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DIAGNOSTIC ACCURACY OF PLASMA cfDNA (CELL-FREE DNA) IN TUBERCULOSIS

By

DR. YOGESHWAR KALLA

Dissertation submitted to

BLDE (DEEMED TO BE UNIVERSITY)

Vijayapura, Karnataka

In partial fulfilment of the requirements for the award of the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

Under the Guidance of

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LIST OF ABBREVIATIONS

Abbreviation	Full form
cfDNA	Cell free deoxyribonucleic acid
CBNAAT	Cartridge based nuclei acid amplification test
AFB	Acid-fast bacilli
TB	Tuberculosis
MTB	*Mycobacterium tuberculosis*
PTB	Pulmonary tuberculosis
EPTB	Extrapulmonary tuberculosis
MTB-cfDNA	*Mycobacterium tuberculosis* cell free deoxyribonucleic acid
LTBI	Latent tuberculosis infections
CSF	Cerebrospinal fluid
BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
LRTI	Lower respiratory tract infection
URTI	Upper respiratory tract infection
PCR	Polymerase chain reaction
ddPCR	Droplet digital polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
NGS	Next generation sequencing
CRISPR	Clustered regularly interspaced short palindromic repeats
IGRA	Interferon-gamma release assay
WHO	World Health Organization

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QFT	QuantiFERON-TB Gold
HIV/AIDS	Human immunodeficiency virus/Acquired immunodeficiency syndrome
ZN stain	Ziehl-Neelsen stain
COVID-19	Coronavirus disease 2019
BCG	bacille Calmette-Guérin
MA	Mycolic acid
NTM	Non-tubercular mycobacteria
MPT-64	Mycobacterium tuberculosis protein 64
MTB/RIF	Mycobacterium tuberculosis/Rifampicin
rpoB gene	RNA polymerase beta subunit gene
LAMP	Loop-mediated isothermal amplification
16S rRNA	16S ribosomal ribonucleic acid
LPA	Line probe assay
LAM	Lipoarabinomannan
PPD	Purified protein derivative
TST	Tuberculin skin testing
T-SPOT	T-cell spot test
ELISPOT	Enzyme-linked immunospot assay
DS-TB	Drug-susceptible tuberculosis
INH	Isoniazid
RIF	Rifampicin
PZA	Pyrazinamide
EMB	Ethambutol
DOT	Directly observed treatment

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ctDNA	Circulating-tumoral deoxyribonucleic acid
n-cfDNA	nuclear cfDNA
mt-cfDNA	mitochondrial cfDNA
PET	Positron emission tomography
FAM	6-Carboxyfluorescein
Ct	Cycle threshold
CFU	Colony forming unit
CT	Computed tomography
OPD	Outpatient department
IPD	Inpatient department
EDTA	Ethylene diamine tetra acetic acid
Rpm	Rotations per minute
S. No.	Serial number
Page No.	Page number
F	Female
M	Male
M:F	Male-to-female ratio
SPSS	Statistical package for the social sciences
PLR	Positive likelihood ratio
NLR	Negative likelihood ratio
PPV	Positive predictive value
NPV	Negative predictive value
CRS	Composite reference standard
AI	Artificial intelligence

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ABSTRACT

Background: Tuberculosis (TB) remains a significant global health burden, requiring rapid and accurate diagnostic methods. Conventional diagnostic techniques such as Ziehl Neelsen stained (ZN-stained) acid-fast bacilli (AFB) smear test has limitations in sensitivity and culture has long turnaround times, while Cartridge based nucleic acid amplification test (CBNAAT/GeneXpert) has variable sensitivity, especially in extrapulmonary tuberculosis (EPTB). This study evaluates the diagnostic accuracy of plasma cell-free DNA (cfDNA) in detecting *Mycobacterium tuberculosis* (MTB) and its correlation with ZN-stained AFB smear test and GeneXpert assay.

Methods: A cross-sectional study was conducted on 94 clinically suspected TB patients. Plasma cfDNA was extracted and analyzed using quantitative PCR for the IS6110 gene (MTB). Results were compared with Ziehl-Neelsen stained AFB smear test and taking CBNAAT (GeneXpert) as reference standards. Diagnostic parameters such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy were assessed.

Results: Plasma MTB-cfDNA testing showed a sensitivity of 93.3% and specificity of 95.3%, with a PPV of 90.3% and an NPV of 96.8%. The overall diagnostic accuracy was 94.68%. In comparison, ZN-stained AFB smear test showed lower sensitivity (53.3%) but high specificity (95.3%). Plasma cfDNA testing was particularly useful in smear-negative and EPTB cases, detecting TB in samples that were negative on GeneXpert assay.

Conclusion: Plasma cfDNA is a highly specific and sensitive diagnostic tool for TB, offering a minimally invasive and rapid alternative to traditional diagnostics. Its ability to detect TB in smear-negative and EPTB cases makes it a valuable adjunct to CBNAAT/GeneXpert assay.

Keywords: Tuberculosis, cfDNA, GeneXpert, AFB smear, CBNAAT, diagnostic accuracy

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"DIAGNOSTIC ACCURACY OF PLASMA cfDNA (CELL-FREE DNA) IN TUBERCULOSIS"

INTRODUCTION

Tuberculosis (TB) continues to pose a considerable challenge to global health, with an estimated 10.8 million individuals (95% uncertainty interval: 10.1–11.7 million) affected by the disease worldwide in 2023. India is responsible for 26% of this substantial global tuberculosis burden, followed by Indonesia, which contributes 10%.[1] In 2019, prior to the COVID-19 pandemic, approximately 1.6 million deaths were attributed to tuberculosis.[2] During the pandemic, there was a reported decline in TB-related mortality, likely due to underreporting and the overshadowing of TB deaths by COVID-19 fatalities.[1] According to the WHO Global TB Report 2024, there has been a resurgence in TB-related deaths in the post-COVID-19 context, underscoring the ongoing public health significance of this disease.[1]

Tuberculosis (TB) is a persistent infectious disease attributable to the bacterium *Mycobacterium tuberculosis* (MTB), which has the potential to affect various organs and systems within the human body.[3] The lungs are the primary site of infection, leading to the manifestation of pulmonary tuberculosis (PTB); conversely, infections occurring in organs outside of the pulmonary system are classified as extrapulmonary tuberculosis (EPTB). In India, EPTB accounts for approximately 24% of the total TB cases reported.[3,4,5]

The prompt identification of pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) is essential for effective disease management and the prevention of transmission, as delays in diagnosis result in postponed treatment and facilitate the spread of the disease.[6] However, achieving rapid early diagnosis poses significant challenges due to the limited sensitivity of Ziehl Neelsen-stained acid-fast bacilli (AFB) smear tests, the protracted duration

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required for *Mycobacterium tuberculosis* (MTB) culture, and the often atypical findings observed in diagnostic imaging.[7]

Although modern immunology and molecular biology techniques have significantly improved diagnostic sensitivity and detection speed compared to traditional microbiological methods, numerous challenges persist concerning the standardization of test results and their applicability across diverse populations.[8] To effectively interrupt the transmission cycle of tuberculosis (TB), it is imperative to develop a diagnostic test that is rapid, accurate, and highly effective.

Cell-free DNA (cfDNA) refers to extracellular DNA fragments that are released from their originating cells and exist in a free state within various body fluids.[9] Initially identified in human plasma by Mandel and Metais in 1948,[10] cfDNA can be found in a range of biological fluids, including human plasma, synovial fluid, cerebrospinal fluid (CSF), pleural fluid, urine, prostate fluid, saliva, and others.[10] The ability to detect cfDNA has significantly advanced prenatal diagnostics, as well as the diagnosis and therapeutic monitoring of cancer and other medical conditions. Recent research has demonstrated that circulating cell-free DNA (cfDNA) is detectable in a range of pathogenic infections caused by bacteria, fungi, and parasites, highlighting its significance in the diagnosis and management of infectious diseases.[11,12]

MTB-cfDNA can be identified with significant diagnostic efficacy within a matter of hours in human plasma, as well as various body fluids through the application of polymerase chain reaction (PCR).[13] This advancement facilitates a novel methodology for the diagnosis of tuberculosis (TB).

The diagnostic effectiveness of circulating cell-free DNA (cfDNA) applications in the context of tuberculosis (TB) remains a subject of debate. The detection of *Mycobacterium tuberculosi*s (MTB) cfDNA has demonstrated a sensitivity between 50% and 79.5% for the diagnosis of

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pleural TB, and a sensitivity ranging from 56.5% to 100% for the diagnosis of tuberculous meningitis, with a specificity of at least 92% when utilizing polymerase chain reaction (PCR).[14]

In 2016, Ushio et al. demonstrated the presence of *Mycobacterium tuberculosis*-specific insertion sequence 6110 (IS6110)-circulating free DNA (cfDNA) in the plasma of patients diagnosed with pulmonary tuberculosis (PTB) through the application of droplet digital polymerase chain reaction (ddPCR).[15]

Subsequently, Click et al. employed quantitative PCR (qPCR) techniques to illustrate that MTB-cfDNA was detectable in the plasma of nearly 50% of patients diagnosed with pulmonary tuberculosis (PTB), even in the absence of *Mycobacterium tuberculosis* (MTB) bacteremia.[16]

In 2021, researchers Guocan Yu, Yanqin Shen, Bo Ye, and Yan Shi performed a meta-analysis and systematic review that assessed the efficacy of circulating free DNA (cfDNA) testing for the tuberculosis (TB) diagnosis, encompassing extrapulmonary TB (EPTB) and pulmonary TB (PTB). Their findings indicated that cfDNA testing demonstrated commendable diagnostic performance when evaluated against the composite reference standard and culture methods. However, the study also highlighted considerable variability in the sensitivity and specificity of the tests. Overall, the diagnostic capability of cfDNA testing was found to be effective for both PTB and EPTB, with comparable diagnostic efficacy observed for each TB type.[17]

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AIM AND OBJECTIVES

AIM:

To evaluate the diagnostic accuracy and to compare plasma cfDNA (cell-free DNA) test with Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test and GeneXpert assay in tuberculosis.

OBJECTIVES:

1. To assess the diagnostic accuracy of plasma *Mycobacterium tuberculosis* cell-free DNA (MTB-cfDNA).
2. To correlate the level of plasma MTB-cfDNA with the Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test and GeneXpert assay in clinically suspected tuberculosis cases.

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REVIEW OF LITERATURE

HISTORY:

In the year 1882, the German physician and microbiologist Robert Koch made a landmark discovery by identifying the tubercle bacillus, subsequently named *Mycobacterium tuberculosis* (MTB)—as the etiological agent responsible for tuberculosis (TB), marking a pivotal moment in the history of medical microbiology.[18] Since this pivotal discovery, the TB epidemic has persisted, continuing to proliferate across all regions of the world. TB is recognized as a highly transmissible aerosol-borne infectious disease and ranks among the major causes of death worldwide.[1,19] While the disease predominantly manifests in the lungs, termed pulmonary TB, it is also capable of disseminating to other areas of the body, a condition referred to as extrapulmonary TB.[3,5]

Mycobacterium tuberculosis (MTB) has the capacity to remain dormant within the human body for extended periods, often without manifesting any clinical symptoms, leading to a significant number of individuals becoming asymptomatic carriers of inactive tuberculosis. The 2022 Global TB report from the World Health Organization (WHO) indicated that approximately one-quarter of the global population, equating to around 2 billion individuals, were latently infected with MTB.[1]

Individuals with latent tuberculosis infections (LTBI) have an estimated lifetime risk of reactivation to active tuberculosis (TB) ranging from 5% to 10%.[1] In immunocompromised individuals especially individuals with human immunodeficiency virus (HIV) coinfection, the dormant mycobacteria can become active. The risk of developing TB in HIV infected individuals is estimated to be 18 times greater than that of individuals without HIV. [1,19] TB reactivation occurs when the equilibrium is disrupted, leading to a significant increase in bacterial load, at which point the disease manifests with symptoms.

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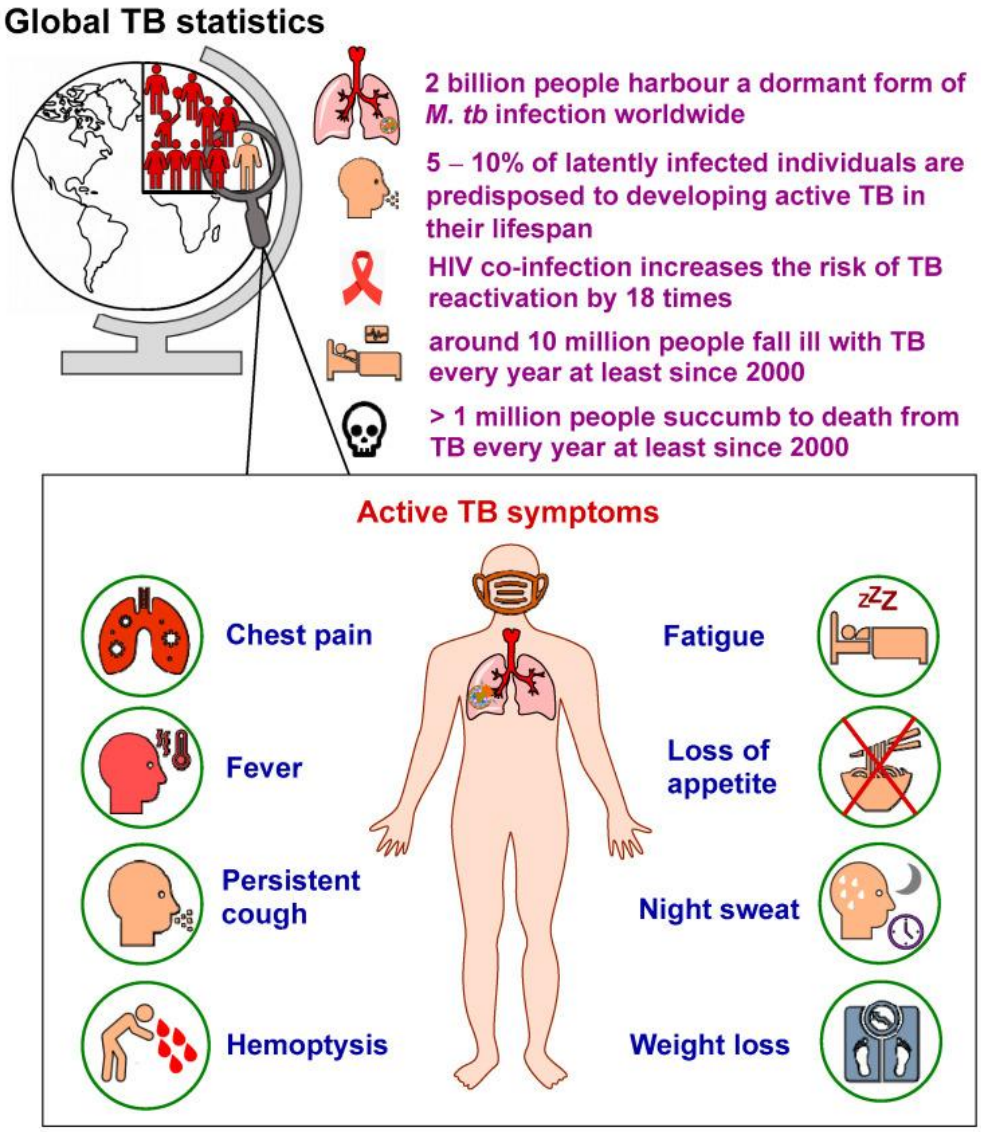

Figure 1: General tuberculosis statistics and main symptoms of pulmonary tuberculosis.[20]

It is essential to diagnose tuberculosis (TB) early and provide effective treatment to mitigate the further dissemination of the bacteria and the emergence of drug-resistant strains. A variety of diagnostic methodologies are routinely utilized, including immunological, radiographic, microscopic and culture, along with clinical approaches. Tuberculin skin test (Mantoux test) and QuantiFERON-TB Gold (QFT) are the two immunological assays which are primarily employed for screening purposes and for excluding infection by TB. Radiographic imaging, particularly chest X-rays, can help in screening of active pulmonary TB but are not effective in identification of latent TB infection. Sputum smear microscopy stained with Ziehl-Neelsen

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stain is a widely utilized diagnostic technique for TB but it cannot distinguish between *Mycobacterium tuberculosis* (MTB) and other acid-fast bacilli thereby having low sensitivity. In contrast, sputum culture utilizing Löwenstein–Jensen medium to cultivate the bacteria, is a highly sensitive and specific diagnostic method for TB. However, it takes a minimum of two weeks, and in some cases, up to 6–8 weeks for the appearance of MTB colonies, which can further postpone diagnosis and treatment. Finally, clinical diagnosis may be established in 5–10% of individuals infected with TB, based on the presentation of various signs and symptoms. The clinical manifestations of active pulmonary TB may encompass pleuritic chest pain, low-grade fever, a prolonged productive cough, hemoptysis, fatigue, loss of appetite, night sweats, and weight loss.[21]

EPIDEMIOLOGY:

"World Health Organization" (WHO) "Global TB report" in 2021 stated that South-East Asian region had the greatest number of TB cases (45% of global cases). The African region followed it with 23% of global cases, while the Western Pacific region had 18% of global cases. Over half of the worldwide TB burden is contributed by India (28%), Indonesia (9.2%) China (7.4%) and the Philippines (7.0%).[19] Approximately 10 million individuals have been reported to develop active TB each year since 2000.[19]

From the year 2000 to 2021, it is estimated that between 1.4 million and 2 million individuals succumbed to tuberculosis (TB) annually, with the decade of year 2000 to 2010 having the peak mortality rates. As per the 2022 report on TB by "World Health Organization" (WHO), tuberculosis was responsible for the deaths of more than one million individuals globally in 2021, specifically accounting for approximately 1.4 million deaths among HIV-negative individuals and deaths among HIV-positive individuals amounting to an estimated 0.2 million.

