also it is observed that patients with Diabetes mellitus, hypothyroidism, gastrectomy patients have an increased risk of developing TB. Patients with leukemia, lymphoma, disseminated malignancy predispose to develop pulmonary TB.

PATHOGENESIS OF PULMONARY TUBERCULOSIS

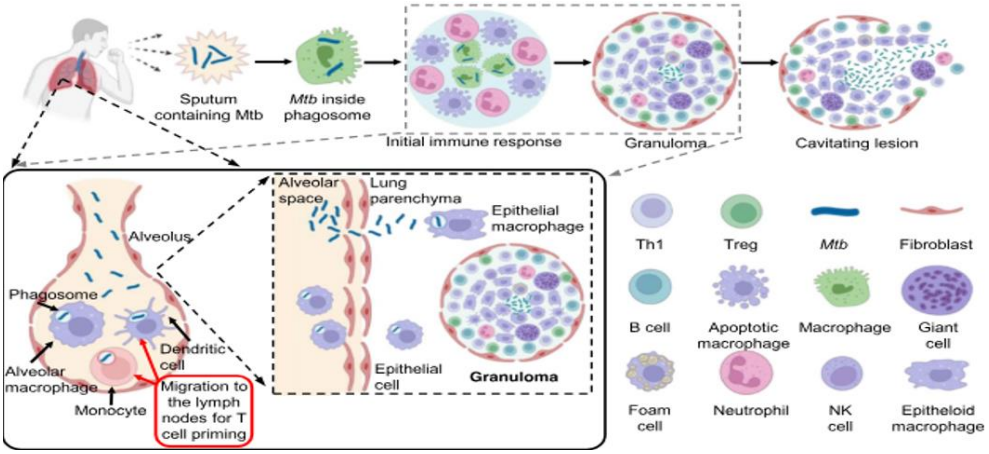


Fig. 2 : https://biorender.com Yan et al. Molecular Biomedicine (2022) 3:48 https://doi.org/10.1186/s43556-022-00106-yThe pathogenic mechanism of Mycobacterium tuberculosis: implication for new drug development Weizhu Yan1†, Yanhui Zheng1†, Chao Dou1†, Guixiang Zhang2*, Toufc Arnaout3,4* and Wei Cheng1*

The pivotal event in the pathogenesis of tuberculosis (TB) is the initial deposition of *Mycobacterium tuberculosis* within the pleural membrane. The primary route of entry occurs through the nasal passages upon inhalation of airborne droplets expelled by untreated infectious patients or those experiencing a relapse. Infection commences with MTB uptake via aerosols or sputum by alveolar macrophages and dendritic cells residing within lung tissues. Subsequently, MTB undergoes an initial phase of unhindered intracellular replication, during which infected cells migrate towards regional lymph nodes. Within these nodes, MTB can disseminate further within the lungs by infecting additional host cells.

Upon the advent of cellular immunity, a localized proinflammatory response ensues, triggering recruitment of monocytes and lymphocytes that congregate around infected macrophages to form granulomas. Granuloma formation represents a pathological hallmark of TB, characterized by MTB persisting in a state of arrested or delayed replication within these structures. Should MTB replication escalate unchecked, granulomas may fail to contain the infection, facilitating dissemination to other organs, including the brain. At this juncture, MTB may enter the bloodstream or re-enter the respiratory tract, thereby perpetuating further infections.

Occasionally, TB can manifest via direct inoculation of MTB into the skin through cuts or abrasions, a hazard faced particularly by healthcare professionals and laboratory staff handling infectious materials. These lesions, historically termed "prosector's warts," present a unique occupational risk, exemplified by the unfortunate demise of Laennec, the pioneer of the stethoscope, who succumbed due to such an exposure.[24]

CHRONOLOGY OF IMMUNOPATHOGENESIS OF TUBERCULOSIS:

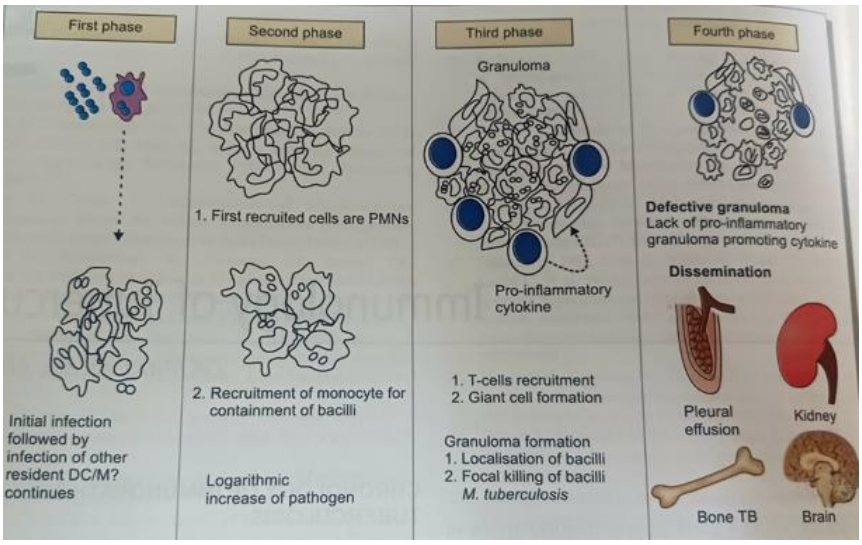
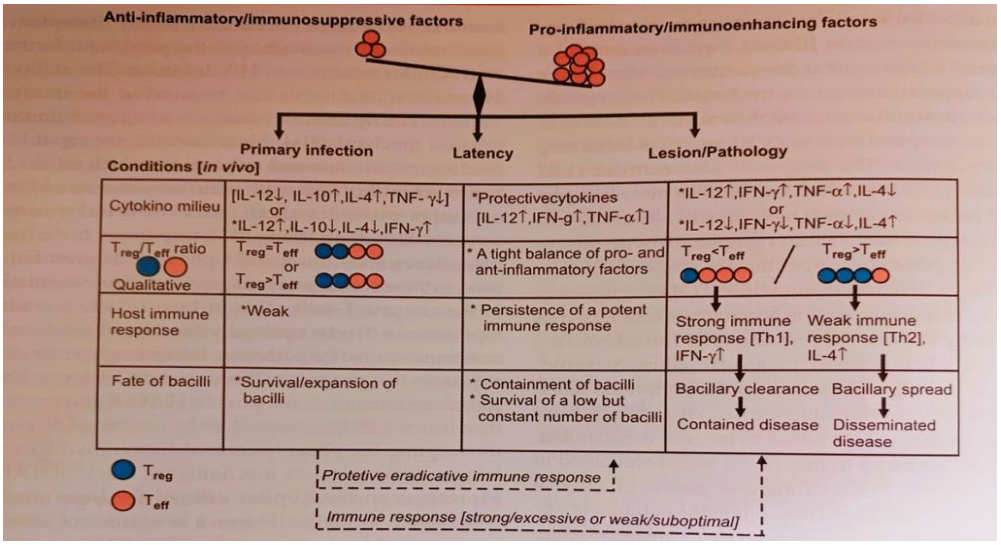


Fig. 3 Reference fig. DK Mitra, AK Rai ,Immunology of tuberculosis, chapter 7, pg.109, Alladi mohan,2nd edition, Jaypee bros.

DC=dendritic cells; M_{φ}= macrophages; PMNs= polymorphonuclear leucocytes; TB=Tuberculosis.

During tuberculosis (TB) infection, several distinct phases unfold sequentially. Initially, *Mycobacterium tuberculosis* invades alveolar macrophages, marking the first phase of infection. This is followed by an influx of polymorphonuclear leukocytes (PMNs), which helps to contain the bacteria within the lungs and prevents their escape from the initial immune response. In the second phase, monocytes are recruited to the site of infection, contributing to the inflammatory response. The third phase is characterized by the formation of granulomas, structured with multinucleated giant cells and elongated epithelioid cells at the core, surrounded by T-cells. This granuloma formation aims to encapsulate and isolate the bacilli, preventing their spread. However, in the fourth and final phase, if granuloma formation is defective or insufficient, M. tuberculosis can disseminate from the lungs to other organs. The organs commonly targeted after dissemination include the brain, among others. This progression underscores the complex interplay between the bacterium and the immune system, crucial for understanding TB pathogenesis and developing effective treatment strategies. [25]

IMMUNOMODULATORS IN TUBERCULOSIS:



TNF-alpha, IFN -gamma, IL= interleukin; T_{reg}= regulatory T-cells; T eff= effector T-cells; ↑ = increased , ↓ = decreased.

Fig. 4 Reference fig. DK Mitra, AK Rai ,Immunology of tuberculosis, chapter 7, pg.120, Alladi mohan,2nd edition, Jaypee bros. Comparative charts showing different components of immune system involved in various stages of infection with Mycobacterium Tuberculosis; primary infection, latency and active disease/pathology.

In the first exposure, weak host immune status facilitates the expansion of tubercle bacilli and helps in establishment of infection due to delayed immune recognition [primary infection]. The delayed immune recognition caused by several immune escape mechanisms adapted by bacilli, develops low level immunity helping in establishment of infection. Later on, ensuing effector immune response dictates the disease course. Firstly, protective and eradicative immune response helps in containment of bacilli leading to persistence of bacilli without any sign of pathology [latent infection]. The tight balance of pro and anti-inflammatory factors in restricting the bacilli below a threshold number are the major characteristics of latent infection. Secondly a non-optimal [excessive and sub-optimal] immune response developed by host against bacilli may lead to the two extremes / poles of the disease: [i] contained disease; and [ii] disseminated disease. Excessive or strong immune response facilitates clearance of bacilli and disease is contained or localized. In this form, the pathology is mostly associated with Th1 like response. On the other hand, disseminated disease is characterized by Th2 associated weak immune response resulting in bacillary spread.

HOST IMMUNE RESPONSE AGAINST MYCOBACTERIUM TUBERCULOSIS:

Mycobacterium tuberculosis exemplifies a pathogen where protection cruxes significantly on cell-mediated immunity. Given its residence within macrophage vacuoles, the presentation of mycobacterial antigens via MHC class II to CD4+ T-cells is a direct consequence of infection. It is well-established that individuals with impaired T-cell responses face heightened susceptibility to primary or reactivated TB. The interplay between T-cells and infected macrophages plays a pivotal role in fostering protective immunity against M. tuberculosis. While CD4+ T-cells play a critical role, they collaborate with other T-cell subsets including CD8+ T-cells, γδ T-cells, and CD1-restricted T-cells. Within tuberculosis granulomas, both CD4+ and CD8+ T-cells are prominently involved in mounting an anti-pathogenic response to confine the infection within the granuloma and suppress reactivation. Recent research has demonstrated the first instance of a recall T-cell response to a CD-restricted antigen in individuals exposed to Mycobacterium tuberculosis purified protein derivative (PPD). Peripheral blood mononuclear cells (PBMCs) from PPD-positive subjects showed proliferation in response to an isoprenoid glycolipid, contrasting with those from PPD-

negative subjects, and this proliferation was inhibited by anti-CD1c antibodies. [26]

Primary Tuberculosis

Definition: Primary tuberculosis refers to the initial infection and dissemination of Mycobacterium tuberculosis from the site of entry into the body.

Description: In primary tuberculosis, the organisms spread from the implantation site through the lymphatic system to the regional lymph nodes. This process forms a primary complex, which includes the lesion at the primary site, the draining lymphatics, and the inflamed regional lymph node. When the primary site of infection is in the lungs, it is referred to as Ghon's focus. The combination of Ghon's focus, the draining lymphatics, and the involved lymph nodes is known as the Ghon's complex.

Post-Primary Tuberculosis

Definition: Post-primary tuberculosis, also known as secondary tuberculosis, occurs when the initial infection progresses or reactivates, leading to more severe forms of the disease.

Description: In rare cases, the primary lesion can directly advance to the post-primary form, which is marked by extensive caseation necrosis and cavitation. While reactivation of the infection can occur at any site, post-primary TB typically affects the apical posterior segments of the upper lobes or the superior segments of the lower lobes in over 95% of cases (Balasubramanian et al.). This form of tuberculosis is more severe and is characterized by significant tissue destruction.[27]

GENETIC SUSCEPTIBILITY TO TUBERCULOSIS.

1. Twin studies-Pulmonary TB has been reported to occur more commonly in fraternal twins even when they are living independently[28] It has been suggested that the disease expression rate of TB is significantly higher in monozygotic twins [33.3%]

than dizygotic twins [15.7%][29]. Among household contacts, the disease is more likely to occur in siblings than in the spouse sharing the same bed.

2. Racial differences- Racial variances influence the degree of resistance to mycobacterial diseases. In one study Jews have been relatively found to be immune to pulmonary TB compared to the Africans, Americans and certain African tribes who were found to be susceptible to pulmonary TB [30,31,32]. Yet in another study, the disease prevalence in Gurkhas of Nepal has been found to be higher than other ethnic groups in the same topographical area[33]
3. ABO and Rh Blood Groups- A significant increase of pulmonary TB in persons with blood groups 'O' and 'AB' has also been conveyed in sputum positive Danish patients as compared to those with group 'A' or 'B'[34].

CLINICAL FEATURES:

A] SYMPTOMS:

The patient may develop symptoms insidiously or may remain asymptomatic. Usually, patients of pulmonary TB present with two types of symptoms:

1) **Constitutional symptoms**: fatigue, cephalgia, weight loss, fever (late afternoon or evening rise of temperature), afebrile, hoarseness, , night sweats, loss of appetite, amenorrhea in severe diseases
2) **Respiratory symptoms**: Primarily Cough which may be dry or productive, sometimes mislabeled as 'smoker's cough' lasting for 3 or more weeks, Sputum may be mucoid, mucopurulent, purulent or blood streaked usually scanty, hemoptysis (severity varies from blood tinged to massive; from rupture of a bronchial artery, chest uneasiness-dull aching, acute chest pain, severe pain at the height of inspiration, breathlessness, wheeze can occur due to endobronchial TB or because of heaviness of enlarged lymph nodes pressing on the bronchus .[35]

B] SIGNS:

General physical examination: Anemia, cachexia, tachycardia, digital clubbing with superadded suppuration, increased respiratory rate, EPTB foci such as cold abscess, cervical lymphadenopathy, enlarged mesenteric lymph nodes, localized immobility of spine and or deformity, epididymitis, etc. Sometimes phlyctenular conjunctivitis or keratitis, meningeal irritation, anasarca, changes in hair color and leukonychia, lower BMI can be seen.

Respiratory examination: Displacement of trachea, heart, Chest wall retraction, fibrosis, collapse and distinction of pleural effusion, emphysema or pneumothorax. Undue unilateral prominence of the clavicular head of the sternocleidomastoid muscle [Trail's sign] may be indicative of apical fibrosis due to TB. Mobility restriction of the affected chest wall, a dull percussion note due to consolidation, collapse of the lung or thickened pleura or infiltration seen in TB. A stony dull note can be elicited over a pleural effusion or empyema. Cracked-pot sound may be appreciated in cases where percussion is practiced over a cavity which communicates with bronchus of moderate size and is most distinct when the mouth is wide open. It results due to a sudden expulsion of air through a constricted orifice. It has a hissing character, combined with a clicking sound like that produced by shaking coins together which is a rare finding. High pitched tubular bronchial breathing heard in TB pneumonia, increased vocal fremitus in case of large cavity, *post tussive crepitations,* an important sign of TB infiltration.[36]

DIFFERENTIAL DIAGNOSIS OF PULMONARY TB:

The following diseases can mimic Pulmonary tuberculosis, hence should be ruled out with appropriate investigations post general and systemic examinations. These differential diagnoses include Bacterial pneumonia, lung abscess fungal and miscellaneous bacterial infections, bronchogenic carcinoma, Bronchiectasis, bronchial asthma, Sarcoidosis, Pneumoconiosis, Cardiovascular disease, congenital abnormalities, Hyperthyroidism, diabetes mellitus.[37]

SEQUELAE AND COMPLICATIONS OF PULMONARY TUBERCULOSIS:

1) Parenchymal Complications - thin-walled cavities, fibrotic bands, cicatrization, end-stage lung destruction and aspergilloma. Hemoptysis being the clinically most important consequence of these sequelae.
2) Airway Complications – Bronchiectasis, tracheobronchial stenosis and broncho lithiasis (presence of calcified or ossified material within the lumen of the tracheobronchial tree.
3) Pleural Complications- Chronic empyema, fibrothorax, bronchopleural fistula, and pneumothorax leading to significant loss of thoracic volume.

4) Vascular complications – Pulmonary or bronchial arteritis and thrombosis, bronchial artery dilatation and Rasmussen aneurysm (seen as an enlarging mass or rapidly enlarging consolidation due to hemorrhage, which is seen in 4-5% of autopsy cases in patients with chronic TB.[38]

LABORATORY DIAGNOSIS:

In pulmonary TB :[39]

1. Specimen of choice - is thick, purulent, sufficient quantity (at least 5 ml), specimen containing only saliva to be discarded.
2. Container of choice – [i] wide mouthed, [ii] volume capacity of 25 ml approx. [iii] made of transparent material clear enough to observe the content. [iv] screw - capped watertight seal, reduce transportation leakage, [v]easily labelled to allow permanent identification, [vi] rigid, to avoid breakage during transit.
3. Collection procedure of choice- [i] Clear instruction to the patient-
   - ✓ Inhale deeply 2-3 times,
   - ✓ cough out deep from the chest
   - ✓ open the container and spit sputum into the bottle
   - ✓ avoid saliva or nasal secretions
   - ✓ Close the container

- ✓ [ii] patient is advised to produce sputum in the open , away from the crowd, avoid toilets due to aerosol formation , [iii] one spot specimen on attending health center [iv] next day early morning specimen, [v] Next day spot specimen, while submitting the early morning specimen, [vi] pooling avoided, sent separately.

4. Transportation of choice- [i] specimen to be transported asap preferably in triple layer packaging [ii] in case of delay keep in a cool place

[iii] If refrigerator unavailable and specimen to be transported in hot climate then specimen to be preserved by adding equal volume of one percent cetyl pyridinium chloride in 2% saline.

5. Receiving sample in lab-
   [i] The sample received should be refrigerated at -80°C if processing is postponed.:

[ii] The sample should be decontaminated and digested to remove unwanted growth of bacteria

[iii] NALC liquifies the mucus by splitting the disulfide bonds

[iv] Concentration done by high-speed centrifugation 2000 to 3000 g for 15 minutes

[v] Decant all the supernatant carefully, add PBS and store at 4°C prior to DNA extraction

[vi] Inoculate the concentrate in liquid /solid culture media.

SPECIMENS WHICH CAN BE COLLECTED OTHER THAN SPUTUM

- Fiberoptic Bronchoscopy- Bronchial aspirate and post- scopy sputum specimen
- Gastric Lavage
- Urine
- CSF
- Serous Fluids
- Tissue
- Pus
- Bronchial secretions
- Bone marrow aspirates

Aseptically collected fluids like urine, CSF, Synovial etc. don't require decontamination. For other specimen such as sputum, NaOH in the final concentration of 2% in the diluted specimen is the commonly used liquefying agent and digestant. The decontaminated specimen is concentrated by sedimentation in a refrigerated centrifuge at 3000 g for 30 minutes. The sediment can be used for inoculating media and preparation of smears while the supernatant can be used for biochemical and / or immunological investigations. [40]

DIRECT DEMONSTRATION OF MYCOBACTERIA BY STAINING TECHNIQUES:

1) Ziehl-Neelsen staining
2) Kinyoun's / Gabett's staining
3) Fluorescent staining [auramine- O, Rhodamine, auramine-rhodamine, acridine orange , etc.]
4) Novachrom staining.

TABLE 3: THE VARIOUS AGENTS FOR DECONTAMINATION TECHNIQUES ARE:

AGENT	COMMENTS
N-Acetyl-l-cysteine plus 2% NaOH	Mild decontamination solution with mucolytic agent NALC to free mycobacteria entrapped in mucus. Limit exposure to NaOH to 15 minutes.
Dithiothreitol plus 2% NaOH	Very effective mucolytic agent used with 2% NaOH. Trade name of dithiothreitol is Sputolysin. Reagent is more expensive than NALC. Limit exposure to NaOH to 15 minutes.

Trisodium phosphate, 13% plus benzalkonium chloride (Zephiran)	Preferred by laboratories that cannot carefully control time of exposure to decontamination solution. Specimens treated with TSP-Zephiran should be inoculated onto an egg-based culture medium to neutralize the growth inhibition of the Zephiran. If agar-based media are used, neutralization of the Zephiran can be accomplished by adding lecithin.
NaOH, 4%	Traditional decontamination and concentration solution. Time of exposure must be carefully controlled to no more than 15 minutes. NaOH, 4% effects mucolytic action to promote concentration by centrifugation.
Trisodium phosphate, 13%	Can be used for decontamination of specimens when exposure time can be completely controlled. Not as effective as TSP-Zephiran mixture.
Oxalic acid, 5%	Most useful in processing specimens that contain Pseudomonas aeruginosa as a contaminant.
Cetylpyridium chloride, 1%, plus 2% NaCl	Effective as a decontamination solution for sputum specimens mailed from outpatient clinics. Tubercle bacilli have survived 8-day transit without significant loss.

Reference: Shah RR, Dye WE. The use of dithiothreitol to replace N-acetyl-L-cysteine for routine sputum digestion-decontamination for the culture of mycobacteria. Am Rev respir Dis 1966;94:454

Smithwick RW, Stratigos CB, David HL. Use of cetylpyridinium chloride and sodium chloride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of

Mycobacterium tuberculosis. J Clin Microbiol 1975;1:411-413.

TABLE 4: ISOLATION OF *Mycobacterium tuberculosis* BY CULTURE METHODS:

Broadly two types of media can be used for the growth of Mycobacterium Tuberculosis. These are solid and liquid media

SOLID MEDIA **Functions:** **Isolation of organism** **Antigen preparation** **Chemical tests**	**LIQUID MEDIA** **Functions:** **Sensitivity testing**
1. Lowenstein-Jensen Media	Dubo's
2. Egg Based- Petragnini, Dorset	Middlebrooks
3. Blood based- Tarshis	Proskauer
4. Serum based- Loeffler	Becks
5. Potato based- Pawlowski	Sula
	Sautons

Reference: Bhatia R Tuberculosis. In: Sharma SK, editor. Laboratory Diagnosis.2nd ed. New Delhi: Jaypee; year of publication 2011. p. 164.

