COMPARATIVE ANALYSIS OF GENEXPERT AND LINE PROBE ASSAY FOR DETECTING *MYCOBACTERIUM TUBERCULOSIS* AT THE NATIONAL TUBERCULOSIS CONTROL CENTER IN NEPAL

A Dissertation Submitted to the **Department of Microbiology, National College,** Tribhuvan University, Kathmandu, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology

(Medical)

By

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DECLARATION

This dissertation entitled "**Comparative Analysis of GeneXpert and Line Probe Assay for Detecting *Mycobacterium tuberculosis* at The National Tuberculosis Control Center in Nepal**" has been submitted to the Central Department of Microbiology/Campus/College/ Institute of Science and Technology, Tribhuvan University (T.U.), for the partial fulfilment of the requirements to the degree of Master of Science in Microbiology. This dissertation is conducted under the supervision of Dr Era Tuladhar. This is an original report of my own research, has been conducted entirely by myself, and has not been submitted for any other degree or professional qualification. I have followed Tribhuvan University's current research ethics guidelines and accept responsibility for the conduct of the procedures in accordance with the University's rules and regulations.

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RECOMMENDATION

This is to certify that Mr. Soyuz Baral has completed this dissertation work entitled "**Comparative Analysis of GeneXpert and Line Probe Assay for Detecting *Mycobacterium tuberculosis* at the National Tuberculosis Control Center in Nepal**" as a partial fulfillment of the requirements of M. Sc. degree in Microbiology (Medical) under our supervision. To our knowledge this work has not been submitted to any other degree.

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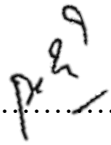

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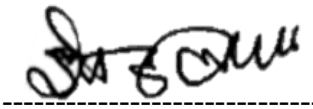
On the recommendation of Dr. Era Tuladhar, this dissertation work of Mr. Soyuz Baral entitled "**Comparative Analysis of GeneXpert and Line Probe Assay for Detecting *Mycobacterium tuberculosis* at the National Tuberculosis Control Center in Nepal** " has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirements for M. Sc. degree in Microbiology(Medical).



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This dissertation work (PRO-406) entitled "**Comparative Analysis of GeneXpert and Line Probe Assay for Detecting *Mycobacterium tuberculosis* at the National Tuberculosis Control Center in Nepal**" by **Mr. Soyuz Baral** (Symbol No. MB 1429/075 and T.U. Registration No. 5-2-366-44-2012 under the supervision of **Dr. Era Tuladhar** in the Department of Microbiology, National College is hereby submitted for the partial fulfillment of the Master of Science (M.Sc.) degree in Medical Microbiology.

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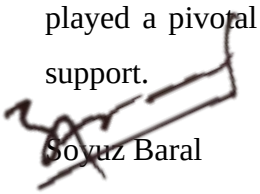

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ABSTRACT

Tuberculosis is a disease caused by *Mycobacterium tuberculosis*; a pathogenic bacterial species primarily known for causing chronic respiratory infections. In Nepal, tuberculosis (TB) remains a critical public health challenge, highlighting the urgent need for effective and rapid diagnostic tools. This study is important as it evaluates the effectiveness of GeneXpert and Line Probe Assay (LPA) compared to the culture method, potentially enhancing rapid TB detection and improving patient management. This hospital-based cross-sectional study was conducted from September 2022 to April 2023 at the National Tuberculosis Control Centre. The objective was to evaluate the diagnostic capabilities of GeneXpert and Line Probe Assay (LPA) for detecting *Mycobacterium tuberculosis* compared to the culture method, which is considered the gold standard. The research method involved collecting and analyzing sputum samples (n=398) using solid culture, GeneXpert and LPA. The culture method identified *Mycobacterium tuberculosis* in 128 cases. GeneXpert detected it in 129 cases, while LPA detected it in 123 cases. Against culture, GeneXpert demonstrated a sensitivity of 95.49% and a specificity of 99.22%, with results available in approximately 2 hours. The positive predictive value for GeneXpert was 98.45%, and the negative predictive value was 97.70%. In comparison, LPA showed a sensitivity of 90.98% and a specificity of 99.18%, with a turnaround time of up to 6 hours. The positive predictive value for LPA was 98.37%, and the negative predictive value was 95.29%. Additionally, Cohen's Kappa value for GeneXpert was 0.95, and for LPA, it was 0.92, indicating almost perfect agreement with the culture method. The findings suggest that GeneXpert offers higher sensitivity and faster results, making it valuable for rapid TB detection. Both assays exhibited high specificity, supporting their integration into routine TB diagnostics. This enhances rapid and accurate detection, thereby improving patient management and treatment outcomes.

Keywords: *Mycobacterium tuberculosis*, Tuberculosis, GeneXpert, Line Probe Assay, Culture

सोध सार

क्षयरोग (टीबी) मायकोब्याक्टेरियम ट्यूबरकुलोसिस (*Mycobacterium tuberculosis*) नामक रोगजनक ब्याक्टेरियाले गराउने रोग हो, जसले मुख्यतया श्वासप्रश्वास संक्रमण गराउँछ। नेपालमा क्षयरोग (टिबी) एक जल्दोबल्दो सार्वजनिक स्वास्थ्य चुनौतीको रूपमा रही आएको छ जसको नियन्त्रण गर्न प्रभावकारी तथा चुस्त निदान उपकरणहरूको आवश्यकता छ । यो अध्ययन महत्त्वपूर्ण छ किनकि यसले जीन एक्सपर्ट (GeneXpert) र लाइन प्रोब असे (Line Probe Assay) को प्रभावकारिता कल्चर विधिको तुलनामा मूल्याङ्कन गर्दछ र द्रुत टीबी पत्ता लगाउने र बिरामीको व्यवस्थापनलाई प्रभावकारी बनाउन मद्दत पुऱ्याउँछ। अस्पताल केन्द्रित यस क्रस-सेक्सनल अध्ययन सेप्टेम्बर २०२२ देखि अप्रिल २०२३ सम्म राष्ट्रिय क्षयरोग नियन्त्रण केन्द्र, थिमि, भक्तपुरमा गरिएको थियो । यसको उद्येश्य *Mycobacterium tuberculosis* पत्ता लगाउन Gold Standard को रूपमा चिनिने कल्चर विधिको तुलनामा GeneXpert र Line Probe Assay (LPA) को डायग्नोस्टिक क्षमताहरूको मुल्यांकन गर्नु रहेको थियो । यस अनुसन्धानमा GeneXpert तथा LPA ठोस कल्चर विधि अवलम्वन गरी ३९८ वटा खकारको नमूनाहरूको संकलन तथा विश्लेषण गरिएको थियो । कल्चर विधिले १२८ वटा माइकोब्याक्टेरियम क्षयरोग पत्ता लगाको थियो । त्यसैगरी GeneXpert ले १२९ तथा LPA ले १२३ वटा नमूनाहरूमा क्षयरोग पत्ता लगाएको थियो। कल्चरमा GeneXpert ले ९५.४९% को संवेदनशीलता (sensitivity) तथा ९९.२२ % को विशिष्टता (specificity) का साथ लगभग २ घण्टा मा परिणाम प्रदान गरेको थियो । GeneXpert को लागि सकारात्मक अनुमानित मुल्य ९८.४५ तथा नकारात्मक अनुमानित मुल्य ९७.७० कायम रहेको थियो । तुलनात्मक रुपमा भन्नुपर्दा LPA मा ६ घण्टा सम्मको टर्नअराउन्ड समयका साथमा ९०.९८ % को संवेदनशीलता र ९९.१८ % को विशिष्टता कायम रहेको थियो । LPA का लागि सकारात्मक अनुमानित मुल्य ९८.३७ तथा नकारात्मक अनुमानित मुल्य ९५.७० कायम रहेको थियो । साथै कोहेन काप्पा मुल्य (Cohen's Kappa value) GeneXpert को ०.९५ तथा LPA का लागि ०.९२ रहेको थियो जुन कल्चर विधिसंग पूरै मिल्दोजुल्दो थियो । यस अध्ययनले के देखाउँछ भने GeneXpert ले LPA को तुलनामा बढी संवेदनशीलता तथा छिटो नतिजा प्रदान गर्दछ रो यो सिघ्र क्षयरोग निदानका लागि महत्वपूर्ण छ । यी दुबै किसिमका परीक्षणहरूले उच्च विशिष्टता (high specificity) प्रदर्शन गर्दै नियमित क्षयरोग निदानका लागि उनीहरूको उपयोगितालाई पुष्टि गरेका छन् । यसले चुस्त तथा शुध्द किसिमले क्षयरोगको निदानका साथै विरामी व्यवस्थापन तथा उपचारको नतिजाहरूमा सुधार ल्याउँदछ ।

Keywords: क्षयरोग, जीन एक्सपर्ट(GeneXpert), Line Probe Assay(लाइन प्रोब असे), मायकोब्याक्टेरियम ट्यूबरकुलोसिस *(Mycobacterium tuberculosis)*

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ABBREVIATION

AFB:	Acid-Fast Bacilli
CDC:	Centers for Disease Control and Prevention
CT:	Computed Tomography
CXR:	Chest X-Ray
DNA:	Deoxyribonucleic Acid
DOTS:	Directly Observed Treatment, Short course
DST:	Drug Susceptibility Testing
EDTA:	Ethylenediaminetetraacetic Acid
EMB:	Ethambutol
HIV:	Human Immunodeficiency Virus
IFN:	Interferon
IGRA:	Interferon-Gamma Release Assays
INH:	Isoniazid
IL:	Interleukin
IV:	Intravenous
LAMP:	Loop-Mediated Isothermal Amplification
LJ:	Lowenstein-Jensen
LPA:	Line Probe Assay
MDR:	Multidrug-Resistant
MTB:	*Mycobacterium tuberculosis*
NAAT:	Nucleic Acid Amplification Tests
NPV:	Negative Predictive Value
PZA:	Pyrazinamide

POC:	Point-of-Care
TB:	Tuberculosis
TNF:	Tumor Necrosis Factor
XDR:	Extensively Drug-Resistant

CHAPTER I
INTRODUCTION AND OBJECTIVES

1.1. Background

Mycobacterium tuberculosis is the infectious agent that causes tuberculosis (TB), an illness that primarily affects the lungs but can also spread to other areas of the body (extra-pulmonary TB). Tuberculosis (TB) is transmitted via airborne particles when individuals with pulmonary TB release bacteria, often through actions such as coughing. Nationally, the predominant diagnostic technique for TB is sputum smear microscopy, which involves identifying bacteria in sputum samples using a microscope. In Nepal, the utilization of rapid molecular tests for the diagnosis of TB and drug-resistant TB is on the rise. There are two national reference laboratories equipped with sputum culture facilities. For new cases of drug-susceptible TB, the recommended treatment involves a six-month regimen of four first-line drugs (isoniazid, rifampicin, ethambutol, and pyrazinamide) administered in a fixed-dose combination. The cure rate for new TB cases reported in FY 2078/79 was 92.0%, with a treatment success rate of 91.5%. Managing multidrug-resistant tuberculosis (MDR-TB), which is resistant to isoniazid and rifampicin—the two most effective anti-TB drugs—requires a longer duration of treatment and involves the use of more expensive and toxic medications. According to the World Health Organization, the recommended regimens for most MDR-TB patients span 20 to 24 months. In FY 2078/79, a total of 659 rifampicin-resistant/multidrug-resistant TB (RR/MDR-TB) cases were registered. The overall treatment success rate for drug-resistant TB, including MDR, pre-XDR, and XDR, was recorded at 67% (Ministry of Health and Population, 2022).

Tuberculosis (TB) remains a significant public health issue in Nepal. Despite numerous efforts to control the disease, TB continues to pose a heavy burden, further complicated by the challenges in diagnosing cases using traditional methods such as Acid-Fast Bacillus (AFB) smear microscopy. The GeneXpert MTB/RIF assay introduces a new approach to overcome these diagnostic challenges. This molecular tool not only enables rapid detection of

Mycobacterium tuberculosis but also identifies rifampicin resistance, marking a substantial improvement over smear microscopy, which is notably less sensitive in HIV patients or those with low bacterial loads. Moreover, additional diagnostic tools like the Line Probe Assay (LPA) have been developed to further enhance TB diagnosis. LPA is a molecular technique that identifies *Mycobacterium tuberculosis* and resistance to key anti-TB drugs, offering high sensitivity and specificity. This complements the diagnostic abilities of GeneXpert, enhancing the overall detection and management of TB, particularly in complicated cases.(National Tuberculosis Control Center, 2020)

The urgency of integrating more effective diagnostic tools such as GeneXpert and LPA is underscored by the high prevalence of TB in Nepal and the strategic goals set forth by the National TB Program, which aligns with the World Health Organization's End TB Strategy. The guidelines emphasize the necessity of early detection and appropriate management to improve treatment outcomes and reduce transmission rates, particularly in AFB smear-negative cases which are often overlooked yet contribute substantially to the disease burden. (National Tuberculosis Center, 2019)

The National Tuberculosis Management Guidelines 2019 highlight several key advancements and strategic shifts in the approach to TB diagnosis and treatment in Nepal. These include reducing the number of required sputum samples for initial diagnosis, adopting same-day diagnosis protocols, and emphasizing the use of GeneXpert where available. Such advancements are pivotal in contexts where delayed diagnosis and treatment initiation significantly impede TB control efforts.

This research aims to evaluate the diagnostic accuracy and effectiveness of the GeneXpert MTB/RIF assay and the Line Probe Assay (LPA) in detecting tuberculosis and drug-resistant tuberculosis among the Nepalese population, thereby suggesting the possibility of integrating these methods into the healthcare system as alternatives to the slow gold standard culture method.

1.2. Objective

1.2.1. General Objective

- To compare the performance and feasibility of GeneXpert and Line Probe Assay (LPA) for the detection of *Mycobacterium tuberculosis* (MTB) at the National Tuberculosis Control Center in Nepal.

1.2.2. Specific Objectives

- To determine the diagnostic accuracy of GeneXpert and LPA in identifying *Mycobacterium tuberculosis* and rifampicin mono resistance using culture methods as the gold standard for validation.
- To analyze the sensitivity and specificity of GeneXpert and LPA in detecting *Mycobacterium tuberculosis* and rifampicin resistance.
- To investigate the agreement between GeneXpert and LPA with culture methods through statistical measures.

CHAPTER II
LITERATURE REVIEW

2.1. Tuberculosis

2.1.1. Introduction to Mycobacterium tuberculosis

Mycobacteria is an ancient genus of bacteria that has existed for millions of years, adapting to diverse environments such as water, soil, dust, and air (Bates & Stead, 1993; Donoghue, 2009; Ghodbane et al., 2014; Hruska & Kaevska, 2012). The analysis of mummified ibises and cats from Egyptian tombs supports their ancient origins and age, enhancing our understanding of historical practices and the significance of these animals in ancient Egyptian culture (Daniel, 2006; Konomi et al., 2002). The genus Mycobacterium includes several clinically significant species responsible for chronic pulmonary and extrapulmonary diseases (Barksdale & Kim, 1977; Hartmans et al., 2006; Koch, 1882; Saviola & Bishai, 2006). Named for their mold-like growth on liquid culture media, mycobacteria are non-motile, slightly curved rods that resist acid-alcohol decolorization after staining with phenolated fuchsin (Rastogi et al., 2001; Wayne, 1982). They belong to the Mycobacteriaceae family within the order Actinomycetales, which includes diverse microorganisms with significant ecological and morphological variation (Goodfellow & Wayne, 1982; Stackebrandt, 2002; Wayne, 1982). *Mycobacterium* is closely related to Corynebacterium, Nocardia, and Rhodococcus (Jarlier & Nikaido, 1994; Lechevalier et al., 1971; Wards et al., 1995), and differentiation involves analyzing carbon backbone length, unsaturated bonds, oxygenated functional groups, and esters produced during pyrolysis (Jarlier & Nikaido, 1994; Rastogi et al., 2001; Vincent Levy-Frebault & Portaels, 1992). The Ziehl-Neelsen stain, testing for acid-fastness, is widely used for rapid identification of *Mycobacterium* (Barksdale & Kim, 1977). On solid culture media, *Mycobacterium* species are distinguished by their color and morphology, with most forming whitish or cream-colored colonies, while rapid growers may produce bright yellow or orange colonies due to carotenoid pigments (Juhlin, 1967; McCready & Ratledge, 1978; Tarnok & Tarnok, 1970). Classification of pathogenic Mycobacterium relies on the presence of these pigments and their

ability to produce them either in the dark (scotochromogenic species) or in response to light (photochromogenic species) (Tarnok & Tarnok, 1970).

2.1.2. General characteristics of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis, responsible for tuberculosis, is characterized by its acid-fast, non-motile, and non-spore-forming nature (WHO, 2022b). It has a slow growth rate and thrives best at human body temperature, affecting various organs and causing both pulmonary and extra-pulmonary tuberculosis (WHO, 2022a). The bacterium's ability to persist latently in hosts contributes to its global challenge, with a large genome and diverse strains influencing its virulence and drug resistance (Cole et al., 1998; Coscolla & Gagneux, 2014). Its unique lipid-rich cell wall, including mycolic acids, plays a crucial role in its survival and pathogenicity (Brennan & Nikaido, 2003; Kaur et al., 2009). M. tuberculosis's complex metabolism and dormancy mechanisms further complicate treatment efforts (Beste et al., 2013; Wayne & Hayes, 1996). Understanding these characteristics is vital for combating tuberculosis effectively.

2.1.2.1. Historical background and discovery of *Mycobacterium tuberculosis*

Tuberculosis, historically known as phthisis, consumption, and the white plague, has long been associated with symptoms like wasting, cough, and fever, often proving fatal (WHO, 2021b) Early treatments emphasized rest, fresh air, and a nutritious diet, with the discovery in the late 1800s that UV rays could kill bacteria leading to the use of heliotherapy in sanatoriums (Loddenkemper et al., 2016). In 1882, Robert Koch's application of his postulates confirmed *Mycobacterium tuberculosis* as the causative agent, a landmark discovery aided by advancements in staining techniques (Hata & Ehrlich, 1910; Koch, 1882). Despite initial skepticism, Koch's findings established the bacterial nature of tuberculosis, revolutionizing its understanding and paving the way for diagnostic tests, sanatoriums, and effective treatments like antibiotics (Dubos & Dubos, 1987; Lönnroth & Raviglione, 2008).

2.1.3. Transmission and Infection of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis spreads through the air via respiratory droplets when infected individuals cough or sneeze, posing a risk of infection to susceptible persons (Van Zyl-Smit et al., 2012). Close contact with infectious individuals, crowded living conditions, poor ventilation, and compromised immune systems increase the transmission risk (Nahid et al., 2016). Once inhaled, *Mycobacterium tuberculosis* can infect the lungs and other organs, but not all exposed individuals develop active disease, influenced by factors like immune status and underlying health conditions (CDC, 2023). The bacterium's pathogenesis involves evading macrophage defenses, forming granulomas that can become necrotic and spread infection to other organs (Barry et al., 2009).

2.1.3.1. Mode of Transmission of Mycobacterium tuberculosis

Mycobacterium tuberculosis primarily targets the lungs but can infect any body part (Wells, 1934). The bacteria spread through airborne droplets from actions like coughing, sneezing, and speaking (Knechel, 2009; Loudon & Roberts, 1967). The most infectious droplets are about 5 μm in diameter and can stay airborne for extended periods. Factors influencing TB transmission include droplet bacilli count, virulence, UV light, ventilation, and aerosolization (Riley, 1957). Larger droplets settle in the upper respiratory tract, while 1-10 μm droplet nuclei can reach the lungs (Loudon & Roberts, 1967). Droplet nuclei from smear-positive TB patients can infect others, with infection rates of 25-50% among close contacts (Loudon & Roberts, 1967; Riley, 1957; Wells, 1934). Uncommon transmission methods include *Mycobacterium bovis* via unpasteurized milk and *Mycobacterium africanum* in West Africa, particularly affecting HIV patients (Ayele et al., 2004; Harris et al., 2007). Infection through contaminated fomites is rare, but handling infected tissues poses a risk to healthcare workers (Rouillon et al., 1976).

