DEVELOPMENT OF A REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR RAPID DETECTION OF *Mycobacterium Tuberculosis*

A THESIS SUBMITTED BY

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TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

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DECLARATION

I, Swathy S Nair hereby certify that I had personally carried out the work depicted in the thesis titled, **"Development of a Real-Time Loop-mediated isothermal amplification assay for rapid detection of *Mycobacterium Tuberculosis*"**

No part of this thesis has been submitted for the award of any other degree or diploma prior to this date.

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The thesis entitled, "**Development of a Real-Time Loop-mediated isothermal amplification assay for rapid detection of *Mycobacterium Tuberculosis***" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

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The thesis entitled

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SWATHY S NAIR

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LIST OF ABBREVIATIONS

S No	Abbreviation	Full Form
1	MTB	*Mycobacterium tuberculosis*
2	TB	Tuberculosis
3	AFB	Acid Fast Bacillus
4	LJ	Löwenstein- Jensen
5	MGIT	Mycobacteria Growth Indicator Tube
6	NAAT	Nucleic Acid Amplification Test
7	CB-NAAT	Cartridge Based Nucleic Acid Amplification Test
8	rpoB	RNA Polymerase β subunit
9	RIF	Rifampicin
10	Bst. Pol	Bacillus stereo thermophilous Polymerase
11	DOTS	Directly Observed Treatment Short-course
12	MDR-TB	Multi Drug-Resistant Tuberculosis
13	XDR-TB	Extensively Drug-Resistant Tuberculosis
14	SDG	Sustainable Development Goals
15	PC	Parliamentary Constituency
16	TNFα	Tumor Necrosis Factor
17	LTBI	Latent Tuberculosis Infection
18	TST	Tuberculin Skin Testing
19	IGRAs	Interferon-gamma Release Assays
20	PPD	Purified Protein Derivative

21	DTH	Delayed-Type Hypersensitivity
22	BCG	Bacille Calmette- Guerin
23	IFNγ	Interferon Gamma
24	ESAT-6	Early Secreted Antigenic Target 6
25	CFP-10	Culture Filtrate Protein 10
26	ELISA	Enzyme Linked Immunosorbant Assay
27	ELISPOT	Enzyme Linked Immunosorbant Spot
28	DST	Drug Susceptibility Testing
29	LPA	Line Probe Assay
30	WGS	Whole Genome Sequencing
31	ART	Anti- Retroviral Therapy
32	IUATLD	International Union against Tuberculosis and Lung Disease
33	COPD	Chronic Obstructive Pulmonary Disease
34	PPV	Positive Predictive Value
35	NPV	Negative Predictive Value
36	Ct	Cycle Threshold

SYNOPSIS

Tuberculosis (TB) is an infectious disease caused by the airborne bacteria *Mycobacterium tuberculosis* (MTB), which remains a serious public health concern. Pulmonary tuberculosis is the most common disease that primarily affects the lungs and those who suffer from this condition may experience coughing, chest pain, and difficulty in breathing symptoms. On the other hand, extra-pulmonary tuberculosis affects other parts of the body, such as the kidneys or spine.

The most common pulmonary TB disease is highly preventable; however, it remains a serious public health problem due to the number of undiagnosed and untreated individuals, leading to community infection transmission and an increase in TB cases. In 2021, the World Health Organization (WHO) estimated an increase of 4.5% in TB new cases from 2020 reversing the decline of about 2% per year incidence rate due to the rapid spread of TB disease. Such an incidence rate resulted in an increased mortality level among the affected population. An effective strategy to decrease the rate of transmission of disease would be to diagnose and treat infected individuals at the earliest stage. The current diagnostic methods involve microscopic examination of smears, bacterial culturing procedures and Polymerase Chain Reaction (PCR) based nucleic acid tests from patient sputum samples.

The smear test is the primary method for diagnosing pulmonary tuberculosis, particularly in areas with high TB prevalence. It is an inexpensive and easy-to-perform tool for early diagnosis of TB disease. However, the sensitivity of the smear test varies from 20% to 60% depending on the burden of bacteria present in the sputum.