TABLE 5: BIOCHEMICAL TESTS FOR CONFIRMATION OF IDENTITY AND DIFFERENTIATION OF MYCOBACTERIA

Tests	**Mycobacterium tuberculosis**	**Mycobacterium bovis**	**Nontuberculous mycobacteria**
Production of niacin	+	-	-
Binding of neutral red	+	+	+/-
Hydrolysis of Tween 80	-	-	+

Production of enzymes			
Nitrate reduction	+	-	+/-
Arylsulphatase	-	-	-/+
Catalase **At room temperature** **At 68°C**	- -	- -	+ +
Catalase-Peroxidase	**Weakly +**	**Weakly +**	**Strongly +**
Nicotinamidase	+	-	-
Pyrazinamidase	+	-	+/-
Susceptibility to: **Pyrazinamide** **Uptake of Iron**	+ -	- -	- -/+

+ = Positive; - = Negative

Reference: Bhatia R Tuberculosis. In: Sharma SK, editor. Laboratory Diagnosis.2nd ed. New Delhi: Jaypee; year of publication 2011. p. 167.

ANIMAL INOCULATION: Guinea pig inoculation by intra dermal and intra nasal route once a popular way of diagnosing TB , has now been regarded as obsolete. It has clearly been demonstrated that the use of this animal offers no practical advantage over the invitro culture.

IMMUNODIAGNOSIS: Antibody Detection Tests; The antigen A60, the most extensively used antigen for both pulmonary and extra-pulmonary, adult and childhood TB have been evaluated. IgG and IgM detection has been evaluated. The sensitivity of these tests has ranged between 30 to 100 per cent[41,42,43]. Tests are also available which use purified antigens mainly 38kDa and 30kDa. Antibody detection by enzyme linked

immunosorbent assay [ELISA] or other serological tests are of limited use. The conclusion of a review of several studies showed that none of the assays perform well enough to replace microscopy. [44]

ANTIGEN DETECTION TEST:

Lipoarabinomannan Urine Test- The test detects lipoarabinomannan [LAM] in urine as a surrogate marker for MTB infection, which is a component of the TB bacterial cell wall. The test exists in ELISA and simplified tube format. Clinical trials to develop dipstick format are ongoing.

Flow-through Filter Tests – These tests rely on detection of Mycobacterium tuberculosis in sputum or body fluids with a polyclonal antibody, using a flow-through device[45].

GENOTYPE ASSAYS:

Two Genotype assays are commercially available. The first is [Genotype Mycobacteria Assay] for TB diagnosis, the second is [Genotype MTBDR Assay] for detection of Rifampicin and Isoniazid resistance. Isolation is commonly done by PCR amplification of the 16S-23S ribosomal DNA spacer region followed by hybridization of the biotinylated amplified DNA with 16 specific oligonucleotide probes. The specific probes being mobilized as parallel lines on a membrane strip.

IDENTIFICATION OF MYCOBACTERIA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In the recent past, several newer rapid methods have become available to achieve reliable results. One such rapid method, developed at the Centre for disease control [CDC], Atlanta, USA, is the use of high-performance liquid chromatography [HPLC]for the analysis of species-specific mycolic acids present in the cell walls of mycobacteria.

DRUG SUSCEPTIBILITY TESTING:

With the rising threat of multidrug resistance in mycobacteria, it necessitates DST on the tubercle bacilli isolates as a guide to therapy. DST are performed on original specimen or subcultures. It is performed in the following cases [i] For the relapsed cases, [ii] for changing drug regimen in suspected drug

resistance, [iii]Undertaking drug-resistance surveillance studies in a region or country.

Direct method: In this case the inoculum is a digested and decontaminated sputum or other clinical specimen in which AFB have been demonstrated in stained smears. In this method the inoculum is a true representative of bacillary population present in the specimen. The advantage being the DST results are available along with the culture results [by 3-4 weeks].

Indirect method: The indirect test are used for specimens that are smear negative but culture-positive or when the growth in the control slope of the direct test is inadequate. Further, in the indirect test, the inoculum is standardized but at the same time is not truly representative and hence there is a chance of selecting a proportion of susceptible or resistant bacilli from the slope. For this reason, the inoculum is prepared by using a representative sweep of the entire surface of the growth on the slope.

There are three general methods used for determining drug susceptibility of mycobacteria by the indirect method:

1) The absolute concentration method also called Minimum Inhibitory Concentration [MIC method]- This method uses a standardized inoculum grown on drug free media and media containing graded concentrations of the drugs to be tested. Here several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth, i.e., MIC. This method is greatly affected by inoculum size and by the viability of the organisms.
2) The Resistance Ratio Method [RR method]- The resistance ratio method compares the growth of unknown strains of tubercle bacilli with that of a standard laboratory strain [$H_{37}R_v$]. Parallel sets of media, containing two-fold dilutions of the drug, are inoculated with a standard inoculum prepared from both the unknown and standard strains of tubercle bacilli. Resistance is expressed as the ratio of the MIC of the test strain to the MIC of the standard strain in the same set. This test is greatly affected by the inoculum size as well as the viability of the

strains. In addition, any variation in the susceptibility of the standard strain also affects RR of the test strain.

3) Proportion Method – The proportion method enables a precise estimation of the proportion of mutants resistant to a given drug. Around 10-fold dilutions of inoculum are planted on to both control [drug free] and drug containing media, at least one dilution should yield isolated countable [50-100] colonies. When these numbers are adjusted by multiplying by the dilution of inoculum used, the total number of viable colonies on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of bacilli resistant to the given drug is then determined by expressing the resistant proportion as a percentage of the total population tested. The proportion method is currently the method of choice. [46]

E – test:

This technique is based on determination of drug susceptibility testing using strips containing gradients of impregnated antibiotics. The E test strip is placed on the surface of the solid culture medium and MICs a measure of the susceptibility of a strain to an antibiotic are determined by interpreting the point at which the ellipse of inhibition crosses the strip.[47] .

Microscopic-Observation Drug – Susceptibility assay:

The [MODS] assay [48,49,50] enables the detection of MTB, directly from the sputum. This innovative technique utilizes a 7H9 liquid medium which facilitates faster growth of the TB bacillus and aids in early microscopic visualization of characteristic cord [microcolonies] formation. The inverted light microscope that is required for MODS assay is expensive. In the study reported by Moore et al , out of 3760 sputum samples evaluated, 401 [10.7%] yielded positive cultures for Mycobacterium tuberculosis. The sensitivity of MODS assay was 97.8 % compared with 89 % for automated mycobacterial culture, and 84% for LJ culture. The MODS assay yielded the culture results much faster [median time 7 days] compared with MBBacT system [BioMerieux] and LJ method which required a median time of 13 and 26 days, respectively. [51]

BIOCHEMICAL AND IMMUNOLOGICAL MARKERS

Adenosine deaminase and interferon- γ

Measurement of Adenosine deaminase [ADA] is a relatively sensitive and specific test for the diagnosis of TB pleurisy. A recent meta-analysis [52] indicated sensitivity of 0.92, specificity 0.90, positive likelihood ratio 9.03, negative likelihood ratio 0.10, and diagnostic odds ratio110.08. In peritoneal TB, IFN- γ and ADA assays showed equal sensitivity of 0.97 and differed marginally in specificity of 0.97 and 0.94 respectively.

DIAGNOSIS OF LATENT TUBERCULOSIS INFECTION:

Unfortunately, MTB infection, in most population is contained by the host immune defenses, and the infection remains latent which is referred by the term latent tuberculosis infection [LTBI]. In this quiescent state, the Mycobacterium tuberculosis bacilli that persists in asymptomatic individuals can reactivate and cause active disease in about 5-10 % of those infected over a lifetime. Globally around 1/3rd of the population is estimated to be victimized with *Mycobacterium tuberculosis* [53]. This enormous pool of individuals with Latent tuberculosis infection [LTBI] poses a threat for TB control universally, because it is from this vast reservoir that new TB cases will be surfacing in the future. Hence the following diagnostic tests are in use to control the menace:

TUBERCULIN SKIN TEST:

Until recently, the only diagnostic test available to detect LTBI was the TST. Being introduced in 1890, the TST is probably the oldest diagnostic test in clinical use[54]. Mantoux is referred to the technique of administering and reading a TST, developed by Charles Mantoux in around 1908. PPD is a crude cocktail of several antigens, many of which are shared among *Mycobacterium tuberculosis*, *Mycobacterium bovis* Bacille Calmette-Guerin [BCG], and several nontuberculous mycobacteria [NTM]. The TST detects cell mediated immunity [CMI] in the form of a delayed type of hypersensitivity [DTH] response to the purified protein derivative [PPD]. The TST has lower specificity in populations with high BCG coverage and

NTM exposure.[55,56,57]. The following table shows the common causes of false-positive and false-negative TST results.

Table 6: Showing interpretation of Tuberculin skin test results:

SIZE OF INDURATION	SIGNIFICANCE
15 mm and above	Signifies infection with tubercle bacilli, irrespective of BCG vaccination status.
10 mm to 14 mm	Attributable to one or more of the following: Cross-sensitivity induced by environmental mycobacteria BCG induced sensitivity Infection with *Mycobacterium tuberculosis*
5mm to 9 mm	Majority of such reactions are attributable to cross-sensitivity induced by environmental mycobacteria And/or BCG vaccination Could be attributable to infection with tubercle bacilli in the presence of immunosuppressive conditions.
Less than 5 mm	Absence of any type of Mycobacterial infection
	Exception: Individuals with severe degree of immunosuppression.

Reference: VK Chada, VK Challu. Tuberculosis. In: Sharma SK, editor. The tuberculin skin test .2nd ed. New Delhi: Jaypee; year of publication 2011. p. 180.

Table 7: False positive and false negative skin test results

TST result	Possible reasons for such a result	Individuals who are likely to have such a result
False-positive	BCG vaccination	Who received BCG vaccination in the past, if they were vaccinated after

		infancy, or if they received repeated BCG vaccinations
	NTM	Individuals who were exposed to NTM or had infection or disease with NTM species [e.g. *Mycobacterium avium*]
False-negative	Anergy	HIV infection, certain viral infections [e.g. measles], live virus vaccinations, immunosuppressive medications, such as steroids, patients on cancer chemotherapy, malnutrition, elderly, renal failure, severe TB disease, lymphoma or leukemia
	Recent TB infection	Individuals who were recently infected [within the past 10-12 weeks]
	Very young age	Children younger than 6 months
	Technical reasons	Inadequate dose of PPD or poor quality PPD, poor Mantoux technique, early or late reading.

Reference: Madhukar Pai, Rajnish Joshi, Sriprakash Kalantri. Tuberculosis. In: Sharma SK, editor. Diagnosis of Latent Tuberculosis Infection: Recent advances and future directions.2nd ed. New Delhi: Jaypee; year of publication 2011. p. 188.

Limitations of TST: accuracy, inter and intra – reader variability [i.e., reproducibility], lack of trained personnel, prolonged waiting time 48-72 hrs. for results, homeless individuals and i.v drug abusers often are reluctant to come back, repeat TST can complicate the interpretation of results.[58]

INTERFERON-GAMMA RELEASE ASSAYS:

Because of the recent advances in molecular biology and genomics such as development of genomic tools like [DNA] microarrays, genome wide scans, proteomics which helps in the identification and cloning of highly specific proteins, an

alternative to the TST has emerged in the form of a new class of in vitro assays that measure interferon- ɣ [INF-ɣ] released by sensitized T- lymphocytes after stimulation with Mycobacterium tuberculosis antigens. These assays are now called INF-ɣ release assays [IGRAs]. Interferon - ɣ is a cytokine, and a classic marker of Th-1 type cellular immune response Two commercial kits namely 1) the QuantiFERON® - TB Gold® [QFT-G] Cellestis Ltd, Carnegie, Victoria, Australia] assay, and the T-SPOT.TB® test [Oxford Immunotec, Oxford, UK]. The QFT-G assay is available in two formats, a 24-well culture plate format [approved by the US Food and Drug Administration [FDA] and a newer, simplified *In Tube* format also FDA approved. Several studies have shown discordance between TST and IGRA and their interpretation, discordance ranging between 10 to 40 % in most studies. Prior BCG vaccination was probably one of the causes in certain studies, other studies found no clear explanation for the discrepancies.[59,60,61,62,63,64]

MOLECULAR TESTS

The World Health Organization (WHO) now recommends molecular tests such as Xpert MTB/RIF, Xpert MTB/RIF Ultra, and Truenat for initial tuberculosis (TB) diagnosis due to their superior sensitivity and specificity. However, challenges persist in diagnosing and treating TB, especially in developing countries like India. The advent of the polymerase chain reaction (PCR) in 1985 revolutionized the diagnosis of infectious diseases in clinical laboratories by enabling rapid, sensitive, and specific detection of pathogens directly from clinical specimens, bypassing the need for culture. These PCR applications are gradually supplementing or replacing traditional culture-based, biochemical, and immunological assays in routine diagnostic settings.[65]

A conventional PCR reaction mixture typically includes target DNA, two primers, heat-stable DNA polymerase, deoxynucleotide triphosphates (dNTPs), and a buffer . Primers are short oligonucleotides (20-30 base pairs) designed to bind to specific sequences at the ends of the target DNA. In our study, conventional PCR was utilized for Mycobacterium tuberculosis detection due to its numerous advantages over traditional culture methods. PCR is highly sensitive, capable of amplifying even minute quantities of specific DNA. It is also highly specific, as it uses primers designed to target unique

DNA [66]sequences of the organism. While traditional methods like Ziehl-Neelsen staining and Auramine O' staining are inexpensive and rapid, they suffer from lower sensitivity and inability to differentiate between different mycobacterial species.

During the COVID-19 pandemic, conventional PCR was extensively used across India for testing throat swab samples, demonstrating its scalability and applicability. In our study, we employed this conventional PCR to test the sputum samples in bulk for detecting MTB, by targeting the IS6110, under specific PCR conditions with the primers described herein. All the amplified products showing a band at 123 bp were identified as Mycobacterium tuberculosis[67].

Furthermore, by utilizing larger screens for imaging the pathogen, unlike those that is experienced during microscopy, reduces strain on the eyes and allows simultaneous review of bulk samples by multiple professionals, enhancing accuracy in MTB detection. Coupled with internet connectivity, results can be swiftly shared among the various stakeholders.

Despite various studies on PCR for MTB detection, none have comprehensively evaluated the efficacy of molecular methods compared to conventional staining techniques in the rural Belagavi district, Karnataka. Therefore, our study aimed to precisely and efficiently identify MTB in new sputum samples from suspected pulmonary TB patients using conventional PCR.

Table 8: Current medications in use for TB treatment.

CURRENT MEDICATIONS IN USE FOR TB TREATMENT AND THEIR NOTABLE SIDE-EFFECTS	
Drug	**Notable side - effects**

Rifampicin (Rif)	Hepatotoxicity, nausea, dyspepsia, abdominal pain, rash, CYP 450 interactions.
Isoniazid (INH)	Hepatotoxicity, peripheral neuropathy, optic neuritis, CYP 450 interactions
Pyrazinamide (PZA)	GI Disturbance, hepatotoxicity, gout.
Ethambutol (EMB)	Optic neuropathy
Levofloxacin (LFX) Moxifloxacin (MFX)	QT Prolongation, tendonitis, hypoglycaemia, psychiatric disturbance.
Bedaquiline (BDQ)	QT Prolongation, CYP 450 interactions.
Linezolid (LZD)	Myelosuppression, dysglycaemia, peripheral neuropathy, optic neuropathy
Clofazimine (CFZ)	Hepatotoxicity, GI Disturbance, neurological disturbance, QT Prolongation, Altered skin pigmentation.
Cycloserine (CYS)	Psychiatric disturbance, peripheral neuropathy
Delamanid (DLM)	QT Prolongation, CYP 450 interactions.
Aminoglycosides (AMK, CAP, STR)	Nephrotoxicity, Ototoxicity
Ethionamide (ETH)/ Prothionamide (PRO)	Psychiatric disturbance, peripheral neuropathy, hepatotoxicity, hypothyroidism, dysglycaemia.
p- Aminosalicylic acid (PAS)	Hepatotoxicity, dysglycaemia, hypothyroidism.
Amoxycillin-Clavulanate with Meropenam or Imipenam Cilastin.	Diarrhea, candidiasis
Given with Pyridoxine prophylaxis to ameliorate risk, Beta lactams must be given with Clavulanate	For success in TB treatment however the only preparation available is Amoxycillin.

Reference: Gill CM, Dolan L, Piggott LM, et al. New developments in tuberculosis diagnosis and treatment. Breathe 2022; 18: 210149 [DOI: 10.1183/20734735.0149-2021].

Table 9: Current guidelines on treating drug resistant Tuberculosis.

CURRENT ATS/CDC/ERS/IDSA CONSOLIDATED GUIDELINES ON TREATING DRUG RESISTANT TB
RR-TB
As per MDR-TB
INH-resistant TB
RIF + PZA + ETM + FLQ for 6 months (can discontinue PZA after 2 months; FLQ only required in patients with extensive disease, i.e cavitary or bilateral infiltrates)
MDR-TB
First line: Levofloxacin or Moxifloxacin with all 4 of: Bedaquiline+Linezolid + Clofazimine + Cycloserine.
Second line: Consider Delamanid or Pyrazinamide or Ethambutol or Amikacin or Streptomycin.
Third line: Consider ETH or Prothionamide or Imipenem-Cilastin /Clavulanate or Meropenem/ Clavulanate or p-Aminosalicylic Acid or High Dose Isoniazid
ATS: American Thoracic Society; CDC: US Centres For Disease Control and Prevention; ERS: European Respiratory Society; IDSA: Infectious Diseases Society of America.

Reference: Gill CM, Dolan L, Piggott LM, et al. New developments in tuberculosis diagnosis and treatment. Breathe 2022; 18: 210149 [DOI: 10.1183/20734735.0149-2021].

DS-TB tends to follow a standard 6 – month regime. This comprises an intensive phase with 2 months treatment consisting of RIF, INH, pyrazinamide (PZA) and ETM, followed by a continuation phase with 4 months treatment of RIF and INH.[97] If the isolate is susceptible to both RIF and INH, ETM can be stopped. The continuation phase should be extended to 7 months in the presence of: cavitation on the initial chest radiograph; persistent sputum growth at 2 months; or if PZA cannot be used due to monoresistance or drug side – effects. Consideration should also be given to extending this phase to 7 months in patients who are otherwise immunosuppressed, such as patients with HIV, Diabetes mellitus, malignancy or medications associated with immunosuppression [97]. Unfavourable outcomes are most associated with high grade smear positivity (at least 3+)

and dependent on the size of cavities, as well as extent of disease on chest radiographs. [98]

Table 10: Treatment is given in two phases. [99]

Type of TB case	Treatment regimen in IP	Treatment regimen in CP
New and previously treated cases (H and R sensitive/ Unknown)	2 HRZE	4HRE

Prefix to the drugs stands for number of months.

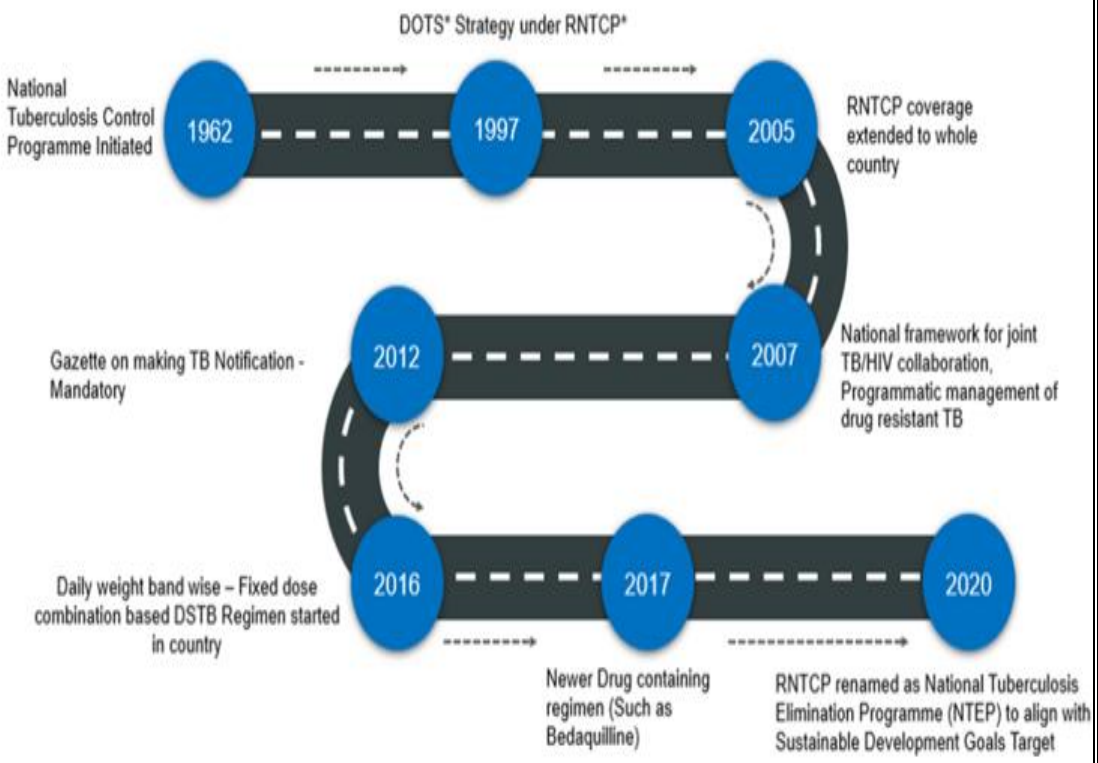
Table 11: Drug dosages for FIRST LINE ANTI TB drugs [99]

Drugs	Adults	Children	Maximum in children
Isoniazid	10mg/kg daily (7-15 mg/kg)	5 mg/kg daily (4-6 mg/kg)	300 mg
Rifampicin	15 mg/kg daily (10-20 mg/kg)	10 mg/kg daily (8-12 mg/kg)	600 mg
Pyrazinamide	35 mg/kg daily (30-40 mg/kg)	25 mg / kg daily (20-30 mg/kg)	2000 mg
Ethambutol**	20 mg/kg daily (15-25 mg/kg)	15 mg /kg daily (12-15 mg/kg)	1500 mg
Streptomycin*	20 mg/kg daily (15-20 mg/kg)	15 mg / kg daily (15 -20 mg/kg)	1000 mg

** Ethambutol is given separately for children to monitor ophthalmic ADR.

* Streptomycin is administered only in certain situations, like TB meningitis or if any first line drug need to be replaced due to ADR as per weight of the patient.

EVOLUTION OF NATIONAL TUBERCULOSIS ELIMINATION PROGRAMME (NTEP) IN INDIA


DOTS* Strategy under RNTCP*
National Tuberculosis Control Programme Initiated
1962
1997
2005
RNTCP coverage extended to whole country
2007
National framework for joint TB/HIV collaboration, Programmatic management of drug resistant TB
2012
Gazette on making TB Notification - Mandatory
2016
Daily weight band wise – Fixed dose combination based DSTB Regimen started in country
2017
Newer Drug containing regimen (Such as Bedaquilline)
2020
RNTCP renamed as National Tuberculosis Elimination Programme (NTEP) to align with Sustainable Development Goals Target


METHODOLOGY

METHODOLOGY

Study Methodology Overview

Study Setting: 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.