The deposition of M. tuberculosis in the lungs can result in four possible outcomes:

- rapid clearance of the bacteria,
- development of primary disease,
- latent infection, or

- reactivation of the disease after several years.

The outcome depends on factors like the number of organisms, their virulence, host factors, T-cell response, delayed immunity, and nutritional status (Schlossberg, 2017).

2.1.3.2. Risk factors for *Mycobacterium tuberculosis* infection

Tuberculosis (TB) can develop shortly after infection, especially in individuals with weakened immune systems. Approximately 5-10% of those with latent TB will develop active disease if untreated. Conditions such as HIV, diabetes, severe kidney disease, cancer treatments, and malnutrition increase the risk of TB. Those living in or traveling to areas with poor living conditions or drug-resistant strains face higher risks (CDC, 2023a; WHO, 2021a; Erhabor et al., 2010; Lönnroth et al., 2010; Zumla et al., 2015). Poverty and substance abuse also elevate TB risk due to weakened immune systems (Hossain et al., 2012; Oeltmann et al., 2009). Unstable living conditions and lack of employment further increase TB exposure (B. Chen et al., 2019). Healthcare workers are particularly susceptible due to frequent patient contact, especially with drug-resistant TB (Pai et al., 2016; Pai & Schito, 2015; WHO, 2021). Effective infection control and protective measures are crucial in healthcare settings (WHO, 2021).

2.1.3.3. Pathogenesis of *Mycobacterium tuberculosis* infection

Tuberculosis is characterized by granulomatous inflammation driven by type IV hypersensitivity reactions, involving activated T lymphocytes and their cytokines, which play a crucial role in halting disease progression by destroying bacilli (Ahmad, 2011; Algood et al., 2003a; Boyd, 1995; Thurlbeck & Churg, 1995). Upon inhalation, tubercle bacilli reach the terminal alveoli, triggering an immune response recognized by innate host defenses and involving T lymphocytes, macrophages, and other immune cells (Algood et al., 2003b; Thurlbeck & Churg, 1995). These cells collaborate to form granulomas, encapsulating necrotic tissue and restricting tubercle bacilli replication and spread.

Macrophages employ various mechanisms like reactive oxygen and nitrogen species production and autophagy to eliminate mycobacteria, influencing their

intracellular fate (Kundu & Thompson, 2008; van Crevel et al., 2002). Recognition of M. tuberculosis by the host involves Toll-like receptors, NLRs, and C-type lectins, initiating specific immune responses crucial for pathogen control (Harding & Boom, 2010; Jo et al., 2007). CD4+ T cells within granulomas regulate infection by inducing apoptosis of infected macrophages, producing cytokines such as IL-2, TNF-α, IL-10, IL-12, and IL-15, and activating macrophages via CD40 ligand (Algood et al., 2003a; Cooper, 2009). IFN-γ, essential in mycobacterial defense, is critical, as deficiencies in its production or receptors can lead to severe systemic infections (Dorman & Holland, 1998; Frucht & Holland, 1996).

2.1.4. Clinical Features of Mycobacterium tuberculosis

Tuberculosis, caused by *Mycobacterium tuberculosis*, progresses through several stages and presents varied clinical manifestations depending on immune status. Latent tuberculosis involves persistent bacilli in necrotic tissue, potentially reactivating under immune compromise (Jensen et al., 2005). Primary pulmonary tuberculosis is often asymptomatic, with diagnostic tests crucial for detection (American Thoracic Society and Centers for Disease Control and Prevention, 2000). Active tuberculosis develops in a minority, characterized initially by nonspecific symptoms and progressing to include cough, hemoptysis, and systemic signs (American Thoracic Society and Centers for Disease Control and Prevention, 2000). Extrapulmonary tuberculosis, affecting over 20% of immunocompetent patients and more in immunosuppressed individuals, includes severe forms like tubercular meningitis and miliary tuberculosis, requiring rapid diagnosis (American Thoracic Society and Centers for Disease Control and Prevention, 2000; CDC, 2023a). Lymphatic tuberculosis is common, with other sites including bones, joints, pleura, and genitourinary system (CDC, 2023a).

2.1.4.1. Types of Tuberculosis

The medical field classifies tuberculosis into two main types, namely pulmonary and extra-pulmonary. This division results in a total of 11 different types of tuberculosis, with four being attributed to pulmonary tuberculosis and the remaining seven to extra-pulmonary tuberculosis. Individuals with a

weakened immune system are more susceptible to extrapulmonary tuberculosis, which primarily affects this population.

- Pulmonary Tuberculosis
- Laryngeal Tuberculosis
- Cavitary TB
- Miliary TB
- TB Pleurisy
- Extrapulmonary Tuberculosis
- Adrenal Tuberculosis
- Lymph Node TB
- Osteal TB
- TB Peritonitis
- Renal TB
- TB Meningitis
- Pericardial TB(Gengenbacher & Kaufmann, 2012)

2.1.4.2. Symptoms of Mycobacterium tuberculosis infection

Depending on the disease's stage and the patient's immune system, the signs and symptoms of MTB infection can vary. Cough, exhaustion, fever, chest pain, weight loss, shortness of breath, night sweats, and loss of appetite are typical symptoms. Hemoptysis, joint discomfort, headaches, and confusion are less frequent symptoms. It is crucial to remember that TB symptoms might resemble those of other illnesses, therefore a doctor should be consulted for an accurate diagnosis and course of treatment.(CDC, 2023)

A persistent cough lasting over two weeks is a common symptom (Dheda et al., 2019), often producing sputum or blood and worsening in the morning (Escombe et al., 2007). Chronic fatigue and weakness are also common, leading to decreased daily activities (Dheda et al., 2019). A low-grade fever is another common symptom (Getahun et al., 2015), often accompanied by chills and night sweats (Lönnroth & Raviglione, 2008).

Chest pain, described as tightness or discomfort, is common and can be associated with coughing or breathing difficulties (Escombe et al., 2007). Unintentional weight loss often accompanies loss of appetite (Getahun et al., 2015; Lönnroth et al., 2009). Shortness of breath is also common and often

associated with coughing (Escombe et al., 2007). Night sweats can cause sleep disturbances (Kasper et al., 2015).

Hemoptysis, or coughing up blood, is less common but often associated with advanced disease (Hwang et al., 2022). Joint pain can occur in patients with extrapulmonary TB. Headache and confusion are also rare symptoms (Kasper et al., 2015).

The symptoms of TB vary depending on the individual's condition and the location of the infection in the body. Additionally, not all people infected with TB will exhibit symptoms. Regular screening and testing are recommended for individuals who are at high risk of TB infection.(CDC, 2023)

2.1.5. Diagnostic methods for *Mycobacterium tuberculosis* infection

2.1.5.1. Common Diagnostic Technique for Diagnosis of Tuberculosis

- Microscopic examination of sputum

Microscopic examination of sputum is a common diagnostic method for TB. This method involves staining sputum with the Ziehl-Neelsen (ZN) stain, which helps identify the presence of acid-fast bacilli (AFB). The test is simple, inexpensive, and can provide a result within hours. However, it has low sensitivity, and multiple samples may be required for an accurate diagnosis. (Zumla et al., 2015)

Auramine O fluorescent staining is another method for the detection of acid-fast bacilli in sputum samples. This technique has been shown to have higher sensitivity compared to ZN-staining, making it a preferred method for TB diagnosis in some settings. The technique involves the use of a fluorescent dye, Auramine O, which binds to AFB and emits bright yellow-green fluorescence under UV light. However, the technique requires fluorescent microscope and trained personnel, making it less accessible in resource-limited settings (N. P. Singh & Parija, 1998).

- Culture of *Mycobacterium tuberculosis*

Culture of *Mycobacterium tuberculosis* is an important diagnostic method for TB, as it is considered the gold standard for diagnosis. It involves growing the

bacteria on a specialized culture medium which can take several weeks to produce results. This method has high specificity and sensitivity, making it very reliable for TB diagnosis. However, it requires specialized laboratory infrastructure and expertise which may not be available in all settings. Additionally, it is time-consuming and can cause delays in treatment initiation, which can result in disease progression and transmission. Despite these limitations, culture remains an important diagnostic tool, particularly for confirming cases of drug-resistant TB and for monitoring treatment response (Boehme et al., 2010). Radiological imaging is often used in combination with other diagnostic methods such as microscopy and nucleic acid amplification assays to enhance diagnostic accuracy and expedite the diagnostic process.

Solid culture on Lowenstein-Jensen (LJ) media is a commonly used method for culturing *Mycobacterium tuberculosis*. The medium contains various nutrients, including glycerol, malachite green, and egg yolk, which provide an environment that supports the growth of the bacterium. The LJ method is inexpensive and easy to perform, but it can take up to 6-8 weeks to obtain results.

Another culture method is liquid culture, which involves using automated systems such as the BACTEC system. This method uses a liquid medium containing nutrients and growth factors that promote the growth of *M. tuberculosis*. The BACTEC system allows for rapid and automated detection of TB and can produce results within 2-3 weeks. However, it requires specialized equipment and is more expensive than solid culture methods (Martin et al., 2008).

Both solid and liquid culture methods have their advantages and disadvantages. Solid culture is simple and inexpensive, but it takes longer to obtain results. On the other hand, liquid culture is more rapid, but it requires specialized equipment and is more expensive.

Rageade et al., (2014) compared various culture media for TB detection and found that the BACTEC MGIT 960 System performed the best in terms of sensitivity, while Löwenstein–Jensen medium (LJ) had the best specificity. Liquid media showed faster times to positivity compared to solid media, but

different suppliers' media showed variations in performance, which could bias validation studies of new diagnostics. They also mentioned recent developments in TB culture, such as a colorimetric nitrate reductase assay and a novel biphasic culture medium that showed promising results for TB detection. (Rageade et al., 2014) emphasized the need for continued research and improvements in TB culture methods to improve the accuracy and speed of TB diagnosis.

- Nucleic acid amplification tests (NAATs)

Nucleic acid amplification tests (NAATs) are molecular diagnostic tools that detect the DNA of *Mycobacterium tuberculosis* in samples such as sputum, blood, or urine. These tests are rapid and highly sensitive, capable of providing results within hours. However, they are more costly than microscopy and have lower specificity compared to culture methods. (Steingart et al., 2014).

Nucleic acid amplification tests (NAATs) have transformed tuberculosis (TB) diagnosis by enabling rapid, accurate, and sensitive detection of *Mycobacterium tuberculosis* DNA in various clinical samples, including sputum, blood, and urine. NAATs operate by amplifying small amounts of TB DNA to detectable levels using techniques such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) (Horne et al., 2019; Zifodya et al., 2021).

- GeneXpert:
- LPA

- Serological Tests

Serological tests for tuberculosis, designed to detect antibodies against *Mycobacterium tuberculosis*, operate on the principle of antigen-antibody reaction. Despite being simple and quick, these tests suffer from low specificity, leading to false positives and an inability to distinguish between active and latent infections. Due to these limitations, the World Health Organization advises against using serological tests for TB diagnosis, as their use could increase costs and false-positive diagnoses. Consequently, the Indian health ministry has banned their import and sale, advocating for WHO-endorsed diagnostics instead (Steingart et al., 2012).

- Chest X-ray (CXR)

Chest X-ray (CXR) is a non-invasive, quick, and cost-effective tool for detecting pulmonary tuberculosis (TB), identifying lung abnormalities such as cavities, consolidation, nodules, infiltrates, and lymph node enlargement, and monitoring treatment response (Harris et al., 2019). However, CXR cannot differentiate between active and latent TB, may miss up to 25% of cases, and can yield false positives in non-TB lung diseases or prior TB scarring (WHO, 2016). Despite these limitations, CXR is widely used in resource-limited settings, often alongside other diagnostic methods like sputum smear microscopy and GeneXpert to improve accuracy (WHO, 2016). Ultimately, CXR's diagnostic accuracy relies on the radiologist's expertise and image quality, necessitating its use in combination with other tools and clinical information (Harris et al., 2019).

- Other diagnostic tools for tuberculosis (TB):
- Computed tomography (CT) scans:

CT scans offer detailed images of the lungs, which assist in identifying the location and extent of TB lesions. CT scans can also be used to detect extra-pulmonary TB, such as TB meningitis or TB in the spine (WHO, 2013).

- Bronchoscopy

Bronchoscopy implies the insertion of a compliant tube into the lungs to collect sputum or tissue samples for laboratory testing. This procedure can be useful for people who are unable to produce sputum or have sputum that is difficult to collect. It can also be used to diagnose TB in people with normal chest X-rays who have signs of TB or who have a positive interferon-gamma release assay (IGRA) test or TB skin test(CDC, 2022).

- Biopsy

Biopsy requires the removal of a small piece of tissue from the affected section for laboratory testing. This diagnostic tool is usually reserved for cases where other diagnostic methods have failed or where there is a need for a more accurate diagnosis, such as in the case of suspected TB lymphadenitis (Mayo Clinic, 2021).

2.1.5.2. Challenges and limitations in diagnosing *Mycobacterium tuberculosis* infection.

Diagnosing tuberculosis (TB) presents formidable challenges, primarily due to the limitations of traditional diagnostic methods like sputum smear microscopy and culture, which often yield low sensitivity and cannot distinguish between active and latent TB infections, thereby delaying treatment and increasing transmission risks (Migliori et al., 2020). Extrapulmonary TB (EPTB) diagnosis further complicates matters, requiring invasive procedures with lower sensitivity compared to pulmonary TB (PTB) diagnostics, such as AFB smear microscopy of cerebrospinal fluid (WHO, 2022a). Despite advancements like the GeneXpert system, access to reliable TB diagnostics remains constrained in resource-limited settings due to infrastructure challenges, funding gaps, and a shortage of trained personnel (Dheda et al., 2019; WHO, 2022b) The emergence of drug-resistant TB and its co-infection with HIV exacerbate diagnostic complexities, necessitating improved tools accessible across diverse healthcare settings (WHO, 2022a). Social stigma also hampers TB management efforts, highlighting the need for community-based interventions to address these barriers (Abebe et al., 2010; Nepal et al., 2012; Putera et al., 2015; Sharma et al., 2007)

2.1.6. Treatment and Prevention of *Mycobacterium tuberculosis*

Tuberculosis standard treatment lasts six months with a combination of drugs to prevent resistance (Gyselen, 2010). Drug susceptibility testing is essential for previously treated patients to identify and treat multidrug-resistant TB (MDR-TB), which has poor outcomes (WHO, 2021a). MDR-TB, resistant to isoniazid and rifampicin, requires complex and costly treatment with a success rate below 60% (Espindolaa et al., 2017). Healthcare settings must implement administrative controls to minimize TB transmission risk, including education and airborne precautions (CDC, 2023b). The rise of drug-resistant TB necessitates new antimycobacterial drugs and rapid molecular diagnostics to improve treatment access and outcomes (Allué-Guardia et al., 2021). However, drug resistance remains a significant obstacle, with many patients facing economic and social burdens (WHO, 2022a).

2.1.6.1. Anti-tuberculosis drugs and treatment regimens

The World Health Organization (WHO) recommends a combination of four first-line drugs—isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA)—for drug-susceptible tuberculosis (TB), while fluoroquinolones like levofloxacin and moxifloxacin are reserved for other TB types (CDC, 2023b). Treating drug-resistant TB involves second-line drugs, which are less effective, more toxic, and expensive (Ginsberg & Spigelman, 2007; Sotgiu et al., 2015). Drug resistance arises from poor adherence, incorrect prescriptions, and transmission of resistant strains, leading to multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) (Alipanah et al., 2018; Sotgiu et al., 2015). Mismanagement, poor quality drugs, and inadequate absorption contribute to resistance (WHO, 2022a). Adherence challenges necessitate new drugs like bedaquiline, pretomanid, linezolid, and moxifloxacin for MDR and XDR TB (Dartois & Rubin, 2022; Wallis et al., 2016) The bacille Calmette-Guérin (BCG) vaccine prevents severe TB in children but is less effective for adult pulmonary TB and has limited impact on transmission (CDC, 2023b; WHO, 2022a).

2.1.6.2. Adverse effects of anti-tuberculosis drugs

Anti-tuberculosis drugs, essential for treating TB, can cause adverse effects ranging from mild gastrointestinal disturbances and skin reactions to severe conditions like hepatitis and renal failure (Forget & Menzies, 2006; Saukkonen et al., 2006). Hepatotoxicity is particularly concerning, with symptoms such as nausea, jaundice, and dark urine necessitating prompt medical attention (Saukkonen et al., 2006). Early recognition of these side effects is crucial to reduce morbidity and mortality, as treatment interruptions can lead to failure (Saukkonen et al., 2006). Monthly evaluations are recommended to monitor for adverse events during TB treatment (CDC, 2023b). Studies indicate a high prevalence of adverse drug reactions among TB patients, with gastrointestinal intolerance, cutaneous reactions, and hepatotoxicity being most common. Severe side effects are more frequent in older patients, those with liver disease, or HIV infection. Awareness and monitoring of these side effects are vital, especially for first-line drugs like isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin (WHO, 2022a; Gyselen, 2010). Serious side

effects, including thrombocytopenia and renal failure, underscore the need for vigilance in TB treatment (Saukkonen et al., 2006; CDC, 2023b).

2.1.6.3. Preventive measures for *Mycobacterium tuberculosis* infection

Preventive measures for *Mycobacterium tuberculosis* infection aim to reduce the risk of initial infection and the progression to active tuberculosis (TB), which is crucial in high TB burden settings. The BCG vaccine is recommended for children in high TB burden countries to prevent severe forms of TB. Isoniazid preventive therapy (IPT) is advised for high-risk individuals, such as those with HIV and young children exposed to TB patients, reducing the risk of active TB by 60-90%. Effective infection control in healthcare settings, including rapid diagnosis, proper ventilation, and protective measures for healthcare workers, is essential. Active case finding and managing contacts of TB patients help detect and treat TB early, preventing further transmission. These multifaceted strategies, supported by WHO guidelines, are fundamental for global TB management (Stefan H. E. Kaufmann & Eric Rubin, 2008).

2.1.7. Epidemiology and Global Burden of *Mycobacterium tuberculosis*

Tuberculosis (TB) remains a significant global health challenge, with an estimated 10 million new cases and approximately 1.5 million deaths annually as of 2021 (WHO, 2022). The disease's global distribution is uneven, with the highest burdens in Asia and Africa, driven by socioeconomic factors, healthcare access, and co-infections like HIV (Zumla et al., 2015). Countries such as India, China, Indonesia, the Philippines, Pakistan, Nigeria, and South Africa account for about 60% of global TB cases, with India alone contributing nearly a quarter of the total cases (WHO, 2022a). These regions experience high TB incidence, primarily affecting adults in their most productive years, thereby perpetuating poverty and disease cycles (WHO, 2022a).

Controlling TB globally faces significant challenges, especially in high-burden countries. Strategies recommended by the World Health Organization (WHO) include enhancing TB surveillance, improving diagnostics, and expanding access to preventive and curative treatments. Recent advancements like point-of-care diagnostics, more effective drug regimens, and vaccines are crucial for

reducing TB transmission. Active case finding in high-burden settings is essential for early detection and treatment (WHO, 2022a). Integrated approaches that combine health system strengthening, social support, and technological innovations are necessary to overcome barriers to effective TB control (Alipanah et al., 2018; Jamison et al., 2017).

2.2. Prevalence of tuberculosis in Nepal

According to the National Strategic Plan to End Tuberculosis 2021/22-2024/26, the prevalence of tuberculosis (TB) in Nepal is significantly higher than previously estimated. The National Tuberculosis Prevalence Survey conducted in 2017/18 revealed a TB prevalence rate of 416 per 100,000 population, which is 1.8 times higher than earlier estimates by WHO. The revised incidence rate was 245 per 100,000, 1.6 times higher than previously estimated. Additionally, TB mortality rates were re-estimated to be 3.3 times higher than earlier figures (National Tuberculosis Control Center, 2022).