The culture test is considered the gold standard for the confirmation of TB disease. In comparison with the smear test, it has higher sensitivity and specificity for TB detection. However, because the test is labour-intensive and time-consuming, it

delays appropriate treatment in the absence of a confirmed diagnosis that results in the potential spread of TB infection. Therefore, it would be advantageous to develop a test that combines the rapidity of microscopy and the sensitivity of bacterial culture methods to facilitate the initiation of an effective treatment for tuberculosis.

Regarding these factors, an advanced detection method called the nucleic acid amplification test (NAAT), improves TB diagnosis by allowing rapid detection with high specificity and sensitivity that would enable early diagnosis of TB. The cartridge-based Xpert MTB/RIF test developed by Cepheid Inc. is a real-time PCR-based technique that simultaneously detects TB and rifampicin (RIF) resistance of MTB from unprocessed sputum samples within 2 hours. The overall sensitivity and specificity of Xpert MTB/RIF were reported as 85% and 98%, respectively, compared to the culture test. Molbio Inc recently developed a PCR-based technique called TrueNat with similar sensitivity and specificity to that of Xpert MTB/RIF assay. In addition, to detect antibiotic resistance of the bacterium a method called Line probe assay (LPA), another robust PCR-based technique, is used. All the above-mentioned NAAT-based techniques require specialized laboratory settings, which makes them challenging to implement in resource-poor settings.

Loop-mediated isothermal amplification (LAMP) is a potential alternative to PCR-based assays. A LAMP assay is easy to set up with a heating block or water bath, which minimizes the requirement for expensive laboratory facilities and can produce a large number of amplified products in less than an hour for rapid detection. There are several methods available for the detection of amplification in the LAMP assay, including turbidity measurement, measuring changes in pH, the detection of amplification using DNA intercalating fluorescent dyes, and measuring the change in Mg^{2+} levels to make it robust. However, each of these detection techniques alters the

sensitivity of the assay. Some of these methods require opening the tube after the assay is completed, which leads to cross-contamination due to the high copy numbers of the amplified products in the LAMP reaction. Therefore, a method that can reduce the risk of cross-contamination could increase the sensitivity of the LAMP assay for diagnostic purposes. To achieve this, real-time monitoring of the LAMP reaction could provide a reliable way to determine an effective dye suitable for detecting amplified DNA products.

The study aims to develop a detection protocol in Real-time Loop-mediated Isothermal Amplification (RT-LAMP) assay with a suitable DNA binding dye to achieve the maximum sensitivity and high specificity to diagnose pulmonary TB with the simplest configuration.

AIM

The study aims to develop a Real-time Loop-mediated Isothermal Amplification (RT-LAMP) of the *mpt64* gene of *Mycobacterium tuberculosis* (MTB) for the rapid detection of TB infection with high specificity and sensitivity by using an efficient DNA intercalating fluorescent dye.

RESULTS

For the study, the *mpt64* gene specific to MTB, which encodes a strain-conserved secreted protein, was used as the amplification target for the LAMP assay. The LAMP reaction primers were specific to the *mpt64* gene and were designed using the criteria necessary to follow for the assay. The assay was carried out at 65ºC for 45 minutes, with optimum concentrations of the primers and WarmStart LAMP master mix reagents required for the LAMP reaction. The results showed that the primers designed are following the eligibility criteria for the efficient amplification of the *mpt64* gene by LAMP reaction.

Next, to find a favourable DNA binding dye for the LAMP assay, two DNA intercalating fluorescent dyes SYTO 16 and SYBR Green 1 with an optimum concentration were compared and evaluated. For the comparison, the fluorescent intensity of the SYTO 16 and SYBR Green 1 after the LAMP assay was measured. The results showed that the fluorescence intensity differences between SYTO 16 and SYBR Green 1 were significant, in which SYTO 16 showed a higher intensity.

To evaluate the efficiency of these two fluorescent dyes on the LAMP reaction, a real-time LAMP (RT-LAMP) assay was performed with SYTO 16 and SYBR Green 1 for the real-time monitoring of the reaction with these dyes.