Study Clearance: The proposed research study is ethical and justifiable and has been cleared by the JNMC Institutional Ethics Committee, Belagavi on 27.09.2022.

Source of Data: Sputum samples were collected from suspected pulmonary TB patients attending the OPD of Pulmonary Medicine Department, of Dr Prabhakar Kore Hospital and Medical Research Centre, Belagavi.

Study Design: Analytical cross-sectional study conducted over one year from January 2023 to December 2023.

Study Population: The study included fresh sputum samples from suspected pulmonary TB patients in the rural population of Belagavi.

Inclusion Criteria: All new suspected pulmonary TB patients attending the respiratory OPD of Dr Prabhakar Kore Hospital and Medical Research Centre.

Exclusion Criteria: Patients already diagnosed and under treatment for pulmonary TB, and those with extra-pulmonary TB.

Sample Size Calculation: Sample size was determined based on prevalence and statistical criteria.

Sample size : $n = [4pq / d^2]$

$= 4x (38.51) x (100-38.51)/ (0.20 x 38.51)^2$

$= 161$

P = 3 year mean of our hospital

= 38.51 %

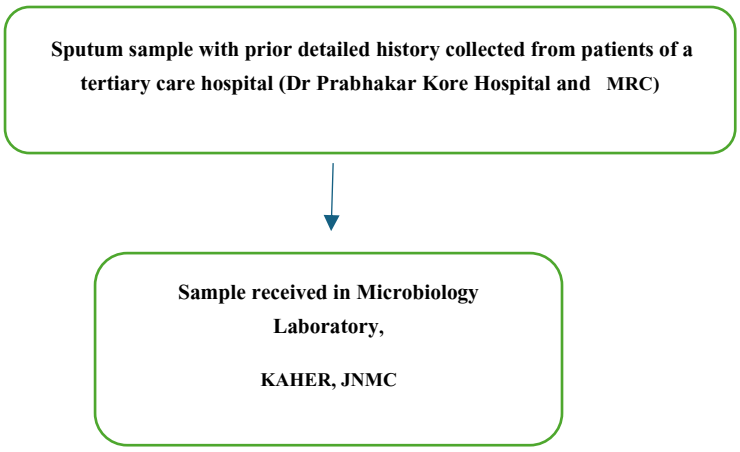
Sampling Procedure: Sputum samples were collected under sterile conditions after obtaining consent from patients, ensuring proper labeling and documentation of clinical history.

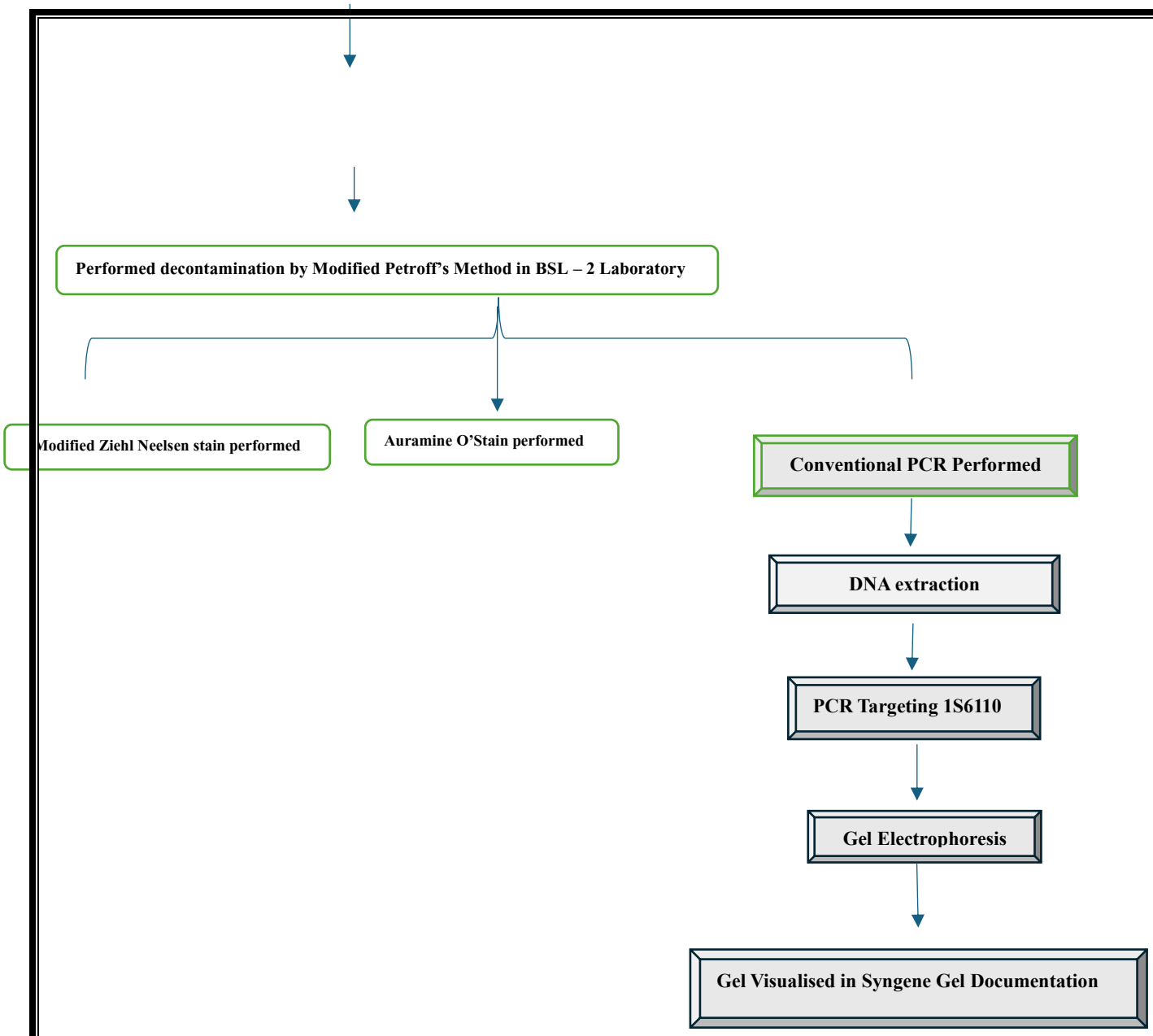
Statistical Analysis: Prevalence was calculated and expressed as a percentage using appropriate statistical methods. p-value is obtained by Cochran's Q test'.

Data Collection: Data were collected using a standardized proforma.

This study aimed to utilize conventional PCR to detect *Mycobacterium tuberculosis* from new sputum samples, providing a reliable diagnostic approach for TB in the rural population of Belagavi district, Karnataka.

METHODOLOGY OVERVIEW:





Refer Annexure - IV for Procedures and Annexure – V for Methods of Preparation.

Table 12: Biochemical tests

Biochemical tests to differentiate mycobacteria:			
Tests	**Mycobacterium tuberculosis**	**Mycobacterium bovis**	**Nontuberculous mycobacteria**
Production of niacin	+	-	-
Binding of neutral red	+	+	+/-
Hydrolysis of Tween 80	-	-	+
Production of enzymes			
Nitrate reduction	+		+/-
Arylsulphatase	-	-	-/+
Catalase **At room temperature** **At 68°C**	- -	- -	+ +
Catalase-Peroxidase	**Weakly** +	**Weakly** +	**Strongly** +
Nicotinamidase	+	-	-
Pyrazinamidase	+	-	+/-
Susceptibility to:	+		

Pyrazinamide **Uptake of Iron**	-	- -	- -/+

+ = Positive; - = Negative

Although these tests were not performed, but this table can help in the identification of *Mycobacterium tuberculosis, Mycobacterium bovis, Non tuberculous mycobacteria.*

RESULTS

IDENTIFICATION OF MYCOBACTERIUM TUBERCULOSIS:

ZN- Stain appearance - The tubercular bacillus manifested as lean, straight, and occasionally slightly curved rod-shaped entities, measuring 2-4 micro metre in length and 0.2 to 0.8 micro metre in breadth, appearing individually, in pairs, and in tiny clusters.

Beaded or barred forms of bacilli were frequently seen which is characteristic of *Mycobacterium tuberculosis*. *Mycobacterium bovis* on the other hand will stain more uniformly.

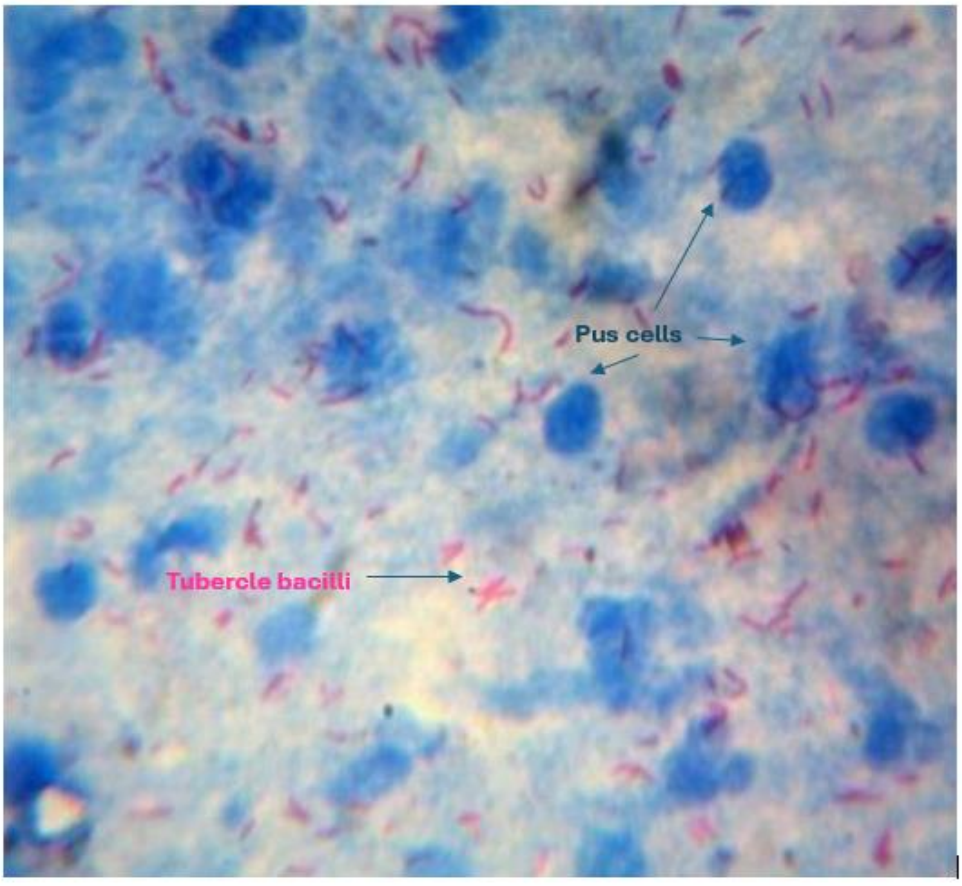


Fig. 1 Showing *Mycobacterium tuberculosis* bacilli and pus cells (arrow marks) from a suspected PTB patient.

Auramine O' Stain appearance – Here the bacilli appeared as bright yellow fluorescence, with a dark background very short and slightly curved organism.

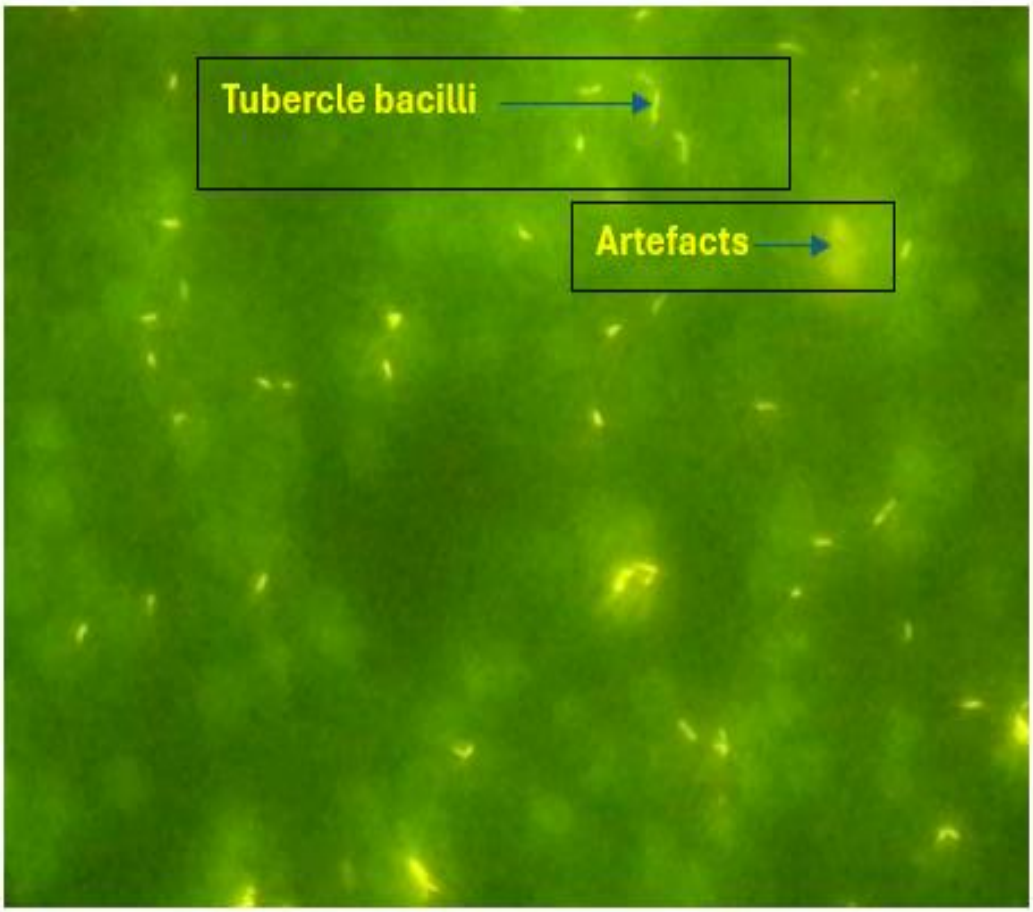


Fig.2 Depicting *Mycobacterium tuberculosis* bacilli and Artefacts (arrow marks) in the same suspected PTB patient.

Conventional PCR appearance – The PCR products were analyzed in 1% Agarose Gel Electrophoresis followed by Ethidium bromide stain. The *Mycobacterium tuberculosis*

target gene IS6110 appeared as a bright amplicon at 123 bp (base pair).

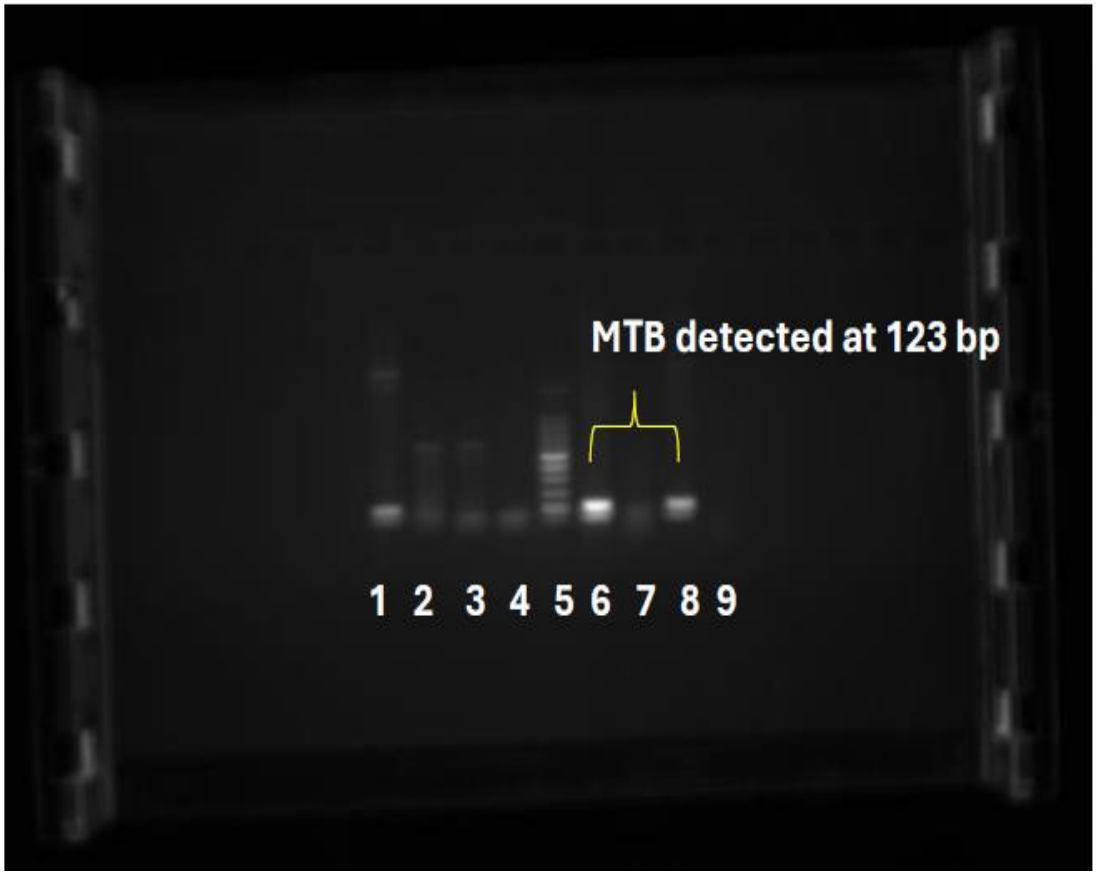


Fig. 3 Lane 1 shows positive control $H_{37}R_v$ strain, lanes 2,3,4,7 are negative samples, lane 5 shows 100 bp ladder, lane 6 and 8 shows MTB detected at 123 bp of two different patients' sputum samples, lane 9 is the negative control with water.

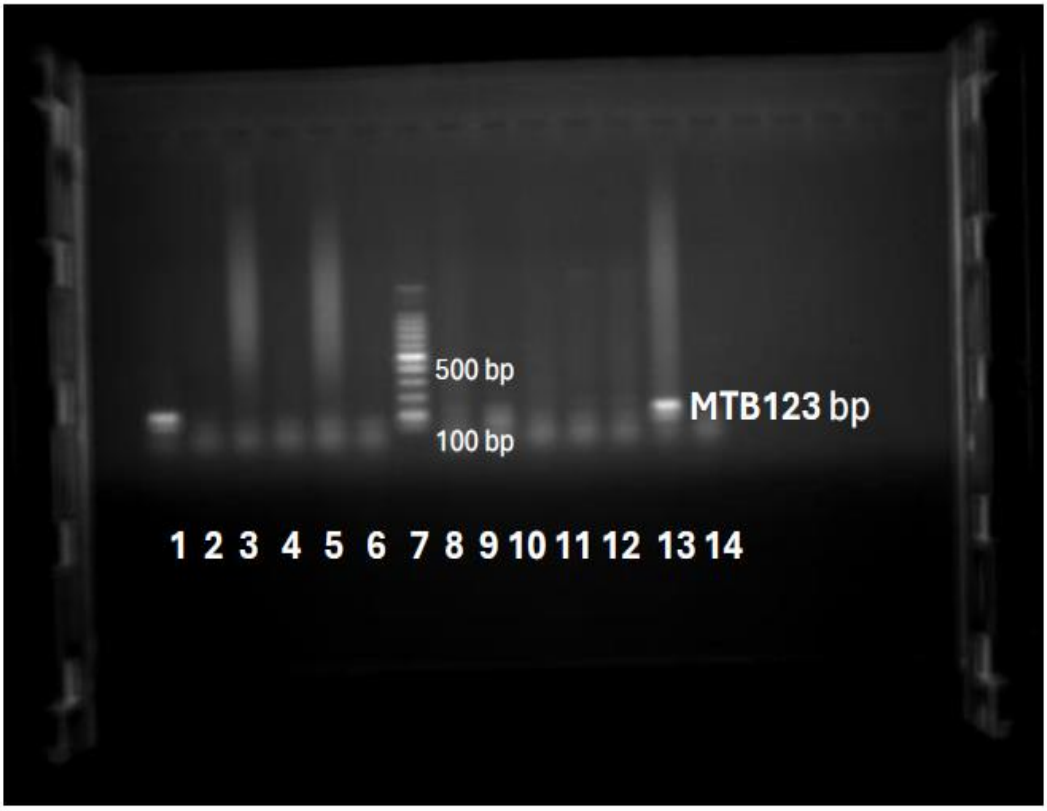


Fig.4 Lanes 1 and 14 are the positive and negative controls for MTB respectively. Lane 7 is the 100 bp ladder, lane 13 is positive for PTB and lanes 2,3,4,5,6,8,9,10,11,12 are negative PTB samples.