The high burden of TB in Nepal is attributed to several factors, including inadequate access to healthcare, social stigma, and the presence of multidrug-resistant TB strains. These factors make difficult the efforts to control and eliminate TB from the region.

Epidemiological Data:

Prevalence: 416 per 100,000

Incidence: 245 per 100,000

Mortality: Significantly higher than previously estimated

Drug Resistance: 1.57 times higher than previous estimates

The survey also pointed out that the notification rates of new TB cases vary significantly across different geographic areas in Nepal, with the lowest rates found in the mountain areas and the highest in the Terai plains. This geographic variance in TB incidence suggests the need for region-specific strategies to manage and control TB effectively.

The 2018/19 survey data underscores the importance of utilizing more effective technologies for the diagnosis of TB to support the national strategy to end TB.

These technologies include the use of digital X-rays as a primary screening tool and rapid diagnostic tools like Xpert MTB/RIF testing, which can help increase TB case detection, particularly in remote areas where access to quality healthcare is limited (National Tuberculosis Control Center, 2022).

2.3. Culture Method

The culture method for diagnosing tuberculosis (TB) is a traditional yet essential microbiological technique used to isolate and identify *Mycobacterium tuberculosis* (MTB) from clinical specimens. This method involves the cultivation of MTB in a suitable growth medium under controlled laboratory conditions to allow its growth and subsequent detection.

The process typically begins with the collection of respiratory specimens such as sputum, bronchoalveolar lavage fluid, or tissue samples from suspected TB patients. These specimens are then processed to decontaminate and concentrate MTB organisms before inoculating onto solid or liquid culture media.

2.3.1. Principles and Technology

The culture method relies on the principle of cultivating MTB in a controlled environment that supports its growth. Traditionally, solid media such as Lowenstein-Jensen (LJ) or Middlebrook 7H10/7H11 agar and liquid media like Middlebrook 7H9 broth are used. These media provide nutrients and conditions optimal for the slow-growing MTB to proliferate over several weeks.

The detection of MTB growth in culture is primarily based on visual inspection for characteristic colony morphology on solid media or by monitoring turbidity or fluorescence in liquid media. Confirmatory tests, including acid-fast staining and molecular techniques like PCR, are often employed to confirm the presence of MTB and differentiate it from other mycobacterial species.

Advantages of Culture Method over Traditional Methods

The culture method offers several advantages in TB diagnosis compared to methods like sputum smear microscopy:

Sensitivity: It has higher sensitivity, especially for paucibacillary samples and cases missed by smear microscopy.

Specificity: It allows for the identification of MTB species and differentiation from non-tuberculous mycobacteria.

Comprehensive: It provides a platform for drug susceptibility testing (DST), crucial for guiding appropriate treatment regimens.

Precision: Culture techniques ensure reproducibility and reliability in diverse laboratory settings.

Clinical validation has underscored the culture method's utility in TB management. It serves as a gold standard for TB diagnosis, particularly in confirming cases missed by other methods. Culture-based DST guides individualized treatment, crucial for managing drug-resistant TB (WHO, 2021a).

2.4. GeneXpert MTB/RIF assay

The GeneXpert MTB/RIF assay, developed by Cepheid, is a molecular diagnostic test designed for the simultaneous detection of *Mycobacterium tuberculosis* (MTB) and rifampicin resistance. This assay utilizes real-time polymerase chain reaction (PCR) technology and targets specific regions of the *rpoB* gene of MTB, which is linked to rifampicin resistance (Miller et al., 1994).

The assay is fully automated and cartridge-based, meaning that all necessary reagents and controls are contained within a single disposable cartridge. This self-contained nature simplifies the testing process and reduces the risk of contamination. The GeneXpert MTB/RIF assay provides rapid and accurate results in less than 2 hours, making it an invaluable tool for diagnosing tuberculosis and identifying rifampicin resistance, a key indicator of multidrug-resistant TB.

Overall, the GeneXpert MTB/RIF assay offers several advantages over traditional TB diagnostic methods, including speed, accuracy, simplicity, and the ability to detect both MTB and rifampicin resistance in a single test. Its performance characteristics have been extensively validated, demonstrating high sensitivity, specificity, and clinical utility in the diagnosis of TB and drug-resistant TB.

2.4.1. Principles and technology

The GeneXpert MTB/RIF assay is a molecular diagnostic test designed for the simultaneous detection of *Mycobacterium tuberculosis* (MTB) and rifampicin resistance. This assay operates on the principle of real-time polymerase chain reaction (PCR) technology, specifically utilizing hemi-nested real-time PCR. By targeting the *rpoB* gene of MTB, which is associated with rifampicin resistance, the assay amplifies and detects specific regions of the gene to identify the presence of MTB and mutations indicative of rifampicin resistance.

The technology behind the GeneXpert MTB/RIF assay is fully automatic and cartridge-based, requiring minimal hands-on manipulation. The assay is self-contained, meaning that all necessary reagents and controls are included within the cartridge, eliminating the need for complex laboratory procedures. The system provides results in less than 2 hours, making it a rapid and efficient tool for diagnosing TB and rifampicin resistance.

In the GeneXpert system, five probes (A, B, C, D, and E) target specific regions within the *rpoB* gene, which is associated with rifampicin resistance in *Mycobacterium tuberculosis* (MTB).

The system sets a valid maximum cycle threshold (C_t) of 39.0 for Probes A, B, and C, and 36.0 for Probes D and E. The detection and analysis process hinges on the C_t values of these probes. The "earliest probe" is the one that reaches its C_t value first, indicating it detected the target sequence sooner, while the "latest probe" reaches its Ct value last.

"MTB DETECTED" is reported when at least two probes have C_t values within the valid range and a $\Delta C_{t\ min}$ (the smallest C_t difference between any pair of probes) of less than two.

"Rif Resistance NOT DETECTED" is reported if the $\Delta C_{t\ max}$ (the C_t difference between the earliest and latest probe) is $\leq$ 4, whereas "Rif Resistance DETECTED" is reported if the $\Delta C_{t\ max}$ is >4.0.

"Rif Resistance INDETERMINATE" is reported when two conditions are met:

- the C_t value of any probe exceeds the valid maximum C_t (or is zero, i.e., no threshold crossing), and

- the earliest *rpoB* C_t value is greater than the valid $C_{t\,max}$ of the probe that exceeded its limit, minus the $\Delta C_{t\,max}$ cut-off of 4.0.

"MTB NOT DETECTED" is reported when there is only one or no positive probe.

These settings are integrated as automatic calculations in the Xpert® MTB/RIF Assay protocol and cannot be modified by the user .

2.4.2. Advantages of GeneXpert MTB/RIF assay over traditional methods

The GeneXpert MTB/RIF assay offers several advantages over traditional methods of TB diagnosis, such as sputum smear microscopy and culture-based techniques:

Speed: The assay provides rapid results within hours, enabling prompt initiation of appropriate treatment.

Accuracy: The assay demonstrates high sensitivity and specificity for detecting MTB and rifampicin resistance, reducing the likelihood of false-negative or false-positive results.

Simplicity: The assay is user-friendly and requires minimal training, making it suitable for use in resource-limited settings.

Automation: The fully automated nature of the assay reduces the potential for human error and standardizes the testing process.

Simultaneous Detection: The ability to detect both MTB and rifampicin resistance in a single test simplifies the diagnostic process and expedites treatment decisions.

2.4.3. Performance characteristics of GeneXpert MTB/RIF assay

The performance characteristics of the GeneXpert MTB/RIF assay have been extensively studied and validated, demonstrating its reliability and accuracy:

Sensitivity: The assay has shown high sensitivity for detecting MTB, even in smear-negative samples, improving case detection rates.

Specificity: The assay exhibits high specificity for identifying rifampicin resistance mutations, reducing the need for confirmatory testing.

Accuracy: Comparative studies have confirmed the assay's accuracy in detecting both MTB and rifampicin resistance, aligning with reference standard methods.

Precision: The assay's reproducibility and repeatability have been established, ensuring consistent results across different testing conditions.

Clinical Utility: Clinical validation trials have highlighted the assay's clinical utility in diagnosing TB and rifampicin resistance, leading to improved patient outcomes.

The GeneXpert MTB/RIF assay marks a significant advancement in TB diagnostics by offering a reliable, rapid, and comprehensive method for detecting MTB and rifampicin resistance. Its excellent performance characteristics make it an essential tool in the fight against tuberculosis, particularly in environments where timely and accurate diagnosis is critical for effective patient management and disease control (WHO, 2014a).

2.5. Line Probe Assay

2.5.1. Principles and technology

The Line Probe Assay (LPA) applies molecular genetics principles to detect specific mutations in the *Mycobacterium tuberculosis* genome that confer drug resistance. Utilizing polymerase chain reaction (PCR) amplification followed by reverse hybridization, LPA identifies mutations in target genes linked to resistance to antibiotics like rifampicin and isoniazid. This method offers a rapid and accurate approach to guiding effective treatment strategies (Barnard et al., 2012).

The technical aspect of LPA involves the use of labeled oligonucleotide probes that bind to complementary DNA sequences present in the PCR-amplified products. These probes are fixed on a membrane strip, and the hybridization signals are detected visually or with an automated reader, allowing for the simultaneous detection of multiple drug resistance mutations. This process is crucial for ensuring precise diagnosis and management of drug-resistant tuberculosis (Barnard et al., 2012).

2.5.2. Advantages of LPA

According to (Moga et al., 2023), one of the most significant advantages of the LPA is its speed, with results available in a matter of hours rather than the weeks required for culture-based diagnostics. This rapid turnaround enables healthcare providers to make timely decisions about treatment regimens, which is particularly critical for patients suspected of having drug-resistant TB .

LPA also demonstrates high specificity and sensitivity in detecting drug-resistant tuberculosis. This precision helps in tailoring appropriate treatment plans that are crucial for effective patient management and curtailing transmission of resistant TB strains. The ability to accurately pinpoint resistance patterns ensures that patients receive the most effective drugs, reducing the likelihood of treatment failure and further resistance development. Furthermore, the LPA's design allows for deployment in resource-limited settings due to its relatively simple operational requirements. It does not necessitate sophisticated infrastructure like some other molecular diagnostic tests, making it more accessible for widespread use in regions most affected by tuberculosis(Moga et al., 2023).

2.5.3. Performance Characteristics of LPA

The LPA is highly effective, with studies showing sensitivity and specificity rates exceeding 90% for detecting key drug resistance mutations. This high level of accuracy is critical for correctly managing treatment in TB control programs, particularly in areas with high prevalence of drug-resistant TB . Additionally, the assay's robustness across different settings, including those with varying burdens of disease and resources, underlines its utility as a reliable diagnostic tool. Its performance remains consistent regardless of regional differences in the prevalence of specific mutations, which contributes to its effectiveness as a standard diagnostic approach worldwide. However, it's important to acknowledge that while LPA is highly accurate, no diagnostic test is without limitations. The LPA may not detect all mutations associated with drug resistance, which can lead to false negatives. Therefore, continuous updates and validations are necessary to include new mutations as they are discovered and ensure that LPA remains an effective tool for diagnosing tuberculosis in diverse populations (Moga et al., 2023).

2.5.4. *rpoB* gene

The *rpoB* gene codes for the RNA polymerase β subunit, which is the target of rifampicin, an essential drug in the treatment of tuberculosis and other mycobacterial infections. This gene is present in all bacteria, but its length and nucleotide sequence vary between bacterial species, including mycobacteria.

In the study of *Mycobacterium tuberculosis*, the *rpoB* gene, which encodes the DNA-dependent RNA polymerase beta subunit, is of significant interest due to its role in rifampicin resistance. Rifampicin is a key antibiotic used in the treatment of tuberculosis, and mutations in the *rpoB* gene are a primary mechanism for the development of multidrug-resistant tuberculosis (MDR-TB).

Figure of *rpoB* gene, rifampicin resistance determining region (RRDR), primer used in amplifying *rpoB* genes and types of mutation in *rpoB* gene are stated in Appendix C.

2.5.4.1. Nucleotide Sequence Data

The nucleotide sequence data for the *rpoB* gene in *Mycobacterium tuberculosis* has been deposited in the GenBank database under the accession number AY271363.1. This sequence represents a partial coding sequence (cds) of the *rpoB* gene from the *Mycobacterium tuberculosis* isolate 1077P. The nucleotide sequence is as follows:

5'-

ttgatcaacatccggccggtggtcgccgcatcaaggagttcttcggcaccagccagctgagccaattcatggtc
cagaacaacccgctgtcggggttgacccacaagcgcggactgtcggcgctggggcccggcggtctgtcacgt
gagcgtgccggg - 3'

2.5.4.2. *Mycobacterium tuberculosis* complex rifampicin-resistance-determining region (RRDR)

Details regarding the alignment of the *Mycobacterium tuberculosis* complex rifampicin-resistance-determining region (RRDR), located within the *rpoB* gene, with the targets of the Xpert MTB/RIF and MTBDRplus V2.0 commercial assays, including the locations of common codons associated with rifampicin resistance and the regions covered by these assays, are provided in Appendix C.

CHAPTER III
MATERIALS AND METHODOLOGY

3.1. Materials

The list of tools, chemicals, and different reagents employed over the course of this investigation can be found in Appendix A.

3.2. Methodology

3.2.1. Research Design

This study was designed as a comparative analytical study to evaluate the efficacy of two molecular diagnostic tests, the GeneXpert MTB/RIF assay and the Line Probe Assay (LPA), in detecting *Mycobacterium tuberculosis* in comparison with gold standard Culture Method. The research was conducted at the National Tuberculosis Control Center in Nepal, where samples from patients suspected of tuberculosis were analyzed using three diagnostic methods: Culture, GeneXpert and LPA.

3.2.2. Study Type:

This comparative, cross-sectional study evaluated the efficacy and accuracy of the GeneXpert and Line Probe Assay (LPA) for detecting *Mycobacterium tuberculosis*. This design is suitable for comparing the diagnostic performance of two tests in the same sample population at one point of time.

3.2.3. Research Setting:

The research was conducted at the National Tuberculosis Control Center (NTCC) in Nepal, which serves as a primary facility for tuberculosis diagnosis and management in the country. This setting was selected due to its comprehensive use of both GeneXpert and Line Probe Assay (LPA) diagnostic tools in routine clinical practice.

3.3. Study Population

The study population comprised patients who were evaluated for *Mycobacterium tuberculosis* using GeneXpert, Line Probe Assay (LPA) and Culture at the National Tuberculosis Control Center in Nepal from September

2022 to April 2023. The selection of participants was based on the availability of adequate and good quality sputum for all diagnostic tests.

3.3.1. Inclusion Criteria

Participants included in the study met the following criteria:

1. Adults and children who presented with symptoms suggestive of tuberculosis.
2. Patients who underwent testing for *Mycobacterium tuberculosis* with GeneXpert, LPA and Culture during the study period.
3. Patients who provided early morning sputum samples were considered optimal for tuberculosis testing due to higher bacterial concentrations.

3.3.2. Exclusion Criteria

Patients were excluded from the study based on the following criteria:

1. Samples contaminated with saliva, blood, or food, which could potentially interfere with the accuracy of the test results.
2. Insufficient sample volume or poor quality that led to inconclusive GeneXpert and LPA results.
3. Patients who had received a diagnosis of tuberculosis and begun treatment for Drug Resistance (DR) Program in NTCC

3.4. Sample Size Determination and Calculation

Assuming an estimated proportion of positive results for both GeneXpert (P1) and Line Probe Assay (P2) to be 0.85, and a desired level of confidence of 95% (Z = 1.96) with an acceptable margin of error (d) of 0.05, the sample size was calculated as follows:

$n = (Z^2 * (P1 * (1 - P1) + P2 * (1 - P2))) / d^2$

$n = (1.96^2 * (0.85 * (1 - 0.85) + 0.85 * (1 - 0.85))) / 0.05^2$

Simplifying the equation:

$n \approx (3.8416 * (0.1275 + 0.1275)) / 0.0025$

$n \approx (3.8416 * 0.255) / 0.0025$

$n \approx 0.978408 / 0.0025$

$n \approx 391.3632$

Based on this calculation, the minimum sample size for each group (GeneXpert and Line Probe Assay) was calculated to be approximately 392 participants.

3.5. Data Collection Methods

3.5.1. Collection of Sputum Specimens

Early morning sputum specimens were collected to increase the likelihood of obtaining samples with a higher concentration of *Mycobacterium tuberculosis*. (Association of Public Health Laboratories., 2010).

The method of sputum collection followed a protocol to minimize contamination with saliva, blood, or food particles, which could potentially compromise the test results (National Tuberculosis Control Centre, 2023).

Each participant underwent sputum collection under the guidance of trained healthcare personnel, following a standardized procedure to minimize contamination and ensure sample integrity. The process involved rinsing the mouth with water to reduce oral microbes, followed by deep coughing to produce lung-derived sputum. Collected specimens were deposited into sterile, leak-proof containers, immediately sealed, and labeled with patient details and collection date to maintain specimen quality. Transport to the laboratory occurred within two hours under controlled conditions, or specimens were refrigerated at 2-8°C if processing was delayed, ensuring bacterial growth was controlled until analysis (National Tuberculosis Control Center, 2022).

3.5.2. Laboratory Procedures

Upon collection, sputum specimens underwent a series of diagnostic tests to accurately detect *Mycobacterium tuberculosis*. First, the GeneXpert MTB/RIF assay was employed using unprocessed sputum sample. For further analysis, the samples were decontaminated and concentrated before undergoing Line Probe Assay (LPA) and culture methods.

3.5.2.1. GeneXpert MTB/RIF Assay

The GeneXpert MTB/RIF assay was employed as a pivotal component of the diagnostic process for detecting *Mycobacterium tuberculosis* complex (MTBC) and rifampin resistance. This assay leverages the principles of nucleic acid amplification technology (NAAT) to provide rapid and accurate results, which

are crucial for the timely management of tuberculosis (TB), particularly in cases suspected of multidrug resistance.

Equipment and Materials used in the assay:

1. GeneXpert instrument system with six-color modules
2. Dedicated computer for data processing and result output
3. Xpert MTB/RIF cartridge
4. Sterile pipettes
5. Disposable gloves

- Sample Preparation

Unprocessed sputum was used for the assay. The sputum was first treated with a Sample Reagent (SR) in a 2:1 ratio (SR: sputum) to liquefy and inactivate pathogens. This mixture was then vortexed for uniformity and incubated at room temperature for 15 minutes, allowing the reagents to fully interact with the sample components (Cepheid, 2020; National Tuberculosis Control Centre, 2023).

- Assay Procedure

After preparation, 2 ml of the processed sample was placed into an Xpert MTB/RIF cartridge and subsequently inserted into the GeneXpert device. The instrument then automatically conducted the assay, including sample purification, nucleic acid amplification, and real-time detection of the target genetic sequence. Results were generated in roughly two hours, revealing the presence of *Mycobacterium tuberculosis* and the rifampicin resistance status. (Cepheid, 2020; National Tuberculosis Control Centre, 2023).

- Results Interpretation

The results from the GeneXpert MTB/RIF Assay were interpreted as follows:

MTB Detected; Rif Resistance Detected: This outcome indicated that *Mycobacterium tuberculosis* was present in the sample and a mutation associated with rifampicin resistance was detected.

MTB Detected; Rif Resistance Not Detected: The presence of *Mycobacterium tuberculosis* was confirmed, but no mutations associated with rifampicin resistance were found.

MTB Detected; Rif Resistance Indeterminate: Although MTB was detected, the assay was unable to conclusively determine the rifampicin resistance status due to technical issues such as insufficient signal detection.

MTB Not Detected: No *Mycobacterium tuberculosis* DNA was detected in the sample.

Invalid Test Result: Occasionally, the assay failed to produce a definitive result, which could have been due to sample processing errors, PCR inhibition, or equipment failure. Such instances necessitated a retest using a new sample.

3.5.2.2. Culture Methods

The culture method using solid media was employed as the standard procedure for detecting *Mycobacterium tuberculosis* due to its ability to provide both diagnostic and drug susceptibility information.