Compared to SYBR Green 1, SYTO16 showed a better signal-to-noise ratio and a rapid amplification without inhibiting the reaction. The results, therefore, concluded that SYTO 16 is an effective DNA intercalating dye for the performance of LAMP assay.

Next, to determine the limit of detection of the RT-LAMP assay with SYTO 16, a plasmid pL4440 cloned with the *mpt64* gene sequence was used as the target for

the reaction. The results showed that the RT-LAMP assay can detect < 10 copies of DNA at the same level as the quantitative PCR (qPCR) method.

The further objective of the study is to test the specificity of the RT-LAMP assay for MTB, by using various non-mycobacterial DNA extracts as templates. The results showed that the RT-LAMP assay designed for the purpose is precise for the amplification of the *mpt64* gene from MTB. The resulting data from these experiments ensured that the TB LAMP assay with SYTO 16 can be considered a reliable diagnostic tool for TB clinical samples.

To clinically confirm, a cross-sectional study was conducted to compare the efficacy of TB RT-LAMP assay by concurrently performing the smear, Xpert MTB/RIF, and culture tests on 350 presumptive TB clinical samples. The early morning patient sputum samples were collected with the approval of the Ethics committee and with informed consent from the patients. The study collected the epidemiological, demographical and clinical data of patients by following the WHO national guidelines.

A new protocol for DNA extraction from patient sputum samples was standardised. To collect and decontaminate the sputum samples, the N-acetyl-l-cysteine–sodium hydroxide (NALC-NaOH) solution was used. The decontaminated samples were then centrifuged, and the pellets were incubated at 95^0C in lysis buffer containing 0.4 M NaCl, 2mM EDTA, 10mM Tris-Cl, and 0.5 % Triton X-100. After neutralising the solution with 10mM Tris-HCl at pH7.5, the tubes were sealed and stored at 4^0C until the next use.

Alongside the TB RT-LAMP assay, an in-house TB-quantitative PCR (TB-qPCR) assay targeting *mpt64*-gene with Fluorescein amidites (FAM) and Black Hole Quencher 1 (BHQ1) probes was developed to confirm the efficiency of MTB DNA

extraction protocol. The TB-qPCR assay was carried out with the Luna Universal probe qPCR mix.

The results obtained from TB RT-LAMP testing on 350 patient samples were compared with the gold standard culture test. As compared to the culture test, the TB RT-LAMP showed 89.36% sensitivity and 94.06% specificity, while a primary test called the smear test, revealed only 59.57% sensitivity and 95.38% specificity. The sensitivity and specificity comparison of Xpert MTB/RIF to the culture test showed 87.23% and 93.73%, similar to the TB RT-LAMP assay results mentioned above.

In addition, our study revealed that TB RT-LAMP had a sensitivity of 93.33% and specificity of 98.62% when compared with the Xpert MTB/RIF assay on patient samples. The results indicated that the TB RT-LAMP assay has a similar efficacy to that of the Xpert MTB/RIF in detecting MTB.

To further confirm, the positive smear and culture samples were grouped, and the detection efficiency of the TB RT-LAMP assay was compared with these results. The data showed that the TB RT-LAMP assay had a sensitivity and specificity of 100% and 95.85%, respectively, similar to those of the Xpert MTB/RIF, which showed a sensitivity and specificity of 100% and 95.5%, respectively. Moreover, the comparison of results from the TB RT-LAMP assay and Xpert MTB/RIF test with culture-positive and smear-negative samples revealed a sensitivity of 73.68% and 68.42%, respectively. The results indicated that the TB RT-LAMP assay with similar detection accuracy as that of Xpert MTB/RIF could be a better option for a primary test or an add-on confirmation method for smear-negative samples with TB symptoms.

The results of the in-house TB-qPCR assay with isolated DNA from patient samples for this study also showed identical results to those of the TB RT-LAMP assay.