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TB had the most fatalities caused by a single pathogen, prior to the emergence of the coronavirus disease 2019 (COVID-19) pandemic.[19]

To date, an efficacious vaccine for the prevention of adult tuberculosis (TB) either before or after exposure to *Mycobacterium tuberculosis* (MTB) remains unavailable. The sole licensed TB vaccine, "bacille Calmette-Guérin" (BCG), was created nearly 100 years ago and offers limited protection, primarily benefiting infants and children by reducing the risk of severe TB forms like TB meningitis and miliary TB.[19] It is noteworthy that while TB can infect individuals of any demographic, approximately 90% of those who develop active TB are adults, with a higher incidence observed in men. Thus, it is of utmost importance to advance the development of a more efficacious vaccine that offers comprehensive protection against all forms of tuberculosis (TB) across distinct demographics of age. In light of ongoing challenges in TB management, the development of new anti-tuberculosis drugs with superior efficacy, safety, and reduced treatment timelines is urgently required to effectively combat and contain the disease.[19]

PATHOGENESIS OF TUBERCULOSIS

Tuberculosis (TB) is transmitted through aerosolized droplets containing *Mycobacterium tuberculosis* (MTB), which are released when individuals with active TB cough, sneeze, or speak.[22] Once inhaled, the bacteria travel through the respiratory tract and reach the lungs, where the host's innate immune system mounts an initial defence.[22] Alveolar macrophages engulf the tubercle bacilli, but if they fail to neutralize them, the bacteria begin to multiply inside the cells. These infected macrophages then release the bacteria, which are taken up by other macrophages, continuing the cycle. Meanwhile, lymphocytes are drawn to the infection site, initiating a cell-mediated immune response aimed at containing the bacteria.[22] At this stage, most individuals remain asymptomatic, and the bacteria may either be cleared or enter a

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dormant state within granulomas. However, in instances of compromised immunity, the condition can swiftly progress to active TB, accompanied by clinical manifestations.[22]

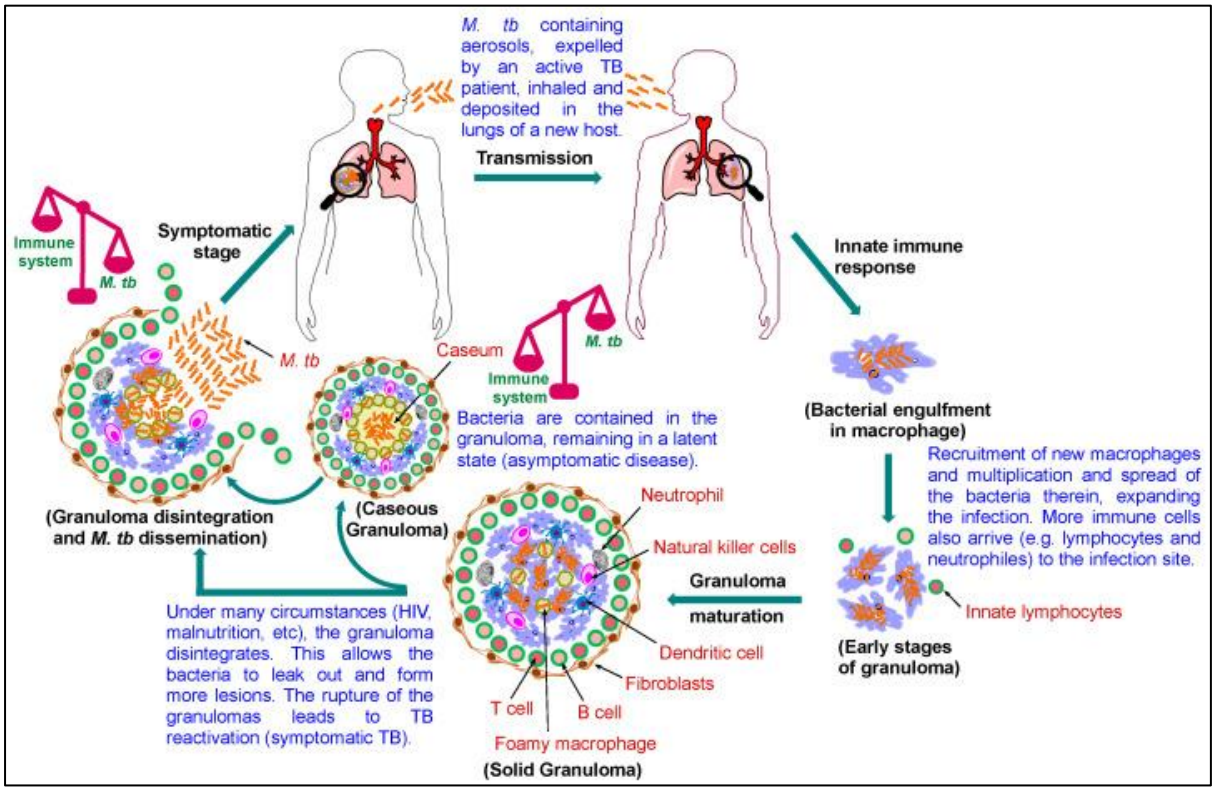

Figure 2: Pathophysiology of pulmonary tuberculosis.[20]

The granuloma represents a fundamental characteristic of pulmonary tuberculosis (TB), consisting of a circumscribed, heterogeneous aggregation of macrophages and various immune cells that function to limit the dissemination of the bacteria. In individuals with competent immune systems, the formation of granulomas helps contain the *Mycobacterium tuberculosis* (MTB) bacteria, preventing progression to active disease, even though the pathogen is not completely eliminated. The bacilli persist by evading immune destruction through strategies such as blocking phagolysosomal fusion and modulating the host immune response. These adaptations enable MTB to survive within the host for prolonged periods, often in a dormant or slow-growing state, effectively avoiding immune detection. Consequently, the patient remains asymptomatic and non-infectious, classified as latently infected. A significant

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challenge in contemporary TB treatment is the effective targeting of this resilient pathogen residing within the granuloma.[23]

Maturation of granuloma happens by differentiation of macrophages into foamy macrophages and other specialized cell types. The center of the granuloma may become necrotic as immune cells undergo lytic death, resulting in the formation of a cheese-like substance known as caseum. This soft, necrotic material accumulates within the granuloma's core, giving rise to the term "caseous granuloma" due to its resemblance to cheese. Foamy macrophages, characterized by the presence of lipid droplets, are distributed around the necrotic foci of the granuloma. Importantly, *Mycobacterium tuberculosis* (MTB) disrupts the host's lipid metabolism by altering the balance between lipid particle uptake, release, and storage. This dysregulation has been recognized as a key contributor to the progression of the disease. This disruption in lipid metabolism facilitates the formation of foam cells, which contribute to bacterial persistence and ultimately lead to the accumulation of caseum within the granuloma.[24]

"Mycolic acids" (MAs), the major lipid constituents of the thick cell wall of *Mycobacterium tuberculosis* (MTB), are essential for the bacteria's growth and survival.[23] Studies have shown that these lipids significantly contribute to the transformation of macrophages into foam cells.[24] The resulting caseous lesions serve as protective niches, harbouring the bacilli and promoting their dormancy. However, in later stages of the disease, the caseous core begins to soften and form cavities, leading to the reactivation of the bacteria. This process leads to the development of active tuberculosis (TB) in the patient, ultimately resulting in the transmission of infectious bacilli to new hosts. The progression to this life-threatening state is heavily dependent on the efficacy of the host's immune response in controlling bacterial replication.[23,24]

While the primary factor contributing to the reactivation of tuberculosis (TB) is often attributed to co-infection with human immunodeficiency virus (HIV), various other conditions can also

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precipitate the transition from a latent to an active infection. These factors include malnutrition, the use of immunosuppressive medications, chemotherapy, poorly controlled diabetes mellitus, sepsis, substance abuse (including drugs and alcohol), chronic renal failure, smoking, and malignancies. In instances where the host's immune system is compromised, dormant *Mycobacterium tuberculosis* (MTB) bacilli, which are typically contained within granulomas, may reactivate and proliferate. This process is accompanied by the liquefaction and cavitation of the granuloma. Consequently, the structural integrity of the granuloma diminishes, leading to the release of infectious bacteria and the subsequent formation of cavitary lesions, which are indicative of lung damage in individuals with TB. Moreover, the caseous material within the granuloma provides a nutrient-rich environment that facilitates the growth of the pathogen, resulting in a significant bacterial load. Eventually, the bacilli spread throughout the lungs and can enter the bloodstream, facilitating transmission to other individuals and enabling dissemination to distant organs. The disease becomes both highly contagious and symptomatic at this stage, marking the onset of active TB. Histological analysis of lung tissue during active infection often reveals granulomas at different developmental stages, indicating a link between granuloma maturation and TB reactivation. Three main types of granulomas have been described. They are solid, necrotic, and caseous granulomas, representing a continuum rather than separate forms. Solid granulomas usually appear during the early phase of infection and are associated with damage to the localized tissue. These structures reflect both disease pathology and bacterial containment. Typically, they are circumscribed by a fibrous tissue, lack central necrosis, and are composed of epithelioid cells (activated macrophages), T lymphocytes, plasma cells and multinucleated giant cells (Langhans type and foreign body type) which play a crucial role in controlling the infection. The MTB burden is relatively low in solid granulomas, making them common in individuals with latent TB infection (LTBI). As the disease advances, the center of the solid granuloma undergoes necrosis (resulting in necrotic

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granulomas), which eventually leads to the reactivation of dormant bacteria as the necrotic center enlarges and liquefies. A comprehensive analysis of granuloma development from latent to active TB has been discussed in other literature.[25]

DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS (MTB):

Microscopy:

Sputum smear microscopy continues to be a fundamental technique for the identification of *Mycobacterium tuberculosis* (MTB) in developing nations. The primary technique employed is acid-fast staining using carbol fuchsin (Ziehl Neelsen stain), which capitalizes on the lipid-rich cell wall of *Mycobacterium tuberculosis* (MTB). This unique composition renders the bacilli resistant to decolorization by acid-containing reagents, enabling the microscopic visualization of acid-fast organisms in smears prepared from sputum, pleural fluid, bronchoalveolar lavage fluid, or other specimens.[26]

Culture:

Solid and Liquid Culture:

The World Health Organization (WHO) continues to endorse culture as the gold standard for tuberculosis (TB) diagnosis, and is crucial not only for confirming the presence of the disease but also for identifying drug resistance. Traditional MTB culture is done using either Lowenstein–Jensen which is a solid media, or Middlebrook 7H9 (liquid media). It is important to note that solid culture is generally more cost-effective and exhibits a lower risk of contamination from other bacterial or fungal species. Conversely, liquid culture offers advantages in terms of speed, sensitivity, and convenience, as it allows for automatic detection of growth.[27]

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Rapid Identification Assays:

"Rapid identification assays" following positive culture results are essential for the prompt initiation of anti-tuberculosis therapy as they can differentiate between various *Mycobacterium tuberculosis* (MTB) and non-tuberculous mycobacteria (NTM) following positive culture results are essential for the prompt initiation of anti-tuberculosis therapy. Conventional biochemical assays are characterized by their protracted turnaround times, often ranging from two to three weeks. One notable MTB-specific antigen, known as MTB protein 64 (MPT-64), is secreted during the growth of the bacteria.[28]

Molecular Tests:

Xpert MTB/RIF:

The "Xpert MTB/RIF test", developed by "Cepheid", represents a widely utilized automated molecular diagnostic tool for the identification of *Mycobacterium tuberculosis* (MTB) and its resistance to "rifampicin" (RIF) directly from clinical specimens.[29] This assay employs a "hemi-nested real-time polymerase chain reaction" (PCR) methodology to amplify a specific sequence of the "rpoB gene" associated with MTB.[29] Notably, the test offers a rapid turnaround time of approximately 2 to 3 hours, and it effectively mitigates the risk of cross-contamination through the use of self-contained cartridges.[29]

Loop-Mediated Isothermal Amplification (LAMP):

"Loop-mediated Isothermal Amplification" (LAMP) is a nucleic acid amplification technique that operates at a constant temperature. It targets the "*gyr*B" and 16S rRNA genes (six distinct regions) by employing an auto-cycling strand displacement mechanism and amplifies DNA. The specificity of TB-LAMP method is comparable to that of Xpert MTB/RIF assay while its sensitivity is marginally lower in comparison.[30]

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Line Probe Assay (LPA):

The "Line Probe Assay" (LPA) identifies the presence of tuberculosis (TB) DNA and genetic mutations linked to drug resistance following the processes of DNA extraction and polymerase chain reaction (PCR) amplification.[31] The "Line Probe Assay" (LPA) works by capturing a pre-labelled amplified DNA product using a specific DNA probe fixed onto a membrane strip. Detection is achieved through colorimetric techniques, with the results appearing as distinct linear bands on the strip.[31]

"Truenat" (Micro RT PCR):

Developed by "Molbio Diagnostics", the "Truenat MTB", "Truenat MTB Plus", and "Truenat MTB-Rif Dx" assays are micro real-time polymerase chain reaction (PCR) tests designed to detect *Mycobacterium tuberculosis* (MTB) in sputum samples within an hour following DNA extraction.[32] The "Truenat MTB-Rif Dx" includes an additional feature of detecting rifampicin resistance sequentially using a supplementary chip.[32]

Immunological Diagnosis:

Antibody Detection:

Serological tests depend on the humoral immune response for the identification of *Mycobacterium tuberculosis* (MTB) antigens. These are not endorsed by the "World Health Organization" (WHO) for the purposes of commercial serological testing in diagnosing TB as they have limited diagnostic sensitivity and specificity.[33]

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Antigen Detection:

The sandwich "enzyme-linked immunosorbent assay" (ELISA) technique enables the detection of circulating *Mycobacterium tuberculosis* (MTB) antigens in various clinical samples such as serum, sputum and urine.[34] As a distinctive component of the *Mycobacterium tuberculosis* cell envelope, "lipoarabinomannan" (LAM) has shown considerable promise as a diagnostic biomarker for tuberculosis[34]

Tuberculin Skin Testing (TST):

The Tuberculin Skin Test (TST) is a traditional diagnostic approach that assesses type IV hypersensitivity through the use of purified protein derivative (PPD) derived from tuberculin. Patients infected with *Mycobacterium tuberculosis* (MTB) are capable of generating sensitized T lymphocytes that can identify MTB antigens. Upon re-exposure to these antigens, the sensitized T lymphocytes release a range of soluble lymphokines, which subsequently enhance vascular permeability and lead to localized erythema, edema, and induration.[35]

T-cell Spot Test for Tuberculosis (T-SPOT):

On the basis of the principle of enzyme-linked immunospot (ELISPOT) method, the T-SPOT assay measures the number of cells that release interferon-gamma (IFN-γ) after exposure to *Mycobacterium tuberculosis*-specific antigens. This assay is currently widely used for detecting MTB infections.[36]

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Comparison of the typical methods for diagnosis of TB disease.

	Microscopy	Xpert MTB/RIF	Culture	T-SPOT.TB
Price	Low	High	Medium	High
Procedure complexity	Low	Low	High	High
Sensitivity	Low (for bacterial-positive TB)	High (for bacterial-positive TB)	High (for bacterial-positive TB)	Relatively high (for both bacterial-positive and bacterial-negative TB)
Specificity	High (in regions with a low incidence of NTM)	High	High	High (for diagnosis of Mtb infection), medium (for diagnosis of active TB in TB-endemic areas)
Advantages	Fast, simple, inexpensive	Fast, simple, low biosafety risk, detecting one drug resistance	Detecting all drug resistances	Detecting bacterial-negative TB, detecting latent TB infection
Shortcomings	Low sensitivity, cannot differentiate between live and dead bacilli	Expensive, cannot differentiate between live and dead bacilli	High complexity, long turnaround time, high biosafety risk	Expensive, high complexity

Figure 3: Comparison of typical methods for diagnosis of tuberculosis.[37]

Treatment Guidelines for Drug-Susceptible Tuberculosis:

The standard treatment for drug-susceptible tuberculosis (DS-TB) involves a four-antibiotic combination—"rifampicin" (RIF), "isoniazid" (INH), "pyrazinamide" (PZA), and "ethambutol" (EMB) — all of which were discovered nearly sixty years ago.[38] This four-drug regimen is to be administered for a minimum duration of six months under "directly observed treatment" (DOT) to achieve optimal treatment success and cure rates.[38] The treatment regimen is structured into two distinct phases: an intensive phase involving the administration of the

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aforementioned four antibiotics for two months, which is followed by dual antibiotic administration i.e. isoniazid (INH) and rifampicin (RIF), for an additional four months constituting the continuation phase so as to ensure the eradication of dormant bacilli.[38]

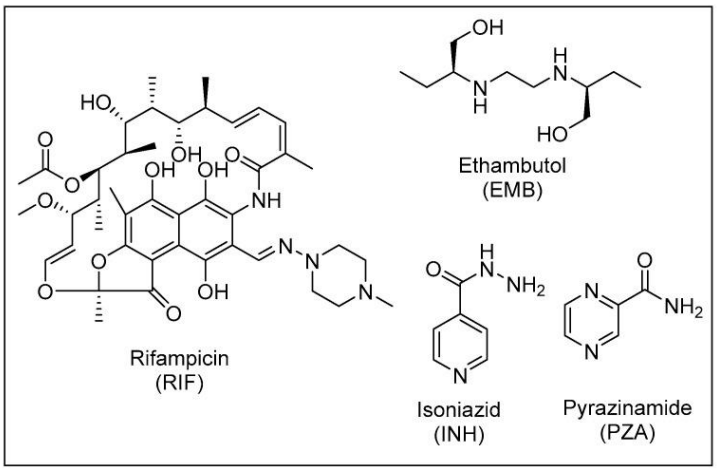

Figure 4: The four front-line anti-tuberculosis drugs.[20]

Cell-free DNA (cfDNA):

"Circulating cell-free DNA" (cfDNA) consists of extracellular DNA fragments present in bodily fluids, originating from both normal and diseased cells. Its presence in human blood was first reported by Mandel and Metais in 1948.[10] Nearly two decades later, in 1965, Bendich and colleagues suggested that cfDNA released from cancer cells may contribute to oncogenesis, particularly by facilitating cancer metastasis. However, for the next 55 years, cfDNA remained relatively underexplored due to limited knowledge about its structure, function, and biological significance. The first association between cfDNA and disease state was established in 1966 when Tan et al. reported elevated levels of cfDNA in the blood of patients with systemic lupus erythematosus.[41]

A decade later, Leon et al. utilized a radio-immunochemistry assay to reveal that cancer patients exhibited elevated levels of circulating cell-free DNA (cfDNA) compared to healthy individuals.[42] Furthermore, they noted that cfDNA levels diminished in response to successful

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anticancer treatments. However, due to technological constraints, it was not until 1989 that Stroun et al. provided the first experimental evidence identifying the origin of cfDNA in cancer patients.[43] The authors observed a specific instability in the double-stranded structure of tumor DNA present in cfDNA, leading to the term circulating-tumoral DNA (ctDNA) being adopted. The advancements in molecular biology techniques during the 1990s, particularly in conjunction with the development of the Human Genome Project, facilitated a more direct elucidation of tumor origins. In 1994, Vasioukhin et al. and Sorenson et al. identified tumor-specific mutations in the N-RAS gene within the plasma samples of patients diagnosed with pancreatic adenocarcinoma and acute myeloid leukemia.[44,45] Concurrently, the analysis of circulating cell-free DNA (cfDNA) garnered significant interest within the clinical realm. In 1997, Lo et al. successfully detected fetal cfDNA in maternal plasma and serum.[46] These findings have paved the way for numerous opportunities, indicating that maternal plasma and serum DNA could serve as a valuable resource for non-invasive prenatal diagnosis of genetic disorders in obstetric practice. In subsequent years, emerging research provided evidence supporting the role of cfDNA methylation as an epigenetic biomarker. In the years that followed, growing research highlighted the potential of cfDNA methylation as a valuable epigenetic biomarker. In 1999, two independent research groups pioneered the study of cfDNA methylation in human clinical samples. Esteller et al. detected hypermethylation of tumor suppressor gene promoters in cfDNA from patients with non-small cell lung cancer, whereas Wong et al. observed abnormal methylation of the *p16* gene in cfDNA obtained from individuals with hepatocellular carcinoma.[47,48] Both studies were pioneering in demonstrating the detection of aberrant promoter methylation in the peripheral circulation of cancer patients, specifically those with hepatocellular carcinoma.[47,48] Earlier studies had confirmed the presence of tumor-derived DNA in the plasma of cancer individuals and fetal DNA in the serum of pregnant women. However, it was not until 2000 that Zhong et al. provided the first evidence

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of plasma and serum mitochondrial cell-free DNA (mt-cfDNA) from both patients and healthy individuals with diabetes.[49] At that time, the term "cell-free DNA" (cfDNA) encompassed both nuclear cfDNA (n-cfDNA) and mitochondrial cfDNA (mt-cfDNA), with each type exhibiting distinct structural and functional characteristics.