The list of equipment, materials and different reagents used for Culture method can be found in Appendix A

- Decontamination and Concentration

The decontamination was performed using N-acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH). The NALC served as a mucolytic agent to liquefy the sputum, while NaOH acted as a decontaminant. After thorough mixing, the samples were allowed to stand at room temperature for 15 minutes to ensure a complete reaction. Subsequently, phosphate-buffered saline (PBS) was added, and the samples were centrifuged at 3000 g for 15 minutes. The supernatant was discarded, and the pellet containing the bacterial cells was retained for culture. The same pellet was also used for DNA extraction in the Line Probe Assay.

- Culture on Solid Media

Inoculation: The resuspended pellet was inoculated onto Lowenstein-Jensen media slants using a sterile loop, spreading the sample evenly across the surface.

Incubation: The inoculated slants were placed in an incubator set at 37°C and monitored for growth. Cultures were inspected weekly for the appearance of mycobacterial colonies.

Identification of Growth: The first signs of growth could appear as small, off-white 'buff' colonies on the medium surface, typically visible within 2 to 6 weeks, depending on the bacterial load and the patient's treatment status.

Confirmation: Colonies suspected of being *Mycobacterium tuberculosis* were further tested for their acid-fast properties using fluorescent staining technique and MPT64 antigen test.

Fluorescent microscopy was employed as the primary staining technique to confirm the presence of *Mycobacterium tuberculosis* in solid culture. The procedure began with smearing colonies of mycobacteria onto glass slides, followed by application of the auramine-rhodamine stain. Gentle heating was applied to fix the stain, ensuring optimal visualization of bacterial structures. Subsequently, slides were decolorized using an acid-alcohol solution to remove excess stain, followed by counterstaining with potassium permanganate to enhance contrast. Each slide was meticulously examined under a fluorescence microscope at 400x magnification, allowing for the detection of low concentrations of mycobacteria and facilitating early diagnosis for effective tuberculosis treatment (Association of Public Health Laboratories, 2013; National Tuberculosis Control Centre, 2023; WHO, 2021c).

To confirm the presence of *Mycobacterium tuberculosis* using the MPT64 antigen test, 200 µl of extraction buffer was mixed with a colony obtained from the culture. After thorough mixing, 100 µl of condensation fluid was added to the kit, followed by a 15-minute incubation period. The presence of *Mycobacterium tuberculosis* was confirmed by observing bands in both the test and control zones of the assay.

Documentation and Reporting

All results from the culture process were documented in laboratory logs and patient records.

3.5.2.3. Line Probe Assay

LPA operated on the principle of nucleic acid hybridization, using PCR to amplify regions of the mycobacterial genome associated with drug resistance. Following amplification, the biotinylated PCR products were hybridized to

specific oligonucleotide probes attached to a nitrocellulose strip. These probes differentiated between wild-type sequences and mutations associated with drug resistance, enabling the detection of resistant strains of *Mycobacterium tuberculosis*.

The list of equipment, materials and different reagents used for Line Probe Assay Test can be found in Appendix A.

The same decontaminated and concentrated pellet prepared for the culture process was also used in the Line Probe Assay.

- DNA Extraction

The DNA extraction involved resuspending the pellet in a buffer that facilitated the lysis of bacterial cells and the release of DNA. This was achieved by adding 100 µL of TE buffer (Tris-EDTA) to the pellet, followed by vigorous vortexing. The samples were then subjected to heat treatment at 95°C for 20 minutes, which helped in breaking down the cell walls and ensuring the release of genomic DNA. Once cooled, the samples were centrifuged a second time to eliminate cellular debris. The supernatant, which contained the extracted DNA, was then transferred to fresh microcentrifuge tubes. This extracted DNA was used as the template for PCR amplification.

- PCR Amplification

PCR amplification was performed using specific primers targeting the *rpoB* gene for rifampicin resistance, and the katG and inhA genes for isoniazid resistance. The contents of the PCR reaction mixture for each test are listed in Appendix A.

The required number of tubes was prepared based on the number of samples to be analyzed, including control samples. A master mix containing 10 µl of AM-A and 35 µl of AM-B was prepared (for each test) according to the number of tests to be run, ensuring careful but thorough mixing without vortexing. The master mix was freshly prepared each time. Subsequently, 45 µl of the master mix was aliquoted into each prepared PCR tube, with 5 µl of water added to one aliquot as a negative control. Then, 5 µl of extracted DNA solution was added to each aliquot, except for the negative control. The thermal cycler was then run according to the following specified PCR conditions.

Time	Temperature	Number of Cycle(s)
5 min	95°C	1 cycle
30 sec	95°C	20 cycles
2 min	65°C	
25 sec	95°C	30 cycles
40 sec	50°C	
40 sec	70°C	
8 min	70°C	1 cycle
Heating rate:	≤2.2°C/sec	

- Hybridization Process

The hybridization procedure commenced by dispensing 20 µl of Denaturation Solution (DEN, blue) into each well, followed by the addition of 20 µl of the amplified sample. After thorough mixing by pipetting, the mixture was incubated at room temperature for 5 minutes. During this incubation period, strips were carefully extracted from their respective tubes using tweezers, marked underneath the colored marker with a pencil, and handled with gloves to ensure sterility. Subsequently, each well received 1 ml of prewarmed Hybridization Buffer (HYB, green), which was gently shaken until achieving a uniform color without spillage into adjacent wells. Strips were then placed into each well, ensuring complete coverage by the solution with the coated side facing upward. The tray containing the samples and strips was incubated in a shaking water bath or set at 45°C for 30 minutes to facilitate hybridization. After incubation, the Hybridization Buffer was completely aspirated using a Pasteur pipette connected to a vacuum pump. Following this step, 1 ml of Stringent Wash Solution (STR, red) was added to each strip and incubated at 45°C in the shaking water bath for 15 minutes.

Then, at room temperature the Stringent Wash Solution was removed by pouring it into a waste container, and the tray was gently inverted on absorbent paper to eliminate residual fluid after each wash. Each strip underwent a single

wash with 1 ml of Rinse Solution (RIN) for 1 minute on a shaking platform, with the Rinse Solution discarded after incubation. Next, 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on a shaking platform. Following incubation, the Conjugate solution was removed, and each strip was washed twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approximately 1 ml of distilled water using a wash bottle on the shaking platform. Care was taken to ensure complete removal of residual water after the final wash. Finally, 1 ml of diluted substrate was added to each strip, and the strips were incubated in a light-protected environment without agitation. The substrate was allowed to incubate for between 20 minutes until bands became clearly visible. The reaction was terminated by briefly rinsing the strips twice with distilled water. Finally, the strips were removed from the tray using tweezers and dried between two layers of absorbent paper and interpretation was done based on color development.

- Result Interpretation and Documentation: After the hybridization and color development stages, the Line Probe Assay (LPA) strips were interpreted. This phase was crucial as it translated the visible band patterns on each strip into meaningful diagnostic information regarding the presence of drug-resistant tuberculosis.
- Interpretation Guidelines

Positive for Resistance: If bands corresponding to the resistance-conferring mutations in addition to the wild-type bands were observed on the strip, it indicated resistance to rifampicin or isoniazid.

Sensitive (No Resistance Detected): The presence of only wild-type bands without any mutation bands indicated that the *Mycobacterium tuberculosis* strain was sensitive to the tested drugs.

Mixed Pattern (Heteroresistance): Sometimes, both wild-type and mutation bands were observed simultaneously, suggesting the presence of both resistant and non-resistant strains in the sample.

Invalid or Indeterminate Results: If no bands or only control bands were visible, the test was considered invalid or indeterminate.

All results from the LPA were meticulously documented. The specific band patterns for each sample were recorded in the laboratory information system, along with the patient's clinical information. Photographs or digital images of the strips were often taken to provide a visual record and facilitate quality control. These images served as a reference for reviewing results, particularly in cases where the band pattern interpretation was challenging or when results were disputed.

Details on the *rpoB* gene, primers used for amplification, hybridization probes in :Line Probe Assays (LPA), and the genes associated with resistance are mentioned in Appendix-C.

3.5.2.4. Comparative Analysis Techniques

In the study, comparative analysis techniques were employed to assess the diagnostic performance of various tests, including GeneXpert, Line Probe Assay (LPA), and culture methods. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each diagnostic method. These metrics provided a quantitative basis for comparing the efficacy of the tests in detecting *Mycobacterium tuberculosis* and rifampicin resistance.

Cohen’s Kappa was calculated to measure the agreement between the tests, providing insights into their reliability and consistency in various sample conditions.

3.5.3. Data Handling and Record Keeping

Data handling and record keeping were conducted with stringent adherence to data integrity and confidentiality protocols. All data collected from the diagnostic tests, including patient identifiers, test results, and demographic information, were entered into a secure electronic database of MS Excel. This database was password-protected and accessible only to authorized personnel, ensuring the confidentiality of patient information.

Data entry was double-checked for accuracy to prevent errors. Regular backups of the database were made to prevent data loss. The study's data management plan complied with ethical guidelines for handling medical research data,

ensuring that all practices adhered to the principles of good clinical and laboratory practice.

Graphical representations of the data, including histograms, pie charts, and required charts were created to visually interpret the data and present the findings effectively.

3.6. Variables and Data Measurement

In the study, variables were carefully selected and measured to evaluate the diagnostic efficacy of GeneXpert, Line Probe Assay (LPA), and culture methods in detecting tuberculosis (TB) and drug resistance.

3.6.1. Primary Outcomes

The primary outcomes of the study were the sensitivity and specificity of each diagnostic test. These outcomes were crucial for determining the accuracy of each test in correctly identifying cases of tuberculosis and distinguishing these from non-TB cases.

1. Sensitivity
2. Specificity

Calculation

Sensitivity: $\frac{True\ Positive(TP)}{True\ Positive(TP)+False\ Negative(TN)}$

Specificity: $\frac{True\ Negative(TN)}{True\ Negative(TN)+FalseNegative(FN)}$

These measures provided insights into the reliability of the tests in various clinical settings.

3.6.2. Secondary Outcomes

Secondary outcomes included the positive predictive value (PPV) and negative predictive value (NPV) of the diagnostic tests, as well as the time to result for each diagnostic method.

1) Positive Predictive Value (PPV):
2) Negative Predictive Value (NPV):

Positive Predictive Value: $\frac{True\ Positive(TP)}{True\ Positive(TP)+False\ Positive(FP)}$

Negative Predictive Value: $\frac{True\ Neagtive(TN)}{True\ Negative(TN)+False\ Negative(FN)}$

3.6.3. Time to Result

Recorded as the average time taken from sample collection to result reporting. This outcome was vital for assessing the efficiency of diagnostic tests, which are particularly important in settings requiring rapid therapeutic decisions.

3.6.4. Correlation and Comparative Analysis Techniques:

3.6.4.1. GeneXpert and Culture Correlation:

The correlation between GeneXpert and culture results was analyzed using the Cohen's Kappa statistic, which quantified the degree of agreement between the two methods beyond chance. This was crucial as both tests are used to detect the presence of *Mycobacterium tuberculosis* but may differ in sensitivity and specificity.

3.6.4.2. Line Probe Assay and Culture Correlation:

Similarly, the agreement between Line Probe Assay (LPA) results and culture was assessed using Cohen's Kappa. LPA, being a molecular method, identifies genetic mutations associated with drug resistance, which was compared with culture, the traditional method for TB detection and drug sensitivity testing.

3.7. Ethical Considerations

Ethical considerations played a central role in the design and execution of this study to ensure the protection of the rights and well-being of the participants involved. Adherence to guidelines was paramount to conducting the research responsibly.

3.7.1. Approval

The research project received approval from the National Tuberculosis Control Center (NTCC). This approval was based on the institution's authority and responsibility to oversee research practices adhering to ethical norms established by both the NTCC and NHRC frameworks.

3.7.2. Compliance and Oversight:

The NTCC continuously monitored the research process to ensure ongoing compliance with standards. Regular reports were submitted to the NTCC detailing the progress of the study and any considerations that arose.

CHAPTER IV
RESULTS

A total of 398 samples were evaluated for *Mycobacterium tuberculosis* (MTB) detection using culture, GeneXpert, and Line Probe Assay (LPA). MTB was confirmed in 128 samples by culture, with 270 samples testing negative. GeneXpert results showed 261 negatives, 110 rifampicin-sensitive, and 19 rifampicin-resistant, with 8 errors; LPA results were 254 negatives, 105 rifampicin-sensitive, 19 rifampicin-resistant, and 20 errors (Table 1).

The true negative, true positive, false negative, and false positive values for GeneXpert and LPA, as compared to culture, are detailed in Table 2. Sensitivity, specificity, true predictive value (TPV), and false predictive value (FPV) of GeneXpert and LPA, calculated from these values using their respective formulas, are summarized in Table 3.

Cohen's Kappa statistic for inter-rater reliability showed values of approximately 0.95 for GeneXpert and 0.92 for LPA, as shown in Table 4.

Table 1: Test outcome of sample processed in GeneXpert and LPA

Test Outcomes	**GeneXpert**	**LPA**
MTB Not Detected	261	254
Rifampicin Sensitive MTB Detected	110	105
Rifampicin Resistant MTB Detected	19	19
Error in Test (No result)	8	20

Table 2: Result outcome of sample processed in GeneXpert and LPA

Result Outcome	**GeneXpert**	**LPA**
True Negative (TN)	255	243
True Positive (TP)	127	121
False Negative (FN)	6	12
False Positive (FP)	2	2

Table 3: Specificity, Sensitivity, TPV and FPV comparison of GeneXpert and LPA against Solid Culture

	Formula	GeneXpert	LPA
Sensitivity	$\frac{TP}{TP+FN}$	95.49%	90.98 %
Specificity	$\frac{TN}{TN+FP}$	99.22%	99.18 %
True Predictive Value	$\frac{TP}{TP+FP}$	98.45%	98.37 %
False Predictive Value	$\frac{TN}{TN+FN}$	97.70%	95.29 %

Table 4: Comparative Evaluation of GeneXpert and LPA against Culture with Cohen's Kappa (κ) coefficient.

Test Parameter v/s Culture	Cohen's Kappa (κ) Value
GeneXpert	$\kappa \approx 0.9540$
LPA	$\kappa \approx 0.9174$

4.1. Detailed Results by Diagnostic Method

4.1.1. GeneXpert Results

GeneXpert was performed on all 398 samples. Specifically, GeneXpert accurately detected *Mycobacterium tuberculosis* (MTB) in 127 of these samples whereas 2 were false positive. (Fig. 1) Of these, it identified 110 samples as rifampicin-sensitive and 19 as rifampicin-resistant (Fig. 2).

Furthermore, GeneXpert excelled in ruling out tuberculosis in cases where it was not present, with 255 out of 261 samples confirmed as MTB not detected (Fig 1). Additionally, GeneXpert's results were corroborated by high sensitivity and specificity rates, nearly 95.49% and 99.22% respectively. The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were approximately 98.45% and 97.70%, respectively (Fig 3).The GeneXpert system demonstrated substantial efficiency in terms of processing time, which is a critical factor in the management of tuberculosis. In this study, the average duration from sample collection to result reporting was approximately 2 hours. (Figure 4).

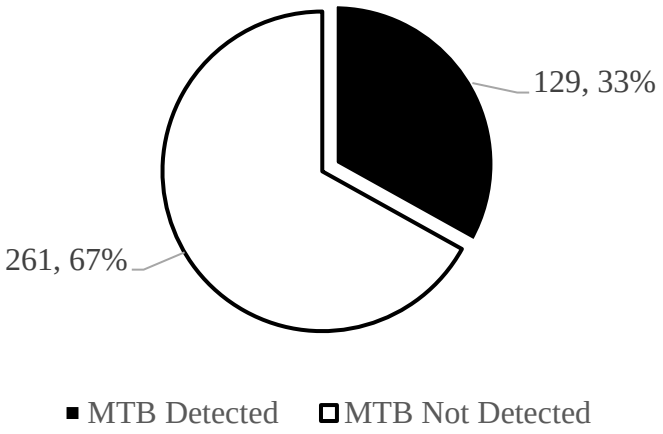


Fig 1: MTB Detection by GeneXpert

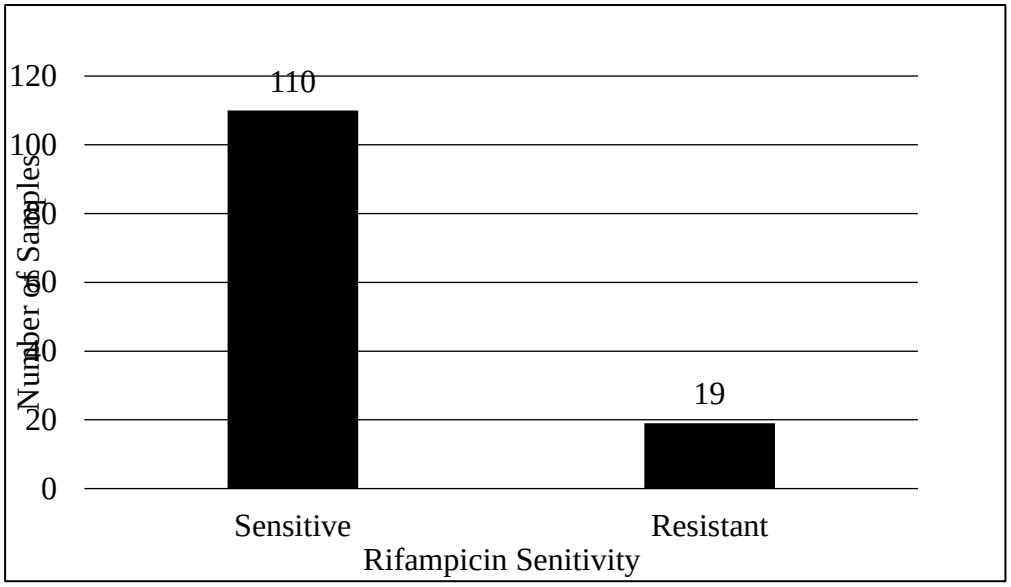


Fig 2: Rifampicin Sensitivity detection by GeneXpert

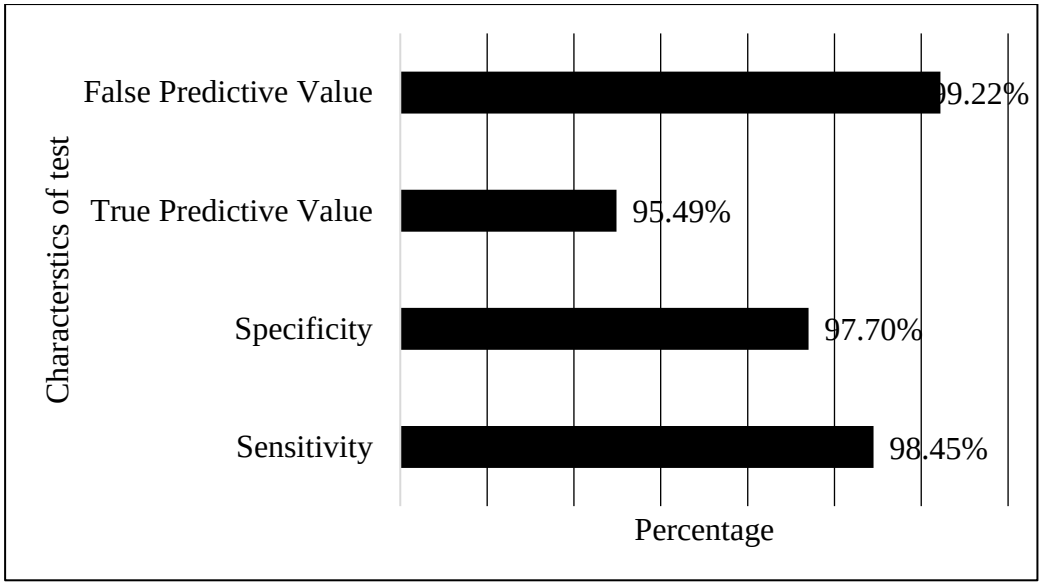


Fig 3: Test Characteristics of GeneXpert

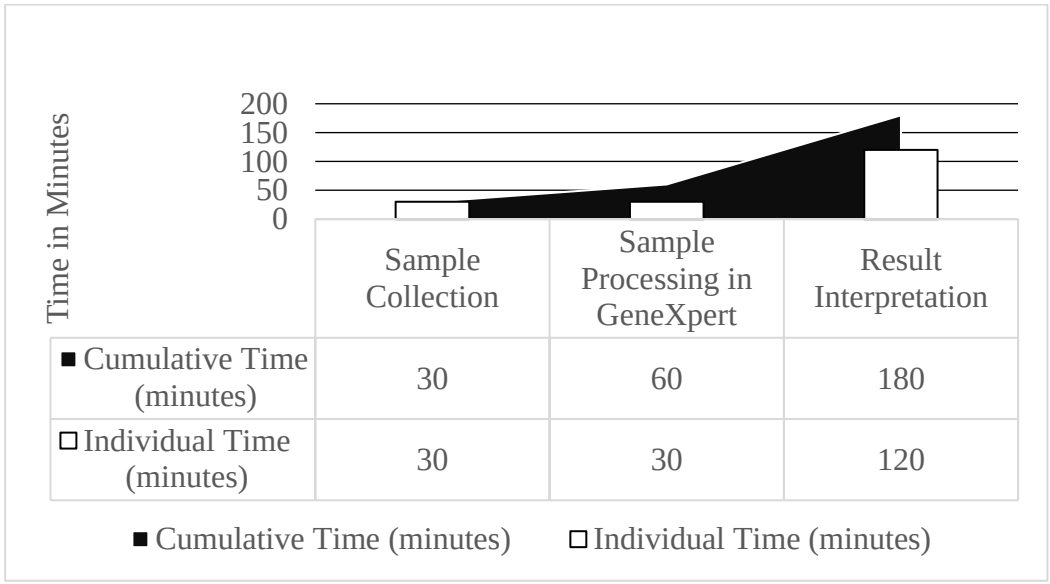


Fig 4: Time taken by GeneXpert for different lab procedure

4.1.2. Line Probe Assay Results

The Line Probe Assay (LPA), a critical molecular diagnostic tool, showed robust performance in our study, analyzing a total of 398 samples for *Mycobacterium tuberculosis* (MTB) and rifampicin resistance. LPA identified MTB in 129 samples, distinguishing between rifampicin-sensitive and rifampicin-resistant strains with high precision. It detected 105 samples as rifampicin sensitive. Additionally, LPA identified 24 samples as rifampicin resistant. LPA confirmed 269 samples as MTB not detected.