This study also attempted to develop an end-point reader to further reduce the cost of conducting TB diagnosis in the field. A working prototype of a device with a heating block to amplify DNA under isothermal conditions and detection at 480 nm excitation and 520nm emission wavelength filters was designed and developed. The data obtained for the TB LAMP assay from the device showed a similar detection sensitivity as compared to the TB RT-LAMP assay. As an alternative end-point reader, we utilised a UV transilluminator, which proved to be a viable option for developing a handheld device. The detailed specifics of this method are discussed in the thesis.

SIGNIFICANCE OF THE STUDY

The study aimed to improve the efficiency and accuracy of TB diagnosis by developing an easy-to-handle method, which contributes to the effectiveness of early TB treatment for patients mainly from low and middle-income countries. The RT-LAMP method used for amplifying the MTB-specific *mpt64* gene using SYTO 16 DNA intercalating fluorescent dye showed similar efficiency and accuracy compared to the currently available NAAT-based diagnostic tests. The results indicated that RT-LAMP assay is an effective tool for rapid diagnosis of TB with high accuracy that can replace or as an add-on with currently used conventional smear tests in resource-limited areas, primarily for in-field diagnosis.

INTRODUCTION

1. INTRODUCTION

Tuberculosis is a contagious airborne infectious disease caused by a bacterial strain named *Mycobacterium tuberculosis* (MTB), which is a major cause of morbidity and mortality worldwide. Even though the disease is known as one of the oldest infections affecting humans, it remains a major threat and the second leading cause of death among the populations with HIV/AIDS (Barberis et al., 2017; Straetemans et al., 2010).

In 2022, an estimated 10.6 million people worldwide were infected with TB, of which the low-middle countries accounted for 87% of the infected cases. The eight countries with the highest number of TB incident cases are India (28%), Indonesia (9.2%), which together accounted for around 38% of all incidences of TB, China (7.4%), the Philippines (7.0%), Pakistan (5.8%), Nigeria (4.4%), Bangladesh (3.6%) and the Democratic Republic of the Congo (2.9%), accounted more two-thirds of global TB cases. About 90% of TB cases among that population were reported in adults, with men having a two-fold higher incidence rate than women (WHO, 2022). Moreover, in such areas, the major risk factors that trebled the TB infection rate reported are malnutrition, air pollution, unhealthy alcohol consumption and smoking (Pai et al., 2016). Concerning this, the classical symptoms of TB present in suspected individuals are persistent coughs, chest pain, weight loss, night sweats, fevers, hemoptysis, fatigue and lack of appetite(Cara M. Gill et al., 2022). Furthermore, the estimated death rate of around 95% due to TB disease was mostly accounted for in low- and middle-income countries, where available healthcare services were

overwhelmed with limited resources (Foo et al., 2022). Though the disease is curable and preventable, TB still poses significant public health risk issues. The most obvious reason for such risk is the extensive reduction in reporting newly diagnosed patients to the clinical spot within the right time to initiate the treatment. Accordingly, the number of undiagnosed and untreated patient levels is increasing, intensifying the community transmission of infection and TB cases (WHO, 2022).

The vital factors for controlling global TB prevalence are early and rapid detection followed by treatment and precautions to limit the transmission of infection from one to another. For that, improving the efficacy of diagnosis would be feasible and reasonable for the timely provision of such treatment for infected patients.

The currently ongoing TB diagnostic methods in most developing countries are the microscopic observation of sputum samples and culturing of bacteria after clinical evaluation, which are literally the conventional methods followed for many years.

The primary smear test for TB diagnosis is performed by collecting patient sputum samples stained with a Ziehl-Neelsen stain for the acid-fast Bacillus (AFB) testing and observed under a microscope (Schmid et al., 2014). The key advantages of the smear diagnostic method for clinical TB testing and evaluation are the rapidity of the analysis, an inexpensive tool for routine testing, the simplicity of performing the test that is vastly effective in regions with high TB incidence and an integral part of monitoring treatment progress and response that helps to predict TB relapse probability in patients. Though the method is a primary test for TB diagnosis, the major drawbacks of the smear test are low and variable sensitivity (ranging from 30-80%),

even though the method is with more than 95% of specificity. Moreover, the smear test results are inconsistent for patient sputum samples containing the burden of bacteria noted as below 10,000 per ml (Yan et al., 2016). Additionally, the results may differ in resource-constrained areas where large samples are collected for analysis, requiring the laboratory technician to confirm smear test grades within 60 seconds. As a result, there is a chance of predicting false negative results for patients with suspected TB.