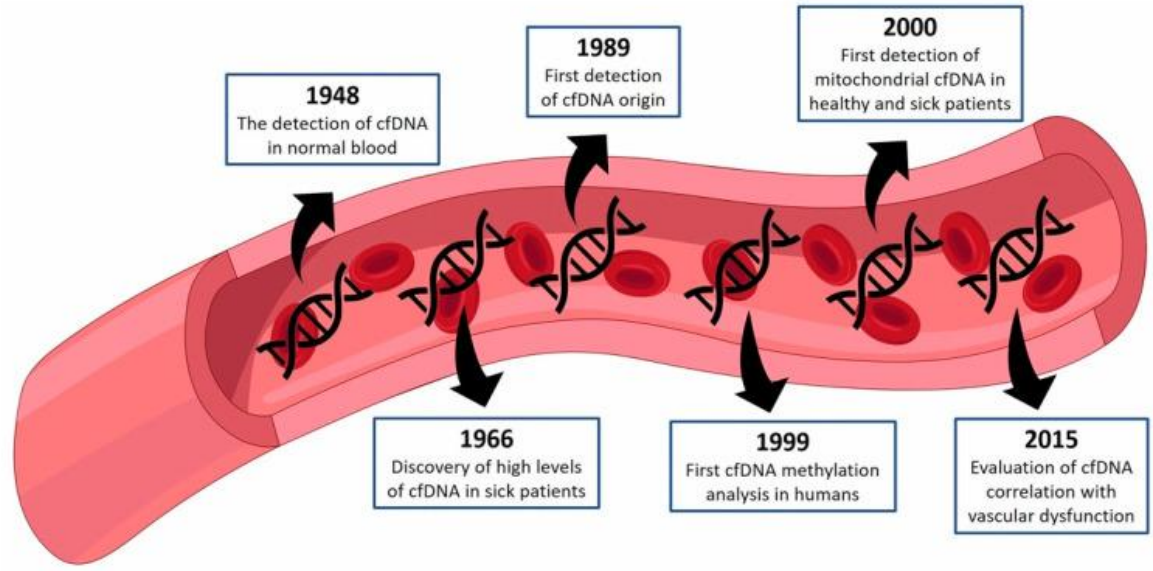

Figure 5: Chronological summary of cell-free DNA (cfDNA)[50]

Cell-free DNA (cfDNA)—Source and Mechanism of Release:

"Circulating cell-free DNA" (cfDNA) is present in various body fluids under both physiological and pathological conditions.[50] The release of DNA fragments from intracellular to extracellular compartments occurs through several mechanisms. In both healthy individuals and those with benign or malignant diseases, the processes responsible for the release of DNA into the bloodstream can be attributed to: (1) necrosis, (2) apoptosis, (3) active DNA release, and (4) exogenous sources.[50]

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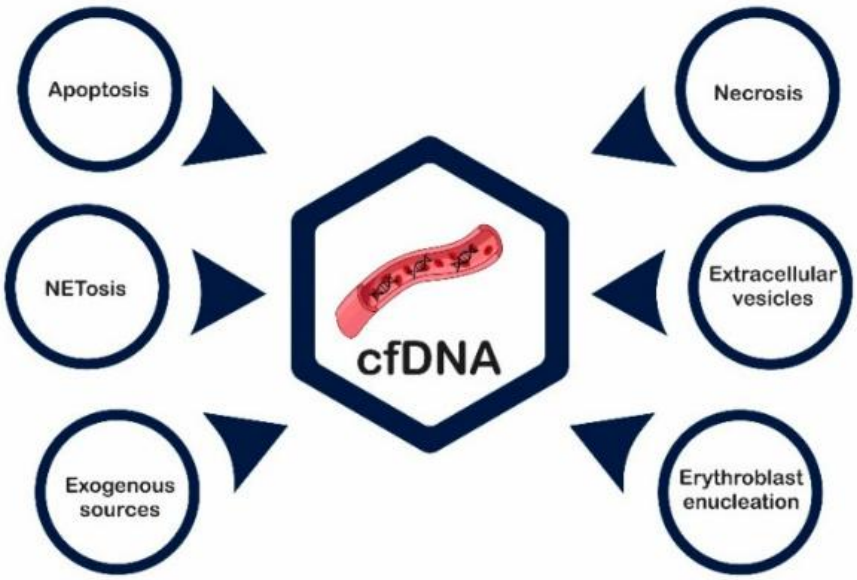

Figure 6: Cell-free DNA (cfDNA) sources.[50]

Cell-Free DNA (cfDNA) Integrity:

The integrity of circulating cell-free DNA (cfDNA) can be assessed through the analysis of its fragmentation levels. Distinct cfDNA molecules of varying sizes can be identified in various biological fluids. The size discrepancies among cfDNA fragments may provide insights into their source or the underlying pathophysiological conditions present in the organism. Specifically, apoptotic cells generate DNA fragments measuring approximately 180–200 base pairs (bp), while necrotic cells release larger DNA fragments exceeding 10 kilobases (kb) in length.[51]

Genetic and Epigenetic Profile:

Cell-free DNA (cfDNA) present in biological fluids exhibits genetic and epigenetic variations analogous to those found in DNA (nuclear and mitochondrial) derived from healthy and diseased cells. These variations may encompass alterations in cfDNA composition, copy-number variations (CNVs), mutations and changes in methylation patterns. Consequently, the analysis of cfDNA facilitates the investigation of a patient's genetic profile.[52]

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Copy-Number Variations (CNVs):

"Copy number variations" (CNVs) play a crucial role in contributing to genomic instability in various diseases. Extensive genomic research has revealed the presence of cfDNA CNVs in multiple cancer types, highlighting their potential utility as biomarkers for cancer detection and monitoring.[53]

Mutations:

Mutation refers to a lasting modification of the nucleotide sequence constituting a gene. These alterations can vary in scale, impacting anything from an individual DNA base pair to extensive regions of a chromosome that encompass multiple genes. cfDNA mutational analysis within specific genes has been shown to possess significant clinical importance. These genes include "KRAS", "TP53", "BRAF", "epidermal growth factor receptor" (EGFR), and "adenomatous polyposis coli" (APC).[54]

Molecular Signatures in Cell-Free DNA (cfDNA):

Variations in an organism's physiological condition can be detected through the nucleic acid profile of circulating cell-free DNA (cfDNA). Research by Natalya Veiko and her team has demonstrated that certain diseases such as myocardial infarction, atherosclerosis and rheumatoid arthritis may cause an increase in guanine-cytosine (GC) content, while cancer, may cause both GC enrichment and oxidative modifications within the cfDNA pool. A significant contributor to apoptotic cell death under conditions of oxidative stress is the increased oxidative modification of cellular DNA. Once released into the bloodstream, this DNA retains the oxidative stress markers, notably elevated levels of 8-oxo-dG, which serves as a biomarker for oxidation.[55]

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Epigenetic Modifications:

"Epigenetic modifications" are stable, heritable changes in gene function that do not involve alterations to the DNA sequence itself.[56] These modifications regulate gene activity and expression through mechanisms such as: **"DNA methylation"** – the addition of methyl groups to cytosine bases, often leading to gene silencing.[56] It is the most extensively researched epigenetic modification.[56] **"Histone modifications"** – chemical changes to histone proteins that influence how tightly DNA is packaged, thereby affecting gene accessibility.[56] **"Regulatory non-coding RNAs"** – RNA molecules that modulate gene expression at the transcriptional and post-transcriptional levels.[56] Epigenetic modifications are essential for normal development, cellular differentiation, and adaptation, and they are increasingly recognized for their role in the onset and progression of various diseases, including cancer.[56]

Cell-Free DNA (cfDNA) Concentration:

An increased concentration of circulating cell-free DNA (cfDNA) may indicate various physiological processes, such as those occurring during physical exercise or in pregnant individuals, as well as pathological conditions, including inflammation, diabetes, tissue injury, sepsis, myocardial infarction, and in patients who have undergone transplantation.[57]

Mitochondrial Cell-Free DNA (mt-cfDNA):

The release of mt-cfDNA into the bloodstream occurs through various mechanisms, including apoptosis, necrosis, and active cellular secretion, as previously outlined. Research indicates that mt-cfDNA exhibits a greater degree of fragmentation compared to nuclear cell-free DNA (n-cfDNA), with fragment lengths typically ranging from 30 to 80 base pairs, and exhibiting peaks between 42 and 60 base pairs. This reduced fragment size is attributed to the lack of nucleosome-associated histone proteins, which makes mt-cfDNA more susceptible to enzymatic degradation.[58]

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Circulating Cell-Free DNA (cfDNA): Biological Characteristics, Clinical Applications, and Emerging Role in Infectious Disease Diagnostics:

Circulating cell-free DNA (cfDNA) are fragments of nucleic acids that are released into the bloodstream and body fluids. They originate from apoptotic human cells and microorganisms during cellular degradation. cfDNA measures around 170bp on an average (70% of which is less than 300bp in size within plasma) and it is significantly smaller than genomic DNA. In the blood of healthy individuals, there is a considerable variability in the concentration of cfDNA, ranging from under 10 ng/ml to over 1500 ng/ml, which is equivalent to an estimated fourteen hundred to two hundred thousand copies of DNA per milliliter of a diploid human genome (based on the diploid human genome size of 6,469.66 megabase pairs and a weight of 650 daltons per base pair, it is fragmented into approximately 170-bp segments). Due to its small size, cfDNA can pass through the renal barrier, allowing it to be detected in urine. Notable variations in the size distribution and quantity of cfDNA have been observed between different individuals and even within the same individual under specific pathological conditions (such as cancer and infectious diseases) and physiological states (including pregnancy).[59]

Circulating cell-free DNA (cfDNA) is currently applied across several clinical domains to support medical decision-making. Its primary applications include noninvasive prenatal testing, where cfDNA from maternal blood is used to detect fetal chromosomal abnormalities early in pregnancy; oncology, where circulating tumor DNA (ctDNA), a tumor-derived subset of cfDNA, serves as a biomarker for cancer diagnosis, monitoring, and treatment response; and organ transplantation, where cfDNA of donor origin helps detect early signs of graft rejection. In oncology, highly sensitive methods such as cancer personalized profiling by deep sequencing (CAPP-Seq), a next-generation sequencing (NGS) approach, can detect ctDNA at extremely low concentrations (as low as 0.00025% of total cfDNA). Additionally, techniques like BEAMing (beads, emulsions, amplification, and magnetics) and digital PCR (dPCR)

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enable detection of ctDNA in circulation at frequencies ranging from 1% to 0.001%. These detection capabilities are expected to extend to other medical conditions as well, including autoimmune disorders, highlighting the growing potential of cfDNA in precision medicine.[59]

Over the past several decades, cell-free DNA (cfDNA) has been used as a diagnostic tool especially in the context of nasopharyngeal carcinoma for EBV (Epstein-Barr virus) detection and more recently, for invasive fungal infections as well as specific infectious agents such as *Plasmodium*, *Trypanosoma*, *Leishmania*, *Schistosoma*, *Leptospira*, and HIV.[59]

Tuberculosis (TB) serves as a pertinent illustration of an infectious disease for which circulating cell-free DNA (cfDNA) holds significant potential. In 2016, it was estimated that there were approximately 10.4 million active cases of TB globally, with around 40% of these cases remaining either undiagnosed or unreported, primarily due to insufficient diagnostic methods. Currently, the majority of TB diagnostic tests necessitate the collection of sputum samples, with sputum microscopy (Ziehl Neelsen stain) being the most prevalent testing method. However, existing sputum-based diagnostics exhibit limited accuracy and are often not suitable for certain population groups, such as children, individuals with HIV-associated TB, or those with extrapulmonary TB, who may struggle to provide sputum samples. Moreover, many blood-based assays under development tend to lack specificity, as they depend on host markers. In contrast, cfDNA can indicate the presence of the pathogen, making it a compelling biomarker for the detection and treatment monitoring of *Mycobacterium tuberculosis* (MTB) in both pulmonary and extrapulmonary TB cases across all age groups, utilizing non-invasive sample types such as urine.[60]

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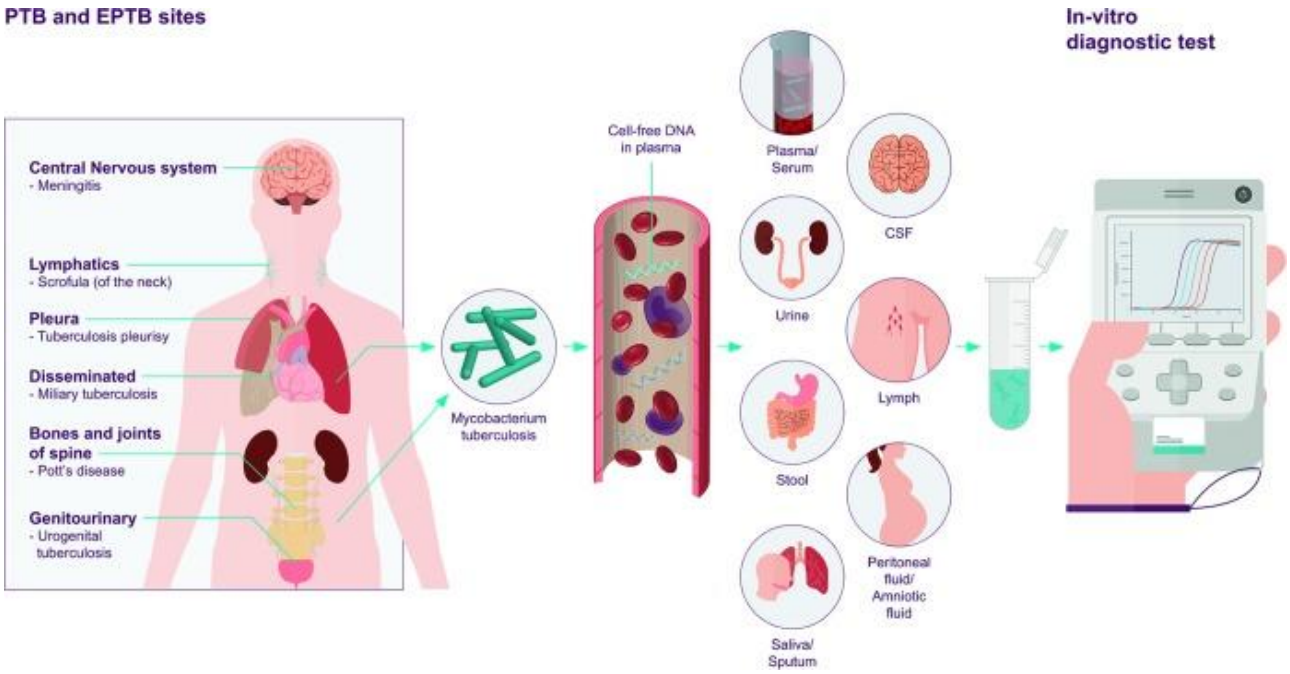

Figure 7: Diagnostic use of *Mycobacterium tuberculosis* cell-free DNA (MTB-cfDNA).[61]

Similar Studies in Literature:

Condos R et al (1996) conducted a study for the diagnosis of *Mycobacterium tuberculosis* examining the efficacy of a blood-based polymerase chain reaction (PCR) assay, within a clinical context. Their findings indicated that the use of peripheral blood for PCR detection represents a technically viable method that may play a significant role in the diagnosis of pulmonary tuberculosis.[62]

Taci N et al (2003) conducted an evaluation of *Mycobacterium tuberculosis* DNA in peripheral blood samples utilizing polymerase chain reaction (PCR) techniques in adult patients who were HIV-negative and newly diagnosed with smear-positive pulmonary tuberculosis. Their findings indicated that the identification of *Mycobacterium tuberculosis* DNA in peripheral blood through PCR is beneficial for the prompt diagnosis of tuberculosis in HIV-negative individuals. However, they emphasized the necessity for standardization of the PCR methodology to ensure accurate tuberculosis diagnosis.[63]

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Che N et al (2017) proposed that cell-free DNA (cfDNA) released from *Mycobacterium tuberculosis* is present in pleural effusion samples from patients with tuberculous pleurisy, and that the detection of this cfDNA could enhance diagnostic sensitivity. The study involved a cohort of 78 patients with pleural effusion, comprising 60 individuals diagnosed with tuberculous pleurisy and 18 patients with alternative conditions. The authors concluded that the assay for cell-free *Mycobacterium tuberculosis* DNA is a rapid and precise molecular diagnostic tool that provides direct evidence of the etiological role of *Mycobacterium tuberculosis*.[10]

Yu G et al (2021) conducted an assessment of the diagnostic accuracy of cell-free DNA (cfDNA) derived from *Mycobacterium tuberculosis* for the diagnosis of tuberculosis (TB). Their analysis incorporated 14 independent studies that compared cfDNA with composite reference standards (CRS), as well as four studies that compared cfDNA with culture methods. The findings indicated a favourable diagnostic accuracy of MTB-cfDNA testing in comparison to both culture and CRS. Consequently, cfDNA has the potential to facilitate rapid and early diagnosis of TB.[17]

Park JH et al (2022) conducted a study to evaluate the efficacy of enriched cell-free DNA (cfDNA) in the diagnosis of pulmonary and extrapulmonary tuberculosis (TB). Their research involved a cohort of 96 patients, of whom 40 (41.7%) were diagnosed with TB, comprising 34 individuals with confirmed TB and six with probable TB. Conversely, 41 patients (42.7%) were found not to have TB. The authors concluded that the plasma cfDNA assay may serve as a valuable supplementary tool to existing diagnostic methods for TB, particularly when utilized in conjunction with interferon-gamma release assays (IGRA) to exclude the presence of the disease.[64]

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Du WL et al (2024) conducted an assessment of the applicability of cfDNA-TB testing across various research sites, utilizing a relatively substantial sample size. Their findings indicate that the efficacy of cfDNA-TB testing is markedly superior to that of Xpert and mycobacterial culture methods. Consequently, they propose that cfDNA-TB testing should be regarded as the primary diagnostic strategy for enhancing the detection of tuberculosis pathogens.[65]

Scope for Further Research:

In order to elucidate the prospective utility of cell-free DNA (cfDNA) in the diagnosis of tuberculosis (TB), the establishment of well-curated biorepositories is essential. These repositories should encompass samples characterized through a comprehensive approach that integrates quantitative microbiological analyses from both sputum and non-sputum specimens, which includes evaluating the time to positivity in culture and the cycle threshold in molecular assays. Furthermore, the incorporation of positron emission tomography (PET) is recommended to detect subclinical or early-stage TB. It is also imperative that these biorepositories collect matched plasma and urine samples. Moreover, there is a significant need for reference materials pertaining to *Mycobacterium tuberculosis* (MTB)-cfDNA, which would serve as quality controls for the validation of various cfDNA methodologies and ultimately facilitate the clinical management of patients through the establishment of absolute standard values.[66]

Within the research agenda focussed on cfDNA in tuberculosis (TB), priority should be to assess the levels of MTB-cfDNA in biological specimens. Accurately estimating the total burden of *Mycobacterium tuberculosis* (MTB) is crucial not only for diagnostic purposes but also for monitoring treatment efficacy. Cell-free DNA (cfDNA) has the potential to reveal the presence of disease throughout the body and may identify pathological conditions that are not detectable through traditional microbiological techniques. Furthermore, it is essential to

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evaluate the relationship between MTB-cfDNA concentration and other non-sputum biomarkers, such as TB antigens like lipoarabinomannan. Preclinical investigations involving novel therapeutic agents could facilitate the calculation of terminal colony-forming units per lesion or lung, allowing for correlation with immediate pre-mortem cfDNA concentrations per milliliter, ideally utilizing positron emission tomography-computed tomography (PET-CT) in non-human primate studies, alongside a longitudinal analysis of cfDNA. Additionally, estimating MTB-cfDNA concentrations in biological samples is critical for the development of assays, encompassing aspects such as sample preparation (including collection volume, cfDNA isolation techniques, and concentration volumes) and the advancement of detection platforms (specifically, the assay limit of detection).[67]

Further research is necessary concerning HIV-positive individuals and pediatric patients. Currently, there exists a paucity of studies focused on the use of cfDNA-based diagnostics for tuberculosis (TB) in HIV-positive populations, and no studies have been identified that address pediatric patients. These demographic groups are likely to derive significant benefits from a cfDNA-based assay, particularly due to the challenges they face in producing sputum samples.