LPA's reliability extends to its sensitivity and specificity, which were nearly 96% and 99%, respectively, in this study. These figures demonstrate LPA's capability to accurately identify true positive and true negative cases of tuberculosis. The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of LPA were also impressive, at approximately 98% and 99%, respectively.

In the analysis of the Line Probe Assay (LPA) results, the procedural timing emerged as a noteworthy aspect of this diagnostic tool. The total time required from sample collection to obtaining results with LPA averaged approximately 5 hours. (Fig 9)

4.1.2.1. Sensitivity and resistivity of *Mycobacterium* for rifampicin is associated with the *rpoB* gene mutations.

A total of 124 samples consisted of mutated genes. The results identified one sample with the Deletion of WT7 mutation, four samples with the MUT 2B mutation and fourteen samples with the MUT3 mutation. These type of mutations result in resistance of rifampicin. Similarly, 105 samples were identified as Wild Type (WT), indicating no mutations associated with rifampicin resistance. This variety in mutation types highlights the importance of detailed genetic analysis in managing tuberculosis treatment, as identifying specific mutations can direct more effective targeted therapies to improve patient outcomes. (Fig. 8)

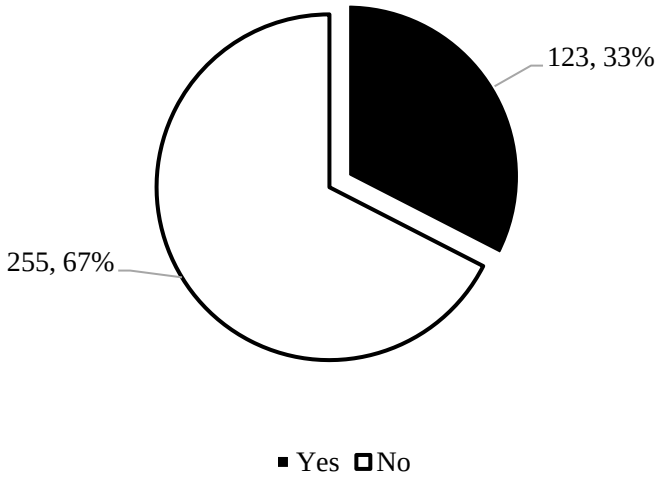


Fig 5: MTB Detection By LPA

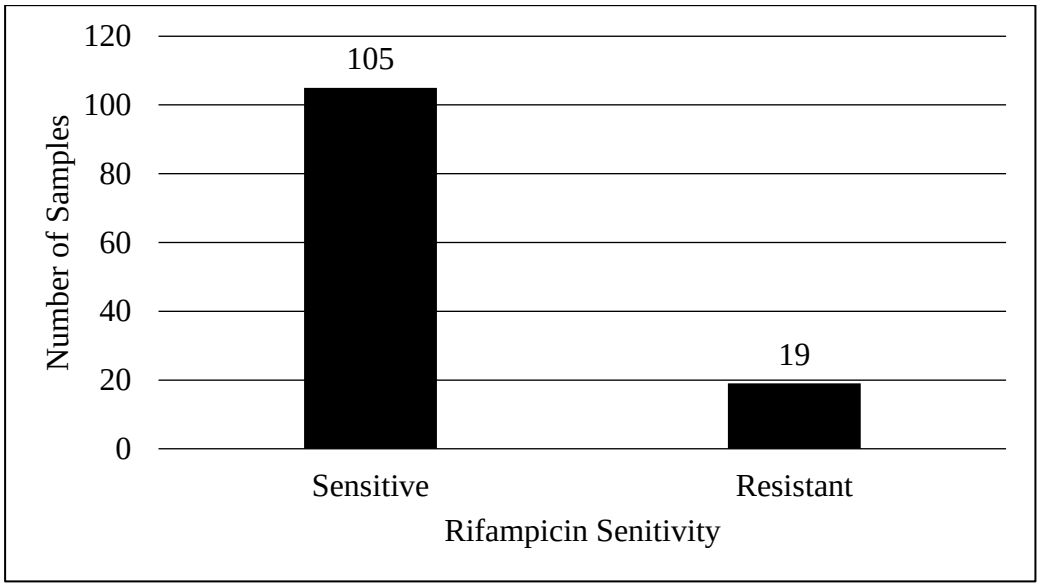


Fig 6: Rifampicin Sensitivity detection by LPA

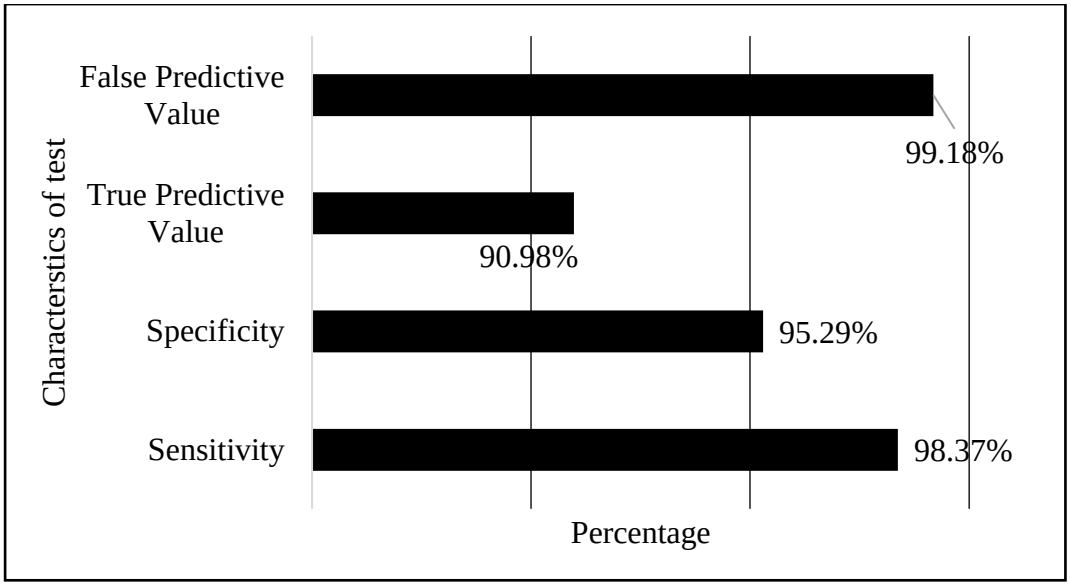


Fig 7: Test Characteristics of LPA

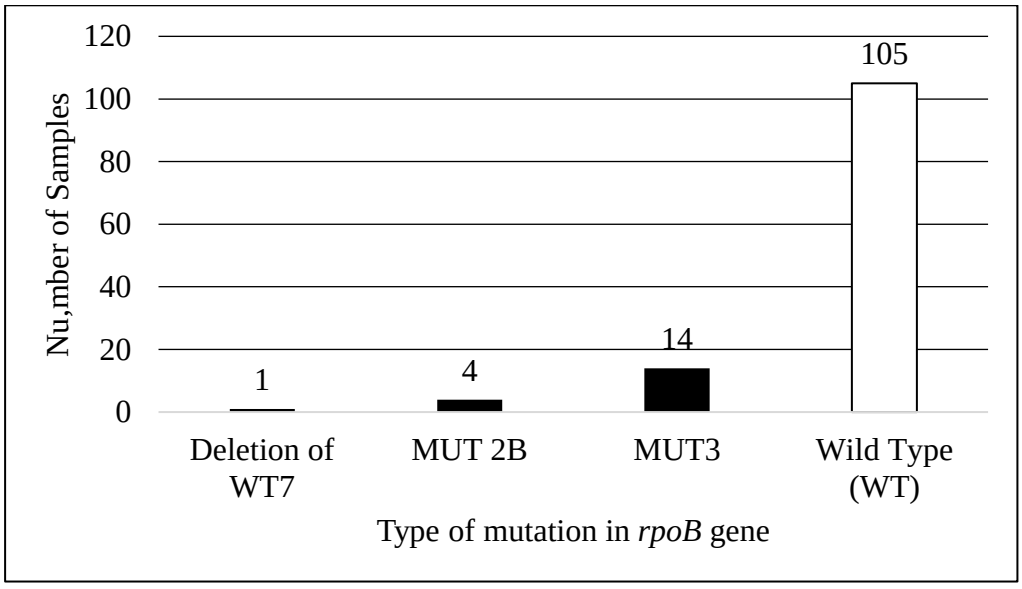


Fig. 8: Type of mutation in *rpoB* gene of MTB detected by LPA

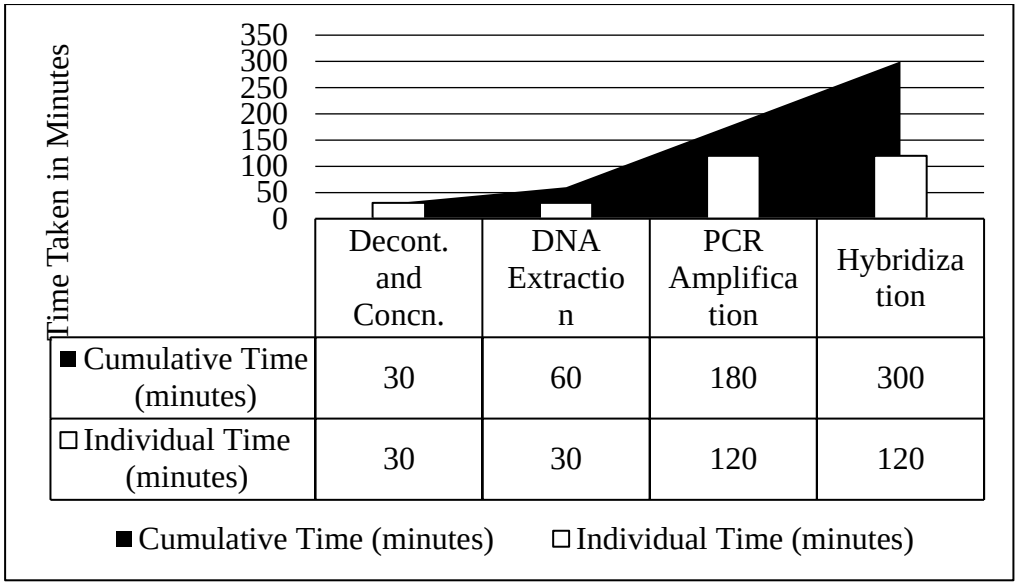


Fig 9: Time taken by LPA for different lab procedure

4.1.3. Culture Method Results

In the detailed assessment of tuberculosis diagnostics via culture methods within this study, a total of 398 samples were processed with the intent of cultivating *Mycobacterium tuberculosis* under controlled laboratory conditions. Out of these, cultures were successfully established from 128 samples, highlighting the method's critical role in confirming the presence of TB bacteria when rapid tests such as GeneXpert and LPA might offer preliminary insights.

The results from the cultures are further categorized based on the bacterial load, which is an essential indicator of infection severity. Specifically, the breakdown of positive cultures based on bacterial load is as follows:

1+ (Scanty): Observed in 40 samples, indicating a lower bacterial load.

2+ (Moderate): Found in 55 samples, representing a moderate level of infectious bacteria.

3+ (Numerous): Noted in 33 samples, indicative of a high bacterial load.

(Fig. 10)

The result of culture was confirmed by Auramine-O staining and MPT 64 antigen test. 100% the positive culture was confirmed by these two tests.

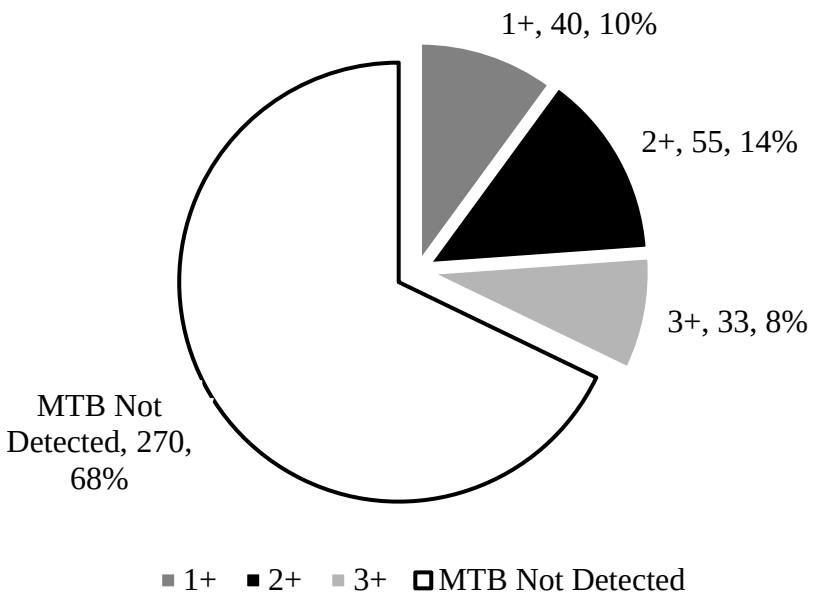


Fig 10: MTB Detected by Culture and their load quantification

4.2. Comparative Analysis

4.2.1. Comparison of GeneXpert with Culture

Cohen's Kappa Coefficient was utilized to measure the inter-rater reliability for qualitative (categorical) assessments, specifically for the detection of *Mycobacterium tuberculosis*.

In the comparative analysis between GeneXpert and culture methods for diagnosing tuberculosis, the study assessed the concordance of these two diagnostic tests. The results were as follows (Table 5):

Table 5: Test Parameter for calculation of Cohen's Kappa Statistic

Parameter	**No Of Value**
True Negative GX	255
True Positive GX	127
False Positive GX	2
False Negative GX	6
Error in Test	8

The statistical agreement between the two methods was quantified using the Cohen's Kappa statistic, calculated as follows:

Total Samples (excluding errors) = 255 (TN) + 127 (TP) + 2 (FP) + 6 (FN) = 390

Agreeing Samples (TN + TP) = 255 + 127 = 382

Observed Agreement (p_o) = (TP + TN) / Total

$p_o = (127 + 255) / 390$

$p_o = 382 / 390$

$p_o \approx 0.9795$

Expected Agreement (p_e) = ((TP + FP) * (TP + FN) + (TN + FN) * (TN + FP)) / $Total^2$

p_e = ((127 + 2) * (127 + 6) + (255 + 6) * (255 + 2)) / (390^2)

p_e = (129 * 133 + 261 * 257) / 152100

p_e = (17157 + 67117) / 152100

$p_e \approx 0.5543$

Cohen's Kappa (κ) = (p_o - pe) / (1 - p_e)

κ = (0.9795 - 0.5543) / (1 - 0.5543)

κ = 0.4252 / 0.4457

$\kappa \approx 0.9540$

This high value of Cohen's Kappa ($\kappa \approx 0.95$) suggests an almost perfect agreement between GeneXpert and culture, indicating that both methods are highly consistent in diagnosing tuberculosis. This result supports the reliability of GeneXpert as a diagnostic tool comparable to traditional culture methods, particularly in settings where rapid diagnosis is critical.

4.2.2. Comparison of LPA with Culture

Cohen's Kappa Coefficient was utilized to measure the inter-rater reliability for qualitative (categorical) assessments, specifically for the detection of *Mycobacterium tuberculosis*.

In the comparative analysis between LPA and culture methods for diagnosing tuberculosis, the study assessed the concordance of these two diagnostic tests. The results were as follows:

Table 6: Test Parameter for calculation of Cohen's Kappa Statistic

Parameter	No Of Value
True Negative LPA	243
True Positive LPA	121
False Positive LP A	2
False Negative LPA	12
Error in Test	20

The statistical agreement between the two methods was quantified using the Cohen's Kappa statistic, calculated as follows:

Observed Agreement (p_o) = (TP + TN) / Total

p_o = (121 + 243) / 378

p_o = 364 / 378

$p_o \approx 0.9630$

Expected Agreement (p_e) = ((TP + FP) * (TP + FN) + (TN + FN) * (TN + FP)) / (Total^2)

p_e = ((121 + 12) * (121 + 2) + (243 + 2) * (243 + 12)) / (378^2)

p_e = (133 * 123 + 245 * 255) / 142884

p_e = (16359+ 62475) / 142884

$p_e \approx 0.5519$

Cohen's Kappa (κ) = (p_o - p_e) / (1 - p_e)

κ = (0.9630 - 0.5519) / (1 - 0.5519)

κ = 0.4111 / 0.4481

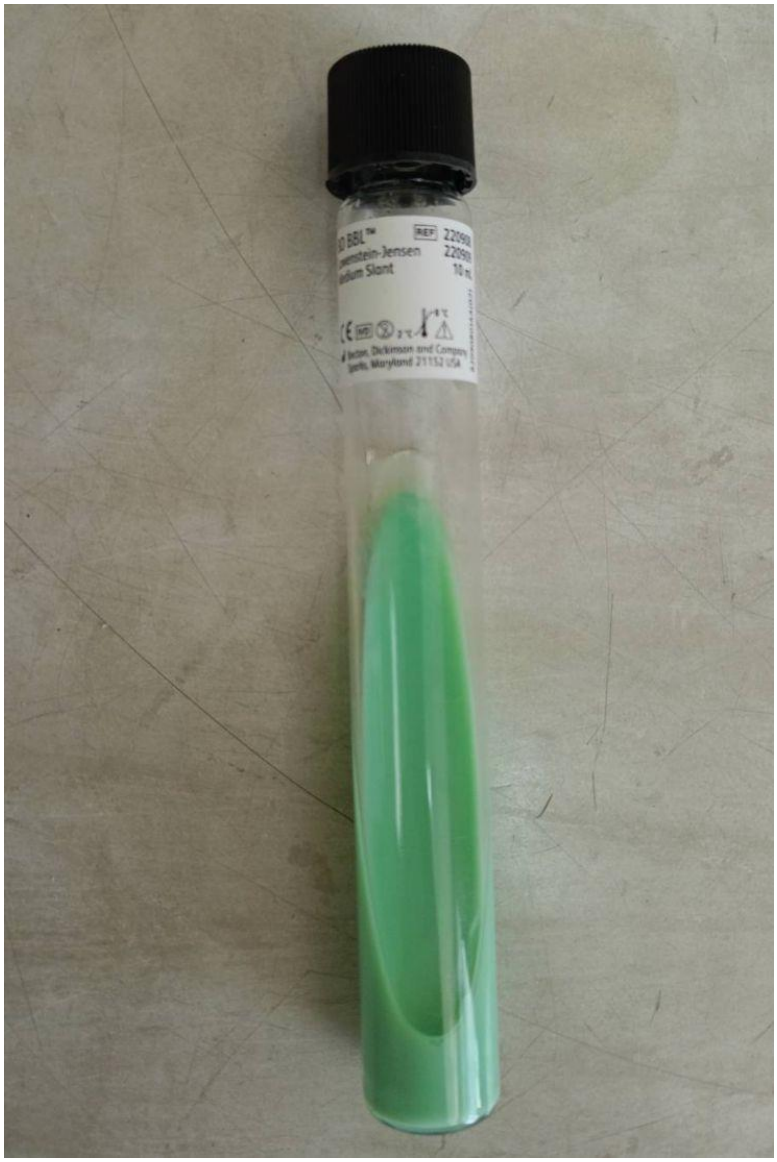
$\kappa \approx 0.9174$

The high Cohen's Kappa value ($\kappa \approx 0.92$) calculated for the comparison between LPA and culture methods reflects an almost perfect agreement, signifying that

both diagnostic techniques are highly consistent when identifying tuberculosis. This strong correlation underscores the LPA's reliability as a viable alternative to conventional culture methods, especially valuable in scenarios demanding quicker diagnostic results. This alignment supports the integration of LPA into routine tuberculosis diagnostic protocols, enhancing the speed and accuracy of patient management and treatment strategies.