Another preliminary clinical test for TB diagnosis is the analysis of Chest X-ray to represent the conditions of the thoracic cavity of patients where the enlargement of the Hilar in the upper zone of the lungs due to swelling of lymph nodes are considered the symptoms of primary TB. Though the test is highly sensitive for the prediction of TB, the method is non-specific for TB disease as the symptoms, as mentioned above, could also be present in other modes of lung diseases. In addition, trained personnel are required to accurately analyse and evaluate the results. Besides, the recurrent analysis of TB patients with Chest X-rays to depict their response to treatment is unfavourable because of their exposure to ionizing radiation (Murphy et al., 2020).

The bacterial culture method is a gold standard for TB diagnosis with accuracy owing to its high specificity and sensitivity for the detection of MTB. The patient sample could be cultured in solid (LJ medium and Middlebrook 7H11) or liquid (MGIT) media for the TB test. The difference in the culture test using these two methods denotes that the detection of TB growth in the LJ medium requires around 3 to 6 weeks for confirmation, whereas, for the MGIT medium, the growth results could

be observed within two weeks. Moreover, the culture method is more convenient for evaluating the viable and non-viable MTB bacteria from the collected sample than the smear test. The major concern of culture tests as a method for timely diagnosis is that the test requires quite a few weeks to confirm the growth of MTB in culture plates as visible colonies because the cell division of MTB requires around 18 to 20 hours of incubation (Asmar and Drancourt, 2015). Therefore, the confirmation of TB via culture method delays the treatment and follow-up of patients on time to control TB infection, which would become a reason for the rapid spreading of the disease. Moreover, the requirement of biosafety parameters and intensive laboratory procedures demands maximum technical ability for the confirmation of TB tests through this method. Regarding this, a rapid diagnostic method with the intention of smear test specificity and sensitivity grade of the culture method would be a reasonable practice for infield TB testing.

The nucleic acid amplification test (NAAT) for the detection of TB is a molecular-based method to identify the specific genomic region of MTB from the collected patient samples. For the rapid detection of MTB and resistance towards rifampicin antibiotics, a NAAT method called Xpert MTB/RIF (Cepheid, USA) is employed to test the sputum samples of patients directly. The method is a cartridge-based nucleic acid amplification test (CB-NAAT) combining automated sample processing and real-time amplification of the rpoB gene, which encodes DNA-dependent RNA polymerase of MTB, with 2 hours of detection time. The primers used for the test comprise five sets of probe primers targeting the Beta subunit, a binding site of rifampicin antibiotics, coding region present in the rpoB (81bp) gene. If there

is a mutation, the failure of amplification from any of the probe primers for the rpoB gene represents the result as MTB positive with rifampicin resistance (Ullah et al., 2016). The TB detection using Xpert MTB/RIF reported an overall sensitivity of around 86- 92% and 97- 99% specificity compared to the culture and smear-positive results. Remarkably, the Xpert MTB/RIF test was found to have a 72- 77% sensitivity for the culture-positive and smear-negative patient samples (Ioannidis et al., 2011). Therefore, this method presents a further advantage for TB diagnostics and treatments in areas with a high HIV burden where patients usually have smear-negative TB results. In that way, the mode of detection could control the TB transmission rate among the global population. Even though the WHO endorsed the Xpert MTB/RIF test as the primary TB diagnostic method in the suspected patient, the implementation of this test is inconvenient for developing countries. The demerit factors of this method, mainly for highly TB-prevalent countries, are the currently available CB-NAAT machinery has only the capacity to hold a maximum of 16 samples at a time; moreover, for its function, it requires sophisticated instrumentation and constant power consumption with critical temperature ceiling settings for the detection of TB from sputum samples. Furthermore, compared to the primary TB diagnostic methods, this test is unaffordable for rapid testing of TB infection, mainly in low- and middle-income countries due to its demand for higher diagnostic costs than smear and culture tests (Yan et al., 2016).