There lies an imbalance as exhibited by many studies that show limitations due to the inclusion of a disproportionately high number of individuals with tuberculosis (particularly those with smear-positive tuberculosis) and a scarcity of control subjects. Thus, hindering the generalizability of the study results to populations with a significantly lower prevalence of the disease, which are likely to present with more paucibacillary forms of the infection. In addition to the identification of *Mycobacterium tuberculosis*, the analysis of cell-free DNA (cfDNA) could enhance our comprehension of infectious processes.

Burnham et al. employed sequencing methodologies for the analysis of cell-free DNA (cfDNA) and documented its utility in quantifying bacterial proliferation, identifying antimicrobial

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resistance genes, and assessing the host's cellular and tissue-level response to urinary tract infections. This approach warrants similar investigation in the context of tuberculosis (TB). Furthermore, cfDNA analysis holds promise for evaluating responses to pharmacological treatment and for providing insights into the disease state. If detection technologies can achieve sufficiently low sensitivity—currently unattainable with existing tools—cfDNA may facilitate the identification of early-stage TB. The implications of such diagnostic tests could be significant. Nonetheless, it remains uncertain whether the burden of *Mycobacterium tuberculosis* (MTB) or the burden of its byproducts, such as cfDNA, is directly associated with the transition from latent to active disease.[68]

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MATERIALS AND METHODS

Source of data:

The study population consisted of 94 patients, both outpatients and patients admitted to BLDE (Deemed to be University) Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura.

Study period: 1st May, 2023 to 31st December, 2024.

Methods of Data Collection:

Study Design: Experimental Study.

Type of Study: Cross Sectional Study.

Study Population: All clinically suspected cases of tuberculosis.

Sample Size: With anticipated sensitivity and specificity of 78% and 97%, respectively, for MTB-cfDNA in plasma, considering the prevalence of tuberculosis as 0.316, at precision of 5% and 95% confidence, the required sample size is minimum of 65.[17, 69]

For this study, a sample size of **94** was considered.

Selection Criteria:

Inclusion Criteria: All clinically suspected cases of tuberculosis who attended the OPD and admitted in IPD of our institute's tertiary care hospital.

Exclusion Criteria: Patients who have taken anti tuberculosis medication in the last three months.

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Data Collection Procedure:

- The institutional ethical clearance certificate [BLDE(DU)/IEC/937/2023-24] was obtained.
- Study purpose was explained to the patient & consent was taken.
- Detailed history was elicited.
- 4 mL of peripheral venous blood was collected into tubes containing EDTA-2K and plasma was separated by light spin (3000rpm, 10 minutes) followed by heavy spin (9000rpm, 10 minutes) and stored in microcentrifuge tubes at -80˚C.
- Extraction of plasma cfDNA was done by using the QIAamp DNA Mini Kit (250, Qiagen) with a final elution volume of 50 µL and stored in microcentrifuge tubes at −80˚C for subsequent qPCR testing.
- Batch-wise cfDNA analysis was performed by qPCR to detect the target MTB-specific cfDNA for IS6110 gene.[13]
- Patients were asked to cough spontaneously and sputum was collected into a sterile container following which smears were prepared. For extrapulmonary samples, smears were prepared from FNAC and body fluid samples. These smears were stained with Ziehl-Neelsen staining for acid-fast bacilli (AFB) following the standard procedure and protocol.[70]
- Results of Ziehl Neelsen-stained AFB smear test for pulmonary and extrapulmonary samples were recorded. GeneXpert assay reports were collected.

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Materials Required:

1. **cfDNA Extraction Kit:**

- **Kit:** Qiagen QIAamp MinElute cfDNA Kit (Cat. No. 55204)
- **Components:**
    - "QIAamp UCP MinElute Columns"
    - Collection Tubes (2 mL & 1.5 mL)
    - "Ultra-Clean Water"
    - "Magnetic Bead Suspension"
    - "Bead Binding Buffer"
    - "Buffer ACS, ACW1, ACW2"
    - "Proteinase K"
    - "Bead Elution Buffer"

2. **PCR Reagents:**

- **Target Gene:** "IS6110" insertion sequence of *Mycobacterium tuberculosis*
- **Primer Sequences:**
    - **Forward Primer:** 5'-CCTGAGGAGTCAAGACGT-3'
    - **Reverse Primer:** 5'-CTCGTCCAGCGCCGCTTCGG-3'
- **Master Mix:** As per Table 1

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TABLE 1: PCR MASTER MIX COMPONENTS FOR 25 µL REACTION

Component	Volume (µL)	Notes
2X PCR Master Mix	12.5	TaqMan™ Universal PCR Master Mix
Forward Primer (10 µM)	0.5	Targeting IS6110
Reverse Primer (10 µM)	0.5	Targeting IS6110
Probe (FAM)	0.05	For TaqMan assay
cfDNA Template (elute)	5.0	Eluted in low-EDTA buffer or water
Nuclease-free Water	6.45 (to 25.0 µL)	Adjustable to final volume

- **Controls:**
    - "No Template Control" (NTC)
    - "Positive Control" (MTB DNA)
    - "Negative Control" (cfDNA from healthy individuals)

3. Ziehl Neelsen Stain:

- **Reagents:**
    - Stain: 1% "carbol fuchsin"
    - Decolouriser: 25% "H_2SO_4"
    - Counterstain: 0.1% "methylene blue"
- **Equipment:**
    - Glass slides (75 mm x 25 mm)
    - Slide staining rack
    - Forceps, cotton swab, filter paper
    - Timer
    - Burner
    - Running water source and water bath

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Methodology:

1. **cfDNA Extraction Process:**
    1. **Sample Preparation:**
        - **Plasma Sample Volume:** 2 mL
        - **Storage Conditions:**
            - Short-term: 2–8°C (up to 24 hours)
            - Long-term: -15°C to -80°C
    2. **Extraction Protocol:**

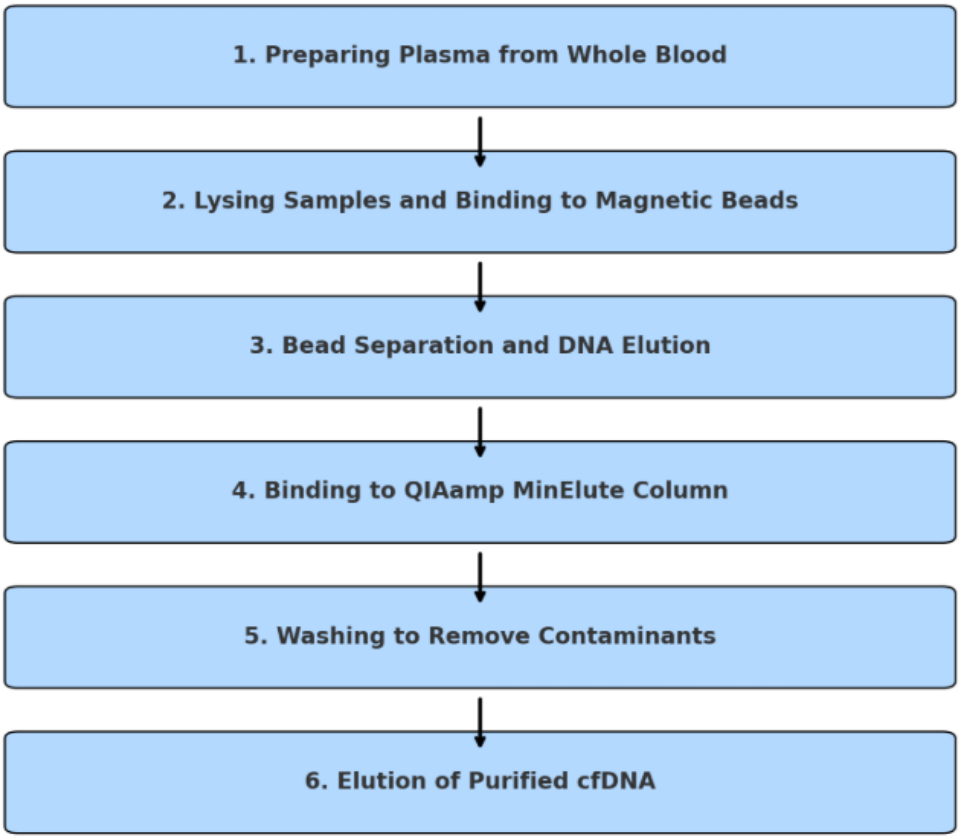

Figure 8: Cell-free DNA (cfDNA) extraction protocol

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3. Plasma cfDNA Extraction Procedure:

1. Mix 1 mL plasma with the following in a 15 mL tube:
    - 30 µL "Magnetic Bead Suspension"
    - 55 µL "Proteinase K"
    - 150 µL "Bead Binding Buffer"
2. Incubate for 10 minutes at room temperature with gentle shaking.
3. Centrifuge briefly at 200 x g (1000 rpm) for 30 sec to remove residual solution.
4. Transfer the sample tube to a magnetic rack and let it stand for 1 minute until solution becomes clear.
5. Discard the supernatant. Add 200 µL "Bead Elution Buffer" and vortex.
6. Pipette up and down to mix and incubate for 5 minutes at 300 rpm.
7. Transfer the mixture (including beads) into a new 2 mL bead elution tube.
8. Add 300 µL "Buffer ACB", vortex and discard the bead pellet.
9. Pipet the supernatant-Buffer ACB mixture into a "QIAamp UCP MinElute column", centrifuge at 6000 x g (5331 rpm) for 1 minutes.
10. Place the "QIAamp UCP MinElute column" into a clean 2 mL collection tube and discard the flow through.
11. Wash with "Buffer ACW2" (500 µL) then centrifuge at 6000 x g (around 5000 to 5500 rpm) for 1 minute.
12. Take a clean 2 mL collection tube and place the "QIAamp UCP MinElute column" into it and discard the flow through.

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13. Centrifuge at full speed at 20000 x g (14000 rpm) for 3 minutes.
14. Place the "QIAamp UCP MinElute column" into a clean 1.5 mL elution tube.
15. Dry the membrane by incubating at 56°C for 3 minutes.
16. Add 20 µL "Ultra Clean Water" onto the membrane and incubate at room temperature for 1 minute.
17. Centrifuge at full speed (20,000 x g, 14,000 rpm) for 1 min to elute the cfDNA.
18. Final cfDNA elute of 25 to 50 µL.
19. "Agilent Bioanalyzer", a microfluidics-based system was used for assessing DNA quality, size, and concentration.

2. PCR Amplification (IS6110 Gene Detection):

1. Instrument Setup (As per manufacturer's protocol):

- PCR System: "Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific)"

2. Experiment Setup:

i. Number of wells/plates selected: 96 well plate or 384 well plate

ii. Detection Channels: "FAM" (6-carboxyfluorescein), VIC/HEX

iii. Choose Taqman assay

iv. Choose cycle threshold (Ct): For MTB-cfDNA assays on QuantStudio 3, targeting IS6110 [16].

- **≤ 40 Ct Value** → Positive
- **> 40 Ct Value** → Negative

v. Select Quantification: Select standard curve.

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3. **Thermal Cycling Conditions:**

   i. Initial incubation at 37°C for 2 minutes

   ii. Initial denaturation at 95°C for 5 minutes.

   iii. 40 cycles of amplification (denaturation, annealing and extension) as per Table 2.

TABLE 2: PCR AMPLIFICATION CONDITIONS

Step	Temperature	Duration	Cycles
Denaturation	95°C	15 sec	40
Annealing	60°C	30 sec	40
Extension	72°C	30 sec	40

4. **Procedure:**

   - Load the samples into the plate. Samples are loaded in duplicates to validate the findings.
   - Seal the plate and load into the instrument.
   - Start the RT-PCR run. Note the results at the end of the 40th cycle.

5. **Data Analysis:**

   - **Threshold Setting-** For MTB-cfDNA assays on QuantStudio 3, targeting IS6110, following are the cycle threshold (Ct) standards.[16]
     - **≤ 40 Ct Value** → Positive
     - **> 40 Ct Value** → Negative
     - If values are within **±0.5 Ct**, they are considered consistent.

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- **Controls Validation:**
    - NTC should remain negative
    - Positive Control must amplify at expected Ct value
    - Negative Control should not amplify

6. Interpretation:

- **Positive Result:** Presence of IS6110 cfDNA in plasma, indicating *Mycobacterium tuberculosis* infection.
- **Negative Result:** Absence of IS6110 cfDNA in plasma, indicating no *Mycobacterium tuberculosis* infection.
- **Indeterminate Result:** If Ct values are more than ±0.5 (borderline) and/or if controls fail. Requires re-testing.

3. Ziehl-Neelsen Stain for Acid-Fast Bacilli:

- **Procedure (Figure 9):**

i. Place the sample smeared slides upwards on a staining rack over the sink.
ii. Cover the slide with 1% carbol fuchsin using a filter.
iii. Heat the slide, moving the flame back and forth, from below until fumes appear, then stop heating.
iv. Leave the heated slide aside for 10 minutes.
v. Gently rinse the slide in water.
vi. Add 25% H_2SO_4 solution on to the slide and leave it for 3 minutes.
vii. Gently rinse the slide in water.
viii. Cover the slide with methylene blue solution for 60 seconds and rinse in water.
ix. Air dry the slide.

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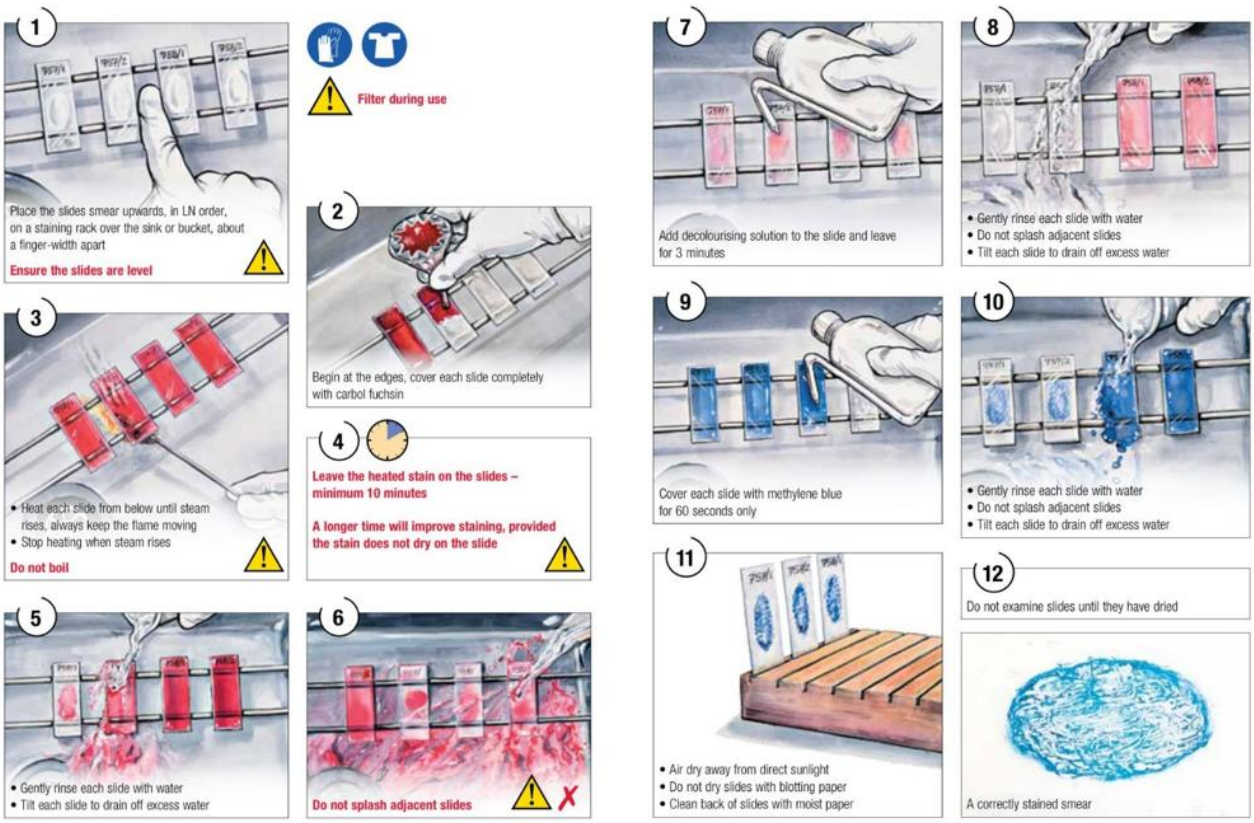

Figure 9: Ziehl Neelsen stain for acid-fast bacilli (AFB) staining procedure.[70]

- **Smear Examination:**
  - Place the smear facing upwards on the stage of light microscope.
  - Use 10x objective lens to focus the smear on purulent/mucoid material.
  - Apply an oil drop on the slide and observe under 100x oil objective lens.
- **Interpretation:**
  - Under oil immersion (100x), acid-fast bacilli (AFB) are red, slender rods occurring singly as V shaped forms or as clumps of bacilli.
  - Reporting and interpretation done as per table 3.