Fig.5

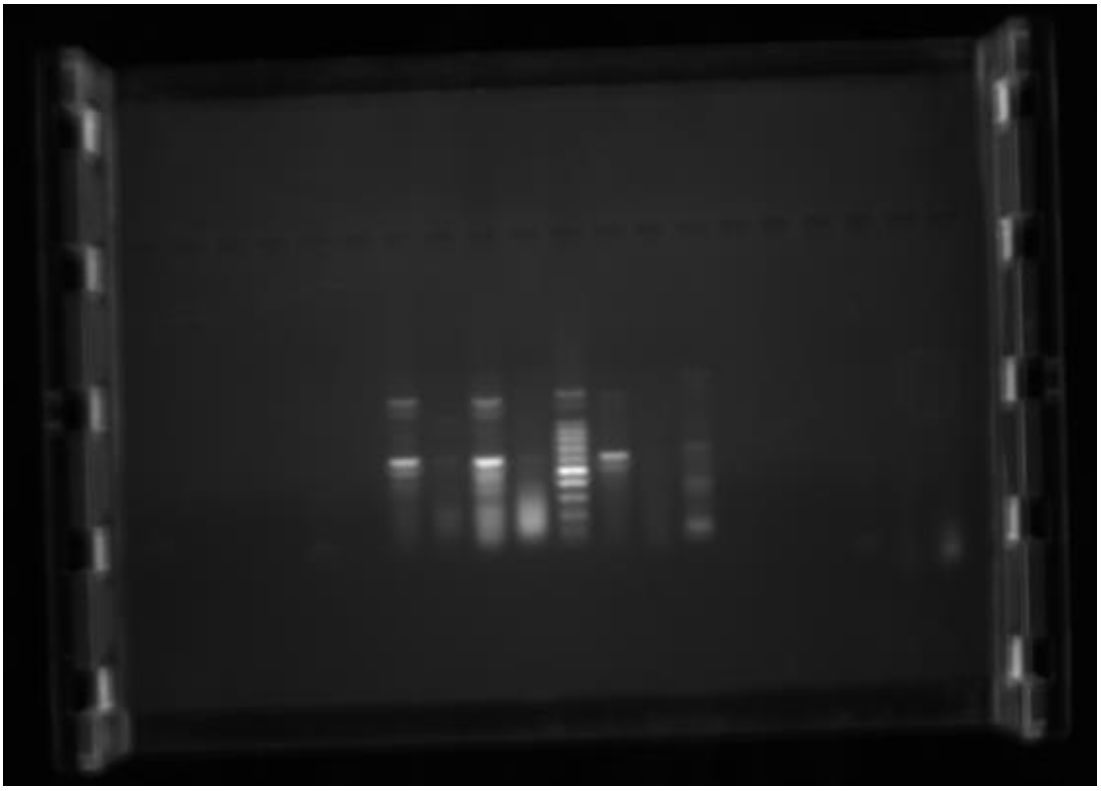


Fig.6

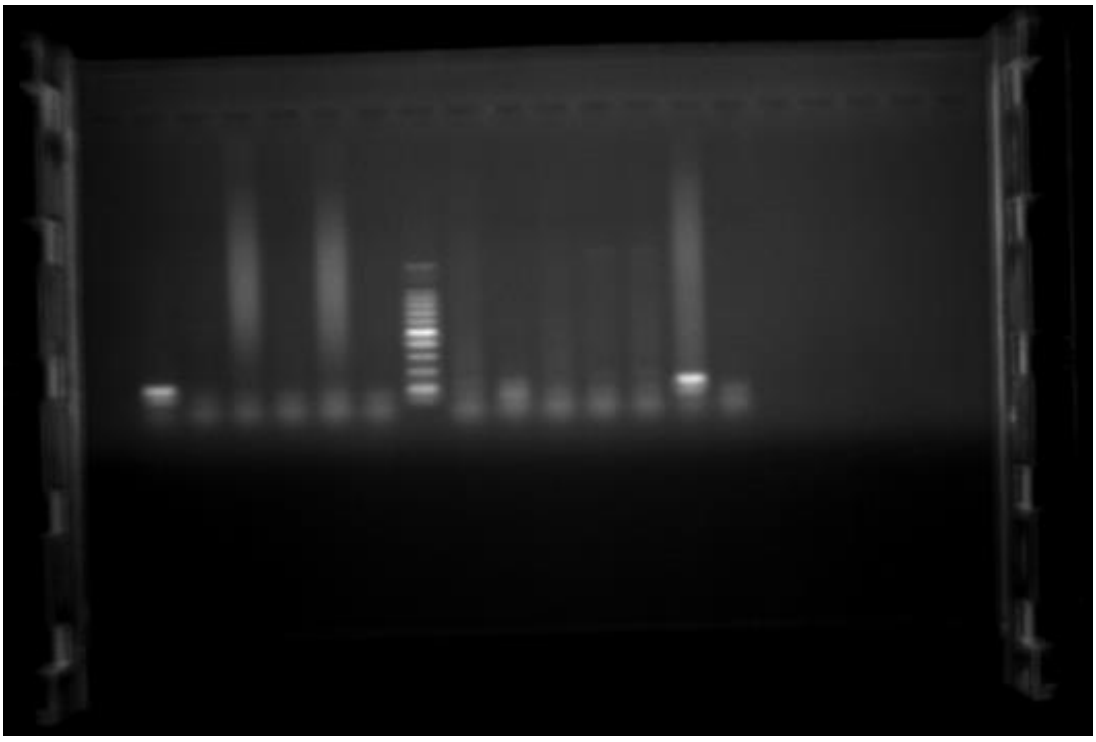


Fig.7

Fig.8

Fig.5 to Fig.8 shows sputum samples taken in bulk which were used to detect MTB from suspected PTB patients, with positive MTB detected at 123bp.

Conventional PCR appearance – The PCR results were examined using 1% Agarose gel-based electrophoresis, subsequently stained with Ethidium bromide (Etbr).

The *Mycobacterium tuberculosis* target gene IS6110 appeared as a bright amplicon at 123 bp (base pair).

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. Samples containing saliva were rejected. Around 2% of the samples were blood tinged.

Sputum sample with prior detailed history collected from patients of a tertiary care hospital (Dr Prabhakar Kore Hospital and MRC)

Sample received in Microbiology Laboratory, KAHER, JNMC

Performed decontamination by Modified Petroff's Method in BSL – 2 Laboratory

Modified Ziehl Neelsen stain performed

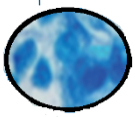
Auramine O'Stain performed

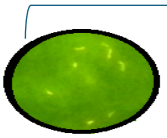
PCR Targeting 1S6110

Conventional PCR Performed

Gel Visualised in Syngene Gel Documentation

Gel Electrophoresis





ZN Stain Positive, 1000 X, magnification,

ZN Stain Negative, 1000 X, magnification,

Auramine O'Stain Positive, 250 X magnification

Auramine O'Stain Negative 250X magnification.

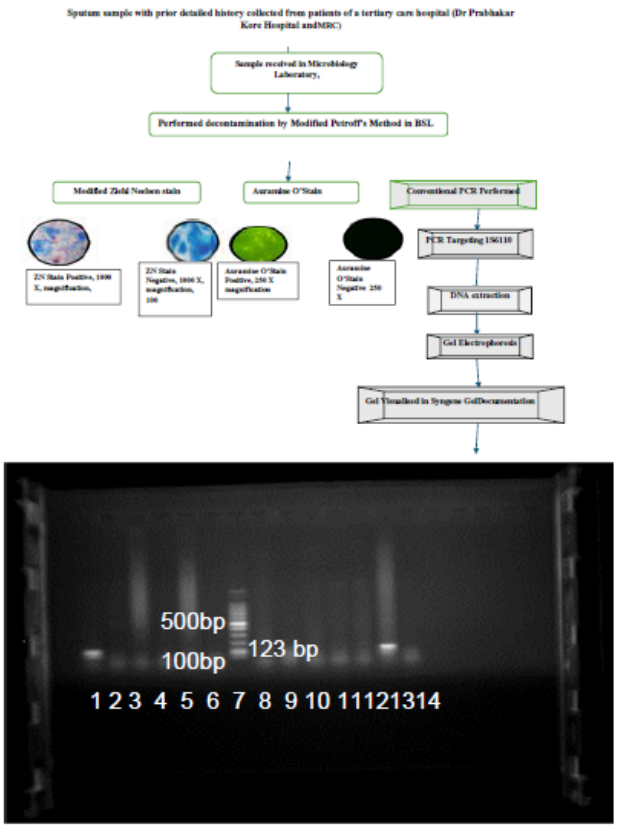


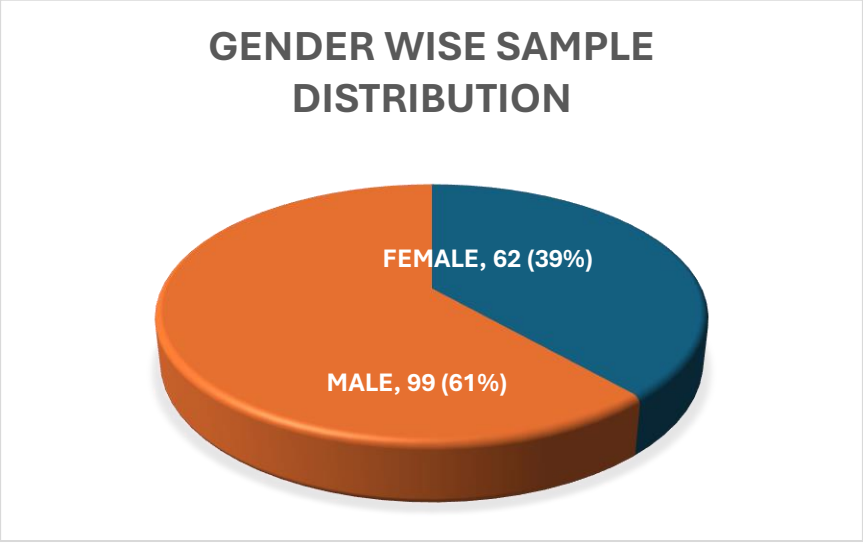
Fig.5 Lane 1 shows the positive control, lane 7 shows a 100 bp DNA ladder, lane 14 is the

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.

Table no. 13: Gender wise distribution of sputum samples:

Gender	No. of Samples	%
Female	62	39%
Male	99	61%
Grand Total	**161**	**100%**

Graph No. 2 Gender wise distribution of sputum samples

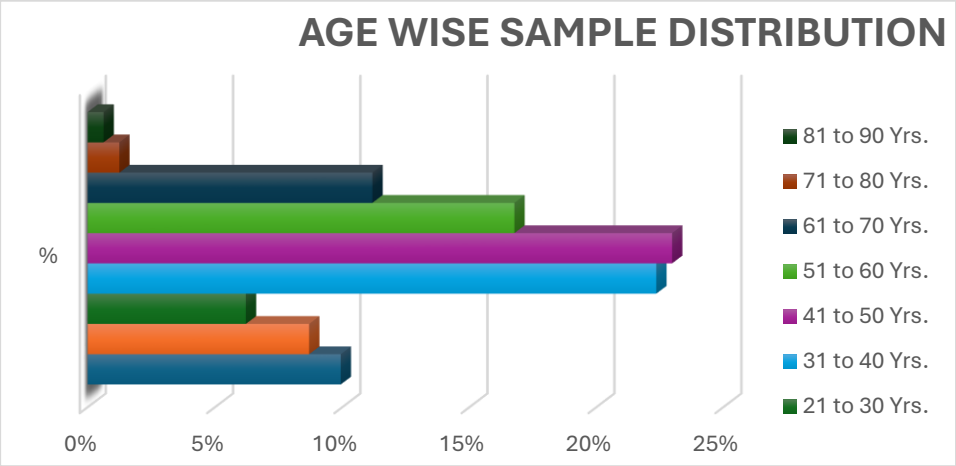


During the study period , the total number of male patients participating in the study constituted around 99 (61%) , whereas the total number of female patients were around 62 (39%).

Table no.14 Age - wise distribution of cases: (n=161)

Age	No. Of Patients	%
01 to 10 Yrs.	16	10%
11 to 20 Yrs.	14	9%
21 to 30 Yrs.	10	6%
31 to 40 Yrs.	36	22%
41 to 50 Yrs.	37	23%
51 to 60 Yrs.	27	17%
61 to 70 Yrs.	18	11%
71 to 80 Yrs.	2	1%
81 to 90 Yrs.	1	1%
Grand Total	**161**	**100%**

Graph no. 3: Age wise distribution of cases (n=161)



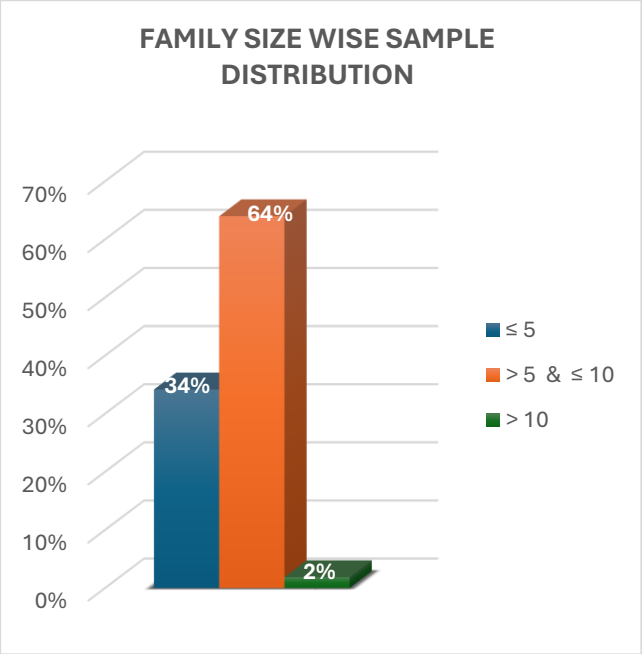
Among the participants of various age groups, maximum patients were belonging to the age group of 41yrs -50yrs (n = 37, 23%), followed by 31yrs – 40 yrs (n= 36, 22%, and 51- 60

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%).

Table no.15: Family size wise patient distribution:

No. Of Family Members	No. of patients	% of patients
≤ 5	55	34%
> 5 & ≤ 10	103	64%
> 10	3	2%
Grand Total	**161**	100%

Graph No.4 Family size wise sample distribution:

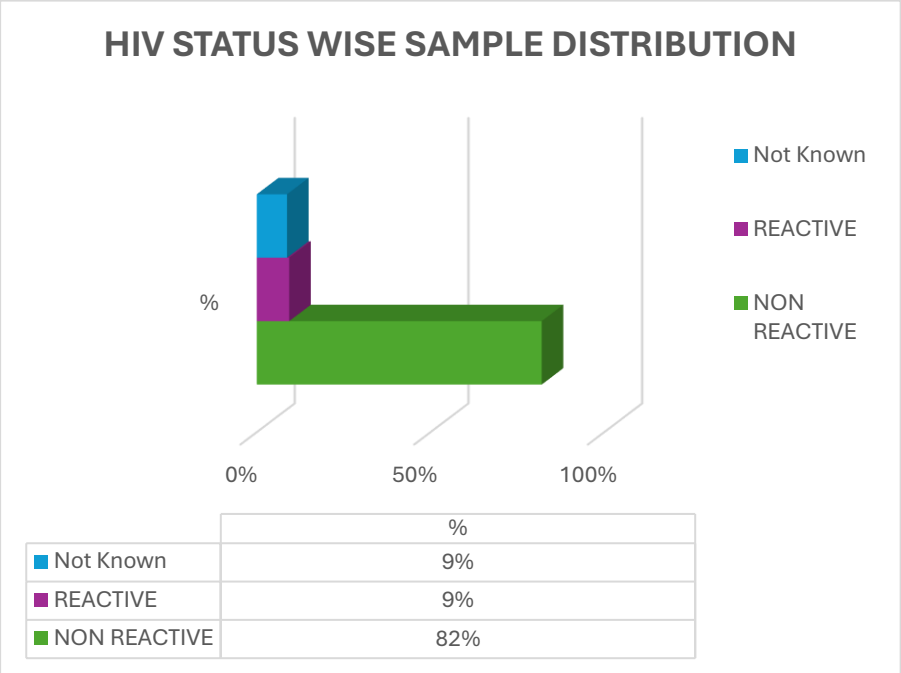


Among the participants, less than or equal to 5 membered family constituted (n=55), 34%, between 5 to less than 10 were (n=103, 64%) and more than 10 family members constituted (n=3, 2%).

Table no.16: HIV status wise sample distribution

HIV - STATUS	No. Of Patients	% of samples
Non-Reactive	132	82%
Reactive	15	9%
Not Known	14	9%
Grand Total	**161**	**100%**

Graph no.5 HIV status wise sample distribution

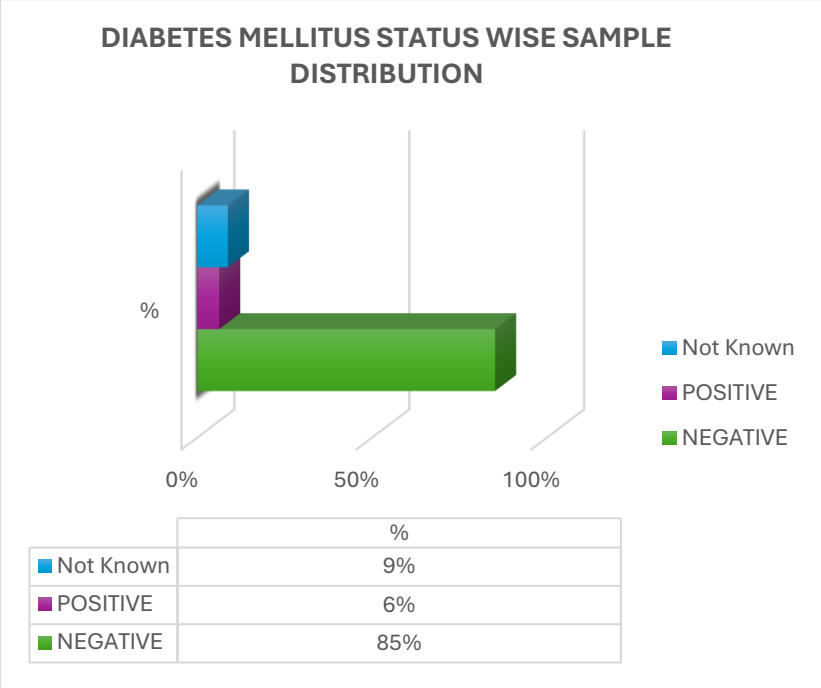


Among 161 patient samples, around (n=132, 82%) , showed HIV Status as non-reactive, followed by 15 patients (n=15, 9%) which showed HIV status as reactive and the rest (n=14, 9%) the HIV status was not known.

Table no.17: Diabetes Mellitus status wise sample distribution:

Diabetes Mellitus	No. Of Patients	% of samples
Negative	137	85%
Positive	10	6%
Not Known	14	9%
Grand Total	**161**	**100%**

Graph no.6 Diabetes Mellitus status wise sample distribution:

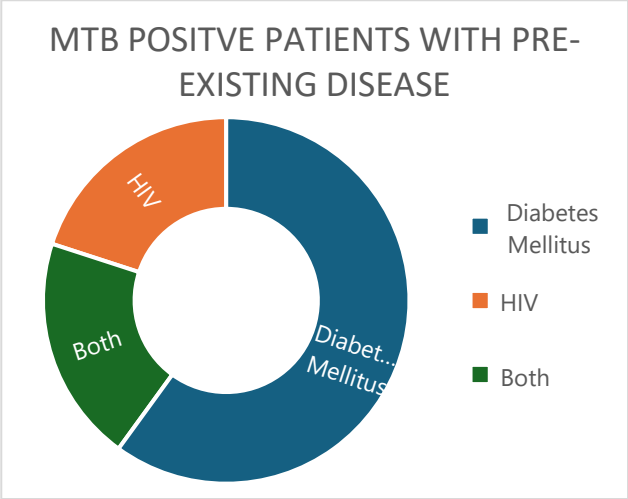


Total number of patients, who were non – diabetic among the 161 patient samples were (n=137, 85%), this was followed by (n=10, 6%) samples which had a positive diabetes mellitus status and the rest (n=14, 9%) , the diabetes mellitus status was not known.

Table no.18: MTB Positive patients with comorbidities:

MTB Tested Positive in PCR		
Pre-existing Disease	**No. Of Patients**	**% Of Samples**
Diabetes Mellitus	6	15.38%
HIV	2	5.13%
Both	2	5.13%

Graph No.7 MTB positive patients with comorbidities:

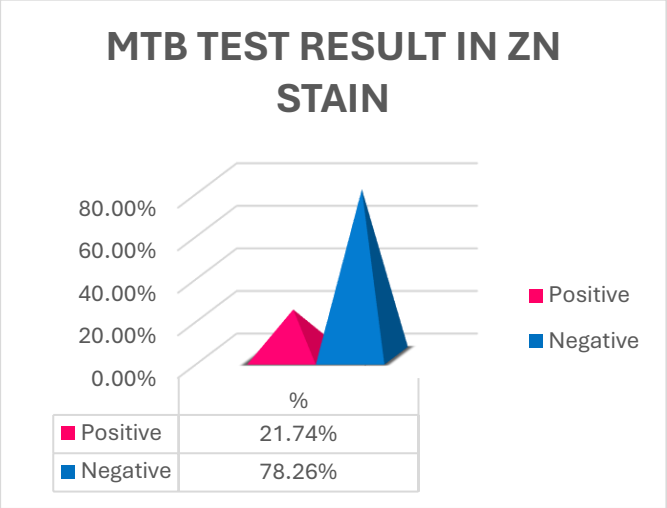


Out of 161 patient samples, MTB positive patients detected by PCR with pre-existing non-communicable disease as Diabetes Mellitus constitute 6 (n=6, 15.38%), this is followed by MTB positive patients with pre-existing communicable disease i.e. HIV constitute around (n=2, 5.13%) and patients presenting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

Table No. 19: MTB Test results in ZN-Staining

ZN Stain		
Result	**No. Of Patients**	**% of samples**
Positive	35	21.74%
Negative	126	78.26%
Grand Total	**161**	**100%**

Graph no.8 MTB Test results in ZN-Staining

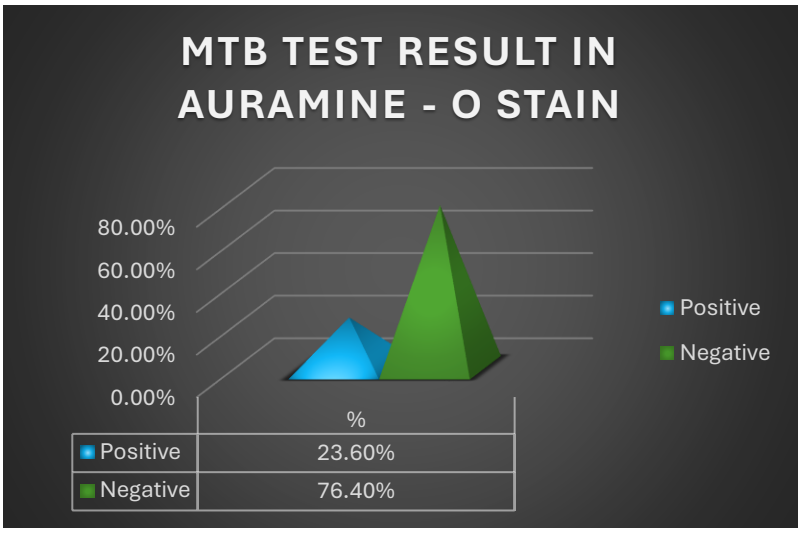


After performing ZN- staining, Out of 161 patients, the number of patients with ZN-positive smear samples were (n=35, 21.74%) and that of negative smear samples were (n=126, 78.26%).