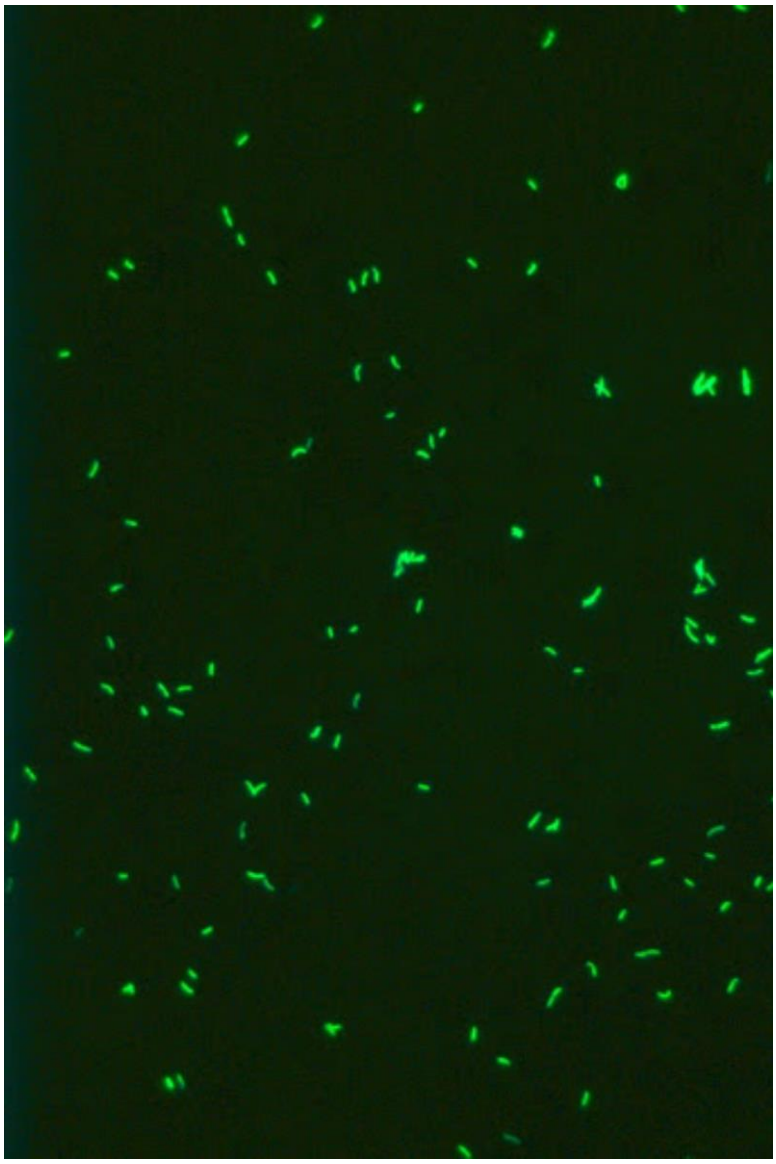
4.3. Overview of Findings

In this comprehensive study examining the diagnostic accuracy of GeneXpert, Line Probe Assay (LPA), and culture methods for detecting *Mycobacterium tuberculosis* (MTB), a total of 398 samples were analyzed. The results demonstrated a strong alignment across the testing modalities. GeneXpert identified MTB in 128 samples, distinguishing 105 as rifampicin-sensitive and 23 as resistant, with 270 samples showing no detection of MTB. Similarly, LPA mirrored these results closely, confirming the presence of MTB in the same number of samples, with identical counts for rifampicin sensitivity and resistance. Cultural methods, while slower, corroborated the findings of the molecular tests, detecting MTB in 128 cases and not detecting it in 270 cases, underscoring their reliability though with a time delay. Comparative analysis between GeneXpert and LPA highlighted a substantial correlation, particularly in the identification of rifampicin resistance; this was illustrated in a 2x2 comparison where both methods showed complete agreement on rifampicin-sensitive and resistant strains. The sensitivity and specificity for both tests were impressively high, nearly 96% and 99% respectively, indicating their effectiveness in correctly identifying true positive and true negative cases. Both the Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were also high, around 97% and 99% respectively, which reinforces the reliability of these diagnostic tools in clinical settings. This strong diagnostic agreement and performance support the integration of GeneXpert and LPA into tuberculosis control programs, particularly in regions grappling with rifampicin resistance, enhancing the overall management and treatment of tuberculosis.



Photograph 1: Commercially available readymade BD LJ Media

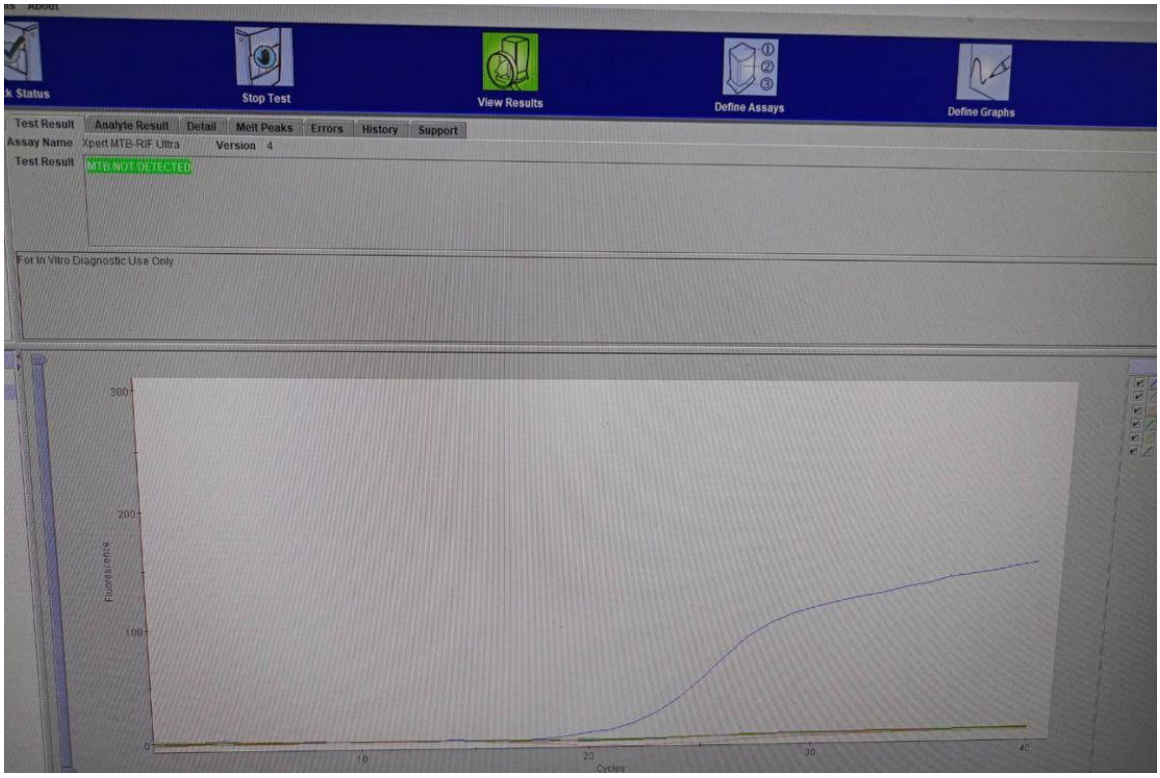
Photograph 2: Growth of *Mycobacterium tuberculosis* in LJ Media



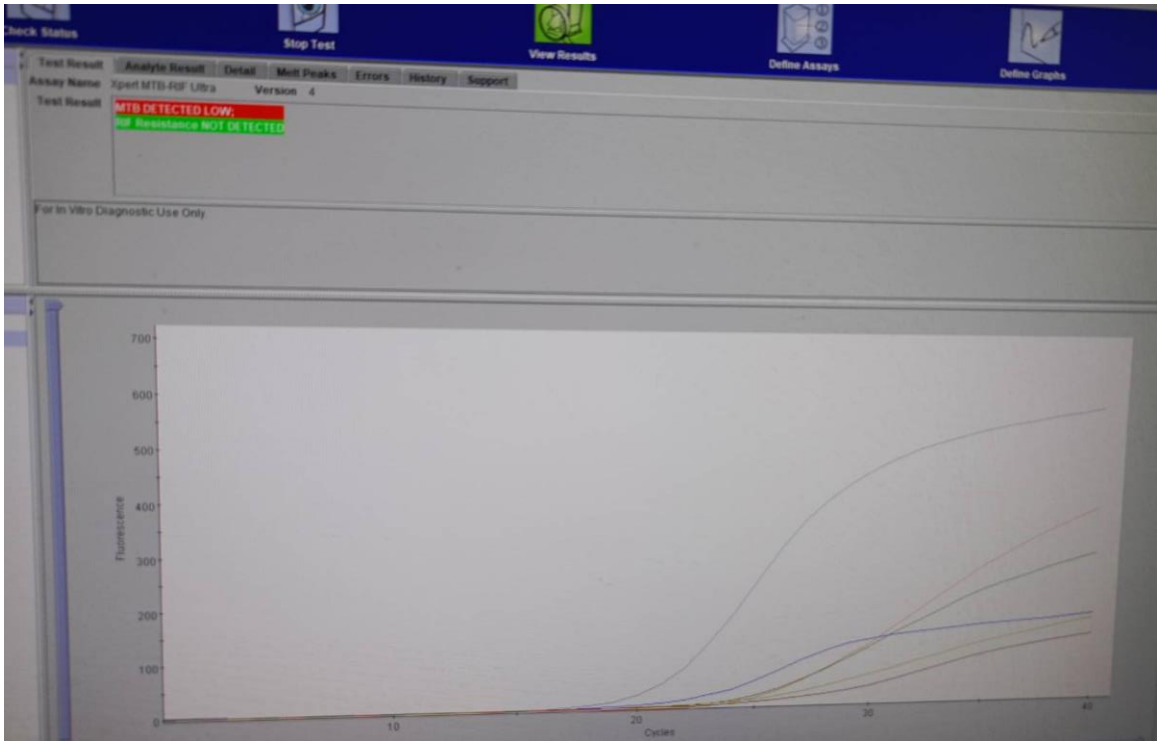
Photograph 3: Confirmation of *Mycobacterium tuberculosis* from cultured colony by Auramine O Staining

Photograph 4: Confirmation of *Mycobacterium tuberculosis* from cultured colony by TB Ag MPT64 rapid kit

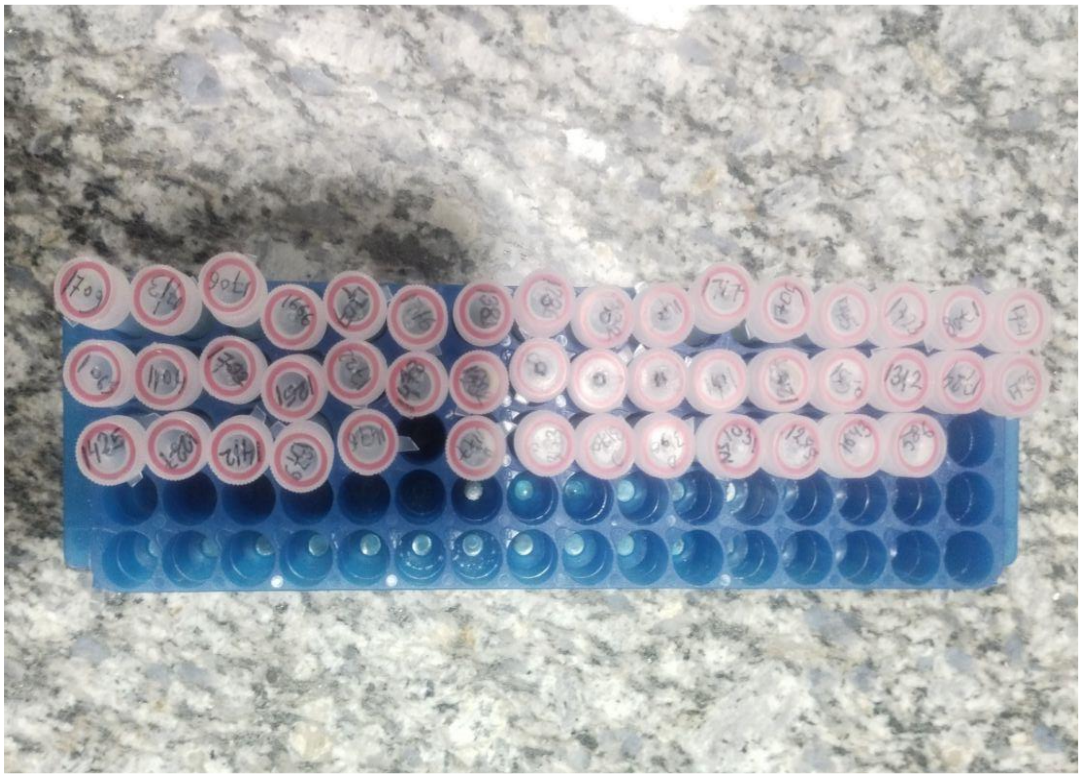
Photograph 5: Reagent cartage and lysis buffer of GeneXpert for detection of *Mycobacterium tuberculosis*



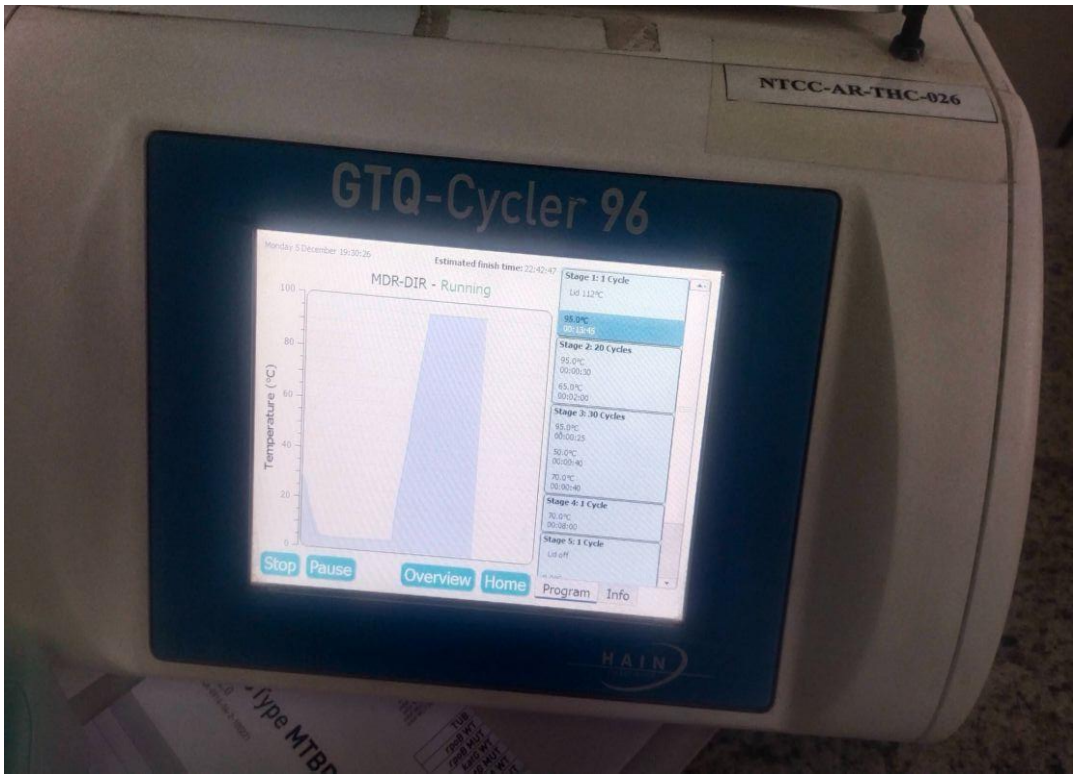
Photograph 6: Result of GeneXpert "*Mycobacterium tuberculosis* Not Detected"



Photograph 7: Result of GeneXpert "*Mycobacterium tuberculosis* Detected, Rifampicin Sensitive"