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TABLE 3: ZIEHL NEELSEN STAIN FOR ACID-FAST BACILLI (AFB) INTERPRETATION

Observation in Oil immersion (100x)	Reporting	Interpretation
No AFB in 100 fields	Negative	Negative
1-9 AFB in 100 fields	Scant	Positive
10-99 in 100 fields	1+	
1-10 AFB/field (in 50 fields)	2+	
>10 AFB/field (in 20 fields)	3+	

Statistical Analysis:

- Data were organized using Microsoft Excel, and statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS), Version 21 (IBM Corp., Armonk, NY, USA).
- Descriptive statistics were employed to summarize the variables: quantitative variables were expressed as mean and standard deviation, while qualitative variables were presented as frequencies and proportions.
- Inferential statistical analysis included the Chi-square test to assess associations between categorical variables.
- Diagnostic performance metrics—sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy—were calculated for both ZN-stained AFB smear test and plasma cfDNA test.
- A p-value of less than 0.05 was considered indicative of statistical significance
- The results of the MTB-cfDNA in plasma, ZN-stained AFB smear test in sputum samples as well as extrapulmonary samples, and GeneXpert assay were correlated.

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RESULTS

The present study was conducted at the Department of Pathology, B.L.D.E. (Deemed to be University), Shri B.M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka.

In this study, we evaluated the plasma collected from peripheral blood samples of 94 patients with a clinical suspicion for tuberculosis.

To determine the diagnostic accuracy of plasma cfDNA in tuberculosis, we compared the results of plasma cfDNA with results of GeneXpert assay (CBNAAT) and Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test in patients suspected for both pulmonary and extrapulmonary tuberculosis.

Here, we present an evaluation of the results of this study.

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TABLE 4: DISTRIBUTION OF THE PATIENTS BASED ON AGE GROUPS

Age Groups	Frequency	Percent
15 to 30 years	31	33
31 to 45 years	27	28.7
46 to 60 years	14	14.9
> 60 years	22	23.4
Total	94	100.0

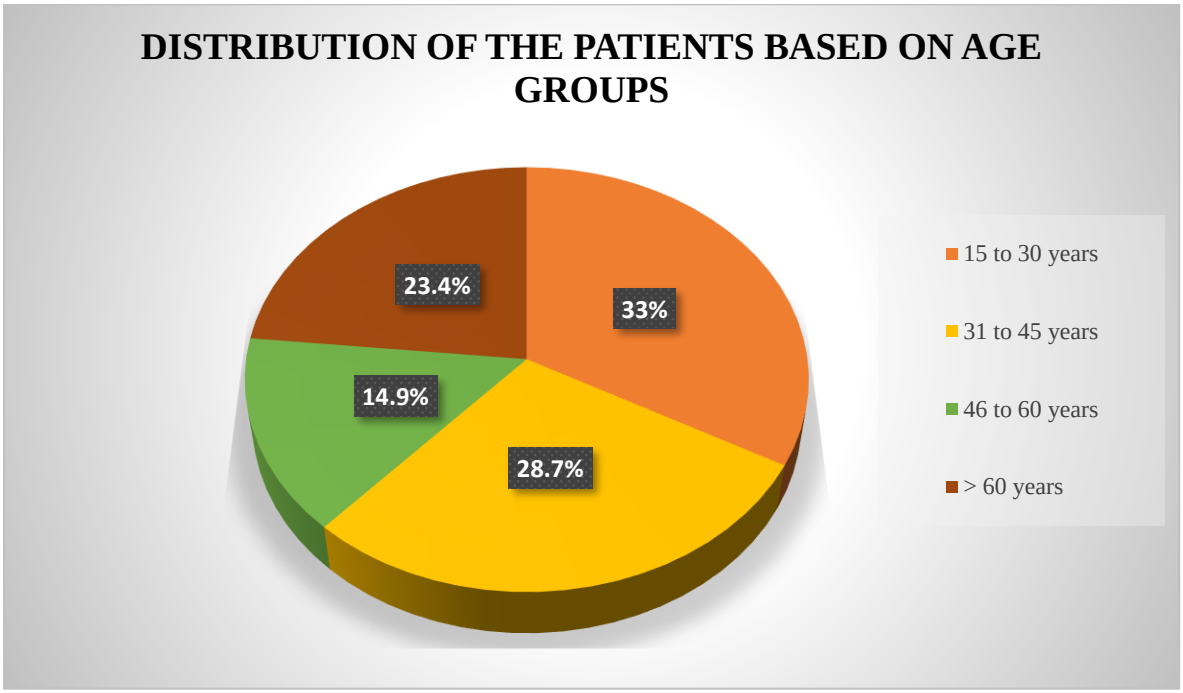

Figure 10: Distribution of the patients based on age groups.

INFERENCE: The majority of the patients (33%) belonged to the age group of 15 to 30 years and the age group of 46 to 60 years had the minority (14.9%).

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TABLE 5: MEAN AGE DISTRIBUTION OF THE PATIENTS

Parameter	N	Minimum	Maximum	Mean	S. D.
Age (years)	94	15.0	87.0	42.87	19.11

INFERENCE: The maximum and minimum ages of the patients are 87 years and 15 years, respectively.

TABLE 6: DISTRIBUTION OF THE PATIENTS BASED ON GENDER

Gender	Frequency	Percent
Females	39	41.5
Males	55	58.5
Total	93	100.0

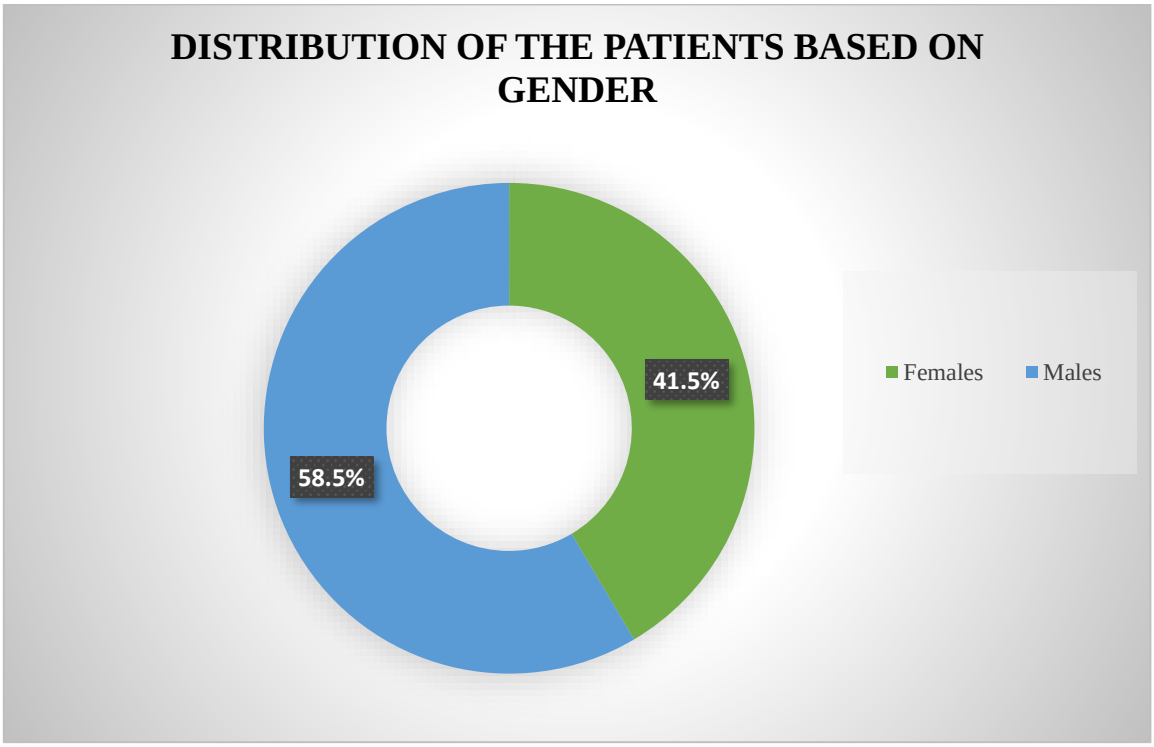

Figure 11: Distribution of the patients based on gender.

INFERENCE: The majority of the patients were males (58.5%) while female patients accounted for 41.5%.

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TABLE 7: DISTRIBUTION OF THE PATIENTS BASED ON SITE

Site		Frequency	Percent
Lung		74	78.7
Extra-pulmonary	Pre-auricular lymph node	1	1.1
	Axillary Lymph node	4	4.2
	Breast	2	2.1
	Cerebrospinal fluid	1	1.1
	Cervical lymph node	5	5.3
	Inguinal lymph node	1	1.1
	Pleural fluid	3	3.2
	Supraclavicular Lymph node	3	3.2
	Total	94	100

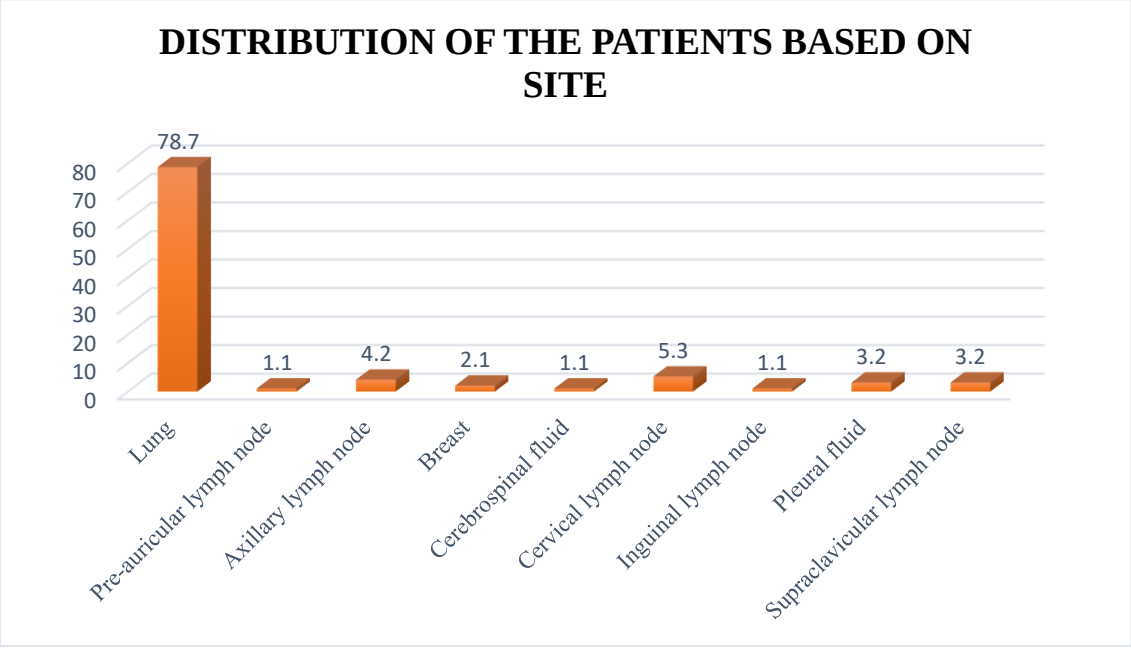

Figure 12: Distribution of the patients based on site.

INFERENCE:

Based on site, 74 cases (78.7%) had lung involvement and 20 cases (21.3%) had extrapulmonary involvement among which most common site was lymph node (14.9%).

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TABLE 8: DISTRIBUTION OF THE PATIENTS BASED ON CLINICAL DIAGNOSIS

Clinical Diagnosis	Frequency	Percent
Acute Mastitis	1	1.1
Chronic Bronchitis	8	8.5
Chronic Mastitis	1	1.1
Pleuritis	3	3.2
COPD	15	16.0
EPTB	7	7.4
LRTI	8	8.5
Pneumonia	13	13.8
PTB	23	24.5
Reactive Lymphadenitis	8	8.5
URTI	7	7.4
Total	94	100.0

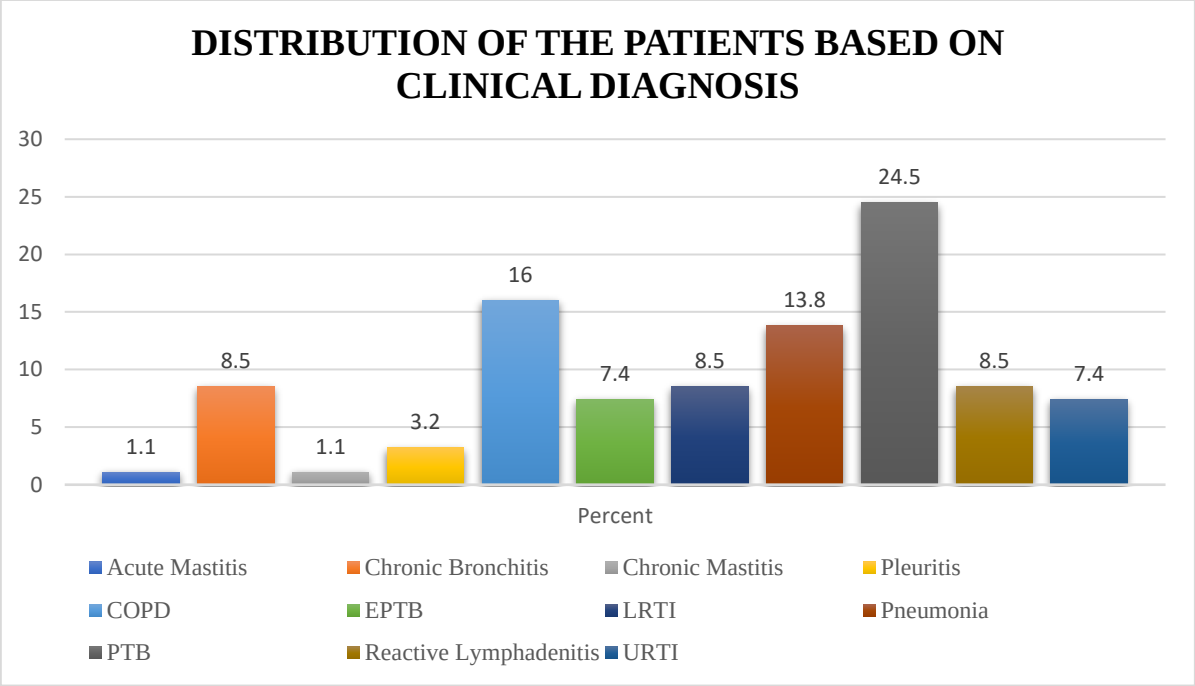

Figure 13: Distribution of the patients based on clinical diagnosis.

INFERENCE: The most common diagnosis was PTB in 23 patients (24.7%). 7 patients had EPTB (7.4%).

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TABLE 9: DISTRIBUTION OF THE PATIENTS BASED ON CBNAAT STATUS

CBNAAT	Frequency	Percent
Negative	64	68.1
Positive	30	31.9
Total	94	100.0

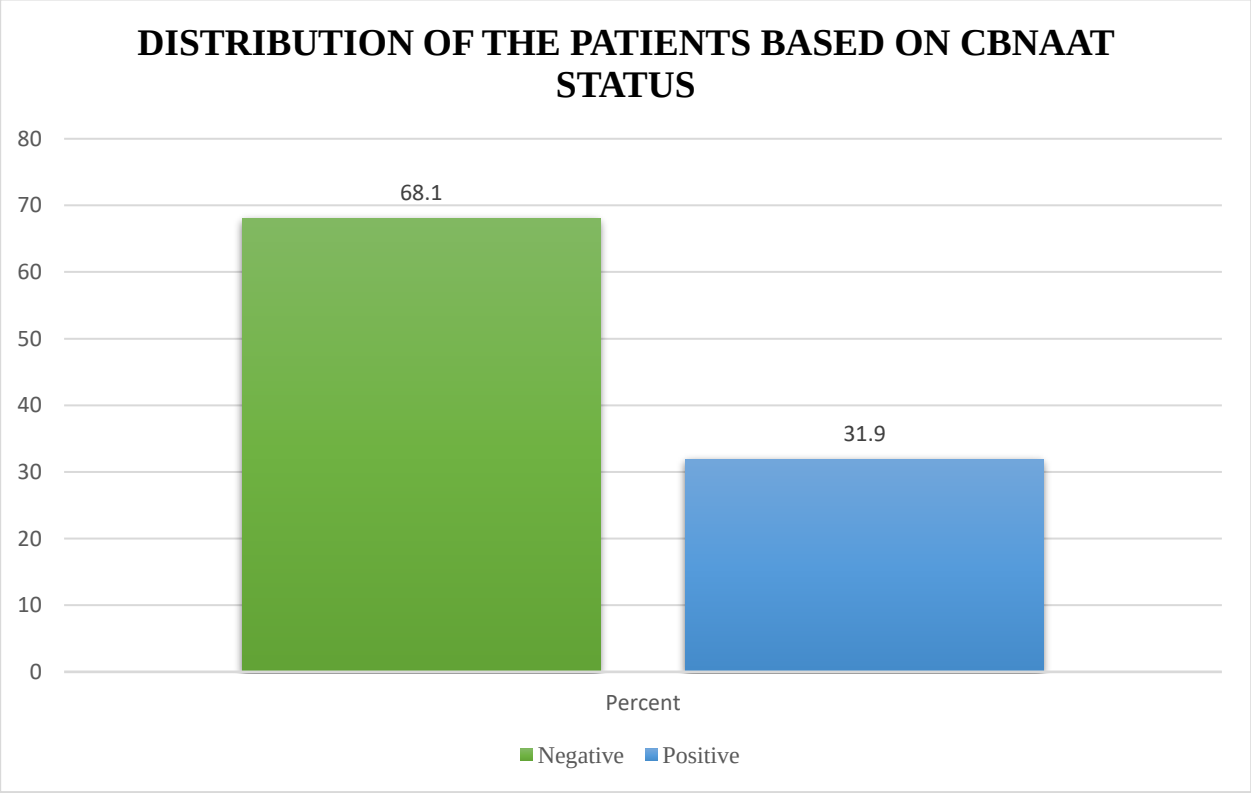

Figure 14: Distribution of the patients based on CBNAAT status.