Table no.20; MTB Test results in Auramine 'O Staining

Auramine-o stain		
Result	**No. Of Patients**	**% of Samples**
Positive	38	23.60%
Negative	123	76.40%
Grand Total	**161**	**100%**

Graph no. 9 MTB Test results in Auramine O Staining

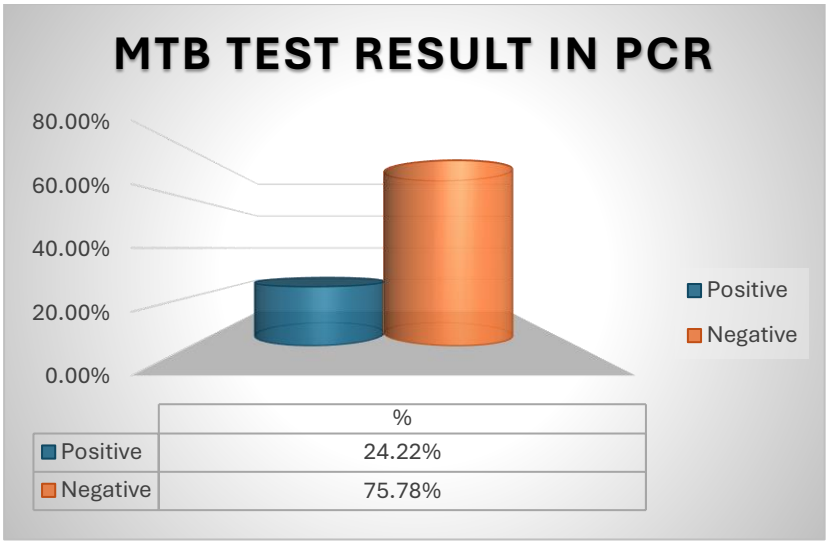


After performing Auramine O' Staining, Out of 161 patients, the number of patients with positive smear samples were (n=38, 23.60%) and that of negative smear samples were (n=123, 76.40%).

Table no.21: MTB Test results in Conventional PCR

PCR		
Result	**No. Of Samples**	**% Of Samples**
Positive	39	24.22%
Negative	122	75.78%
Grand Total	**161**	**100%**

Graph No.10 MTB Test Results in Conventional PCR

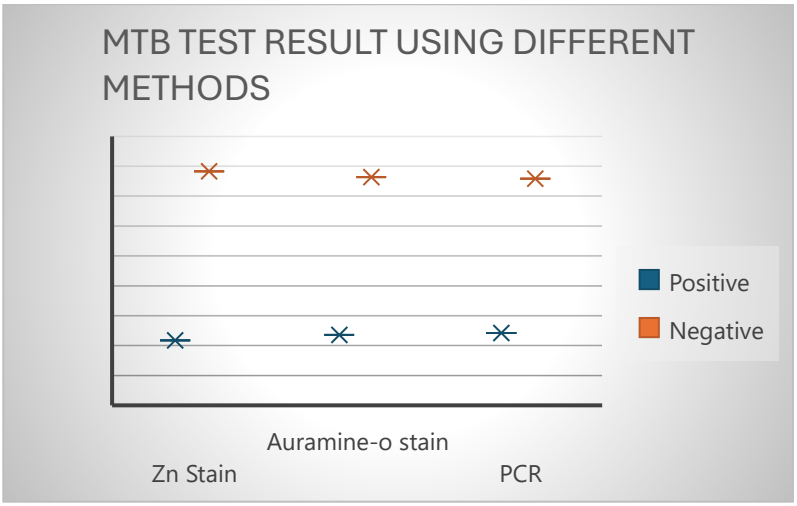


After performing conventional PCR, Out of 161 patients, the number of patients whose MTB was detected at the 123 bp were (n=39, 24.22%) and the number of MTB not detected samples were (n=122, 75.78%).

Table no.22: MTB Test results comparison using different methods

Method	Result	
	Positive	Negative
ZN Stain	21.74%	78.26%
Auramine-o stain	23.60%	76.40%
PCR	24.22%	75.78%

Graph no.11 Showing MTB Test results comparison using different methods

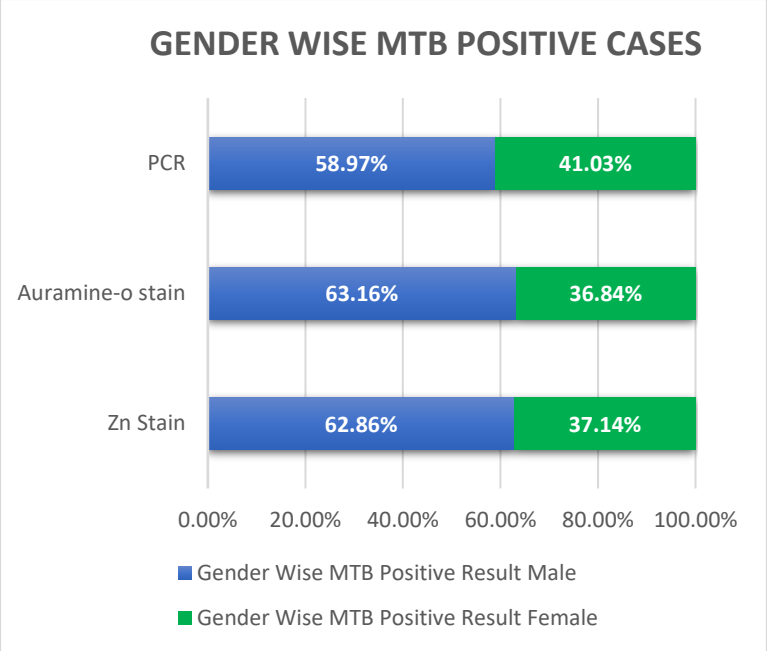


After screening a total of 161 sputum samples, the ZN-stain showed 21.74% Positive results and 78.26% Negative results, Auramine O' Stain showed 23.60% positive results and 76.40% Negative sputum smear, Conventional PCR showed 24.22% positive and 75.78% negative results.

Table no.23: Gender Wise MTB Positive Cases

Method	Gender Wise MTB Positive Cases	
	Male	Female
ZN Stain	62.86%	37.14%
Auramine-O' stain	63.16%	36.84%
PCR	58.97%	41.03%

Graph no.12 Gender Wise MTB Positive Cases

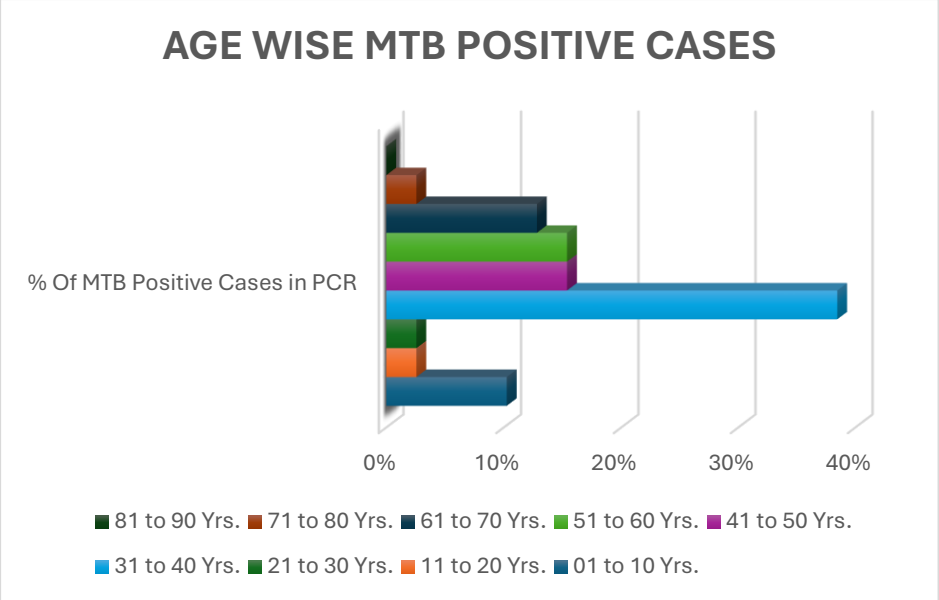


ZN - Stain positive males were 62.86% and ZN- Stain positive females were 37.14% , Auramine O' Stain smear positive males were 63.16% and females were 36.84% , PCR positive males were 58.97% and females were 41.03%.

Table No.24: Age Wise MTB Positive Cases (n=161)

Age	No. Of MTB Positive Cases in PCR	% Of MTB Positive Cases in PCR
01 to 10 Yrs.	4	10%
11 to 20 Yrs.	1	3%
21 to 30 Yrs.	1	3%
31 to 40 Yrs.	15	38%
41 to 50 Yrs.	6	15%
51 to 60 Yrs.	6	15%
61 to 70 Yrs.	5	13%
71 to 80 Yrs.	1	3%
81 to 90 Yrs.	0	0%
Grand Total	**39**	

Graph no.13 Age Wise MTB Positive Cases (n=161)



The highest number of positive cases in conventional PCR was shown in the age group of 31yrs to 40 yrs which is (n=15, 38%). This was followed by 41 yrs to 60 yrs age group(15%). The lowest number of positive cases were seen in 71 yrs to 80 yrs age group (n=1, 3%).

Table No.25: Comparison of Diagnostic Methods for

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

***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 methods. Therefore, the effectiveness of these three diagnostic methods appears to be similar.


DISCUSSION

DISCUSSION

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.[68]

Covering a total area of 13,433 square kilometres as per the 2011 census, Belagavi district is home to a population 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, 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. Notably, HIV represents a particularly potent risk factor for the progression of TB infection into active disease.[69]

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 et al. at BHU, India, where they received a total of 721 clinical specimens from patients suspected of pulmonary TB. 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).[70] 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.

Gene detected:

In our study, we targeted the IS6110 gene, with the gene product size being 123 bp. The standard H37Rv strain was used as the positive control, while H2O was used as the negative control. Various targets have been described for the detection of Mycobacterium tuberculosis, including genes encoding 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 gene, and 16S rRNA. IS986 and IS6110 are among the most prevalent repetitive elements in M. tuberculosis strains, typically present in 10-16 copies. According to a study by de Lassence and Walker DA et al., IS6110 was found to be more sensitive and specific than IS986. Therefore, in our study, we targeted the IS6110 gene in uncultured specimens from suspected MTB patients, as it is a valuable marker for MTB detection. However, the presence of this target in Mycobacterium bovis can lead to false positive results, as observed in a study by Bauer J, Cowan et al.[71]

In another investigation conducted by Farzam and Imami et al., they utilized PCR to target the cyp 141 gene for detecting Mycobacterium tuberculosis in clinical 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 known to play a significant role as a virulence factor.[72] 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.

Gender wise distribution:

In our study, out of the 161 newly detected sputum samples, 99 (61%) samples were from male patients and 62 (39%) 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 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. Studies have shown that tuberculosis disproportionately affects women, particularly in the reproductive 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 to 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.[73,74]

Globally, the incidence of tuberculosis varies significantly by region, with lower rates in Europe (55 per 100,000) and the USA (8 per 100,000), compared to higher rates in Africa (317 per 100,000) and South-East Asia (536 per 100,000), according to the World Health Organization's Global TB Control Report 2006.[75]

The WHO's END TB strategy aims to eradicate tuberculosis by 2035, targeting a 95% reduction in TB deaths and a 90% reduction in incidence compared to 2015, with a goal of 90% coverage in treatment and prevention.[76]. 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 [77]. Belagavi district in North Karnataka ranked as the 4th highest TB burden district in Karnataka based on notified TB cases in 2019. [78]. To control the spread of tuberculosis and reduce the mortality rate, rapid and sensitive laboratory detection tests are crucial. Darban-Sarokhalil et al. developed the first rapid detection method for M. tuberculosis using amplification of the cyp141 gene. In our study, we targeted the IS6110 gene, with the gene product size

being 123 bp. The standard H37Rv strain was used as the positive control, while H2O was used as the negative control.

Socio-economic Conditions:

Similarly, a study by Chakraborty et al. in Wardha district, Maharashtra, which links TB prevalence to socio-economic factors, observed variations based on literacy, employment, and housing conditions. TB prevalence was lowest among graduates and highest among illiterates. Professionals had the highest TB cases, followed by cultivators and agricultural labourers. Those living in 'kutcha' houses had a higher TB prevalence compared to 'pucca' house dwellers. Among females, 48% of TB cases were among the unemployed, including homemakers. Another study by Chakravarty et al. found that TB rates were generally lower in females compared to males across all demographic variables. In another study by Dholakia et al., it was found that among the workers in India estimated to have TB, about 52% were aged 15 to 44 years. Among these workers, around 40% were women in urban areas, whereas in rural areas, the proportion of women workers with TB was only 17.9%. The Wardha survey also showed that urban professionals and rural service workers had a higher prevalence of TB but a low proportion of females, resulting in a lower overall number of female TB cases. Various studies indicate that TB morbidity is a significant public health issue in India, particularly among males in the economically active age group and females in the reproductive age group [79].

Tuberculosis and Diabetes Mellitus:

Diabetes mellitus is the most common co-morbidity in pulmonary TB, followed by malignancy, and is associated with a higher likelihood of cavitary nodules according to a study by Wang JI and Lee et al. In our study, around 137 (85%) of patients had a negative diabetes mellitus status. 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 no. 26: 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

Source: The Union – WDF report, 2014

Recently, a study by Stevenson, Dye C et al., estimated the impact of diabetes mellitus as a risk factor for incident pulmonary TB cases, using India as an example. DM accounted for 14.8% of pulmonary TB and 20.2% of sputum smear-positive TB cases, with the incidence of smear-positive cases being about 15% higher in urban areas compared to rural ones. These observations suggests that DM significantly contributes to the burden of TB cases in India, especially the infectious form. [80,81]

A study by Faruqi et al. indicated that the burden of DM in India is likely to increase, highlighting the need for stringent TB control activities in both urban and rural areas. Al Webel et al. suggested that in patients with DM, TB predominantly occurs in the lower lobes with frequent cavitary lesions. Another study by Bashar et al. found a significant increase in multidrug-resistant TB in patients with DM (36% vs. 10%) compared to those without DM. However, a study by Kameda et al. showed no relationship between the degree of glycaemic control and relapse rate, indicating that TB patients with DM have similar response and long-term relapse rates as those without DM, although they have a poorer prognosis once relapse occurs [82,83,84,85,86]

The widespread involvement of endocrine glands in clinical TB is an important consideration for physicians

managing TB. Further studies are needed to conclusively explore the reported predisposition of certain endocrine disorders, including the increased prevalence of DM in TB patients. Several hypotheses have been proposed to explain the increased prevalence of diabetes mellitus (DM) in tuberculosis (TB). A study by Nichols et al. suggests that when two diseases are associated, a reciprocal relationship is expected. Bloom JD et al. proposed that occult glucose intolerance may predispose individuals to TB infection. Tripathi, Das et al. linked TB with malnutrition, postulating that malnutrition may modulate the pathogenesis of DM. Additionally, Roychowdhury and Sen suggested that TB of the pancreas could be a possible cause of glucose intolerance, although pancreatic involvement in TB is rare. In another study by Mugusi Swai et al., it is suggested that stress-induced diabetes mellitus (DM) can occur during major illnesses like tuberculosis (TB). Abnormalities in glucose tolerance seen in TB patients tend to improve after the initiation of anti-tuberculosis treatment. Atkin SL, Masson EA et al. 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 et al. [87,88,89,90,91,92]

Tuberculosis and Human Immunodeficiency Virus:

In many developing countries, including India, Human Immunodeficiency Virus (HIV) infection and tuberculosis (TB) are causing significant health challenges. In our study, 132 (82%) patients tested negative for HIV, 15 (9%) were HIV-positive, and the HIV status of 14 (9%) patients was unknown. We found that 2 (5.13%) patients had TB-HIV co-infection, and another 2 (5.13%) had a syndemic infection of HIV, diabetes mellitus (DM), and TB. TB is the most common opportunistic infection (OI) in HIV-infected patients in India, which has the third highest number of people living with HIV (PLHIV) globally. India accounts for about 9% of the global burden of HIV-associated TB, making it the second highest in the world. HIV-TB co-infection has a high mortality risk and is responsible for about 25% of global HIV/AIDS deaths each year, as noted in a study by Lawn et al. Most patients diagnosed with TB-HIV co-infection have advanced HIV disease, characterized by low CD4+ T-cell counts, high viral loads, or WHO clinical stage 3 and 4 disease.

In areas with high HIV prevalence, TB programs should prioritize identifying infectious sputum smear-positive cases through microscopy. Diagnosing TB in patients with advanced HIV infection is challenging because HIV-positive individuals with pulmonary TB often have a higher frequency of negative sputum smears, necessitating sputum culture for confirmation. The tuberculin skin test frequently yields false negative or invalid results due to immune system failure, and chest radiography may be less useful in HIV patients as they exhibit fewer cavitations. Additionally, extra-pulmonary tuberculosis is more common in TB-HIV co-infection. Antiretroviral therapy (ART) should be offered to all patients with HIV and TB, regardless of CD4 cell count. Anti-tuberculosis treatment should be initiated first, followed by ART once TB treatment is tolerated, typically between 2 weeks and 2 months. The use of Highly Active Anti-Retroviral Therapy (HAART) in TB patients can lead to a sustained reduction in HIV viral load. [93,94,95,96]

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 results indicated that 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.


CONCLUSION

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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.

Key findings from our study highlight:

1. A predominance of male TB cases, attributed to occupational hazards and societal dynamics.

2. Higher TB positivity rates among larger families, highlighting household transmission dynamics.

3. Significant DM prevalence among TB patients, emphasizing the interplay of comorbid conditions.

4. The potential of Conventional PCR in rural TB diagnostics, utilizing pre-existing infrastructure and expertise.

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

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. This study 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.

Key Findings:

1. Demographics: Analysis of 161 sputum samples revealed a predominance of TB positivity among males (61%) compared to females (39%), influenced by societal factors including occupational hazards and health behaviors.

2. Age Distribution: The majority of TB cases occurred within the 30-60 years age group, highlighting distinct epidemiological patterns between developed and developing countries.

3. Comorbidities: Diabetes mellitus (DM) was prevalent among TB patients (15.38%), emphasizing the compounding effect of communicable and non-communicable diseases in disease progression.

4. Family Size Impact: Higher TB transmission rates were observed in larger families, underscoring the role of household dynamics in disease dissemination.

5.Diagnostic Potential: Conventional PCR, previously deployed for COVID-19 diagnostics, demonstrated potential for repurposing in MTB detection, leveraging existing infrastructure and expertise.

6.These results suggests no significant difference in detection rates among the three methods, for ZN- Stain, Auramine-O' stain, and PCR, implying similar effectiveness in diagnosing *Mycobacterium tuberculosis* from newly diagnosed uncultured sputum samples.

7. The time taken is also considerable as we could do PCR in just 2 hours for 36 samples total Turnaround time being 4-5 hours.

8. Human error which is experienced in traditional techniques could be minimized in this method.

9. The potential of Conventional PCR in rural TB diagnostics, utilizing pre-existing infrastructure and already acquired expertise in PCR could prove to be beneficial in combating this ancient public health problem if Government and Stakeholders starts working at the grass root level.

Recommendations:

Research Focus: Future studies should prioritize the development of affordable and portable diagnostic solutions tailored for rural settings, addressing the challenges such as cost, sensitivity, 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 acknowledges limitations such as sample size constraints, exclusive focus on MTB, necessitating broader scope in future research endeavors.
- £ Limitations of conventional PCR are many like , each individual PCR requires assay, requires careful optimisation of reagents and amplification conditions.
- £ Primer design is extremely important for effective PCR amplification as cross – reaction with non-target DNA can result in non - specific products.
- £ Laboratories performing these assays need to invest considerable amount in dedicated "DNA – free" laboratory space and equipment.
- £ " DNA – free " laboratory space and equipment is essential to minimise contamination of subsequent specimens by PCR amplicons that can lead to false positive results.

Inspite of these limitations, conventional PCR assays will continue to have a role in smaller, rural, regional diagnostic laboratories.


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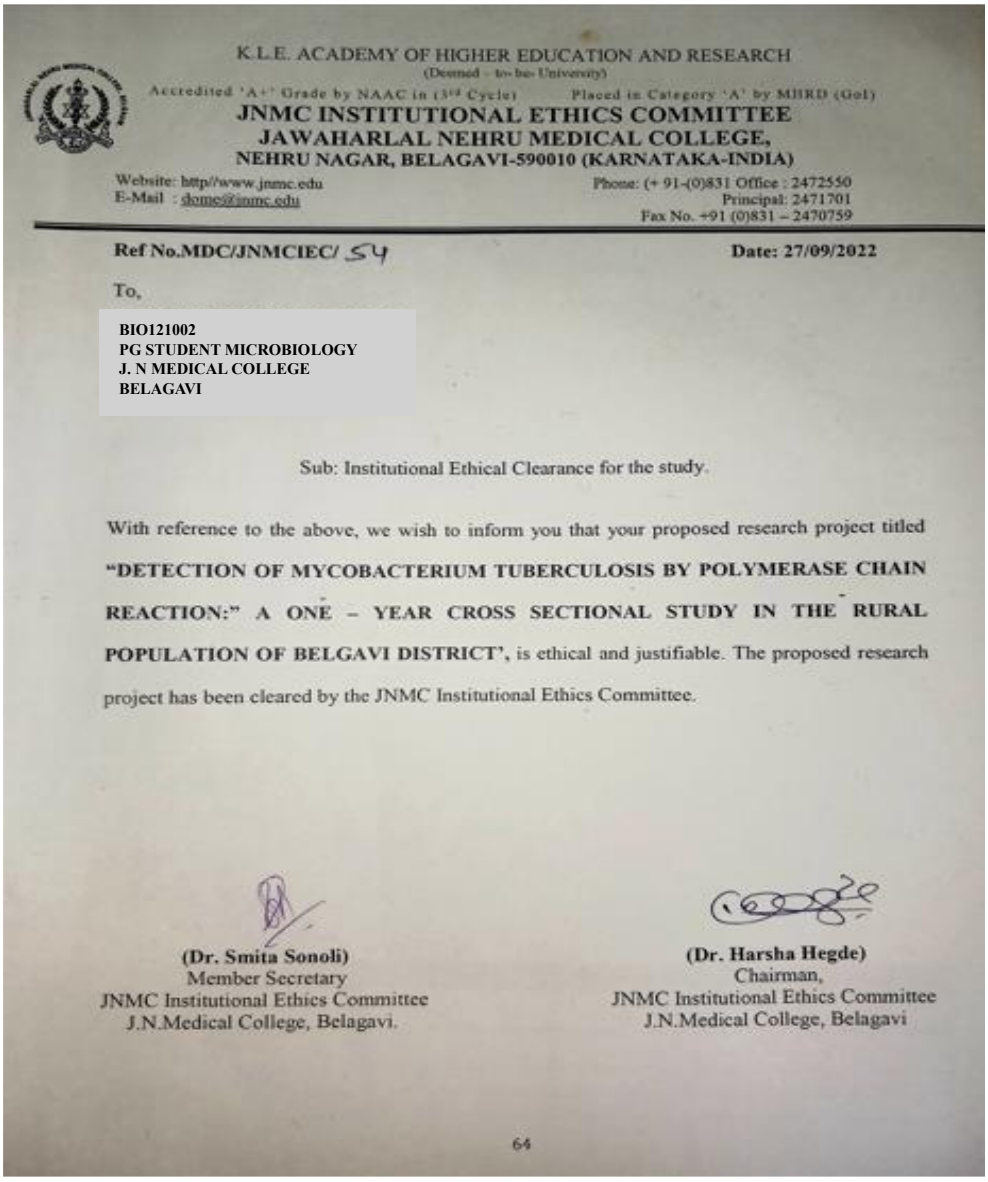
ANNEXURES - I TO IX

ANNEXURE – I

ETHICAL CLEARANCE LETTER



K.L.E. ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed – to- be- University)
Accredited 'A+' Grade by NAAC in (3rd Cycle) Placed in Category 'A' by MHRD (GoI)

JNMC INSTITUTIONAL ETHICS COMMITTEE
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)

Website: http//www.jnmc.edu
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Phone: (+ 91-(0)831 Office : 2472550
Principal: 2471701
Fax No. +91 (0)831 – 2470759

Ref No.MDC/JNMCIEC/ 54 **Date: 27/09/2022**

To,

BIO121002
PG STUDENT MICROBIOLOGY
J. N MEDICAL COLLEGE
BELAGAVI

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"DETECTION OF MYCOBACTERIUM TUBERCULOSIS BY POLYMERASE CHAIN REACTION:" A ONE – YEAR CROSS SECTIONAL STUDY IN THE RURAL POPULATION OF BELGAVI DISTRICT',** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee.