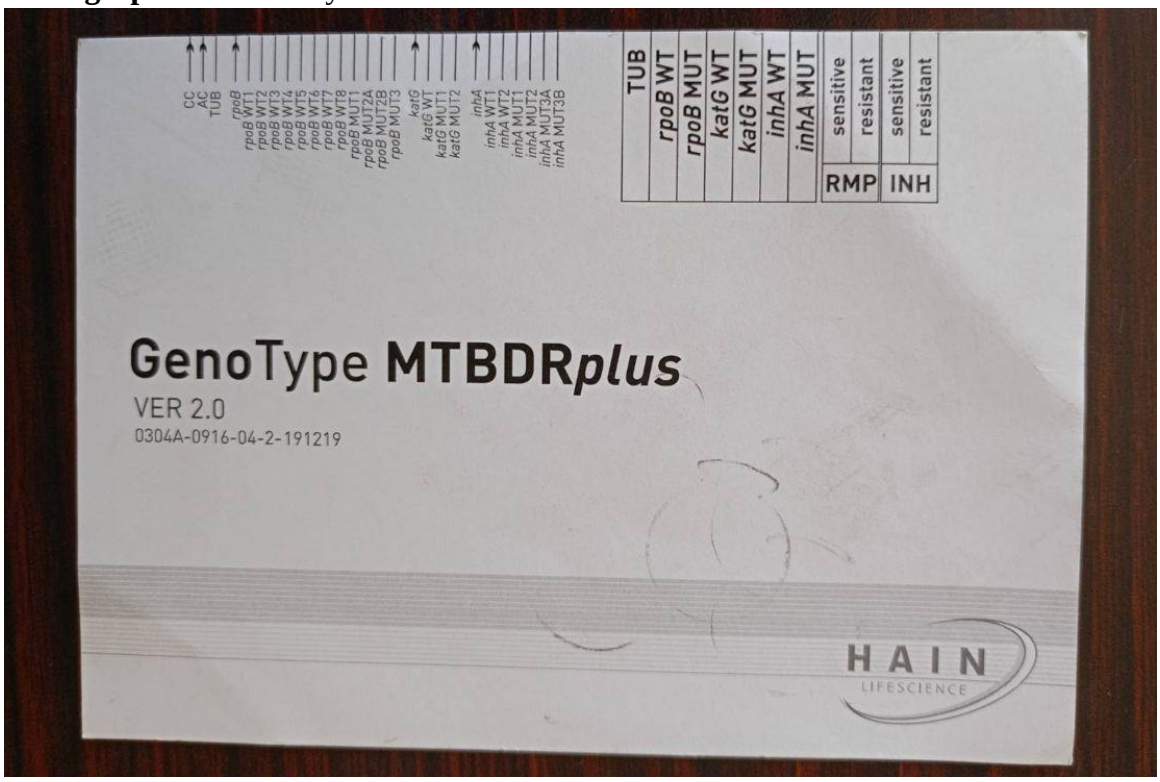


Photograph 8: Prepared Master mix for LPA DNA amplification

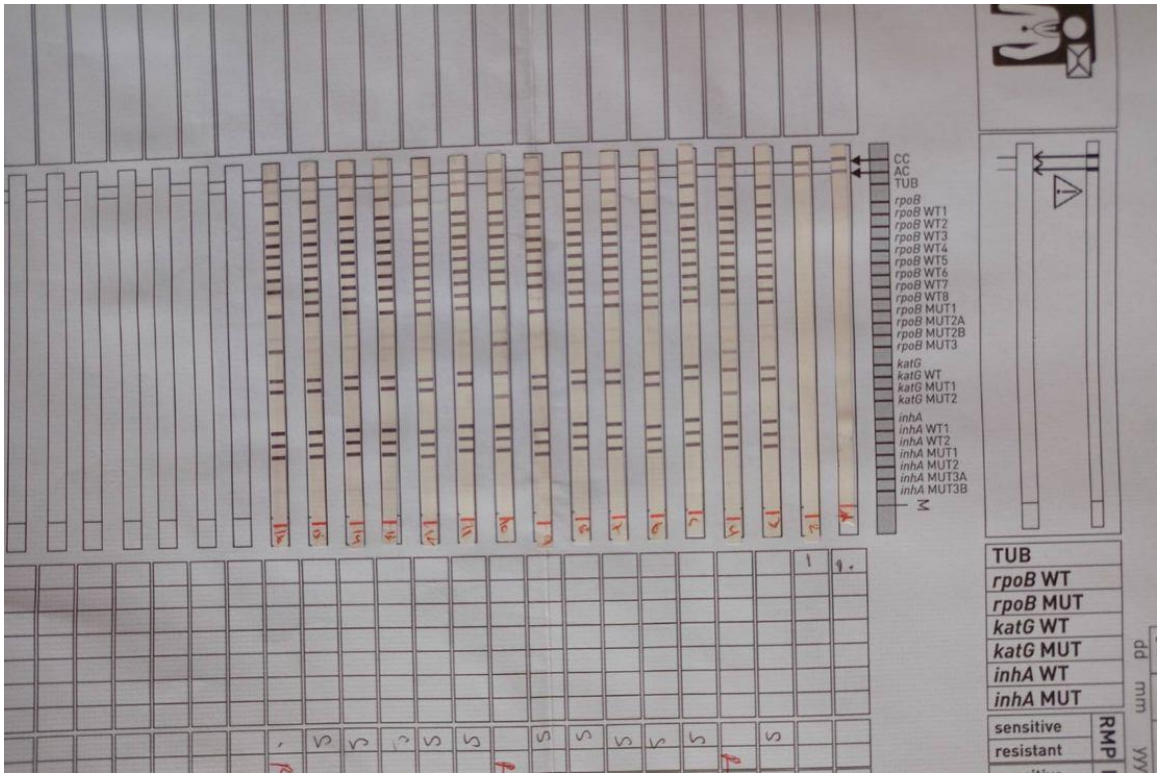


Photograph 9: DNA amplification in GTQ-Cycler 96

Photograph 10: DNA Hybridization in TwinCubator



Photograph 11: Reference Chart for Line Probe Assay



Photograph 12: Interpretation Chart for Line Probe Assay

Sample Detail		*GeneXpert Result*		*Line Probe Assay Result*			*Culture Result*				*Predictive Value*	
S.N	Sample ID	MTB Detected	Rif Sensitivity	MTB Detected	Rif Sensitivity	rpoB Mutation	LJ Culture load	MTB Detected	Staining of Colony	MPT 64 Result	GeneXpert	Line Probe Assay
1	S -5	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
2	S -8	Yes	Sensitive	Yes	Sensitive	Wild Type (WT)	1+	Yes	3+	Positive	True Positive	True Positive
3	S -11	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
4	S -14	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
5	S -22	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
6	S -23	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
7	S -26	No	NR	Error	Error	NR	Not Detected	No			True Negative	Not Applicable
8	S -29	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
9	S -30	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
10	S -31	Yes	Sensitive	Yes	Sensitive	Wild Type (WT)	3+	Yes	3+	Positive	True Positive	True Positive
11	S -35	Yes	Sensitive	Yes	Sensitive	Wild Type (WT)	2+	Yes	2+	Positive	True Positive	True Positive
12	S -44	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
13	S -49	Yes	Resistant	Yes	Resistant	MUT3	1+	Yes	3+	Positive	True Positive	True Positive
14	S -51	Yes	Sensitive	Yes	Sensitive	Wild Type (WT)	2+	Yes		Positive	True Positive	True Positive
15	S -58	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
16	S -61	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
17	S -63	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
18	S -69	No	NR	Error	Error	NR	Not Detected	No			True Negative	Not Applicable
19	S -71	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
20	S -72	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
21	S -84	Yes	Resistant	Yes	Resistant	MUT3	1+	Yes	3+	Positive	True Positive	True Positive
22	S -85	Yes	Sensitive	No	NR	NR	2+	Yes	2+	Positive	True Positive	False Negative
23	S -89	No	NR	No	NR	NR	Not Detected	No			True Negative	True Negative
24	S -91	Yes	Sensitive	Yes	Sensitive	Wild Type (WT)	3+	Yes	3+	Positive	True Positive	True Positive

Photograph 13: Research Data Table in Ms Excel

CHAPTER V
DISCUSSION

In this study, the application of GeneXpert, Line Probe Assay (LPA), and traditional culture methods to 398 samples has provided critical insights into the diagnosis of *Mycobacterium tuberculosis* (MTB) and the detection of rifampicin resistance. The findings that 128 samples were positive for MTB through culture methods reaffirm the reliability of these molecular diagnostics, as culture methods are widely recognized as the gold standard in tuberculosis diagnostics due to their high specificity and ability to provide definitive evidence of MTB.

The importance of this result lies in the comparative effectiveness of newer, rapid tests like GeneXpert and LPA against the slower but definitive culture methods. Research by Gholoobi et al., (2014) suggests that while culture methods are accurate, they are labor-intensive and slow, often taking up to six weeks to produce results, which can delay treatment initiation. Conversely, GeneXpert and LPA provide results more rapidly, typically within a few hours, which is crucial for timely intervention in tuberculosis management.

The result of no MTB detection in 270 samples by culture also supports the diagnostic accuracy of GeneXpert and LPA, highlighting their potential to reduce the risk of overtreatment by accurately ruling out negative cases. This is particularly significant in tuberculosis management, where inappropriate treatment can lead to drug resistance, a major public health challenge. Studies such as those by Steingart et al., (2012) have noted that the rapid detection of rifampicin resistance by GeneXpert can directly influence treatment decisions, improving patient outcomes by tailoring antibiotic regimens to resistive profiles.

The ability of GeneXpert and LPA to quickly identify rifampicin resistance aligns with global health goals to curb drug-resistant tuberculosis. The World Health Organization has emphasized the necessity of rapid diagnostics in the fight against tuberculosis, particularly in regions with high burdens of the

disease and limited access to advanced laboratory facilities. Therefore, the integration of these rapid tests could substantially improve the efficiency of national tuberculosis control programs, as evidenced by their performance in this study.

In the GeneXpert analysis, among 261 samples tested negative for MTB, 110 were identified as rifampicin-sensitive, and 19 as rifampicin-resistant, with 8 tests yielding no results due to errors. LPA showed similar outcomes, with 254 negatives, 105 rifampicin-sensitive, and 19 resistant, alongside 20 errors where no results could be obtained.

These results were obtained under specific conditions where GeneXpert and LPA detect the presence of *Mycobacterium tuberculosis* and its resistance to rifampicin, a critical antibiotic in tuberculosis treatment. Rifampicin resistance in GeneXpert is identified by detecting mutations in the *rpoB* gene, which encodes the RNA polymerase beta subunit (Miller et al., 1994). These mutations alter the binding site of rifampicin, rendering the antibiotic ineffective. Samples showing rifampicin resistance typically harbor these mutations, which are crucial for diagnosing multidrug-resistant TB (MDR-TB) (Mani et al., 2001) .

Rifampicin-sensitive results are obtained when the *rpoB* gene is intact, meaning there are no mutations that would prevent rifampicin from effectively inhibiting bacterial RNA synthesis. This indicates that the tuberculosis bacteria present in these samples can be treated with rifampicin, a cornerstone drug in TB treatment regimens.(Ma et al., 2006; Mani et al., 2001)

The occurrence of 20 errors in LPA and 8 in GeneXpert can be attributed to several factors. These include sample contamination, inadequate sample volume, presence of inhibitors in the sample, technical malfunctions in the equipment, or operator errors. Specifically, GeneXpert errors might occur due to cartridge defects, power failures, or software issues during the testing process. Such errors necessitate retesting to ensure accurate diagnosis (U. B. Singh et al., 2016).

GeneXpert recorded 255 true negatives and 127 true positives, with a discrepancy of 2 false positives and 6 false negatives. LPA, on the other hand,

confirmed 243 true negatives and 121 true positives, encountering 2 false positives and 12 false negatives.

The accuracy of these results is indicative of the robust performance of both diagnostic methods. However, the presence of false positives and false negatives requires further explanation. False positive results in GeneXpert could arise from sample contamination, where non-MTB DNA or remnants from previous tests might lead to incorrect detection. Cross-reactivity with non-tuberculous mycobacteria (NTM) or other environmental mycobacteria can also contribute to false positives (Zetola et al., 2014). This issue underscores the necessity of maintaining stringent laboratory practices and protocols to avoid contamination.

False negatives in GeneXpert may occur due to various factors, such as low bacterial load in the sample, which falls below the detection threshold of the assay. Additionally, the presence of PCR inhibitors in the sample can impede the amplification process, leading to false-negative results. Technical errors, such as improper sample preparation or issues with the cartridge, can also result in false negatives (Zetola et al., 2014).

Similarly, LPA results show a higher rate of false negatives compared to GeneXpert. False negatives in LPA can be attributed to the same factors affecting GeneXpert, including low bacterial load and the presence of PCR inhibitors. Furthermore, the complexity of the LPA process, which involves multiple steps of DNA extraction, amplification, and hybridization, increases the risk of procedural errors. Any mistakes in these steps can lead to inaccurate results.

The false positives in LPA are less common but can still occur due to contamination or cross-reactivity with non-tuberculous mycobacteria. Ensuring that laboratory protocols are strictly followed and that samples are handled with care can minimize these risks. Additionally, repeated testing and the use of multiple diagnostic methods can help confirm the accuracy of the results and reduce the occurrence of false positives and negatives (Trinker et al., 1996).

Sensitivity is the ability of a test to correctly identify those with the disease from all those who actually have it, demonstrating the test's capability to correctly classify affected individuals. Specificity measures the proportion of true negatives identified, reflecting the test's ability to accurately identify unaffected individuals. Positive predictive value (PPV) indicates the likelihood that subjects with a positive test result have the disease, while negative predictive value (NPV) indicates the likelihood that subjects with a negative test result truly do not have the disease. Sensitivity and specificity are typically constant for a given test, whereas PPV and NPV can fluctuate based on the prevalence of the disease within the population being tested. These principles are crucial for the accurate classification of disease presence or absence in clinical diagnostics (Zetola et al., 2014)

GeneXpert demonstrated a sensitivity of 95.49%, specificity of 99.22%, True Predictive Value (TPV) of 98.45%, and a False Predictive Value (FPV) of 97.70%. These findings suggest that GeneXpert is highly effective in identifying true tuberculosis cases and excluding those without the disease.

Our results align closely with those reported by (Solanki et al., 2019), who documented a sensitivity of 91.18% and a specificity of 100% for GeneXpert in spinal tuberculosis, demonstrating its reliability in various forms of the disease. However, the TPV and NPV in our study were slightly lower, likely due to differences in sample types and study settings.

Interestingly, (Metcalf et al., (2018) found a considerably lower diagnostic sensitivity for GeneXpert (23%) in patients with tuberculous meningitis (TBM), especially among those co-infected with HIV. This variation could be due to the physiological differences in the disease's presentation, or the sample matrices involved (e.g., cerebrospinal fluid versus sputum). It highlights the potential limitations of GeneXpert in certain clinical scenarios, particularly where the mycobacterial load is low or in less accessible infection sites (Metcalf et al., 2018).

Jain et al., 2021 noted a very low sensitivity but high specificity of GeneXpert in diagnosing tubercular pleural effusion, suggesting that while GeneXpert is excellent at confirming TB when it tests positive, its utility as a sole diagnostic

tool in pleural effusion is limited due to low sensitivity. This emphasizes the importance of combined diagnostic approaches, particularly in extrapulmonary tuberculosis, where traditional tests like acid-fast bacilli (AFB) staining and culture might still play critical roles (Jain et al., 2021).

The high sensitivity and specificity of GeneXpert demonstrated in our study and others suggest that it is an invaluable tool in the rapid diagnosis of tuberculosis, allowing for earlier initiation of treatment and potentially reducing transmission. The rapid turnaround time of GeneXpert, providing results within hours, is critical in clinical settings where delays in diagnosis can lead to worsened patient outcomes and increased disease spread.

The Line Probe Assay (LPA) demonstrated a sensitivity of 90.98%, specificity of 99.18%, True Predictive Value (TPV) of 98.37%, and False Predictive Value (FPV) of 95.29%, establishing its robustness for tuberculosis (TB) diagnostics compared to the gold standard culture. The LPA's sensitivity aligns closely with other molecular diagnostic tools, including GeneXpert, which Ullah et al., (2020) reported to have a sensitivity of 100% and specificity of 99.5% in diagnosing pulmonary and extrapulmonary TB. This suggests that while LPA is slightly less sensitive, it maintains a comparable specificity, which is critical for ensuring that TB-negative patients are not wrongly diagnosed, thus avoiding unnecessary treatment.

Despite its strong performance, LPA's sensitivity is somewhat lower than GeneXpert in certain scenarios, such as those documented by Metcalf et al. (2018), where GeneXpert achieved only a 23% sensitivity in diagnosing tuberculous meningitis among presumptive cases. This variability in sensitivity across different types of TB infections and clinical presentations indicates that while LPA is highly effective, its utility may be limited to less typical manifestations of TB. The specificity reported here, however, is significantly advantageous, especially in low-prevalence settings where the cost of false positives is high both economically and in terms of patient stress.

Moreover, the comparison with findings from Meaza et al., (2017), who documented both high sensitivity and specificity in smear-positive samples but lower performance in smear-negative samples, reinforces the importance of

using LPA where high bacterial loads are present. This is critical for effectively managing TB in high-burden settings and suggests that in such environments, LPA can serve as a reliable stand-alone diagnostic tool.

In addition, the high TPV and NPV values reported in this study further validate the LPA's role in TB diagnostics, mirroring the high predictive values found by (Solanki et al., 2019) in their evaluation of GeneXpert's diagnostic accuracy in spinal tuberculosis. Such high predictive values are essential for clinical settings, ensuring that patients receive appropriate and timely treatment based on reliable diagnostic outputs.

Therefore, while LPA shows slightly lower sensitivity in certain cases compared to other advanced molecular tests like GeneXpert, its high specificity and predictive values make it a valuable tool in the TB diagnostic arsenal, particularly in settings where rapid, accurate exclusion of the disease is crucial. Future research should aim to further explore the performance of LPA across diverse clinical settings and against newer diagnostic technologies, to fully ascertain its optimal use in global TB control strategies.

The utilization of Cohen's kappa statistic in the comparative evaluation of GeneXpert and LPA with culture is essential for determining interrater reliability, which assesses the agreement between different diagnostic tests against the gold standard (McHugh, 2012). Comparative evaluation is critical, especially in healthcare, to confirm that new diagnostic tests align closely with established methods, ensuring their reliability and accuracy in clinical settings (Hazra & Gogtay, 2017)

Cohen's kappa is particularly valuable because it adjusts for chance agreement, which is a significant consideration in medical diagnostics where random chance can skew results, making it a robust measure for comparing new tests like GeneXpert and LPA to traditional culture methods (McHugh, 2012). This statistical approach ensures that evaluations of diagnostic tools are not only based on their concordance but also reflect true diagnostic agreement beyond chance, which is crucial for making informed clinical decisions (Dhamnetiya et al., 2022).

Using Cohen's kappa for evaluating GeneXpert and LPA against culture provides a reliable measure of agreement that is necessary for integrating these diagnostic tools into routine clinical practice, ensuring they meet the stringent accuracy requirements necessary for effective disease management.

The Cohen's Kappa (κ) value of approximately 0.9540, indicating near-perfect agreement between GeneXpert and culture methods, strongly supports GeneXpert's reliability and accuracy in diagnosing tuberculosis. This agreement is critical, especially considering the urgent need for rapid diagnostics in managing TB outbreaks and treatment initiation.

Comparatively, other studies also affirm GeneXpert's high diagnostic accuracy. For instance, Khan et al. (2020) reported that GeneXpert achieved a sensitivity of 100% and a specificity of 99.5% in diagnosing tuberculosis, closely aligning with the culture method's established reliability. This similarity underscores the potential of GeneXpert to serve effectively in diverse clinical settings. Additionally, studies like that by Meaza et al. (2017) and (Tang et al., 2017) highlight both the high sensitivity and specificity of GeneXpert, reinforcing its diagnostic precision, similar to what is observed in the culture method, across different types of TB manifestations.

The high kappa value aligns with findings from Metcalf et al. (2018), where GeneXpert's utility in diagnosing tuberculous meningitis, despite the challenging nature of the disease, showcases its robustness in various clinical conditions. The consistent performance of GeneXpert as detailed in these studies supports its integration into standard TB diagnostic protocols, as echoed by the comprehensive review in the study by (Sorsa & Kaso, 2021), which showed nearly twice the detection rate of GeneXpert over smear microscopy in TB-HIV co-infected patients.

Furthermore, the diagnostic efficacy of GeneXpert in settings where rapid, accurate testing is essential has been documented by (Pandey et al., 2017), who compared it directly with conventional drug susceptibility testing for MDR-TB, finding no significant difference in performance. This finding is crucial as it positions GeneXpert not only as a rapid diagnostic tool but also as a reliable component of multidrug-resistance screening programs.

The near-perfect kappa statistic observed in this analysis is corroborated by extensive research illustrating GeneXpert's alignment with culture methods and its enhanced diagnostic capabilities across various settings and patient populations. These comparisons highlight the vital role of GeneXpert in modern TB management strategies, ensuring rapid and accurate diagnosis essential for effective disease control.

The high Cohen's Kappa value ($\kappa \approx 0.92$) observed for the comparison between LPA and culture methods signifies an almost perfect agreement, indicating that both diagnostic techniques are highly consistent in identifying tuberculosis. This strong correlation underscores the reliability of LPA as a viable alternative to conventional culture methods, particularly valuable in scenarios demanding quicker diagnostic results.

Comparing these findings with other studies, the performance of LPA is reinforced by several research outcomes. For instance, Meaza et al. (2017) reported high sensitivity and specificity for LPA in detecting MDR-TB from smear-positive sputum samples, although its performance was lower in smear-negative samples. This aligns with the high kappa value found in our study, demonstrating that LPA can be exceptionally reliable in cases with higher bacterial loads.