INFERENCE:

Out of 94 patients, CBNAAT was positive in 30 patients (31.9%) and negative in 64 patients (68.1%).

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TABLE 10: DISTRIBUTION OF THE PATIENTS BASED ON SITE AND TUBECULOSIS (CBNAAT) STATUS

Site involved	TB (CBNAAT) Status	Frequency	Percent
Extra Pulmonary	Positive	7	7.4
	Negative	13	13.8
Pulmonary	Positive	23	24.5
	Negative	51	54.3
Total		94	100.0

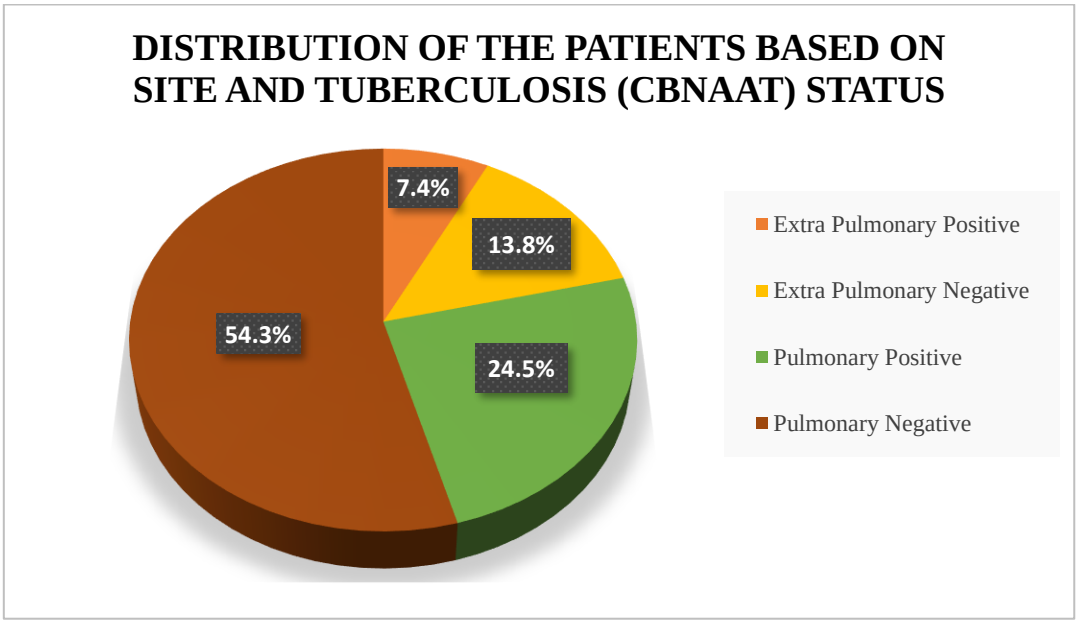

Figure 15: Distribution of the patients based on site and Tuberculosis (CBNAAT) status.

INFERENCE:

Out of 94 cases, 30 were positive on CBNAAT. Among these, 7 cases were EPTB and 23 cases were PTB.

Remaining 64 cases were negative on CBNAAT among which 51 were pulmonary cases and 13 were extrapulmonary cases.

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TABLE 11: DISTRIBUTION OF THE PATIENTS BASED ON ZN-STAINED AFB SMEAR TEST STATUS

ZN for AFB	Frequency	Percent
Negative	75	79.8
Positive	19	20.2
Total	93	100.0

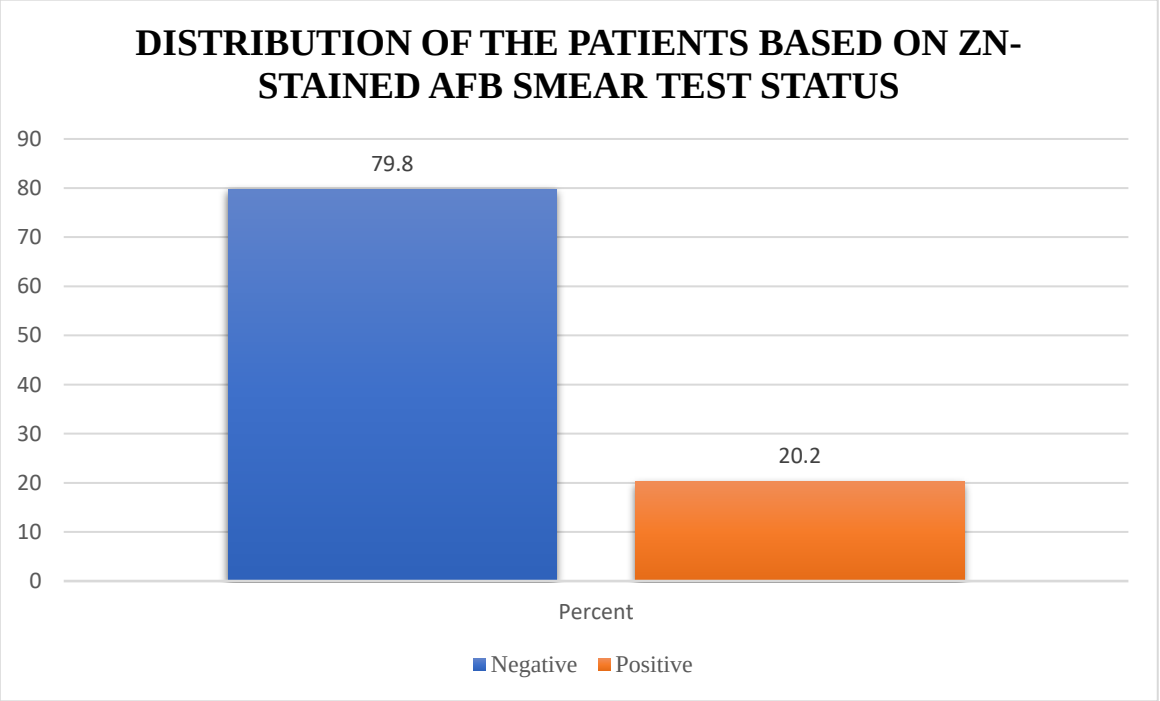

Figure 16: Distribution of patients based on ZN-stained AFB smear test status.

INFERENCE:

Out of 94 patients, Ziehl Neelsen stain for AFB was positive in 19 patients (20.2%) and negative in 75 patients (79.8%).

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TABLE 12: DISTRIBUTION OF THE PATIENTS BASED ON PLASMA cfDNA TEST STATUS

Plasma cfDNA	Frequency	Percent
Negative	63	67
Positive	31	33
Total	94	100.0

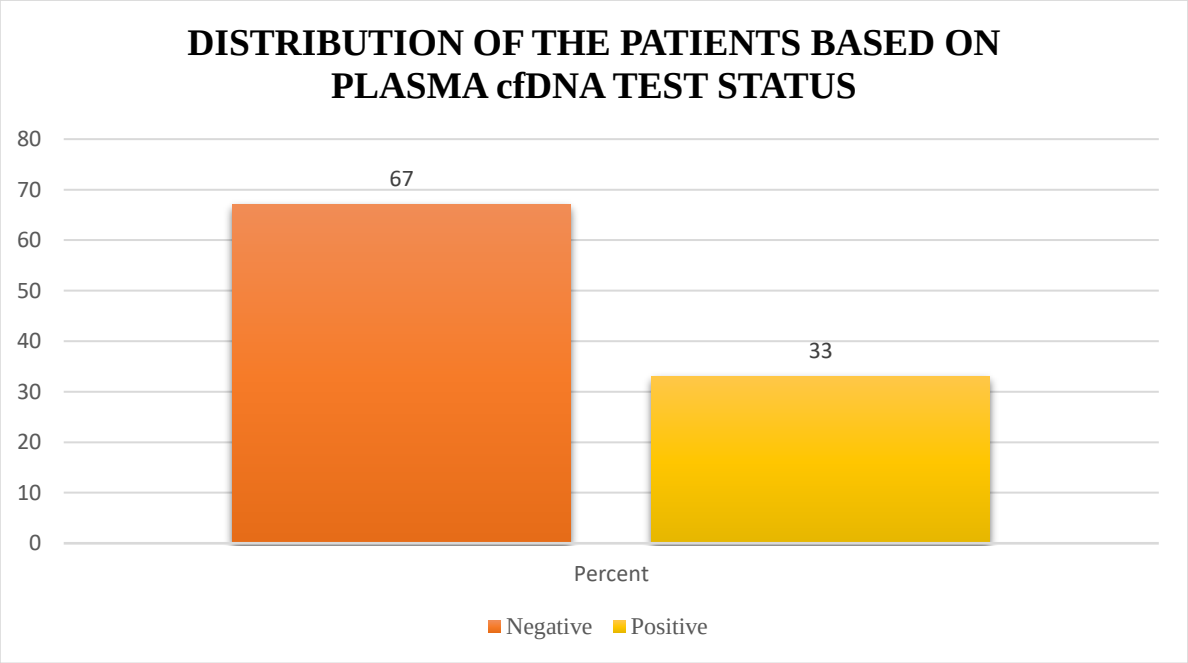

Figure 17: Distribution of the patients based on plasma cfDNA test status.

INFERENCE:

Out of 94 patients, plasma cfDNA test was positive in 31 patients (33%) and negative in 63 patients (67%).

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TABLE 13: CROSS TABULATION OF RESULTS: ZN-STAINED AFB SMEAR TEST AND CBNAAT

ZN for AFB	CBNAAT		Total
	Negative	Positive	
Negative	61	14	75
Positive	3	16	19
Total	64	30	94
Chi-square value-27.03			
p value-0.001*			

***significant**

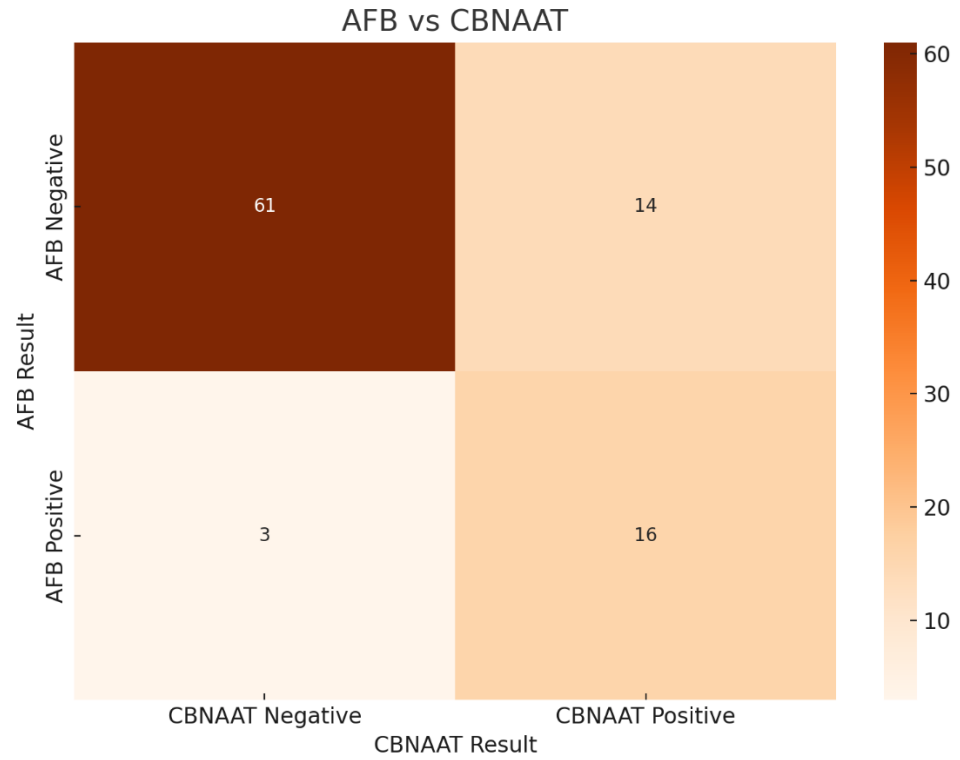

Figure 18: Distribution of results: ZN-stained AFB smear test vs CBNAAT.

INFERENCE: Out of 64 CBNAAT negative cases, 61 cases were ZN stain-negative for AFB.

Out of 30 CBNAAT positive cases, 16 cases were ZN stain-positive for AFB.

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TABLE 14: ZIEHL NEELSEN-STAINED AFB SMEAR TEST RESULT SUMMARY

Statistic Parameter	Value	95% CI
Sensitivity	53.3%	35.69% to 73.55%
Specificity	95.3%	86.91% to 99.02%
Positive Likelihood Ratio	11.34	3.72 to 37.26
Negative Likelihood Ratio	0.49	0.31 to 0.71
Positive Predictive Value	82.3%	62.75% to 94.41%
Negative Predictive Value	81.3%	75.74% to 87.58%
Accuracy	81.9%	73.57% to 89.83%

INFERENCE:

The cross-tabulation of Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test result and CBNAAT results demonstrates a significant association between the two diagnostic tests ($\chi^2 = 27.03$, $p = 0.001$).

Among the 94 cases, 75 were ZN stain-negative for AFB, 19 were ZN stain-positive for AFB.

Among the 64 CBNAAT-negative cases, 61 were ZN stain-negative for AFB. The specificity of ZN-stained AFB smear test was 95.3% (95% CI: 86.91% to 99.02%) indicating that it effectively ruled out tuberculosis in majority of the CBNAAT-negative cases.

Among the 30 CBNAAT-positive cases, 16 were ZN stain-positive for AFB. The sensitivity of ZN-stained AFB smear test was 53.3% (95% CI: 35.69%–73.55%), indicating that it correctly identified approximately half of CBNAAT-positive cases.

The diagnostic performance of ZN-stained AFB smear test compared to CBNAAT demonstrates its effectiveness in tuberculosis detection.

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The positive likelihood ratio (PLR) of 11.34 (95% CI: 3.72–37.26) suggests that a ZN-positive AFB result significantly increases the probability of true tuberculosis infection.

Conversely, the negative likelihood ratio (NLR) of 0.49 (95% CI: 0.31–0.71) implies that a negative result reduces the likelihood of disease but does not completely rule it out.

The positive predictive value (PPV) of 82.3% (95% CI: 62.75%–94.41%) indicates that most ZN-positive for AFB smear test cases are true positives, while the negative predictive value (NPV) of 81.3% (95% CI: 75.74%–87.58%) suggests that a ZN-negative for AFB smear test result is reliable.

Overall, Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test demonstrates an accuracy of 81.9% (95% CI: 73.57%–89.83%).

Docusign Envelope ID: 75DEF99A-38BB-47D8-9FA3-E7DC3D5B96BF

TABLE 15: CROSS TABULATION OF RESULTS: PLASMA cfDNA TEST AND CBNAAT

Plasma cfDNA	CBNAAT		Total
	Negative	Positive	
Negative	61	2	63
Positive	3	28	31
Total	64	30	94
Chi-square value-68.66			
p value-0.001*			

***significant**

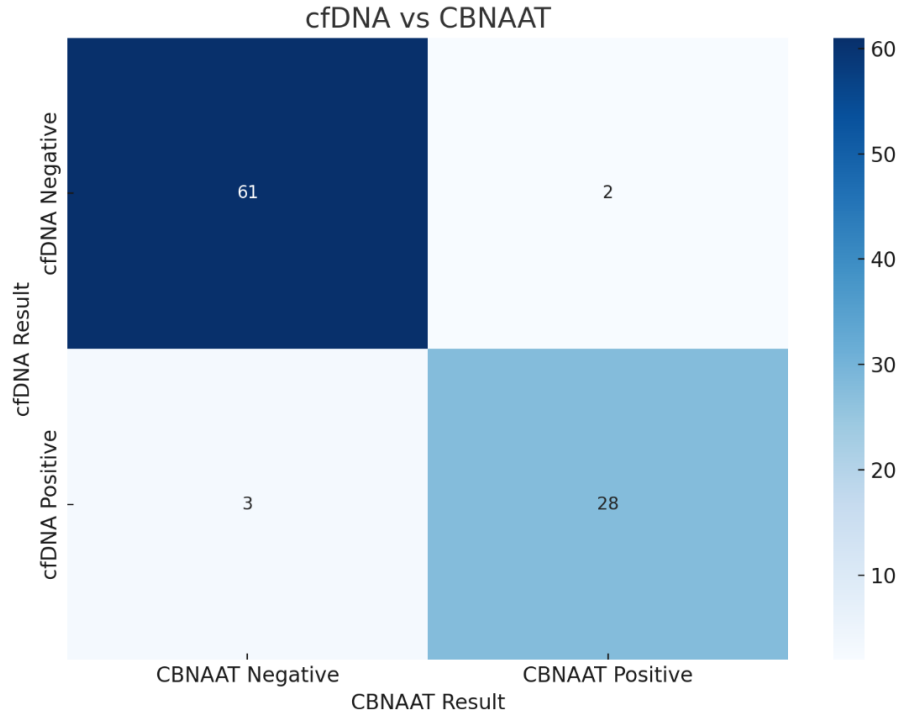

Figure 19: Distribution of results: Plasma cfDNA test vs CBNAAT.

INFERENCE:

Out of 64 CBNAAT negative cases, 61 were negative on plasma cfDNA test. 1 pulmonary case and 2 extrapulmonary cases that were negative on CBNAAT were positive on plasma cfDNA test. Out of 30 CBNAAT positive cases, 28 were positive on plasma cfDNA test. 2 CBNAAT-positive pulmonary cases were negative on plasma cfDNA test.

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TABLE 16: PLASMA cfDNA TEST RESULT SUMMARY

Statistic Parameter	Value	95% CI
Sensitivity	93.3%	77.23% to 99.15%
Specificity	95.3%	86.91% to 99.02%
Positive Likelihood Ratio	19.85	6.55 to 60.22
Negative Likelihood Ratio	0.07	0.02 to 0.28
Positive Predictive Value	90.3%	74.80% to 96.46%
Negative Predictive Value	96.8%	88.89% to 99.15%
Accuracy	94.68%	87.90% to 98.23%

INFERENCE:

The cross-tabulation of plasma cfDNA test and CBNAAT results shows a strong and significant association between the two diagnostic tests ($\chi^2 = 68.66$, $p = 0.001$).

Among the 94 cases, 63 were plasma cfDNA-negative and 31 were plasma cfDNA-positive.

Among 64 CBNAAT-negative cases, 61 were negative on plasma cfDNA test. The specificity of plasma cfDNA test was 95.31% (95% CI: 86.91%–99.02%), suggesting a strong ability to rule out tuberculosis in most CBNAAT-negative cases.

Additionally, among 30 CBNAAT-positive cases, 28 were positive on plasma cfDNA test. The sensitivity of plasma cfDNA test was 93.1% (95% CI: 77.23%–99.15%), indicating that it correctly identifies most CBNAAT-positive cases.