(Dr. Smita Sonoli)
Member Secretary
JNMC Institutional Ethics Committee
J.N.Medical College, Belagavi.

(Dr. Harsha Hegde)
Chairman,
JNMC Institutional Ethics Committee
J.N.Medical College, Belagavi

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ANNEXURE II – CONSENT FORM

CONSENT FOR PARTICIPATION IN RESEARCH

TITLE: "Detection of Mycobacterium tuberculosis by Polymerase Chain Reaction"- A one – year cross sectional study in the rural population of Belagavi District.

PURPOSE OF THE STUDY:

The purpose of the study is to detect Mycobacterium tuberculosis by polymerase chain reaction from the sputum samples of newly suspected pulmonary tuberculosis patients attending the respiratory department of Dr Prabhakar Kore Hospital and to study the demographic profile of Tuberculosis patients.

PROCEDURE:

You are requested to participate in this study which will provide appropriate and effective treatment. During the study you will be asked some questions, and you are supposed to answer to the best of your knowledge. The principal investigator of this study is BIO121002 under the guidance of Professor. If you agree to enroll yourself in the study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

RISKS AND BENEFITS:

There are no risks involved and benefits are to know about the causative organism so that appropriate antitubercular treatment can be given.

ALTERNATIVES:

Your participation in research is voluntary. Your decision whether or not to participate in the study will not affect your

relationship with Jawaharlal Nehru Medical College. If you decide to participate you are free to withdraw at any time.

PRIVACY AND CONFIDENTIALITY: All the information collected during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but you or the information provided by you during research will remain confidential. No information about you or information provided by you during this research will be enclosed to others without your written permission except:

1. In an emergency to protect your rights and welfare.

2. If required by law.

FINANCIAL INCENTIVES FOR PARTICIPATION:

You will not be paid / offered any gifts/ incentives for participating in the study. You will not be reimbursed for expenses.

AUTHORIZATION TO PUBLISH RESULTS:

When the results of research are published or discussed in a conference, no information will be displaced that would disclose your identity. Any information that is obtained in this connection with this study and that can be identified with you will remain confidential.

QUESTION: In case you have any questions related to the study, you can contact:

3. In case you have any questions about your rights as a participant you can contact Dr Harsha Hegde, Chairperson, JNMC, IEC and scientist D, ICMR, National Institute of Traditional Medicine, Ph No- 9480422500.

परिशिष्ट 2 - संमती फॉर्म

<u>संशोधनात सहभागी होण्यासाठी संमती</u>

शीर्षक: "पॉलिमरेज चेन रिॲक्शनद्वारे मायकोबॅक्टेरियम क्षयरोगाचे निदान"- बेळगाव जिल्ह्यातील ग्रामीण लोकसंख्येचा एक वर्षाचा क्रॉस सेक्शनल अभ्यास.

अभ्यासाचा उद्देश:

प्रभाकर कोरे रुग्णालयाच्या श्वसन विभागात नव्याने येणाऱ्या फुफ्फुसाच्या क्षयरोगाच्या रुग्णांच्या थुंकीच्या नमुन्यांमधून पॉलिमरेज चेन रिॲक्शनद्वारे मायकोबॅक्टेरियम क्षयरोगाचा शोध घेणे आणि क्षयरुग्णांच्या जनसांख्यिकीय प्रोफाइलचा अभ्यास करणे हा या अभ्यासाचा उद्देश आहे.

प्रक्रिया:

आपल्याला या अभ्यासात भाग घेण्याची विनंती केली जाते जी योग्य आणि प्रभावी उपचार प्रदान करेल. अभ्यासादरम्यान आपल्याला काही प्रश्न विचारले जातील आणि आपण आपल्या माहितीप्रमाणे उत्तरे देणे अपेक्षित आहे. यांच्या मार्गदर्शनाखाली BIO121002 या अभ्यासाच्या प्रमुख संशोधक आहेत. आपण अभ्यासात स्वत: ची नोंदणी करण्यास सहमत असल्यास, आपला वर्तमान, भूतकाळ आणि कौटुंबिक इतिहास आणि आपल्या क्लिनिकल अभिव्यक्तींबद्दल आपली मुलाखत घेतली जाईल.

जोखीम आणि फायदे:

यात कोणताही धोका नाही आणि फायदे कारक जीवाबद्दल जाणून घेणे आहे जेणेकरून योग्य अँटीट्यूबरक्युलर उपचार दिले जाऊ शकतात.

पर्याय:

संशोधनात आपला सहभाग ऐच्छिक आहे. अभ्यासात भाग घ्यायचा की नाही याचा निर्णय जवाहरलाल नेहरू मेडिकल कॉलेजशी असलेल्या

आपल्या संबंधांवर परिणाम करणार नाही. आपण सहभागी होण्याचे ठरविल्यास आपण कोणत्याही वेळी माघार घेण्यास मोकळे आहात.

गोपनीयता आणि गोपनीयता: या अभ्यासादरम्यान गोळा केलेली सर्व माहिती कायद्याने परवानगी दिलेल्या मर्यादेपर्यंत गोपनीय ठेवली जाईल. कोड नंबर आपल्याला या रिसर्च रेकॉर्डमध्ये ओळखतील. या अभ्यासातील माहिती प्रकाशित केली जाईल परंतु आपण किंवा संशोधनादरम्यान आपण दिलेली माहिती गोपनीय राहील. या संशोधनादरम्यान आपल्याबद्दल कोणतीही माहिती किंवा आपण प्रदान केलेली माहिती आपल्या लेखी परवानगीशिवाय इतरांना जोडली जाणार नाही:

1. आणीबाणीच्या परिस्थितीत आपले हक्क आणि कल्याण ाचे रक्षण करण्यासाठी.

२. कायद्याने आवश्यक असल्यास.

सहभागासाठी आर्थिक प्रोत्साहन :

अभ्यासात भाग घेतल्याबद्दल आपल्याला कोणतीही भेटवस्तू / प्रोत्साहन दिले जाणार नाही / ऑफर केले जाणार नाही. तुम्हाला खर्चाची प्रतिपूर्ती मिळणार नाही.

निकाल प्रकाशित करण्याची अधिकृतता:

जेव्हा संशोधनाचे निष्कर्ष एखाद्या परिषदेत प्रकाशित किंवा चर्चा केले जातात, तेव्हा आपली ओळख उघड होईल अशी कोणतीही माहिती विस्थापित केली जाणार नाही. या अभ्यासाच्या अनुषंगाने जी काही माहिती प्राप्त होईल आणि जी आपल्याशी ओळखली जाऊ शकते ती गोपनीय राहील.

प्रश्न: आपल्याकडे अभ्यासाशी संबंधित काही प्रश्न असल्यास, आपण संपर्क साधू शकता:

3. सहभागी म्हणून आपल्या हक्कांबद्दल आपल्याला काही प्रश्न असल्यास आपण डॉ. हर्षा हेगडे, अध्यक्ष, जेएनएमसी, आयईसी आणि वैज्ञानिक डी, आयसीएमआर, नॅशनल इन्स्टिट्यूट ऑफ ट्रॅडिशनल मेडिसिन, पीएच क्रमांक 9480422500 यांच्याशी संपर्क साधू शकता.

<u>ಸಂಶೋಧನೆಯಲ್ಲಿ ಭಾಗವಹಿಸಲು ಸಮ್ಮತಿ</u>

ಶೀರ್ಷಿಕೆ: "ಪಾಲಿಮರೇಸ್ ಚೈನ್ ರಿಯಾಕ್ಷನ್ ಮೂಲಕ ಮೈಕೋಬ್ಯಾಕ್ಟೀರಿಯಂ ಕ್ಷಯರೋಗದ ಪತ್ತೆ"- ಬೆಳಗಾವಿ ಜಿಲ್ಲೆಯ ಗ್ರಾಮೀಣ ಜನಸಂಖ್ಯೆಯಲ್ಲಿ ಒಂದು ವರ್ಷದ ಅಡ್ಡ-ವಿಭಾಗ ಅಧ್ಯಯನ.

ಅಧ್ಯಯನದ ಉದ್ದೇಶ:

ಡಾ.ಪ್ರಭಾಕರ್ ಕೋರೆ ಆಸ್ಪತ್ರೆಯ ಉಸಿರಾಟದ ವಿಭಾಗಕ್ಕೆ ಹಾಜರಾಗುವ ಹೊಸದಾಗಿ ಶಂಕಿತ ಶ್ವಾಸಕೋಶದ ಕ್ಷಯರೋಗ ರೋಗಿಗಳ ಕಫದ ಮಾದರಿಗಳಿಂದ ಪಾಲಿಮರೇಸ್ ಚೈನ್ ರಿಯಾಕ್ಷನ್ ಮೂಲಕ ಮೈಕೋಬ್ಯಾಕ್ಟೀರಿಯಂ ಕ್ಷಯರೋಗವನ್ನು ಪತ್ತೆಹಚ್ಚುವುದು ಮತ್ತು ಕ್ಷಯ ರೋಗಿಗಳ ಜನಸಂಖ್ಯಾ ಪ್ರೊಫೈಲ್ ಅನ್ನು ಅಧ್ಯಯನ ಮಾಡುವುದು ಅಧ್ಯಯನದ ಉದ್ದೇಶವಾಗಿದೆ.

ವಿಧಾನ:

ಸೂಕ್ತ ಮತ್ತು ಪರಿಣಾಮಕಾರಿ ಚಿಕಿತ್ಸೆಯನ್ನು ಒದಗಿಸುವ ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುವಂತೆ ನಿಮ್ಮನ್ನು ವಿನಂತಿಸಲಾಗಿದೆ. ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ನಿಮ್ಮನ್ನು ಕೆಲವು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲಾಗುತ್ತದೆ ಮತ್ತು ನಿಮ್ಮ ಅತ್ಯುತ್ತಮ ಜ್ಞಾನದ ಪ್ರಕಾರ ನೀವು ಉತ್ತರಿಸಬೇಕು. ಅವರ ಮಾರ್ಗದರ್ಶನದಲ್ಲಿ ಈ ಅಧ್ಯಯನದ ಪ್ರಧಾನ ಪರಿಶೋಧಕರಾಗಿದ್ದಾರೆ. ನೀವು ಅಧ್ಯಯನದಲ್ಲಿ ನೋಂದಾಯಿಸಿಕೊಳ್ಳಲು ಒಪ್ಪಿದರೆ, ನಿಮ್ಮ ಪ್ರಸ್ತುತ, ಹಿಂದಿನ ಮತ್ತು ಕುಟುಂಬದ ಇತಿಹಾಸ ಮತ್ತು ನಿಮ್ಮ ವೈದ್ಯಕೀಯ ಅಭಿವ್ಯಕ್ತಿಗಳ ಬಗ್ಗೆ ನಿಮ್ಮನ್ನು ಸಂದರ್ಶನ ಮಾಡಲಾಗುತ್ತದೆ.

ಅಪಾಯಗಳು ಮತ್ತು ಪ್ರಯೋಜನಗಳು:

ಇದರಲ್ಲಿ ಯಾವುದೇ ಅಪಾಯಗಳಿಲ್ಲ ಮತ್ತು ಪ್ರಯೋಜನಗಳು ರೋಗಕಾರಕ ಜೀವಿಯ ಬಗ್ಗೆ ತಿಳಿದುಕೊಳ್ಳುವುದು ಇದರಿಂದ ಸೂಕ್ತವಾದ ಆಂಟಿಟ್ಯೂಬರ್ಕ್ಯುಲರ್ ಚಿಕಿತ್ಸೆಯನ್ನು ನೀಡಬಹುದು.

ಪರ್ಯಾಯಗಳು:

ಸಂಶೋಧನೆಯಲ್ಲಿ ನಿಮ್ಮ ಭಾಗವಹಿಸುವಿಕೆಯು ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದೆ. ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಬೇಕೆ ಅಥವಾ ಬೇಡವೇ ಎಂಬ ನಿಮ್ಮ ನಿರ್ಧಾರವು ಜವಾಹರಲಾಲ್ ನೆಹರು ವೈದ್ಯಕೀಯ ಕಾಲೇಜಿನೊಂದಿಗಿನ ನಿಮ್ಮ ಸಂಬಂಧದ ಮೇಲೆ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ. ನೀವು ಭಾಗವಹಿಸಲು ನಿರ್ಧರಿಸಿದರೆ, ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಹಿಂದೆ ಸರಿಯಲು ನೀವು ಸ್ವತಂತ್ರರಾಗಿದ್ದೀರಿ.

ಗೌಪ್ಯತೆ ಮತ್ತು ಗೌಪ್ಯತೆ: ಈ ಅಧ್ಯಯನದ ಅವಧಿಯಲ್ಲಿ ಸಂಗ್ರಹಿಸಿದ ಎಲ್ಲಾ ಮಾಹಿತಿಯನ್ನು ಕಾನೂನಿನಿಂದ ಅನುಮತಿಸಲಾದ ಮಟ್ಟಿಗೆ ಗೌಪ್ಯವಾಗಿಡಲಾಗುತ್ತದೆ. ಈ ಸಂಶೋಧನಾ ದಾಖಲೆಯಲ್ಲಿ ಕೋಡ್ ಸಂಖ್ಯೆಗಳು ನಿಮ್ಮನ್ನು ಗುರುತಿಸುತ್ತವೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಮಾಹಿತಿಯನ್ನು ಪ್ರಕಟಿಸಲಾಗುತ್ತದೆ ಆದರೆ ನೀವು ಅಥವಾ ಸಂಶೋಧನೆಯ ಸಮಯದಲ್ಲಿ ನೀವು ಒದಗಿಸಿದ ಮಾಹಿತಿಯನ್ನು ಗೌಪ್ಯವಾಗಿಡಲಾಗುತ್ತದೆ. ಈ ಸಂಶೋಧನೆಯನ್ನು ಹೊರತುಪಡಿಸಿ ನಿಮ್ಮ ಬಗ್ಗೆ ಯಾವುದೇ ಮಾಹಿತಿ ಅಥವಾ ಈ ಸಂಶೋಧನೆಯ ಸಮಯದಲ್ಲಿ ನೀವು ಒದಗಿಸಿದ ಮಾಹಿತಿಯನ್ನು ನಿಮ್ಮ ಲಿಖಿತ ಅನುಮತಿಯಿಲ್ಲದೆ ಇತರರಿಗೆ ಲಗತ್ತಿಸಲಾಗುವುದಿಲ್ಲ:

1. ನಿಮ್ಮ ಹಕ್ಕುಗಳು ಮತ್ತು ಕಲ್ಯಾಣವನ್ನು ರಕ್ಷಿಸಲು ತುರ್ತು ಪರಿಸ್ಥಿತಿಯಲ್ಲಿ.

2. ಕಾನೂನಿನ ಪ್ರಕಾರ ಅಗತ್ಯವಿದ್ದರೆ.

ಭಾಗವಹಿಸುವಿಕೆಗೆ ಆರ್ಥಿಕ ಪ್ರೋತ್ಸಾಹ:

ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಿದ್ದಕ್ಕಾಗಿ ನಿಮಗೆ ಯಾವುದೇ ಉಡುಗೊರೆಗಳು/ಪ್ರೋತ್ಸಾಹಕಗಳನ್ನು ಪಾವತಿಸಲಾಗುವುದಿಲ್ಲ/ನೀಡಲಾಗುವುದಿಲ್ಲ. ನಿಮಗೆ ಖರ್ಚುಗಳಿಗೆ ಮರುಪಾವತಿ ಮಾಡಲಾಗುವುದಿಲ್ಲ.

ಫಲಿತಾಂಶಗಳನ್ನು ಪ್ರಕಟಿಸಲು ಅಧಿಕಾರ:

ಸಂಶೋಧನೆಯ ಫಲಿತಾಂಶಗಳನ್ನು ಸಮ್ಮೇಳನದಲ್ಲಿ ಪ್ರಕಟಿಸಿದಾಗ ಅಥವಾ ಚರ್ಚಿಸಿದಾಗ, ನಿಮ್ಮ ಗುರುತನ್ನು ಬಹಿರಂಗಪಡಿಸುವ ಯಾವುದೇ ಮಾಹಿತಿಯನ್ನು ಸ್ಥಳಾಂತರಿಸಲಾಗುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನಕ್ಕೆ ಸಂಬಂಧಿಸಿದಂತೆ ಪಡೆದ ಮತ್ತು ನಿಮ್ಮೊಂದಿಗೆ ಗುರುತಿಸಬಹುದಾದ ಯಾವುದೇ ಮಾಹಿತಿಯನ್ನು ಗೌಪ್ಯವಾಗಿಡಲಾಗುತ್ತದೆ.

ಪ್ರಶ್ನೆ : ಅಧ್ಯಯನಕ್ಕೆ ಸಂಬಂಧಿಸಿದ ಯಾವುದೇ ಪ್ರಶ್ನೆಗಳನ್ನು ನೀವು ಹೊಂದಿದ್ದರೆ, ನೀವು ಇವರನ್ನು ಸಂಪರ್ಕಿಸಬಹುದು:

ER433. ಭಾಗವಹಿಸುವವರಾಗಿ ನಿಮ್ಮ ಹಕ್ಕುಗಳ ಬಗ್ಗೆ ನಿಮಗೆ ಯಾವುದೇ ಪ್ರಶ್ನೆಗಳಿದ್ದರೆ ನೀವು ಜೆಎನ್ ಎಂಸಿ, ಐಇಸಿ ಅಧ್ಯಕ್ಷ ಡಾ.ಹರ್ಷ ಹೆಗ್ಡೆ ಮತ್ತು ವಿಜ್ಞಾನಿ ಡಿ, ಐಸಿಎಂಆರ್, ನ್ಯಾಷನಲ್ ಇನ್ಸ್ಟಿಟ್ಯೂಟ್ ಆಫ್ ಟ್ರೆಡಿಷನಲ್ ಮೆಡಿಸಿನ್, ಪಿಎಚ್ ಸಂಖ್ಯೆ 9480422500 ಅವರನ್ನು ಸಂಪರ್ಕಿಸಬಹುದು.

ANNEXURE III - PROFORMA

Name:
IP No:

DoA:

Age:
DoC:

Sex:
Sample:

Address:

Chief Complaints:

H/O Presenting Complaints:

Past History:

Family History:

Vitals:

Temperature:
Pulse: B.P:

Cough:
Duration:

Lymph Node Swelling:

H/o Any Investigations:

Symptoms:

Signs:

Treatment History:

Current Medications:

Provisional Diagnosis:

Associated Illness:

TEST	**RESULT**	**APPROX. TIME REQUIRED / Sample/ Bulk of samples**
ZN-STAIN		
AURAMINE O' STAIN		
CONVENTIONAL PCR		

ANNEXURE IV – PROCEDURES

Conventional staining techniques:

1. Ziehl-Neelsen (ZN) Technique STAIN / Hot method –

Principle – a) 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. b) Mycolic acid (a wax), acid – fast in the free state is found in all acid – fast bacteria. c) 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.

Interpretation

Positive result: Mycobacterium tuberculosis appears as long slender, straight or slightly curved and beaded, red coloured acid-fast bacillus. Other non – acid fast organisms present in the smear and the background take up the counter stain and appear blue.

Negative result: At least 100 oil immersion fields should be examined for 10 -15 minutes before giving a negative report.

Total time required for the preparation of smear till the result interpretation step – 1 hour /sample.

2. 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.

Interpretation

Positive result: Mycobacterium tuberculosis appear brilliant yellow against the dark background.

Negative result: At least 100 fields can be screened for 2 minutes before giving a negative result. Also, non-fluorescing bacillary shapes must be considered as artefacts.

Total time required for the preparation of smear till the result interpretation step – 57 minutes approx. / sample.

Grading scales for bright field (Ziehl – Neelsen) and fluorescence microscopy

Union / WHO scale 1000x field	Bright field (1000x magnification; 1 length= 2	Fluorescence (200-250x magnification;1 length= 30	Fluorescence (400x magnification; 1 length=40

= HPF Result	cm= 100 HPF)	fields=300 HPF)	fields= 200 HPF)
Negative	Zero AFB / 1 length	Zero AFB / 1 length	Zero AFB / 1 length
Scanty	1-9 AFB /1 length or 100 HPF	1 -29 AFB/ 1 length	20- 199 AFB/ 1 length
1+	10 – 99 AFB/ 1 length or 100 HPF	30 – 299 AFB / 1 length	20 – 199 AFB / 1 length
2+	1-10 AFB/ 1 HPF on average	10-100 AFB / 1 field	5 - 50 AFB/ 1 field
3+	>10 AFB/ 1HPF on average	>100 AFB/ 1 field	>50 AFB/ 1 field

HPF = high power field; AFB = acid-fast bacilli.

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

Forward primer:	5’ CCTGCGAGCGTAGGCGTCGG 3’

Reverse Primer:	5’ CTCGTCCAGCGCCGCTTCGG 3’

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°C prior to DNA extraction.

1. Remove Tris HCl from Eppendorf tube

2. To the pellet added 180 µl ATL buffer + 20 µl proteinase K.

3. Mix by vortexing, then incubate at 56°C until completely lysed for 10 minutes. (vortex occasionally during incubation)

4. Add 200 µl buffer AL then mix by vortexing for 15 seconds.

5. Incubate at 70°C for 10 minutes. Briefly centrifuge the tube to remove drops from the lid.

6. Add 200 µl ethanol (96-100%). Vortex for 15 seconds. Briefly centrifuge the tube to remove drops from the lid.

7. 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.

8. 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.

9. 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.

10. 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.

11. Place the QIAamp Mini spin column in a new 1.5 ml microcentrifuge tube, 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.

TOTAL TIME REQUIRED TILL THIS STEP FOR 30 SAMPLES = 2 HOURS.