In another study, Yadav et al., (2021) compared the performance of LPA and GeneXpert for early diagnosis of rifampicin-resistant pulmonary tuberculosis. They found that both tests were effective, with LPA showing high agreement with the culture results, similar to our findings. This comparison highlights LPA's robustness in detecting drug resistance, a critical factor in TB management.

Pandey et al. (2017) also found no significant difference between the performance of GeneXpert and conventional drug susceptibility testing methods for MDR-TB. This finding supports the idea that LPA, like GeneXpert, can reliably be used alongside or as an alternative to culture methods, particularly in resource-limited settings where rapid results are crucial.

Sorsa et al. (2021) demonstrated that GeneXpert had a nearly two-fold detection rate compared to smear microscopy in TB-HIV co-infected patients. While this

study focused on GeneXpert, it underscores the importance of using highly sensitive and specific molecular diagnostic tools like LPA to improve TB detection rates in co-infected patients, further validating the high kappa value seen in our analysis.

Additionally, Solanki et al. (2020) reported that GeneXpert showed a sensitivity of 91.18% and a specificity of 100% in diagnosing spinal tuberculosis, indicating high reliability. This study's findings, together with the high kappa value for LPA, suggest that molecular diagnostics, whether LPA or GeneXpert, provide a dependable alternative to culture methods, ensuring rapid and accurate TB diagnosis.

We can say that the high Cohen's Kappa value ($\kappa \approx 0.92$) for LPA compared to culture methods validates its integration into routine TB diagnostic protocols. This agreement not only enhances the speed and accuracy of TB diagnosis but also supports effective patient management and treatment strategies, as corroborated by multiple comparative studies. These findings highlight the critical role of LPA in modern TB diagnostics, ensuring that it remains a cornerstone in the fight against tuberculosis.

CHAPTER VI
CONCLUSION

This study demonstrates that both GeneXpert and Line Probe Assay (LPA) are highly effective for detecting *Mycobacterium tuberculosis* and rifampicin resistance, with GeneXpert showing slightly higher sensitivity (95.49%) compared to LPA (90.98%), and both exhibiting high specificity (99.22% and 99.18% respectively). The strong agreement with culture methods, indicated by Cohen's Kappa values (0.9540 for GeneXpert and 0.9174 for LPA), confirms their reliability. These findings support the integration of both tools into routine TB diagnostics, enhancing rapid and accurate detection, thereby improving patient management and treatment outcomes.

RECOMMENDATIONS

Building on the findings of this study, the following recommendations are proposed to enhance the scope and impact of future tuberculosis diagnostic research:

1. To conduct studies to evaluate GeneXpert and LPA in diverse populations and clinical conditions, including HIV co-infections.
2. To test GeneXpert and LPA on staining-negative samples for broader applicability.
3. To assess the efficacy of GeneXpert and LPA in patients undergoing TB treatment.
4. To integrate GeneXpert and LPA into routine TB diagnostic protocols to leverage high accuracy and rapid results.
5. To develop comprehensive TB diagnostic strategies combining GeneXpert, LPA, and other diagnostic methods.
6. Undertake cost-effectiveness analyses comparing GeneXpert, LPA, and traditional culture methods.

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APPENDIX A
LIST OF TOOLS, CHEMICALS, AND DIFFERENT REAGENTS USED OVER THE COURSE OF THIS RESEARCH

For Culture:

Sterile sputum collection containers,

N-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) solution,

Phosphate-buffered saline (PBS),

Centrifuge,

Biosafety level 3 (BSL-3) laboratory facilities,

Personal protective equipment (PPE) (gloves, masks, protective gowns),

Lowenstein-Jensen (LJ) slants,

Incubators (37°C),

Florescent microscopes equipped for fluorescent staining materials for AFB confirmation.

For GeneXpert

GeneXpert instrument system,

Xpert MTB/RIF cartridges,

Sterile pipettes,

Disposable gloves,

Sample Reagent (SR),

Vortex mixer,

Computer with programming for result

For LPA

Sterile sputum collection containers

Ice packs for sample transportation

N-acetyl-L-cysteine (NALC)

Sodium hydroxide (NaOH)

Phosphate-buffered saline (PBS)

Centrifuge

Vortex mixer

Pipettes and disposable pipette tips

Biohazard waste containers

TE buffer (Tris-EDTA)

Water bath (95°C)

Microcentrifuge tubes

Microcentrifuge

Thermal cycler for PCR

PCR reaction tubes or strips

Master Mix (Taq DNA polymerase, deoxynucleotide triphosphates (dNTPs), magnesium chloride (MgCl2), PCR buffer)

Primers (specific for *rpoB*, katG, and inhA genes)

Nuclease-free water

Twincubator

Nitrocellulose strips with specific oligonucleotide probes

Wash solutions and buffers

Colorimetric detection reagents

Strip reader

Laboratory coats, PPE

Disposable gloves

Safety goggles

Face shields

Laboratory notebooks

Computer and software for data entry and analysis

APPENDIX B
COMPOSITION AND PREPARATION OF DIFFERENT REAGENTS AND CULTURE MEDIA

Procedure for Preparation of NALC-NaOH Solution

The NALC-NaOH (N-Acetyl-L-Cysteine - Sodium Hydroxide) solution is used for the digestion and decontamination of sputum samples in the diagnosis of tuberculosis.

Ingredients and Composition:

Component	Quantity
N-Acetyl-L-Cysteine (NALC)	0.5 g
Sodium Hydroxide (NaOH), 4% solution	100 ml
Sodium Citrate, 2.94% solution	100 ml
Distilled Water	To prepare the above solutions

Preparation Steps:

Preparation of Sodium Hydroxide (4%) Solution:

4 g of NaOH was dissolved in distilled water, and the volume was made up to 100 ml.

Preparation of Sodium Citrate (2.94%) Solution:

2.94 g of sodium citrate was dissolved in distilled water, and the volume was made up to 100 ml.

Preparation of NALC-NaOH Solution:

In a sterile container, 100 ml of the prepared 4% NaOH solution was added. 0.5 g of N-Acetyl-L-Cysteine (NALC) was added to the NaOH solution. The mixture was thoroughly mixed until the NALC was completely dissolved. 100 ml of the prepared 2.94% sodium citrate solution was added to the above mixture. The solution was mixed well to ensure all components were fully dissolved and combined.

Preparation of Lowenstein-Jensen (LJ) Medium

Ingredients and Composition:

Component	**Quantity**
Potassium dihydrogen phosphate (KH_2PO_4)	2.4 g
Magnesium sulfate ($MgSO_4$)	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol (reagent grade)	12 ml
Malachite green, 2% solution	20 ml
Distilled water	Up to 600 ml
Homogenized eggs	1000 ml (from 25-30 fresh country hen’s eggs)

Preparation Steps:

Preparation of Malachite Green Solution (2%):

2.0 g of malachite green dye was dissolved in 100 ml of distilled water completely. The solution was filtered and stored in the refrigerator.

Preparation of Mineral Salt Solution with Malachite Green:

The following ingredients were dissolved in about 300 ml of distilled water by heating:

- 2.4 g of potassium dihydrogen phosphate
- 0.24 g of magnesium sulfate
- 0.6 g of magnesium citrate
- 3.6 g of asparagine

12 ml of glycerol and 20 ml of the 2% malachite green solution were added. The solution was made up to 600 ml with distilled water. The solution was sterilized by autoclaving at 121°C (15 psi) for 30 minutes. The solution was cooled to room temperature and stored in the refrigerator if required.

Preparation of Homogenized Eggs:

Fresh country hen's eggs, not more than seven days old, were used. The eggs were cleaned by scrubbing thoroughly with water and soap. The eggs were soaked for 30 minutes in a soap solution, rinsed thoroughly with running water, and then soaked in 70% ethanol for 15 minutes. The eggs were dried, and hands were scrubbed and washed with a disinfectant before handling. The eggs were cracked into a sterile flask and beaten in a sterile blender for 30 seconds to one minute.

Preparation of Complete Medium:

In a large sterile flask, 600 ml of the mineral salt solution with malachite green and 1000 ml of homogenized eggs were mixed. The complete egg medium was distributed into 6-8 ml volumes in sterile universal containers. The caps were tightly closed, and the medium was inspissated without delay to prevent sedimentation of heavier ingredients.

Coagulation of Medium:

The inspissator was preheated to 85°C. The bottles were placed in a slanted position in the inspissator, and the medium was coagulated for 50 minutes at

85°C. Proper coagulation temperature and time were ensured to avoid discoloration or formation of bubbles.

Sterility Check:

The media batch was incubated at 35-37°C for 24 hours to check for bacterial sterility. 5% of the slopes were randomly selected and continued incubation for 14 days to check for fungal sterility. The contamination rate did not exceed 10%.

Storage:

The LJ medium was dated and labelled with the batch number. The medium was stored in the refrigerator and kept for up to 4 weeks, ensuring caps were tightly closed to prevent drying.

For Staining

Glass slides, auramine-rhodamine stain, acid-alcohol solution, potassium permanganate, fluorescence microscope (400x magnification).

Preparation of Fluorescence Staining Solution (Auramine Method)

Primary Stain:

1.0 g of Auramine O powder was dissolved in 100.0 ml of ethanol. 30.0 ml of melted phenol was mixed with 870.0 ml of distilled water. Both solutions were combined. The mixture was stored in a dark bottle and labelled appropriately.

Decolourizer:

10 ml of concentrated hydrochloric acid was slowly added to 990 ml of ethanol. The solution was mixed thoroughly.

Counter Stain:

0.5 g of potassium permanganate ($KMnO_4$) was dissolved in 100 ml of distilled water. $KMnO_4$ was kept away from combustible materials due to its explosive nature.

Procedure for Preparation of TE Buffer (Tris-EDTA)

TE buffer (Tris-EDTA) is commonly used in molecular biology to store DNA and RNA, as it helps to prevent degradation by chelating divalent cations.

Ingredients and Composition:

Component	**Quantity**
Tris Base	1.21 g
EDTA (Ethylenediaminetetraacetic Acid)	0.0372 g
Distilled Water	Up to 100 ml
pH Adjuster (HCl or NaOH)	As needed

Preparation Steps:

Preparation of 1X TE Buffer (pH 8.0):

Tris Base: 10 mM

1.21 g of Tris base was weighed.

EDTA: 1 mM

0.0372 g of EDTA was weighed.

Dissolve in Distilled Water:

The weighed Tris base and EDTA were added to approximately 80 ml of distilled water in a beaker. The solution was stirred until both the Tris base and EDTA were completely dissolved.

Adjust pH:

A pH meter was used to check the pH of the solution. The pH was adjusted to 8.0 by adding concentrated HCl or NaOH dropwise. The solution was mixed well, and the pH was measured frequently to avoid overshooting the desired pH.

Make Up to Final Volume:

Once the pH was adjusted, the solution was transferred to a graduated cylinder. Distilled water was added to bring the final volume to 100 ml.

Sterilize:

The TE Buffer was sterilized by autoclaving at 121°C for 15 minutes or by passing it through a 0.22 µm filter.

Storage:

The prepared TE buffer was stored in a clean, labelled bottle. The buffer was stored at room temperature or refrigerated, depending on the laboratory's standard practices.

APPENDIX C

THE *RPOB* GENE

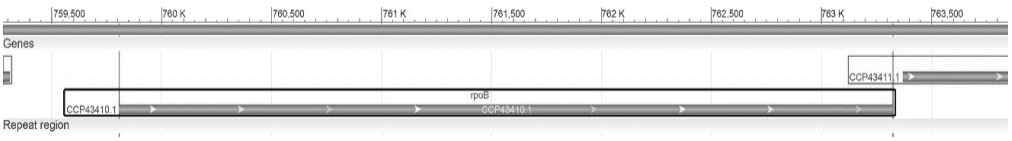

Fig: *rpoB* Gene (Source; *Mycobacterium tuberculosis* H37Rv complete genome,GenBank:AL123456.3m, https://www.ncbi.nlm.nih.gov/nuccore/AL123456.3)

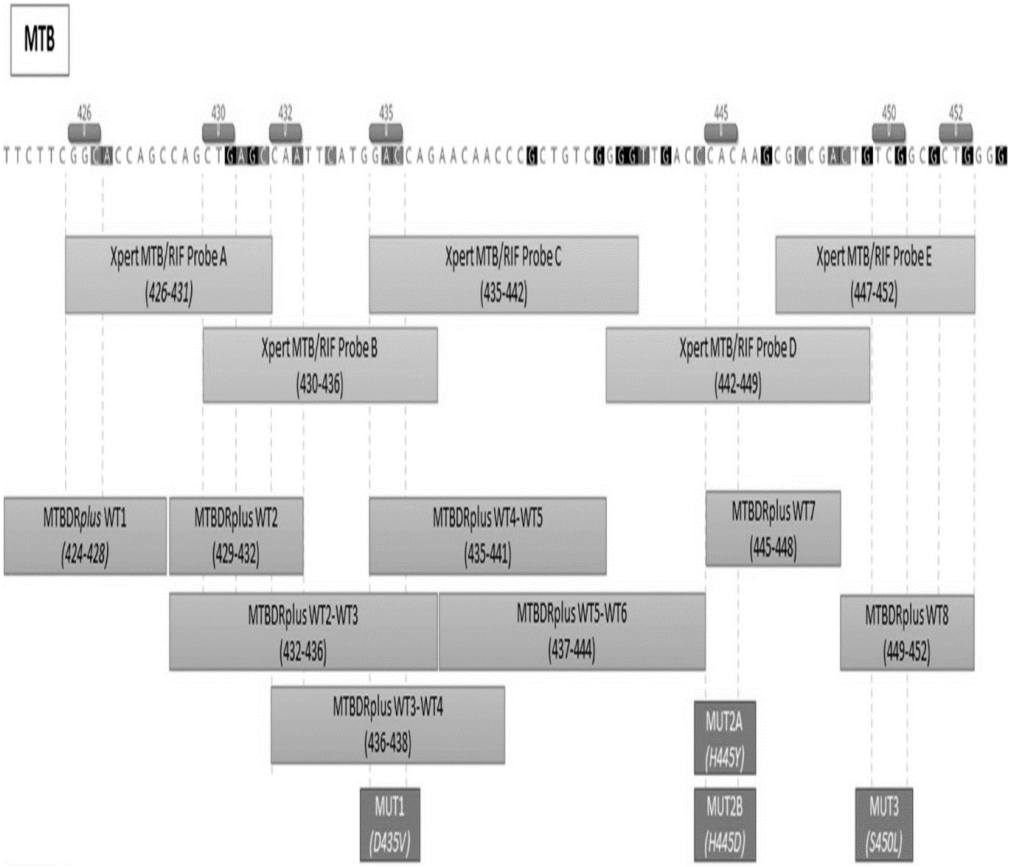

Fig: Alignment of *Mycobacterium tuberculosis* complex rifampicin-resistance-determining region (RRDR) sequence with the targets of Xpert MTB/RIF and the MTBDRplus V2.0 commercial assays. The red bars represent the location of the most common codons associated with rifampicin resistance conferring mutations. The orange bars indicate the regions covered by the five probes of the Xpert MTB/RIF assay. The green bars represent the regions covered by the eight wildtype (WT) and four mutation bands of the GENOTYPE MTBDRPLUS V2.0 assay.(Andre et al., 2017)

Gene	Antibiotic	Primers name	5′ - 3′ sequence	Hybridization temperature	Product size
rpoB	Rifampicin	TR1	TACGGTCGGCGAGCTGATCC	53°C	411 bp
		TR2	TACGG CGTTTCGATGAACC		

Fig: Primers used to amplify and sequence the *Mycobacterium tuberculosis* genes associated with resistance to antibiotics

Table**:** Mutations in the *rpoB* gene and the corresponding wild type and mutation bands.

Failing wild type band(s)	Codons analyzed	Developing mutation band	Mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
rpoB WT2	510-513		E510H
			L511P*
rpoB WT2/WT3	510-517		Q513L*
			Q513P
			del514-516
rpoB WT3/WT4	513-519	*rpoB* MUT1	D516V
			D516Y
			del515
rpoB WT4/WT5	516-522		del518*
			N518I
rpoB WT5/WT6	518-525		S522L
			S522Q
rpoB WT7	526-529	*rpoB* MUT2A	H526Y
		rpoB MUT2B	H526D
			H526R
			H526P*
			H526Q*
			H526N
			H526L
			H526S
			H526C

		rpoB	
rpoB WT8	530-533	MUT3	S531L
			S531Q*
			S531W
			L533P

* These rare mutations have only been detected theoretically (in silico).

Table**:** Mutations in the *katG* gene and the corresponding wild type and mutation bands

Failing wild type band	Codon analyzed	Developing mutation band	Mutation
		katG	
katG WT	315	MUT1	S315T1
		katG	
		MUT2	S315T2

Table**:** Mutations in the *inhA* promoter region and the corresponding wild type and mutation bands

Failing wild type band	Analyzed nucleic acid position	Developing mutation band	Mutation
		inhA	
inhA WT1	–15	MUT1	C–15T
		inhA	
	–16	MUT2	A–16G
		inhA	
inhA WT2	–8	MUT3A	T–8C
		inhA	
		MUT3B	T–8A

(Hain Lifescience, 2015)

APPENDIX D

DATA COLLECTION SHEET

Research No:			Hospital No:	
Name Of Patients:			Sex:	M F
Age:			District	
Contact Number			Sample Type	
Date of Sample Collection:				
GeneXpert Result	Date:			
MTB Detected	YES	NO		
Rifampicin Sensitive	YES	NO		
LPA Result	Date:			
MTB Detected	YES	NO		
Rifampicin Sensitive	YES	NO		
Type of Mutation				
Culture Result	Date:			
MTB Detected	YES	NO		
Load	1+	2+	3+	
Microscopy	AFB	1+	2+	3+
MPT64	+ve	-ve		

APPENDIX E

ETHICAL APPROVAL

Government of Nepal
Ministry of Health and Population
Department of Health Service
National Tuberculosis Control Center
Thimi, Bhaktapur
Phone no. 6630706
6630033
Fax no. 6635986
Website: www.nepalnt.gov.np

Ref. no.

16th Aug 2022
Mr. Soyuz Baral
Principal Investigator

Approval of Research Proposal and Permission for Research

Dear Mr. Baral,

It is my pleasure to inform you that upon departmental review in the National Tuberculosis Control Center, your proposal titled "**Comparative Analysis of GeneXpert and Line Probe Assay for Detecting Mycobacterium tuberculosis at The National Tuberculosis Control Center in Nepal**" has been approved. You are permitted to perform research in the NTCC lab facility, and the researcher must strictly adhere to National Health Research Council guidelines. As per NTCC rules and regulations, the investigator must strictly follow the protocol stipulated in the proposal. Any change in objectives, research objective, methodology and data management that may be necessary in the course of the implementation of the research proposal can only be made and implemented after prior approval from the department. Thus, it is compulsory to submit the details of any such changes intended or desired with justification prior to actual change in the protocol.
Further, the researchers are directed to strictly abide by the guidelines during the implementation of their research proposal and submit a progress report and a full report upon completion.
If you have any questions, please contact the NTCC office.
Thank you,

Sr. Medical Technologist / Clinical Biochemist
Lab Incharge
National Tuberculosis Control Centre
Thimi, Bhaktapur

APPENDIX F

PLAGIARISM REPORT

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Central Library

Kirtipur, Kathmandu, Nepal

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Date: 07/10/2024

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Plagiarism Test Report

The **Master Thesis** titled **"COMPARATIVE ANALYSIS OF GENEXPERT AND LINE PROBE ASSAY FOR DETECTING MYCOBACTERIUM TUBERCULOSIS AT THE NATIONAL TUBERCULOSIS CONTROL CENTER IN NEPAL"** submitted by **SOYUZ BARAL** for a plagiarism test on **July 10, 2024**, has been checked by the iThenticate plagiarism checker software. The software found an overall similarity index of **18%** based on the following criteria.

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Laxmi Bahadur Nemkul

(Section Officer)

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