The positive likelihood ratio (PLR) of 19.85 (95% CI: 6.55–60.22) implies that a positive plasma cfDNA test result significantly increases the probability of true infection, while the negative likelihood ratio (NLR) of 0.07 (95% CI: 0.02–0.28) indicates that a negative result substantially

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reduces the likelihood of disease. The positive predictive value (PPV) of 90.3% (95% CI: 74.80%–96.46%) suggests that most plasma cfDNA test positive cases are true positives, whereas the negative predictive value (NPV) of 96.8% (95% CI: 88.89%–99.15%) highlights the high reliability of a negative result. With an overall accuracy of 94.68% (95% CI: 87.90%–98.23%), plasma cfDNA test proves to be a highly effective diagnostic tool when compared to CBNAAT.

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DISCUSSION

Tuberculosis (TB) remains a global health challenge, with a significant burden in India, where both pulmonary and extrapulmonary TB contribute to disease morbidity. Traditional diagnostic tools such as Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test have limitations in terms of sensitivity and specificity, whereas culture has long turnaround times. While GeneXpert assay (CBNAAT) is effective in diagnosing PTB, it has its own drawbacks especially in EPTB, where sample collection is invasive and it lacks the sensitivity that it has with PTB thus necessitating the need for alternative diagnostic markers such as cell-free DNA (cfDNA).

While MTB-cfDNA can be detected in various body fluids, as per literature review, plasma cfDNA had the highest diagnostic accuracy with the relative ease of sample collection.[64]

TABLE 17: DIAGNOSTIC ACCURACY OF MTB-cfDNA ACROSS SAMPLE TYPES[64]

Sample Type	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic Accuracy (%)
Plasma	78	97	99	82	98
Urine	44	89	86	68	67
CSF	55	100	100	49	77
Pleural Effusion	68	100	100	49	90
BAL Fluid	63	99	100	45	82
Ascitic Fluid	40	90	96	21	65

Hence, the present study was taken up to evaluate the diagnostic accuracy of plasma cfDNA in tuberculosis and correlate its findings with Ziehl Neelsen-stained AFB smear test and GeneXpert assay (CBNAAT).

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Comparison with Other Studies:

Age:

In the present study, the mean age of the patients was 42.87 years with majority belonging to the age group of 30 to 50 years. This was in concordance with the following studies where mean age was between 40 and 50 years.

TABLE 18: COMPARISON OF AGE DISTRIBUTION ACROSS STUDIES

Study	Mean Age (Years)	Age Range (Years)	Age Group Most Affected
Current Study	42	15-87	30-50
Condos R et al. (1996)[62]	50	18-80	50-70
Taci N et al. (2003)[63]	47	25-70	45-65
Che N et al. (2017)[10]	52	30-85	55-75
Yu G et al. (2021)[17]	48	22-78	40-65
Park JH et al. (2022)[64]	49	21-76	45-70
Du WL et al. (2024)[65]	46	20-73	40-60

Gender:

Tuberculosis (TB) continues to be a significant global health concern, disproportionately affecting men more than women. In 2022, adult men (aged ≥15 years) accounted for an estimated 5.8 million TB cases, representing 55% of the total, while adult women comprised 3.5 million cases (33%), and children (0–14 years) made up 1.3 million cases (12%).[71]

This disparity is further highlighted by the male-to-female (M:F) ratio of TB cases. Globally, the M:F ratio was 1.7 in 2020, indicating that 56% of all TB cases occurred in men, 33% in women, and 11% in children. Similarly, in 2023, 55% of the 8.2 million diagnosed TB cases were men, 33% were women, and 12% were children and young adolescents.[72]

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The higher prevalence and incidence of TB among men can be attributed to several factors:

1. **Biological Differences**: Men may have a higher susceptibility to developing active TB disease due to genetic and hormonal factors that influence immune responses.[73]
2. **Behavioural and Social Factors**: Men are more likely to engage in behaviours that increase TB risk, such as smoking and alcohol consumption. Additionally, occupational exposures and larger social networks can elevate the risk of TB transmission among men.[74]
3. **Healthcare Access**: In some regions, men may have better access to healthcare services, leading to higher detection rates. Conversely, in other areas, men might delay seeking care, resulting in more advanced disease at diagnosis.

In the present study, majority of the patients were male with a ratio of 1.41:1 (M:F). This was in concordance with several of the following studies, all of which had a higher male to female ratio of gender distribution.

TABLE 19: COMPARISON OF GENDER DISTRIBUTION ACROSS STUDIES

Study	Male (%)	Female (%)	Male-to-Female Ratio
Current Study	58.5	41.5	1.41:1
Condos R et al. (1996)[62]	65	35	1.85:1
Taci N et al. (2003)[63]	58	42	1.38:1
Che N et al. (2017)[10]	67	33	2.03:1
Yu G et al. (2021)[17]	60	40	1.5:1
Park JH et al. (2022)[64]	63	37	1.7:1
Du WL et al. (2024)[65]	61	39	1.56:1

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Diagnostic Accuracy of Cell-free DNA (cfDNA):

The results of this study indicate that plasma cfDNA testing demonstrated a sensitivity of 93.3% and specificity of 95.3%, making it a highly accurate diagnostic tool for TB detection.

Compared to ZN-stained AFB smear test, which showed a lower sensitivity of 53.3%, plasma cfDNA test exhibited superior performance in detecting *Mycobacterium tuberculosis* DNA.

The high negative predictive value (96.8%) of plasma cfDNA suggests its strong reliability in ruling out TB infection, especially in pulmonary TB where it showed 100% specificity when compared with CBNAAT.

The reference standard in the present study was CBNAAT (GeneXpert assay). To date, no published studies have directly compared diagnostic accuracy of plasma cfDNA testing against the GeneXpert assay alone. Majority of the existing studies have taken culture as the reference standard while few have taken CRS (composite reference standard) as the reference standard. Even though the results from these studies could not be correlated directly with the current study, the diagnostic accuracy of plasma cfDNA, especially, specificity, when compared with CBNAAT depicts a similar picture as with the diagnostic accuracy of plasma cfDNA test in other studies that have taken culture or CRS as reference standard.[17]

Plasma cfDNA test showed 100% sensitivity in extrapulmonary TB cases. It detected all the positive extrapulmonary TB CBNAAT cases. Additionally, there were two CBNAAT negative extrapulmonary TB cases that were positive on plasma cfDNA test. Since CBNAAT has a lower sensitivity for detecting extrapulmonary TB cases as per the current literature,[17] the two cases could very well be positive TB cases that were picked up on plasma cfDNA test.

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TABLE 20: COMPARISON OF DIAGNOSTIC ACCURACY OF MTB-cfDNA ACROSS STUDIES

Study	Sample Type	Reference Standard	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic Accuracy (%)
Current Study	Plasma	CBNAAT	93.3	95.3	90.3	96.8	94.68
Condos R et al. (1996)[62]	Peripheral Blood	Culture	75	92	91	67	88
Taci N et al. (2003)[63]	Peripheral Blood	Culture	72	90	89	64	85
Yu G et al. (2021)[17]	Plasma	CRS & Culture	78	97	96	82	97
Park JH et al. (2022)[64]	Plasma	IGRA & CRS	77	95	94	80	96
Du WL et al. (2024)[65]	Plasma	Xpert & Culture	83	99	98	85	99

TABLE 21: COMPARISON OF DIAGNOSTIC ACCURACY OF MTB-cfDNA IN PTB AND EPTB ACROSS STUDIES

Study	Reference Standard	Pulmonary		Extrapulmonary	
		Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Current Study	CBNAAT	91.3	98	100	84.6
Yu G et al. (2021)[17]	CRS & Culture	78	97	65	97
Park JH et al. (2022)[64]	IGRA & CRS	77	95	72	95
Du WL et al. (2024)[65]	Xpert & Culture	83	99	85	99

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Inference Across Other Studies:

Several other studies have evaluated the role of cfDNA in tuberculosis diagnosis, highlighting its potential as a rapid and non-invasive diagnostic tool:

Ushio et al. (2016) – Demonstrated that MTB-specific insertion sequence 6110 (IS6110)-cfDNA could be detected in plasma using droplet digital PCR (ddPCR), indicating its potential use in TB diagnosis.[15]

Che et al. (2017) – Examined cfDNA in pleural effusion samples and reported that cfDNA detection could improve the sensitivity of TB diagnosis in pleural TB patients.[10]

Click et al. (2018) – Found that cfDNA detection in plasma was feasible for TB diagnosis, but sensitivity improvements were required for clinical application.[16]

Sharma et al. (2020) – Investigated cfDNA in ascitic fluid and found it to be highly effective for diagnosing abdominal TB.[75]

Pollock et al. (2021) – Used metagenomic sequencing to detect cfDNA in plasma, showing its ability to identify M. tuberculosis in pediatric and adult TB cases.[76]

Pan et al. (2021) – Suggested that MTB-cfDNA could serve as an immune-associated microbial biomarker for TB detection and monitoring.[77]

Yu et al. (2021) – Conducted a meta-analysis and systematic review, confirming the high diagnostic accuracy of cfDNA across multiple studies, including both pulmonary and extrapulmonary TB.[17]

Huang et al. (2022) – Reported that CRISPR-mediated cfDNA detection significantly improved TB identification, particularly in pediatric and HIV-associated TB cases.[37]

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TABLE 22: COMPARISON OF DIAGNOSTIC ACCURACY OF PLASMA cfDNA TEST, ZN-STAINED AFB SMEAR TEST WITH CBNAAT

Test Type	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
ZN for AFB	53.3	95.3	82.3	81.3	81.91
cfDNA	93.3	95.3	90.3	96.8	94.68

The above comparison demonstrates that plasma cfDNA test shows superior sensitivity compared to ZN-stained AFB smear test. The likelihood ratios for cfDNA (PLR: 19.85, NLR: 0.07) further establish it as a robust diagnostic tool.

Future Directions:

The ability of plasma cfDNA to detect tuberculosis in a minimally invasive method presents a promising avenue for diagnosis, particularly in cases where sputum samples are difficult to obtain (e.g., pediatric and extrapulmonary TB).

Future studies should explore the role of cfDNA in treatment monitoring and prognosis assessment.

Moreover, standardized methodologies for cfDNA testing across different tuberculosis presentations should be developed to enhance clinical utility and widespread adoption.

While cfDNA-based MTB detection has demonstrated clinical utility, several challenges remain:

1. **Optimization of Sensitivity**:
    - Newer techniques like CRISPR-TB assays have shown higher sensitivity (96%),[78] warranting further exploration.
    - Use of IS6110 and IS1081 digital PCR assays could enhance detection in smear-negative cases.

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2. **Validation in Larger Cohorts**:
    - Future studies should validate cfDNA testing across diverse populations, including HIV-positive individuals and children, where TB diagnosis remains difficult.
3. **Integration with Machine Learning**:
    - AI-driven analysis of cfDNA levels could improve early TB detection and treatment monitoring.
4. **Expansion to Other Biofluids**:
    - Beyond plasma, cfDNA detection in cerebrospinal fluid (CSF), urine, and pleural fluid could expand its diagnostic applications.

Proposed Diagnostic Approach:

Based on our findings, we propose a tiered diagnostic algorithm integrating cfDNA testing into the current TB workflow:

1. **Suspected TB Case (Pulmonary or EPTB):**
    - **Step 1**: AFB Smear Test by Ziehl-Neelsen Stain → Low sensitivity, but rapid.
    - **Step 2**: GeneXpert/CBNAAT → Detects MTB and rifampicin resistance.
    - **Step 3**: cfDNA-PCR Testing → Useful for smear-negative, GeneXpert-negative, or EPTB cases.
2. **Smear-Negative & Extrapulmonary Cases:**
    - cfDNA testing in plasma/CSF/pleural fluid may improve diagnostic yield.
    - If positive → Proceed with TB treatment.
    - If negative but high suspicion → Consider additional tests (culture, biopsy).

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LIMITATIONS OF THE STUDY

1. **Limited Sample Size:**

   The study was conducted on a relatively small cohort of 94 clinically suspected TB cases, which may not fully represent the diversity of TB presentations in larger populations. A larger, multi-center study would strengthen the generalizability of findings.

2. **Lack of Culture-Based Confirmation for All Cases:**

   While CBNAAT (GeneXpert assay) was used as reference standard, culture remains the gold standard for TB diagnosis. The absence of culture-based confirmation for all cases may have influenced sensitivity and specificity estimates.

3. **Challenges in cfDNA Standardization:**

   cfDNA extraction and PCR-based detection require specialized laboratory infrastructure and standardized protocols, which may limit widespread implementation in resource-limited settings. Further standardization is required for routine clinical use.

4. **Limited Comparison with Other Molecular Tests:**

   The study compared cfDNA with AFB smear and CBNAAT/GeneXpert, but a direct comparison with newer technologies like NGS, ddPCR, or CRISPR-based diagnostic**s** was not performed. Such comparisons could better establish cfDNA's relative advantages and limitations.

5. **Limited Pediatric and Immunocompromised Cases:**

   Since these populations have unique diagnostic challenges, further studies are needed to evaluate cfDNA in these groups. This study primarily included adult TB cases.

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CONCLUSION

This study highlights the high specificity and sensitivity of plasma cfDNA testing in tuberculosis diagnosis making it a strong complementary tool rather than a standalone diagnostic method at present.

However, further advancements in NGS, CRISPR, and ddPCR technologies may enhance the sensitivity of cfDNA-based detection for diagnosis of tuberculosis in clinical settings.

While culture remains the gold standard, plasma cfDNA-based detection offers a minimally invasive, rapid, blood-based testing, which can be especially beneficial in pediatric patients, suspected extrapulmonary TB patients and in pulmonary tuberculosis patients with difficult expectoration.

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SUMMARY

Tuberculosis remains one of the leading infectious diseases worldwide, with early and accurate diagnosis being crucial for effective management and control. Traditional diagnostic methods such as Ziehl Neelsen-stained acid-fast bacilli (AFB) smear test and culture have their own limitations, particularly in smear-negative and extrapulmonary TB cases. While CBNAAT/GeneXpert assay has improved TB diagnostics, its sensitivity in detecting extrapulmonary TB remains suboptimal.

This study aimed to assess the diagnostic accuracy of plasma cfDNA in detecting MTB and its correlation with AFB smear test and GeneXpert assay. A total of 94 clinically suspected TB cases were analyzed, with plasma cfDNA extracted and tested for the IS6110 gene using qPCR.

Findings revealed that plasma MTB-cfDNA had a sensitivity of 93.3% and specificity of 95.31%, significantly outperforming ZN-stained AFB smear test (53.3% sensitivity). The study also found that plasma MTB-cfDNA testing could be particularly beneficial for detecting extrapulmonary TB in cases where CBNAAT was negative, making it a promising diagnostic alternative though this needs validation with culture (gold standard).

The results underscore the potential of plasma MTB-cfDNA as a rapid, minimally invasive, and highly accurate diagnostic tool. Its ability to detect both pulmonary TB and extrapulmonary TB cases highlights its utility in clinical settings where obtaining sputum samples is challenging. Future research should focus on standardizing cfDNA detection techniques and evaluating its efficacy in larger, diverse populations to integrate it into routine TB diagnostic workflows.

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ANNEXURE I

BLDE
(DEEMED TO BE UNIVERSITY)
Declared as Deemed to be University u/s 3 of UGC Act, 1956
Accredited with 'A' Grade by NAAC (Cycle-2)
The Constituent College
SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/ 937/2023-24 10/4/2023

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on **Saturday, 18th March, 2023 at 11.30 a.m. in the CAL Laboratory, Dept. of Pharmacology,** scrutinized the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student /Faculty members of this University /Ph.D. Student College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version synopsis of the thesis/ research projects has been accorded ethical clearance.

TITLE: "DIAGNOSTIC ACCURACY OF PLASMA cfDNA (CELL- FREE DNA) IN TUBERCULOSIS".

NAME OF THE STUDENT/PRINCIPAL INVESTIGATOR: DR.YOGESHWAR KALLA

NAME OF THE GUIDE: DR.SUREKHA B. HIPPARGI , PROFESSOR
DEPT. OF PATHOLOGY.

Dr. Santoshkumar Jeevangi
Chairperson
IEC, BLDE (DU),
VIJAYAPURA
Chairman,
Institutional Ethical Committee,
BLDE (Deemed to be University)
Vijayapura

Dr.Akram A. Naikwadi
Member Secretary
IEC, BLDE (DU),
VIJAYAPURA
MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103. Karnataka

Following documents were placed before Ethical Committee for Scrutinization.

- Copy of Synopsis/Research Projects
- Copy of inform consent form
- Any other relevant document

Smt. Bangaramma Sajjan Campus, B. M. Patil Road (Sholapur Road), Vijayapura - 586103, Karnataka, India.
BLDE (DU): Phone: +918352-262770, Fax: +918352-263303, Website: www.bldedu.ac.in, E-mail:office@bldedu.ac.in
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ANNEXURE – II

B.L.D.E. (DEEMED TO BE UNIVERSITY)

SHRI B.M. PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTER, VIJAYAPUR-586103

INFORMED CONSENT FOR PARTICIPATION IN DISSERTATION/RESEARCH

I, the undersigned, _____, S/O D/O W/O _____,aged____years, ordinarily resident of _____do hereby state/declare that Dr. YOGESHWAR KALLA of SHRI B. M. PATIL MEDICAL COLLEGE Hospital has examined me thoroughly on _____ at _____ (place) and it has been explained to me in my own language that I am suffering from _____ disease (condition) and this disease/condition mimic following diseases. Further Doctor informed me that he/she is conducting dissertation/research titled "DIAGNOSTIC ACCURACY OF PLASMA cfDNA (CELL-FREE DNA) IN TUBERCULOSIS" under the guidance of Dr. SUREKHA B. HIPPARGI requesting my participation in the study. Apart from routine treatment procedure, the pre-operative, operative, post-operative and follow-up observations will be utilized for the study as reference data.

Doctor has also informed me that during conduct of this procedure like adverse results may be encountered. Among the above complications most of them are treatable but are not anticipated hence there is chance of aggravation of my condition and in rare circumstances it may prove fatal in spite of anticipated diagnosis and best treatment made available. Further Doctor has informed me that my participation in this study help in evaluation of the results of the study which is useful reference to treatment of other similar cases in near future, and also, I may be benefited in getting relieved of suffering or cure of the disease I am suffering.

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The Doctor has also informed me that information given by me, observations made/ photographs/ video graphs taken upon me by the investigator will be kept secret and not assessed by the person other than me or my legal hirer except for academic purposes.

The Doctor did inform me that though my participation is purely voluntary, based on information given by me, I can ask any clarification during the course of treatment / study related to diagnosis, procedure of treatment, result of treatment or prognosis. At the same time I have been informed that I can withdraw from my participation in this study at any time if I want or the investigator can terminate me from the study at any time from the study but not the procedure of treatment and follow-up unless I request to be discharged.