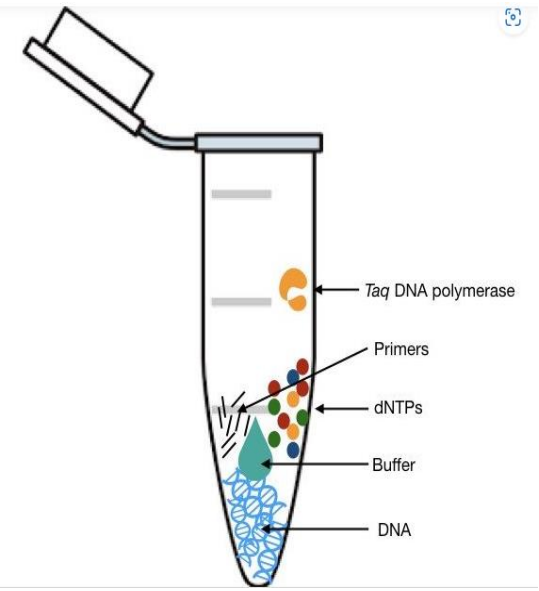
POLYMERASE CHAIN REACTION

We targeted IS6110 by using commercial kit containing Master mix and PCR primers used in the study are:

Forward primer:	5' CCTGCGAGCGTAGGCGTCGG 3'
Reverse Primer:	5' CTCGTCCAGCGCCGCTTCGG 3'

The PCR reactions:

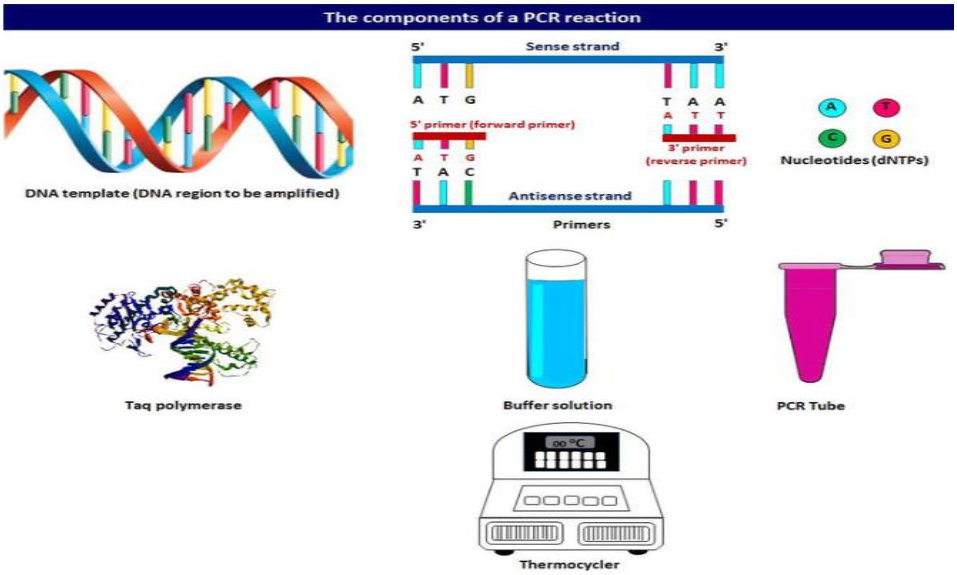
The PCR amplification reactions were performed in a total volume of 25 µl. The reaction mixture consisting of 5 µl of PCR buffer, 0.2µM of each forward and reverse primer 0.2mM of dNTP mixture, 2 mM $MgCl_2$, 0.425 U of Taq Polymerase, and 5.0 µl of template DNA. The PCR reactions were performed at 94°C for 5 minutes (followed by 32 cycles at 94°C for 45s, at 68°C for 45s, at 72°C for 2 min and a final extension at 72°C for 5 minutes, using Genetix (Gradient) thermocycler at Molecular microbiology section of the department.

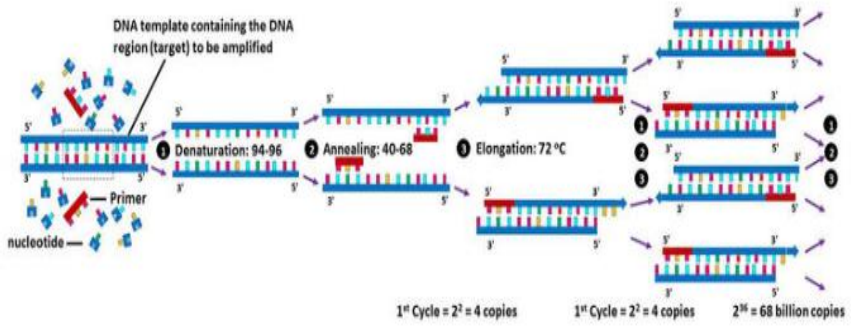


Addition of different components while performing the PCR reaction.

image copyright to ©Genetic Education Inc.

DNA amplification reactions





Reaction preparation 30 minutes.

PCR 1 hour 30 minutes

Total time 3-4 hours. (Depending on efficiency of the TECHNICIAN)

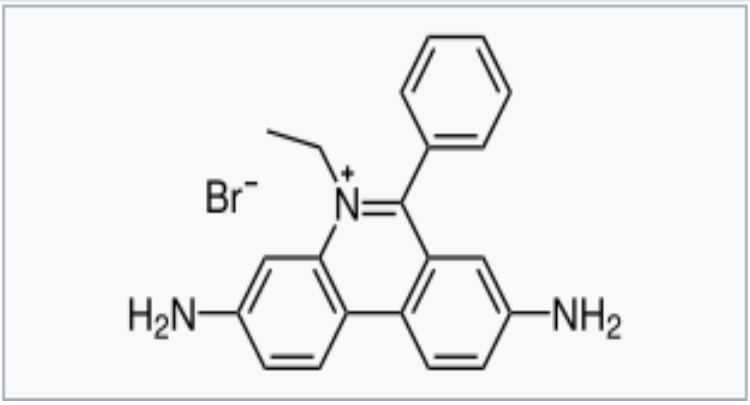
GEL ELECTROPHORESIS

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

Preferred IUPAC name: 3,8-Diamino-5-ethyl-6-phenylphenanthridin-5-ium bromide
Other names:
2,7- Diamino-10-ethyl-6-phenylphenanthridinium bromide
2,7- Diamino-10-ethyl-9-phenylphenanthridinium bromide
3,8- Diamino-1- ethyl-6-phenylphenanthridinium bromide
5- Ethyl-6- phenyl- phenanthridine-3,8-diamine bromide
Ethidium bromide
Homidium bromide
EtBr
EthBr



10-minute gel preparation

50 minutes Gel Run

1 hour

Total turnaround Time for 30 samples – 4 -5 hours approx. till results.

ANNEXURE – V

PREPARATION OF ZN STAINING REAGENTS

A] Preparation of 1% Carbol fuchsin reagent

- Potency correction factor: Note down the dye content – this should be available on the container. The dye content should be approximately 85% - 88%. To calculate the required amount of basic fuchsin, divide the actual amount by the dye content. For example: dye content = 85%, actual amount required = 5 gms, required amount of dye =5/0.85 = 5.88 gms
- Weigh potency corrected amount of basic fuchsin dye (5.88 gms in the above example) in a balance and transfer it to a 250 ml Erlenmeyer glass flask.
- Add 50 ml of methylated spirit and shake to dissolve the dye.
- Heat 25 grams of phenol to melt it and add it to the above solution.
- Heat the flask containing basic dye dissolved in spirit and phenol gently in a water bath at about 60°C. **Do not heat directly on a flame.**
- Transfer the contents into a 500 ml measuring cylinder.
- Add distilled water to make up a final volume of 500 ml.

- Filter the solution through filter paper (Whatmann No. 1) and store Filtered solution in a glass bottle. Label the bottle as 1% Carbol fuchsin with the date of preparation.

Any time particles start to form in the carbol fuchsin solution, the solution must be filtered again.

B] Preparation of 25% sulphuric acid

- Pour 375 ml of distilled water into a 1 litre glass flask
- Measure 125 ml of concentrated sulphuric acid and transfer it slowly into the flask containing water.
- Always add acid to water. Never add water to acid.
- Store the sulphuric acid solution in a glass bottle

C] Preparation of 0.1% methylene blue solution

- Potency correction factor: Note down the dye content – this should be available on the container. The dye content should be approximately 82%. To calculate the required amount of methylene blue, divide the actual amount by the dye content. For example: dye

content = 82 %, actual amount required = 0.5 gms, required amount of dye = 0.5/0.82 =0.61 gms.

- Weigh potency corrected amount of methylene blue (0.61 gms in the above example) in a balance and transfer to a 1 litre glass flask.
- Add 500 ml of distilled water.
- Shake well to dissolve.
- Store in a glass bottle with the label showing name of the reagent and date of preparation.

PREPARATION OF AURAMINE O' STAINING REAGENTS

A) Preparation of Auramine – phenol stain

Concentrated auramine phenol (Pro-Lab PL8008) 100 ml

Distilled water 900 ml

1. Add the auramine phenol to the water in a flask.

2. Mix, place at 37°C overnight to dissolve.

3. Filter through a Whatman No. 1 filter paper into a Winchester bottle.

4. Before use filter through a Whatman No. 1 filter paper into the in- use bottle.

B) Preparation of 1% acid Alcohol

Concentrated hydrochloric acid (BDH 28507) 20 ml

Spirit, methylated industrial (BDH 30244) 1980 ml

1. Pour the methylated spirit into the flask.

2. Place the flask in 2-3 inches (5-8 cm) of water in the sink.

3. Add the hydrochloric acid; leave for 10 min.

4. Decant into a labelled Winchester bottle.

C) Preparation of 0.1% Potassium permanganate

Potassium permanganate (BDH 29644) 2g

Distilled water
2 litres

Add the potassium permanganate to the water and shake to dissolve.

Staining procedure

1. Place the heat – fixed smear(s) on a staining rack.

2. Cover with auramine phenol for 10 minutes.

3. Gently wash with running tap water.

4. Decolourize with 1% acid alcohol for 5 minutes.

5. Gently wash with running tap water.

6. Dry in air.

PREPARATION OF PCR REAGENTS

1. REAGENTS FOR DECONTAMINATION:

A) Preparation of 4% NaOH:

NaOH	4g
Distilled water	100 ml

B) NALC (N-Acetyl-L-Cysteine)

For

Sputum	0.5 ml
NALC	0.005 g

C) 2.9% Tri sodium citrate

Sodium citrate	2.9g
Distilled water	100 ml

D) Tris HCl (pH- 8.3)

Tris base	12.1 g
Nuclease free water	80 ml

Adjust pH (7.4)

Conc. HCl 6-7 ml

E) Preparation of 1 % Agarose gel

1 g Agarose powder (LONZA) dissolved in 50 ml TE buffer was boiled (in microwave oven) 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 bands were visualised in UV Transilluminator (Syngene Gel Documentation). 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 used 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.

GOOD MOLECULAR BIOLOGY LABORATORY PRACTICES.

ANNEXURE - VI

GOOD MOLECULAR BIOLOGY LABORATORY PRACTICES:

Practices that optimize sensitivity include:

1. Use of molecular biology grade reagents and consumables including water, chemicals, enzymes and plastic consumables that have been certified not to contain DNase or RNase (if performing RNA amplification).

2. Collect separate dedicated specimens for PCR in an appropriate manner and transported in a condition and time period that has been proven to maintain the viability of target nucleic acid.

3. Process the specimen to a required level of purity determined by test validation. For some specimens and assay configurations processing may not be necessary other than the initial denaturation step of the PCR. For other specimens and assays multiple processing step may be required to obtain NA in a form sufficiently pure to ensure consistent results.

4. Store samples under conditions that maximize sample integrity and longevity. Archive purified DNA or RNA samples at -70°C for long – term storage.

5. Ensure all equipment is calibrated and operating correctly.

6. Always use the correct pipette for the volume to be dispensed and avoid trying to pipette volumes under 2 µl as dispensing errors increase with smaller volumes.

7. Optimize PCR assay reagent concentrations and thermal cycling conditions to make a robust assay that meets clinical requirements and is validated according to NPAAC Standard

8. Use assay controls to monitor performance such as.

a. Internal control that can be used to monitor the efficiency of sample processing and PCR amplification.

b. Detection limit positive control dilution series that will demonstrate assay reproducibility.

c. Inhibition control to detect samples that contain substances that prevent PCR amplification of target NA.

9. Pulse centrifuge PCR tubes prior to loading into the PCR instrument to ensure all reagents are mixed and in the bottom of the tube for optimum thermal cycling.

Practices that optimize specificity include:

1. Use published primers and probes from studies with the same or similar patient demographics. Validate the assay in accordance with diagnostic criteria.

2. When choosing gene sequence regions for primers and probes use a recognized program for design and ensure the target is unique for the agent and that all strains will be detected. The latter is accomplished by extensive sequence search of microorganisms and human DNA databases.

3. Include no DNA controls (NDCs) that contain all of the components of the PCR except template DNA at a frequency that can be used to determine possible contamination events and rates at both specimen processing and PCR set-up. Add specimen processing NDCs to PCR MM tubes prior to sample

addition at PCR setup to determine if contamination is occurring at PCR set-up. If there is concern of contamination increase the number of NDCs to determine likely source.

4. Carryover contamination is aided by air flow. Specimens must be processed within a BSC to protect the operator from infectious disease agents. Following inactivation of agents at processing, transfers of NA to PCR tubes should be performed in PCR workstations with static air to limit potential for carryover. PCR product analysis should be performed in a static air flow and definitely not within a recirculation BSC as PCR amplified DNA segments pass easily through HEPA filters and the product will be dispersed throughout the laboratory.

5. Use only molecular biology grade plugged filter pipette tips or positive displacement pipettes with disposable tips for all liquid transfers to eliminate carryover.

6. All equipment should remain in the designated laboratory and must be cleaned and decontaminated prior to removal for maintenance. Pipettes should be bagged and returned to the designated laboratory.

7. Consumables must not return to clean laboratories against the workflow.

8.PCR tube racks that must return against the workflow are soaked in 2-10% sodium hypochlorite for 4 h to destroy any contaminating DNA prior to washing and sterilization by autoclave.

9. Pulse centrifuge all tubes before opening to pull liquid away from the lid and reduces the possibility of contaminating pipettes and gloves.

10. Put on a new pair of gloves when starting work in a designated laboratory and change gloves regularly when working.

11. Change your laboratory gown in each designated area and leave it there. Never return a gown from a dirty laboratory to a clean laboratory against the workflow.

12. Wash hands following removal of laboratory gown and prior to leaving each designated laboratory.

13. Store reagents in small aliquots to limit extent of a contamination incident. Always use new plastic ware during preparation and discard remnants of aliquots not totally used in the procedure.

14. Add all components of the PCR into a MM and aliquot into PCR tubes before addition of sample NA. PCR MM must be prepared in the reagent preparation room.

15. Take care during all pipette liquid transfers of samples to limit the potential for aerosol by avoiding bubble formation.

16. Keep tubes closed that are not in immediate use.

17. Add positive controls in amounts that will enable detection at the end of assay set-up following the addition of all samples.

18. Clean cabinets and laboratory surfaces regularly with 2-10% sodium hypochlorite to destroy NA. This is particularly crucial in laboratories analyzing PCR product.

19. Pipette tips used in the PCR process should be discarded in containers containing 1% sodium hypochlorite to disinfect any microorganisms and destroy remaining NA.

20. PCR tubes containing amplified DNA must remain capped and placed into sealed bags for disposal.

21. Great care must be taken for disposal of electrophoresis gels and buffer as they contain large amounts of amplified target DNA. Avoid creation of aerosols and bag gels for disposal. Absorb DNA stain from buffer with activated charcoal (if required) and carefully flush buffer down the sink.

The implementation of PCR testing must be planned by experienced scientists, with appropriate laboratory areas designated and good molecular biology laboratory practices diligently adhered.

Reference:

1. **Burman WJ, Reves RR (2000) Review of false positive cultures for Mycobacterium tuberculosis and recommendations for avoiding unnecessary treatment. Clin Infect Dis 31:1390 – 1395**
2. **National Pathology Accreditation Advisory Council (2006) Laboratory Accreditation Standards and Guidelines for Nucleic Acid Detection and Analysis.**
3. **National Pathology Accreditation Advisory Council (2007) Requirements for the Development and Use of In – House In Vitro Diagnostic Devices (IVDs)**

ANNEXURE – VII

MOLECULAR TERMINOLOGIES

Alignment – Nucleotide sequences from two or more isolates are compared by lining them up above one another such that linear matches between the nucleotide sequences can be identified.

Amplicon- Product of nucleotide amplification.

Amplification- The creation of numerous copies of a given DNA sequence, most frequently with the use of PCR.

Anneal – Binding of two complementary strands of nucleic acid to each other.

Base – The purine or pyrimidine in a nucleotide; represented by A (for adenine), G (for guanine), C (for Cytosine), T (for thymine), and U (for Uracil).

Base pair (bp) – Two complementary nucleotides joined by weak hydrogen bonds; shortest unit of double stranded DNA (the length of a DNA strand is expressed in number of base pairs).

Basic Local Alignment Search Tool (BLAST) – An online computer program that allows comparison of a DNA sequence (from the organism to be identified) to a library of known sequences (of identified organisms) to find the closest matches, or 'hits.

Clade – A group of organisms that are genetically linked by descent from a common ancestor.

Cladogram- A type of phylogenetic diagram (tree).

Complementary – The two strands of DNA are held together by hydrogen bonds between the bases: A to T and G to C; thus, A is complementary to T and G to C. Hence, given the sequence "ATGGAC," the complementary bases would be "TACCTG." The individual strands of DNA run in opposite directions, and to indicate directionality the two ends of the DNA fragment are conventionally termed **5-prime** (5') and **3-prime** (3'). The representation of the sequence and directionality in the above DNA strand would be:

5'- ATGGAC-3'

3'-TACCTG-5'

(When a DNA nucleotide sequence is presented in text, it is always written from the 5' to 3' end.)

Denaturation- The process of breaking the double stranded DNA into two complementary strands; usually performed by heating to 95°C or by use of chemicals.

Deoxynucleotide triphosphate (dNTP) – A nucleotide used by DNA polymerase to add bases to a growing strand of DNA during amplification. There are four dNTPs corresponding to the four bases: dATP, dCTP, dGTP and dTTP.

DNA – Deoxyribonucleic acid; chains of genetic material made of units, each containing one of any of the four bases of either a purine (adenine [A], guanine [G]) or a pyramidine (cytosine [C], thymine [T]). The bases are attached to a five- carbon (pentose) sugar, deoxyribose (a base + the sugar = a nucleoside). A phosphate group joins the sugar backbone of the units, making a chain or strand of nucleotides (base + sugar + phosphate). Bases bind to their complement on another chain of DNA, forming double – stranded DNA.

DNA polymerase – The enzyme that enables DNA replication; it is responsible for adding nucleotides to an existing DNA template segment.

Downstream- Referring to the position on a DNA strand, downstream is the region toward the 3' end of the strand.

Gel electrophoresis – A technique used for determining and separating distinct DNA fragments. An agarose gel matrix is inoculated at one end with material to be analyzed (usually the product of PCR), and an electric current is applied to the gel. DNA fragments migrate various distances, depending upon their size and electrical charge, forming "bands" that are visualized by staining.

Gene – An ordered sequence of DNA on the chromosome that encodes a specific protein or RNA molecule.

Genome – The entire genetic content of an organism.

Genotype – A group of organisms sharing a specific genetic constitution; it is based on the entire genetic identity of the organisms, including gene forms that do not show as phenotypic characteristics.

Housekeeping gene – A gene that is essential to the routine functioning of the cell, is always present, and is expressed (i.e. "turned on") under all conditions. They are considerably conserved within a species.

Hybridization- Binding of complementary strands of nucleic acid. Hybrids can form between DNA-DNA, DNA-RNA, or RNA-RNA; to "anneal" two strands and to " hybridize " them are synonymous.

Master mix- A mixture containing the ingredients or factors necessary for PCR to occur, e.g., MgCl2, buffer, nucleotides, polymerase, and primers. Extracted DNA is added to master mix prior to thermocycling.

Nucleic acid – Deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

Nucleotide sequence- The order of the nucleotides in a strand of DNA or RNA; it is conveyed by the first letter of the base in each nucleotide. A sample sequence is "ACCGT."

Nucleotides – The building blocks (base + sugar + phosphate) of DNA and RNA strands.

Oligonucleotides- Short stretches of chemically synthesized single – stranded DNA (< 50 bp, usually 18 -30 bp); commonly used as primers or probes.

Polymerase Chain reaction (PCR) – A technique involving repeated thermal cycling and use of enzyme DNA polymerase enzyme to create multiple copies of a selected specific template DNA sequence.

Primer – A synthetically constructed short strand of oligonucleotide (~ 18-24 bp long) that can bind to, and

initiate amplification of, an existing longer strand of DNA. Primers are almost always used in pairs: forward primers (complementary to the 5' end of the DNA segment to be amplified) and reverse primers (complementary to the 3' end); DNA amplification occurs by adding complementary nucleotides to the 3' end of the hybridized primers.

Probe- Short segments of nucleotides (of DNA or RNA) synthesized to be complementary (able to hybridize) to a specific region on the single- stranded DNA or RNA from the organism in question. The probe is commonly labelled with a fluorescent or chemiluminescent marker to enable detection of the specific hybridization reaction and identification of the organism.

Query (noun)- The primary mechanism for retrieving information from a database for the identification of the organism under investigation; (verb) to test material (specimen or isolate) for detection or identification of an organism.

Sequencing- Procedure for the determination of the order of nucleotides in a DNA or RNA strand.

Target-The particular gene or DNA sequence being amplified and / or detected. The length of a target could vary from a few to several hundred base pairs, depending on the technique being used.

Template – A nucleic acid strand, on which the complementary primer anneals and extends; it serves as the blueprint for production of the new strand of DNA.

Thermal cycling- Repeated and controlled fluctuation in temperatures, each optimum for a stepwise reaction in PCR.

Reference : Das S,Medically important fungi, In: Larone,editor. Molecular terminology,6th edition, Washinton DC, ASM press, year of publication.- 2018, Pg. 336 - 339

ANNEXURE – V III ABBREVIATIONS

TB – Tuberculosis

COVID -19 – Corona Virus Disease - 19

PCR - Polymerase Chain reaction

MRC – Medical Research Centre

BSL - Bio Safety Level

ZN - Ziehl Neelsen

IS - Insertion Sequence

bp - base pair

DM - Diabetes Mellitus

HIV - Human Immunodeficiency Virus

WHO – World Health Organization

CBNAAT – Chip based Nucleic acid amplification test

LPA – Line Probe Assay

BC - Before Christ

A – Adenine

T – Thymine

G – Guanine

C – Cytosine

DNA – Deoxyribonucleic acid

MAIC – Mycobacterium Avium Intracellular

MA-AG-PG –

LAM – Lipoarabinomannan

Ag – Antigen

pH – Potential of Hydrogen

ART – Anti Retroviral therapy

ATT - Anti Tubercular Therapy

CD – Cluster of Differentiation

Th1 - T-helper 1

Th2 – T-helper 2

B-cell- B Lymphocyte (or B Lymphocyte Cell)

NK Cells – Natural Killer cells

DC – Dendritic Cell

PMNs- Polymorphonuclear leucocytes

TNF- Tumour necrosis factor

IFN- Interferon

IL- Interleukin

Treg- regulatory T cells

Teff- effector T cells

MHC- Major Histocompatibility Complex

T- cells- T Lymphocytes (or T Lymphocyte Cells)

CD-1 – Cluster of Differentiation 1

ABO- ABO Blood Group System

Rh - Rhesus

NALC- N-acetyl-L-Cysteine

CSF- Cerebrospinal fluid

NaOH- Sodium Hydroxide

NaCl- Sodium Chloride

TSP- Trisodium phosphate

IgG- Immunoglobulin G

IgM- Immunoglobulin M

kDa- Kilodalton

ELISA- Enzyme-Linked Immunosorbent Assay

LAM- Lipoarabinomannan

MTBDR- Mycobacterium Tuberculosis Drug Resistance (usually referring to a specific assay for drug resistance in tuberculosis)

16S-23S- 16S-23S rRNA Intergenic Spacer Region (used in bacterial identification and phylogeny)

CDC- Centre of Disease Control

USA- United States of America

HPLC- High Performance Liquid Chromatography

DST- Drug Sensitivity Testing

AFB- Acid Fast bacilli

MIC- Minimum inhibitory Concentration

RR - Resistance Ratio

E-Test- Epsilometer Test; used for antibiotic susceptibility testing)

MODS- Microscopic-Observation Drug – Susceptibility assay

7H9- 7H9 Medium (a type of broth used for culturing Mycobacteria)

LJ- Lowenstein Jensen

MBBACT- Mycobacteria BACTEC (a system for detecting mycobacterial growth)

LTBI- Latent tubercular Infection

IFN-γ- Interferon Gamma

ADA- Adenosine deaminase

TST- Tuberculin Skin Test

BCG- Bacillus Calmette Guerin

NTM- Non tubercular Mycobacteria

CMI- Cell mediated immunity

DTH- Delayed-Type Hypersensitivity

PPD- Purified Protein derivative

IGRAS- Interferon gamma release assays

FDA- Food and drug administration

Xpert- Xpert MTB/RIF (a molecular diagnostic test for tuberculosis and rifampicin resistance)

MTB-Mycobacterium tuberculosis

RIF- rifampicin

dNTPs- deoxynucleotide triphosphate

Rif- Rifampin

INH- Isoniazid

PZA- Pyrazinamide

EMB- Ethambutol

LFX- Levofloxacin

MFX- Moxifloxacin

BDQ- Bedaquilline

LZD- Linezolid

CFZ- Clofazimine

CYS- Cycloserine

DLM- Delamanid

AMK- Amikacin

CAP- Capreomycin

STR- Short Tandem Repeats (also known as microsatellites, used in genetic typing and forensic analysis)

ETH- Ethionamide

PRO- Prothionamide

PAS- para-amino salicylic acid

CYP 450- Cytochrome P 450

GI- Gastrointestinal

QT- Quantitative Test

WGS – Whole genome Sequencing

EtBr – Ethidium bromide

TE – Tris- EDTA buffer

K-FIST – Karnataka fund grants for infrastructure strengthening in science and technology.

VGST – Vision group on science and technology

KAHER – KLE Academy of Higher Education and Research

JNMC- Jawahar Lal Nehru Medical College

HRZE- Isoniazid Rifampicin Streptomycin Ethambutol

WDF- World Diabetes Foundation

HPF - High power field

NaOH – Sodium hydroxide

HCl – Hydrochloric acid

ATL – Lysis buffer with SDS and EDTA

AL – Has guanidine and Hydrochloric acid and malic acid to denature proteins.

AW1- Wash solution that remove contaminants from the DNA attached in the column membrane.

AW2-

AE- Elution buffer

NPAAC- National Pathology Accreditation Advisory Council

NDCs- No DNA Control

MM - Master Mix

BSC - Biosafety cabinet

HEPA - High Efficiency Particulate Air [filter]

dATP - deoxy Adenosine Triphosphate

dCTP – deoxy Cytocine Triphosphate

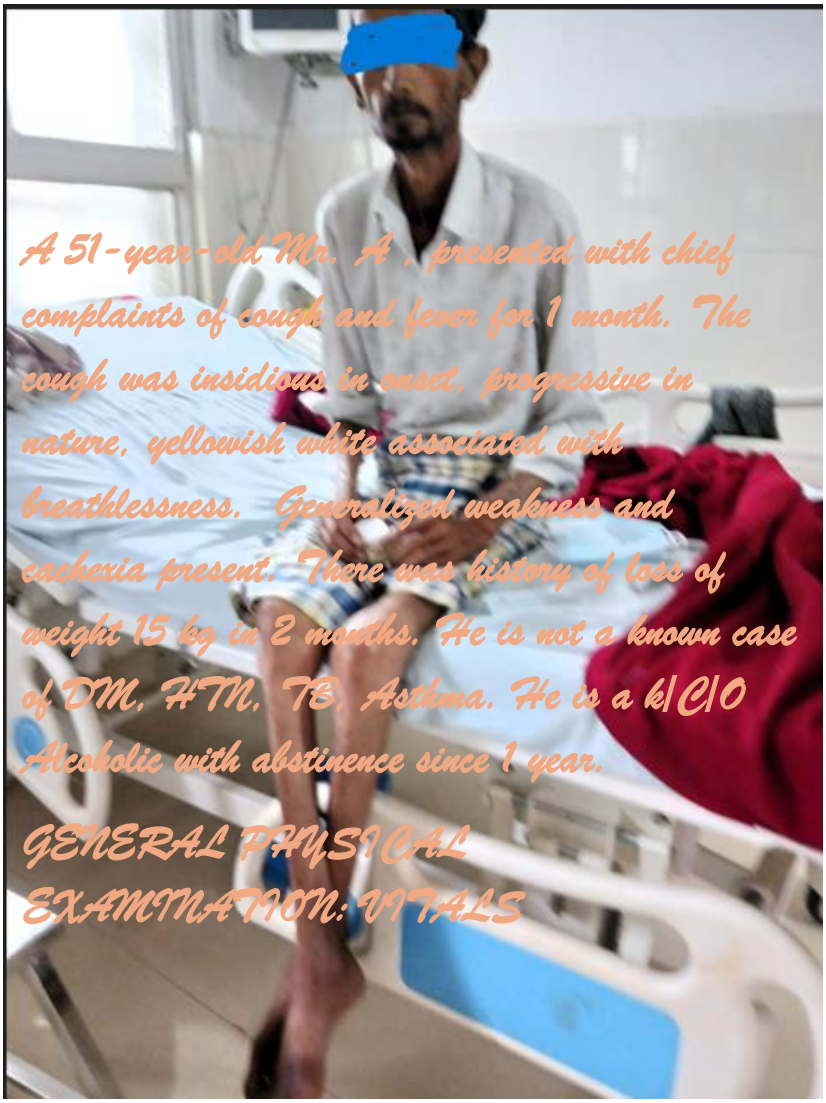
dGTP- deoxy Guanidine Triphosphate

dTTP - deoxy Thymidine Triphosphate

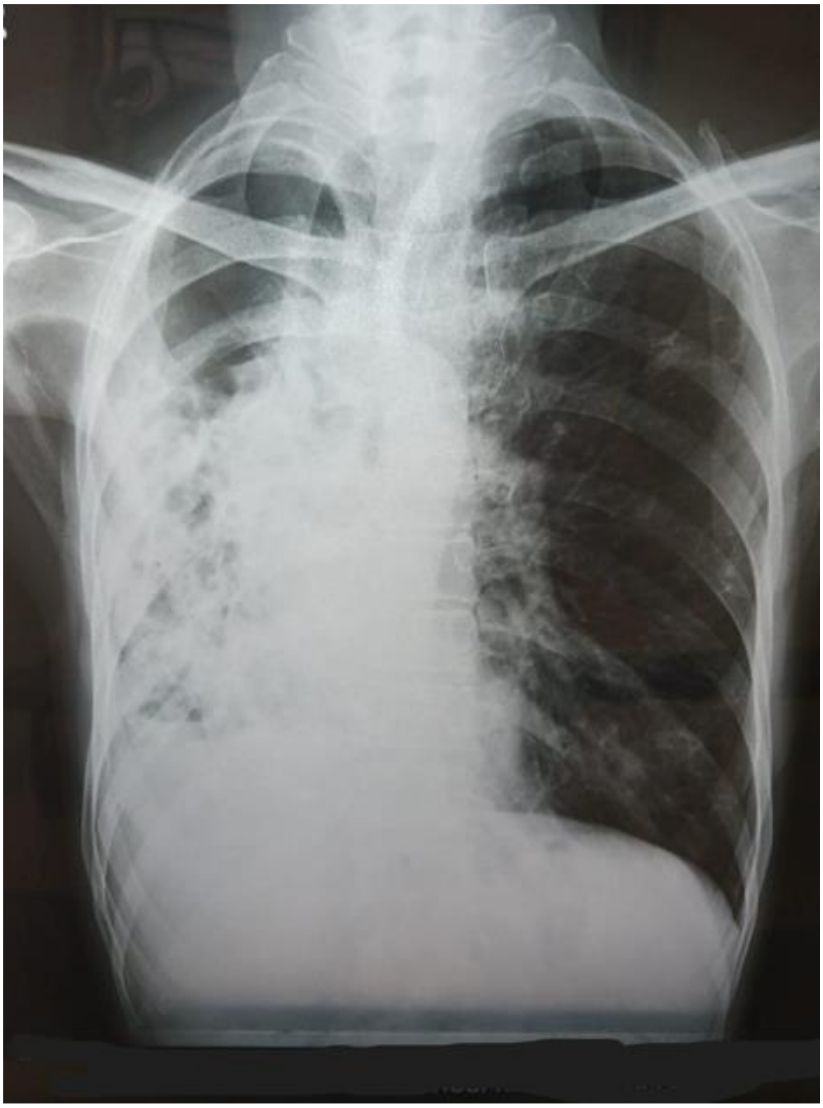

IMAGES

BIOSAFETY CABINET - 2B2

SPUTUM SAMPLES



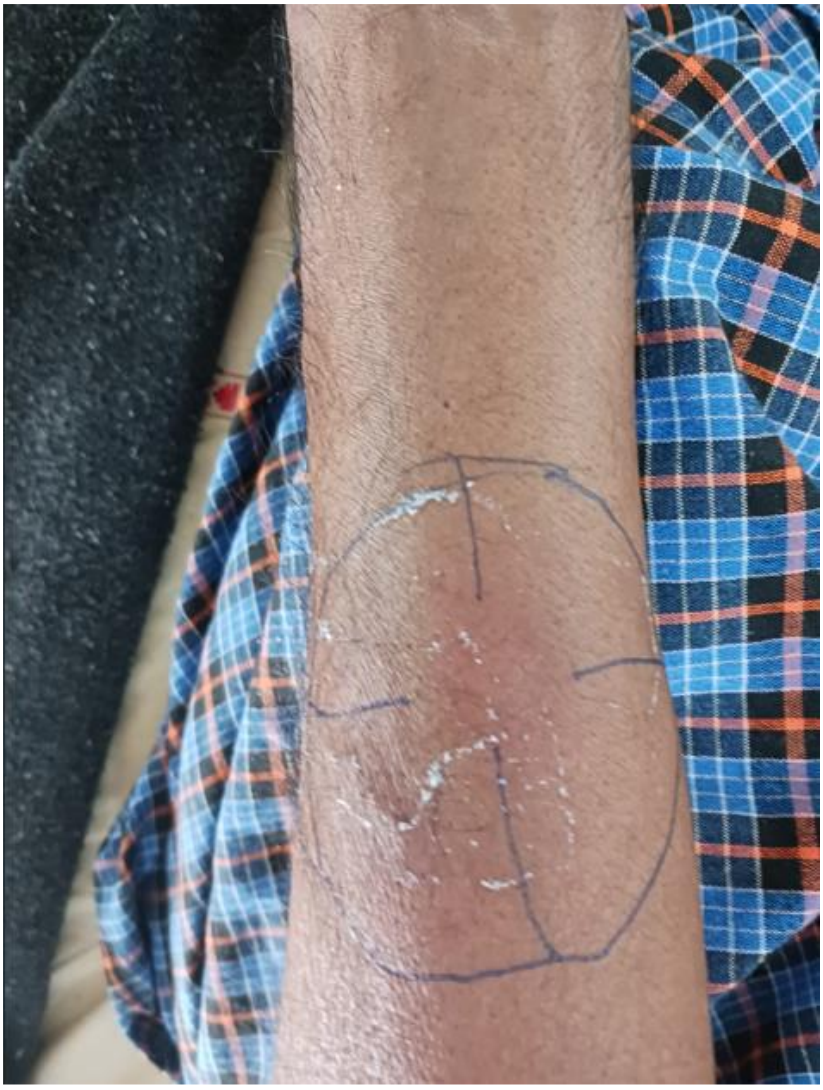
A CACHECTIC PULMONARY TUBERCULOSIS PATIENT.



SYSTEMIC EXAMINATION:

CVS- S1S2+, CNS- WNL, RS- B/L AE +, DECREASED AIR ENTRY ON RIGHT SIDE OF LUNG. PA- SOFT , NON TENDER. AFB / ZN STAIN WAS ADVISED - +VE FOR TB

PCR- MTB DETECTED.



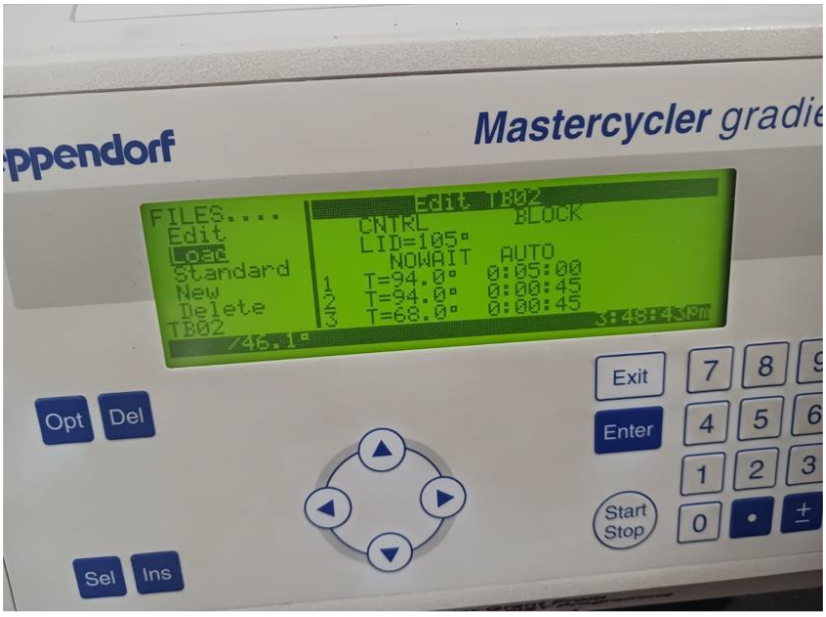
THE SAME PATIENT WITH TUBERCULIN TEST POSITIVE WITH ERYTHEMA AND INDURATION MEASURING MORE THAN 14 MM

REAGENTS USED FOR DNA EXTRACTION.


PCR WORKSTATION
LEAD


JNMC/MICRO/VLL/CENTRIFUGE 03 NACO KSAPS PI: Dr. M B Nagamoti
eppendorf
Centrifuge 5430


eppendorf
Mastercycler gradient
Opt
Del
Sel
Ins
Exit
Enter
Start
Stop
Provided Under Grant From
VGST, DST, GoK Under K-FIST Programme


eppendorf
Mastercycler gradie
FILES....
Edit
Load
Standard
New
Delete
TB02
Edit TB02
CNTRL
BLOCK
LID=105°
NOWAIT
AUTO
T=94.0°
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Del
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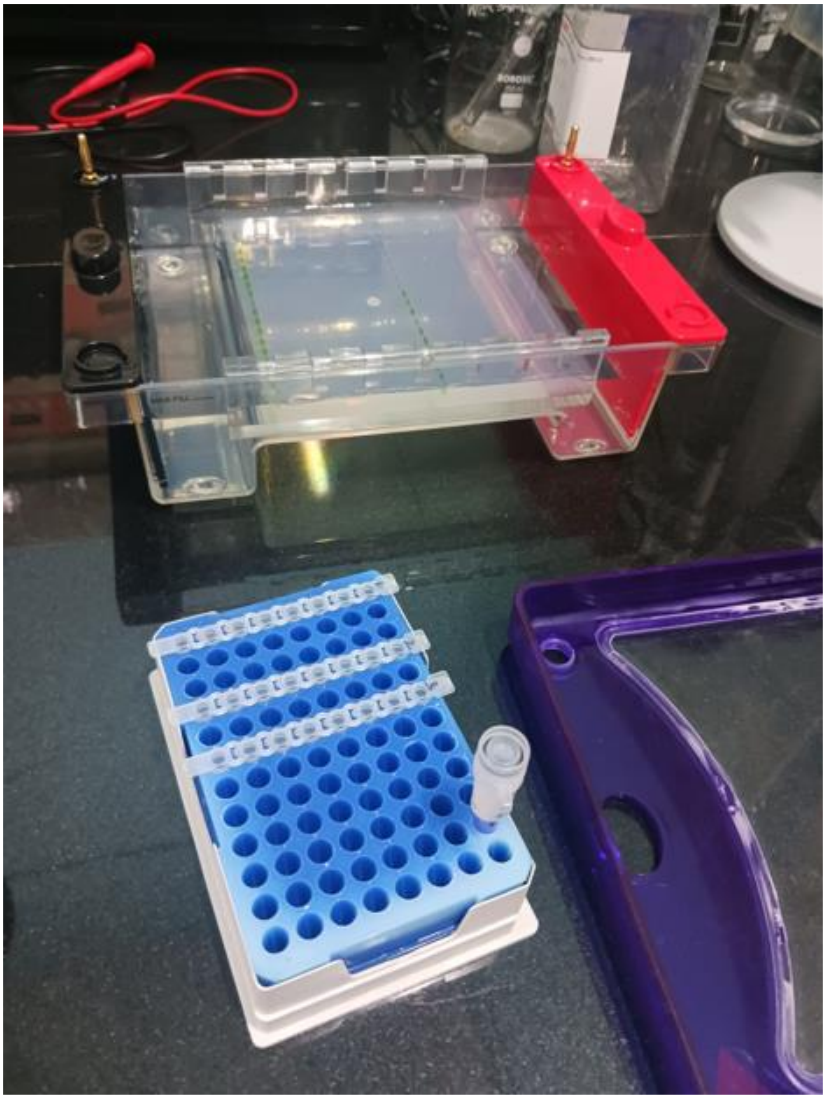
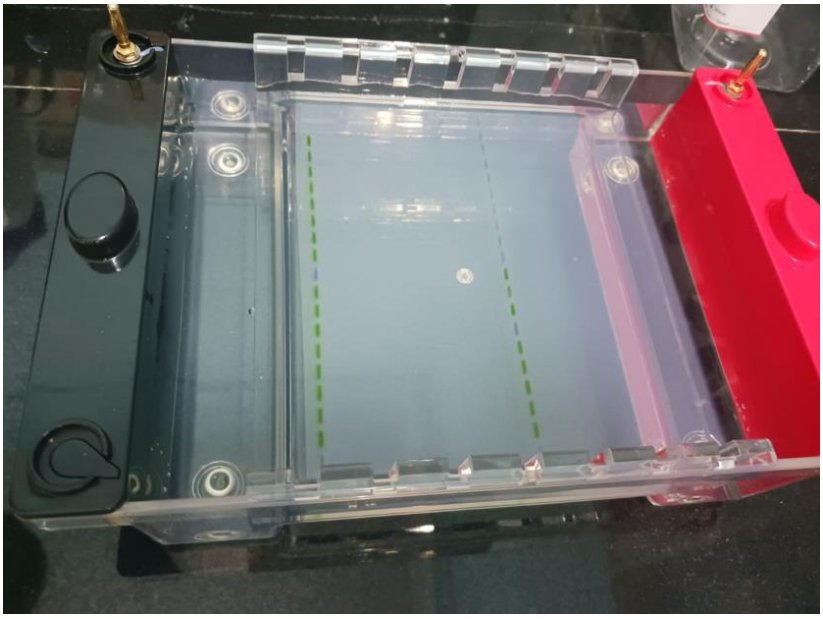
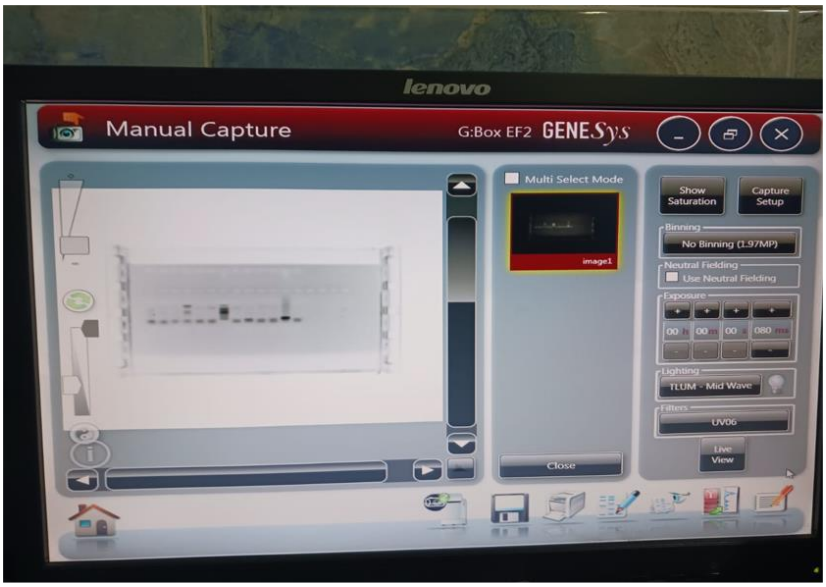
Start
Stop






Provided Under Grant From
SYNGENE
G:BOX


lenovo
Manual Capture
G:Box EF2
GENE.Sys
Multi Select Mode
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Close
Show Saturation
Capture Setup
Binning
No Binning (1.97MP)
Neutral Fielding
Use Neutral Fielding
Exposure
Lighting
TLUM - Mid Wave
Filters
UV06
Live View

MASTER CHART