After understanding the nature of dissertation or research, diagnosis made, mode of treatment, I the undersigned Shri/Smt _____ under my full conscious state of mind agree to participate in the said research/dissertation.

Signature of patient:

Signature of doctor:

Witness: 1.

2.

Date:

Place:

Docusign Envelope ID: 75DEF99A-38BB-47D8-9FA3-E7DC3D5B96BF

ಬಿ.ಎಲ್.ಡಿ.ಇ. (ವಿಶ್ವವಿದ್ಯಾಲಯವೆಂದು ಪರಿಗಣಿಸಲಾಗಿದೆ)

ಶ್ರೀ ಬಿ.ಎಂ. ಪಾಟೀಲ್ ಮೆಡಿಕಲ್ ಕಾಲೇಜು ಆಸ್ಪತ್ರೆ ಮತ್ತು ಸಂಶೋಧನಾ ಕೇಂದ್ರ, ವಿಜಯಪುರ-586103

ಪ್ರಬಂಧ/ಸಂಶೋಧನೆಯಲ್ಲಿ ಭಾಗವಹಿಸಲು ತಿಳುವಳಿಕೆಯುಳ್ಳ ಸಮ್ಮತಿ

ನಾನು, ಕೆಳಗೆ ಸಹಿ ಮಾಡಿರುವ, _____ , _____ ಅವರ ಮಗ/ಮಗಳು/ ಹೆಂಡತಿ,___ವರ್ಷದ ವಯೋಮಾನದ _____ನ ಸಾಮಾನ್ಯವಾಗಿ ನಿವಾಸಿಗಳು ಈ ಮೂಲಕ ಹೇಳುತ್ತೇನೆ/ಘೋಷಣೆ ಮಾಡುತ್ತೇನೆ. SHRI B. M. PATIL MEDICAL COLLEGE ಆಸ್ಪತ್ರೆಯು _____ ರಂದು _____ (ಸ್ಥಳ) ನಲ್ಲಿ ಡಾಕ್ಟರ್ YOGESHWAR KALLA ನನ್ನನ್ನು ಸಂಪೂರ್ಣವಾಗಿ ಪರೀಕ್ಷಿಸಿದೆ ಮತ್ತು ನಾನು _____ ಕಾಯಿಲೆಯಿಂದ ಬಳಲುತ್ತಿದ್ದೇನೆ (ಸ್ಥಿತಿ) ಮತ್ತು ಈ ರೋಗ/ಸ್ಥಿತಿಯು ಈ ಕೆಳಗಿನ ರೋಗಗಳನ್ನು ಅನುಕರಿಸುತ್ತದೆ ಎಂದು ನನ್ನ ಸ್ವಂತ ಭಾಷೆಯಲ್ಲಿ ನನಗೆ ವಿವರಿಸಲಾಗಿದೆ. ಮತ್ತಷ್ಟು ವೈದ್ಯರು ಅವರು ಡಾ SUREKHA B. HIPPARGI ಎಂಬ ಶೀರ್ಷಿಕೆಯಡಿಯಲ್ಲಿ "DIAGNOSTIC ACCURACY OF PLASMA cfDNA (CELL-FREE DNA) IN TUBERCULOSIS" ಎಂಬ ಪ್ರಬಂಧ/ಸಂಶೋಧನೆಯನ್ನು ನಡೆಸುತ್ತಿದ್ದಾರೆ ಎಂದು ನನಗೆ ತಿಳಿಸಿದರು, ನಾನು ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ವಿನಂತಿಸಿದರು. ವಾಡಿಕೆಯ ಚಿಕಿತ್ಸಾ ವಿಧಾನದ ಹೊರತಾಗಿ, ಪೂರ್ವ-ಆಪರೇಟಿವ್, ಆಪರೇಟಿವ್, ಪೋಸ್ಟ್-ಆಪರೇಟಿವ್ ಮತ್ತು ಫಾಲೋ-ಅಪ್ ಅವಲೋಕನಗಳನ್ನು ಅಧ್ಯಯನಕ್ಕಾಗಿ ಉಲ್ಲೇಖ ಡೇಟಾವಾಗಿ ಬಳಸಿಕೊಳ್ಳಲಾಗುತ್ತದೆ.

ಈ ಕಾರ್ಯವಿಧಾನದ ಸಮಯದಲ್ಲಿ ಇಂತಹ ಪ್ರತಿಕೂಲ ಪರಿಣಾಮಗಳನ್ನು ಎದುರಿಸಬಹುದು ಎಂದು ವೈದ್ಯರು ನನಗೆ ತಿಳಿಸಿದ್ದಾರೆ. ಮೇಲಿನ ತೊಡಕುಗಳ ಪೈಕಿ ಅವುಗಳಲ್ಲಿ ಹೆಚ್ಚಿನವು ಚಿಕಿತ್ಸೆ ನೀಡಬಹುದಾದವು ಆದರೆ ನಿರೀಕ್ಷಿತವಲ್ಲ ಆದ್ದರಿಂದ ನನ್ನ ಸ್ಥಿತಿಯು ಉಲ್ಬಣಗೊಳ್ಳುವ ಸಾಧ್ಯತೆಯಿದೆ ಮತ್ತು ಅಪರೂಪದ ಸಂದರ್ಭಗಳಲ್ಲಿ ಇದು ನಿರೀಕ್ಷಿತ ರೋಗನಿರ್ಣಯ ಮತ್ತು ಲಭ್ಯವಿರುವ ಉತ್ತಮ ಚಿಕಿತ್ಸೆಗಳ ಹೊರತಾಗಿಯೂ ಮಾರಕವಾಗಬಹುದು. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಭಾಗವಹಿಸುವಿಕೆಯು ಅಧ್ಯಯನದ ಫಲಿತಾಂಶಗಳನ್ನು ಮೌಲ್ಯಮಾಪನ ಮಾಡಲು ಸಹಾಯ ಮಾಡುತ್ತದೆ ಎಂದು ವೈದ್ಯರು ನನಗೆ ತಿಳಿಸಿದ್ದಾರೆ.

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ನಾನು ನೀಡಿದ ಮಾಹಿತಿ, ತನಿಖಾಧಿಕಾರಿಯು ನನ್ನ ಮೇಲೆ ತೆಗೆದ ಅವಲೋಕನಗಳು / ಛಾಯಾಚಿತ್ರಗಳು / ವೀಡಿಯೊ ಗ್ರಾಫ್‌ಗಳನ್ನು ಗೌಪ್ಯವಾಗಿ ಇರಿಸಲಾಗುತ್ತದೆ ಮತ್ತು ಶೈಕ್ಷಣಿಕ ಉದ್ದೇಶಗಳಿಗಾಗಿ ಹೊರತುಪಡಿಸಿ ನನ್ನನ್ನು ಹೊರತುಪಡಿಸಿ ಇತರ ವ್ಯಕ್ತಿ ಅಥವಾ ನನ್ನ ಕಾನೂನು ಬಾಡಿಗೆದಾರರಿಂದ ಮೌಲ್ಯಮಾಪನ ಮಾಡಲಾಗುವುದಿಲ್ಲ ಎಂದು ವೈದ್ಯರು ನನಗೆ ತಿಳಿಸಿದ್ದಾರೆ.

ನನ್ನ ಭಾಗವಹಿಸುವಿಕೆಯು ಸಂಪೂರ್ಣವಾಗಿ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದ್ದರೂ, ನಾನು ನೀಡಿದ ಮಾಹಿತಿಯ ಆಧಾರದ ಮೇಲೆ, ರೋಗನಿರ್ಣಯ, ಚಿಕಿತ್ಸೆಯ ವಿಧಾನ, ಚಿಕಿತ್ಸೆಯ ಫಲಿತಾಂಶ ಅಥವಾ ಮುನ್ನರಿವುಗೆ ಸಂಬಂಧಿಸಿದ ಚಿಕಿತ್ಸೆಯ / ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ನಾನು ಯಾವುದೇ ಸ್ಪಷ್ಟೀಕರಣವನ್ನು ಕೇಳಬಹುದು ಎಂದು ವೈದ್ಯರು ನನಗೆ ತಿಳಿಸಿದರು. ಅದೇ ಸಮಯದಲ್ಲಿ ನಾನು ಬಯಸಿದಲ್ಲಿ ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಭಾಗವಹಿಸುವಿಕೆಯಿಂದ ನಾನು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಹಿಂದೆ ಸರಿಯಬಹುದು ಅಥವಾ ತನಿಖಾಧಿಕಾರಿಯು ನನ್ನನ್ನು ಅಧ್ಯಯನದಿಂದ ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ವಜಾಗೊಳಿಸಬಹುದು ಆದರೆ ನಾನು ಬಿಡುಗಡೆ ಮಾಡಲು ವಿನಂತಿಸದ ಹೊರತು ಚಿಕಿತ್ಸೆ ಮತ್ತು ಅನುಸರಣೆಯ ಕಾರ್ಯವಿಧಾನವಲ್ಲ ಎಂದು ನನಗೆ ತಿಳಿಸಲಾಗಿದೆ.

ಪ್ರಬಂಧ ಅಥವಾ ಸಂಶೋಧನೆಯ ಸ್ವರೂಪ, ಮಾಡಲಾದ ರೋಗನಿರ್ಣಯ, ಚಿಕಿತ್ಸಾ ವಿಧಾನಗಳನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡ ನಂತರ, ನಾನು ನನ್ನ ಸಂಪೂರ್ಣ ಪ್ರಜ್ಞಾಪೂರ್ವಕ ಮನಸ್ಸಿನ ಅಡಿಯಲ್ಲಿ ಕೆಳಗೆ ಸಹಿ ಮಾಡಿದ ಶ್ರೀ/ಶ್ರೀಮತಿ _____ ಹೇಳಿದ ಸಂಶೋಧನೆ/ಪ್ರಬಂಧದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಒಪ್ಪುತ್ತೇನೆ.

ರೋಗಿಯ ಸಹಿ:

ವೈದ್ಯರ ಸಹಿ:

ಸಾಕ್ಷಿ: 1.

2.

ದಿನಾಂಕ:

ಸ್ಥಳ:

Docusign Envelope ID: 75DEF99A-38BB-47D8-9FA3-E7DC3D5B96BF

ANNEXURE III

PROFORMA

Name : **OP/IP No.** :

Age : **Sex** :

D.O.A. : **D.O.D.** :

Residence : **Occupation** :

Presenting Complaints :

Past history :

Personal history :

Family history :

Treatment history :

General physical examination:

Systemic examination: **Vitals:**

Cardiovascular system : PR: RR: BP:

Respiratory system :

Per Abdomen : **Clinical Diagnosis:**

Central nervous system :

Investigations:

a. AFB smear test :

b. Plasma MTB-cfDNA :

c. GeneXpert assay :

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MASTER CHART

CBNAAT-NEGATIVE PULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
1	74	F	Lung	COPD	Negative	Negative	Negative
2	87	M	Lung	COPD	Negative	Negative	Negative
3	59	M	Lung	Pneumonia	Negative	Negative	Negative
4	51	M	Lung	Pneumonia	Negative	Negative	Negative
5	74	F	Lung	Chronic Bronchitis	Negative	Negative	Negative
6	18	F	Lung	Pneumonia	Negative	Negative	Negative
7	72	F	Lung	Chronic Bronchitis	Negative	Negative	Negative
8	66	M	Lung	COPD	Negative	Negative	Negative
9	38	M	Lung	Pneumonia	Negative	Negative	Negative
10	69	M	Lung	COPD	Negative	Negative	Negative
11	22	M	Lung	Pneumonia	Negative	Negative	Negative
12	70	F	Lung	Chronic Bronchitis	Negative	Negative	Negative
13	37	M	Lung	Pneumonia	Negative	Negative	Negative
14	67	F	Lung	COPD	Negative	Negative	Negative
15	77	M	Lung	Chronic Bronchitis	Negative	Negative	Negative

Docusign Envelope ID: 75DEF99A-38BB-47D8-9FA3-E7DC3D5B96BF

CBNAAT-NEGATIVE PULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
16	75	F	Lung	Pneumonia	Negative	Negative	Negative
17	71	F	Lung	Pneumonia	Negative	Negative	Negative
18	50	M	Lung	Pneumonia	Negative	Negative	Negative
19	71	M	Lung	COPD	Negative	Negative	Negative
20	71	M	Lung	COPD	Negative	Negative	Negative
21	70	M	Lung	COPD	Negative	Negative	Negative
22	65	M	Lung	COPD	Negative	Negative	Negative
23	40	M	Lung	LRTI	Negative	Negative	Negative
24	32	M	Lung	Chronic Bronchitis	Negative	Negative	Negative
25	69	M	Lung	COPD	Negative	Negative	Negative
26	15	M	Lung	URTI	Negative	Negative	Negative
27	20	M	Lung	LRTI	Negative	Negative	Negative
28	38	M	Lung	Pneumonia	Negative	Negative	Negative
29	29	M	Lung	LRTI	Negative	Negative	Negative
30	52	M	Lung	Chronic Bronchitis	Negative	Negative	Negative
31	74	F	Lung	LRTI	Negative	Negative	Negative
32	52	M	Lung	COPD	Negative	Negative	Negative
33	69	F	Lung	COPD	Negative	Negative	Negative

Docusign Envelope ID: 75DEF99A-38BB-47D8-9FA3-E7DC3D5B96BF

CBNAAT-NEGATIVE PULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
34	42	M	Lung	LRTI	Negative	Negative	Negative
35	59	M	Lung	COPD	Negative	Negative	Negative
36	47	M	Lung	URTI	Negative	Negative	Negative
37	22	F	Lung	LRTI	Negative	Negative	Negative
38	39	M	Lung	Pneumonia	Negative	Negative	Negative
39	30	F	Lung	LRTI	Negative	Negative	Negative
40	66	M	Lung	COPD	Negative	Negative	Negative
41	26	M	Lung	URTI	Negative	Negative	Negative
42	37	M	Lung	Chronic Bronchitis	Negative	Negative	Negative
43	33	F	Lung	URTI	Negative	Negative	Negative
44	24	M	Lung	URTI	Negative	Negative	Negative
45	26	M	Lung	LRTI	Negative	Negative	Negative
46	42	M	Lung	Pneumonia	Negative	Positive	Negative
47	36	F	Lung	Pneumonia	Negative	Negative	Negative
48	29	M	Lung	URTI	Negative	Positive	Positive
49	35	M	Lung	Chronic Bronchitis	Negative	Negative	Negative
50	42	F	Lung	COPD	Negative	Negative	Negative
51	21	M	Lung	URTI	Negative	Negative	Positive

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CBNAAT-NEGATIVE EXTRAPULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
52	36	F	Pleural fluid	Pleuritis	Negative	Negative	Negative
53	70	F	Breast	Chronic Mastitis	Negative	Negative	Negative
54	15	F	Cervical lymph node	Reactive Lymphadenitis	Negative	Negative	Negative
55	21	F	Pleural fluid	Pleuritis	Negative	Negative	Negative
56	16	M	Inguinal lymph node	Reactive Lymphadenitis	Negative	Negative	Negative
57	15	F	Supraclavicular lymph node	Reactive Lymphadenitis	Negative	Negative	Negative
58	23	M	Axillary lymph node	Reactive Lymphadenitis	Negative	Positive	Negative
59	40	F	Breast	Acute Mastitis	Negative	Negative	Negative
60	46	F	Pleural fluid	Pleuritis	Negative	Negative	Negative
61	29	F	Cervical lymph node	Reactive Lymphadenitis	Negative	Negative	Negative
62	42	F	Cervical lymph node	Reactive Lymphadenitis	Negative	Negative	Positive
63	32	M	Cervical lymph node	Reactive Lymphadenitis	Negative	Negative	Positive
64	15	M	Auricular lymph node	Reactive Lymphadenitis	Negative	Negative	Negative

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CBNAAT-POSITIVE PULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
65	73	M	Lung	PTB	Positive	Positive	Positive
66	49	F	Lung	PTB	Positive	Positive	Positive
67	24	F	Lung	PTB	Positive	Positive	Positive
68	50	M	Lung	PTB	Positive	Positive	Positive
69	50	M	Lung	PTB	Positive	Positive	Positive
70	53	F	Lung	PTB	Positive	Negative	Negative
71	52	M	Lung	PTB	Positive	Positive	Positive
72	28	F	Lung	PTB	Positive	Positive	Positive
73	35	M	Lung	PTB	Positive	Positive	Positive
74	45	M	Lung	PTB	Positive	Negative	Negative
75	65	M	Lung	PTB	Positive	Positive	Positive
76	26	F	Lung	PTB	Positive	Positive	Positive
77	18	F	Lung	PTB	Positive	Negative	Positive

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CBNAAT-POSITIVE PULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
78	28	F	Lung	PTB	Positive	Negative	Positive
79	30	M	Lung	PTB	Positive	Positive	Positive
80	45	M	Lung	PTB	Positive	Positive	Positive
81	38	M	Lung	PTB	Positive	Negative	Positive
82	19	F	Lung	PTB	Positive	Positive	Positive
83	20	M	Lung	PTB	Positive	Negative	Positive
84	40	F	Lung	PTB	Positive	Negative	Positive
85	38	M	Lung	PTB	Positive	Negative	Positive
86	45	M	Lung	PTB	Positive	Negative	Positive
87	53	M	Lung	PTB	Positive	Positive	Positive

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CBNAAT-POSITIVE EXTRAPULMONARY CASES

S. No.	Age	Sex	Site	Clinical Diagnosis	CBNAAT	AFB	cfDNA
88	32	F	Axillary Lymph node	EPTB	Positive	Negative	Positive
89	38	F	Axillary Lymph node	EPTB	Positive	Negative	Positive
90	24	F	Supraclavicular Lymph node	EPTB	Positive	Positive	Positive
91	22	M	Supraclavicular Lymph node	EPTB	Positive	Negative	Positive
92	23	F	Cerebral	EPTB	Positive	Positive	Positive
93	24	F	Axillary Lymph node	EPTB	Positive	Negative	Positive
94	21	M	Cervical Lymph node	EPTB	Positive	Negative	Positive

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KEY TO MASTER CHART

Abbreviation	Full Form
S. No.	Serial number
CBNAAT	Cartridge based nuclei acid amplification test
cfDNA	Cell free deoxyribonucleic acid
AFB	Acid fast bacilli
F	Female
M	Male
COPD	Chronic obstructive pulmonary disease
LRTI	Lower respiratory tract infection
URTI	Upper respiratory tract infection
PTB	Pulmonary tuberculosis
EPTB	Extrapulmonary tuberculosis

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DR. YOGESHWAR KALLA

DIAGNOSTIC ACCURACY OF PLASMA cfDNA (CELL-FREE DNA) IN TUBERCULOSIS

BLDE University

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