A molecular-based amplification technique known as Loop-mediated isothermal amplification (LAMP) allows the test to be performed in less sophisticated settings than the CB-NAAT method. The LAMP assay is an auto-cycling strand

displacement method using a large fragment of Bacillus stereo thermophilous Polymerase (Bst. Pol) enzyme that can produce non-denatured DNA amplification at a constant temperature (Notomi et al., 2000). Moreover, LAMP requires a specific primer design mode for amplifying DNA sequences to perform the reaction under isothermal conditions along with the enzyme. The LAMP primers named forward and backward inner primers (FIP and BIP, respectively) and outer forward and backward primers (F3 and B3, respectively) are the major sequences initiating the reaction. To design the primers, the factors that need to consider are the size of primers within the range of 130bp and 200bp and the sequences with the melting temperature suitable for LAMP reaction conditions (Notomi et al., 2000). This results in the amplification of the target region with higher sensitivity and specificity, similar to that of the PCR method. For developing an effective diagnostic method for TB, especially for low- and middle-income countries with high TB burden, the demanding factors involve rapid on-site detection with a similar range of sensitivity and specificity of CB-NAAT with an accessible configuration suitable for handling more samples within a limited amount of time and space in an easy-to-perform module. Therefore, the LAMP assay can be considered a diagnostic method for TB detection, which facilitates early detection to improve treatments and prevent disease transmission.

However, the LAMP assay has certain limitations in observing amplified products and interpretation of adequate results. There are several methods reported for the analysis of LAMP products after the assay, which involves turbidity measurement showing the precipitation of magnesium pyrophosphate after the LAMP assay, the intensity of colour change after the reaction depending on pH and the other method of

measuring the fluorescence intensity with DNA intercalating dyes and with calcein fluorescent dye which binds to the metal ions, especially the measuring of Mg^{2+} ions accumulation in the reaction solution (Ma et al., 2019). However, each of these detection methods alters the sensitivity of the LAMP assay. Some of these methods require the opening of the tubes after the reaction, which can result in cross-contamination issues due to the high copy numbers of the LAMP-amplified DNA products. Therefore, a method that can reduce the risk of cross-contamination would be suitable for the detection of LAMP-amplified products, mainly for diagnostic purposes. To obtain this, the real-time monitoring of the LAMP reaction could provide a reliable way to determine a suitable dye for detecting and evaluating the amplified DNA products. Therefore, the Real-time LAMP (RT-LAMP) reaction is a method of DNA amplification that can be performed with a fluorescent DNA-binding dye (Aoi et al., 2006). As the reaction progresses, the fluorescent DNA binding dye is incorporated into the DNA strands, allowing for amplification and detection of the target sequence. Rather than using probes, DNA binding dyes are found to have an excellent alternative method for detecting Real-time LAMP products as they can increase the sensitivity of the assay compared to other detection methods (Li et al., 2014). When choosing an appropriate dye, the criteria that need to be noted include optimal concentration and the ideal combination of shorter amplification time, high fluorescence, and lower reaction inhibition.

For the study, two DNA-intercalating fluorescent dyes, SYTO 16 and SYBR Green1, were evaluated to find a suitable detection method for TB LAMP assay for diagnostic purposes (Abbasi et al., 2016; Gudnason et al., 2007). For the TB LAMP

assay, the primers were designed by targeting an *mpt64* gene sequence specific to MTB which codes for a 24-kDa secreted protein vital for the activation of immune response of the TB-infected individuals, which is found to have a higher sensitivity for TB detection (Balne et al., 2013; G. Sharma et al., 2019).

The study aimed to monitor the amplification of the *mpt64* gene sequence using the TB-LAMP method by evaluating the effect of SYTO 16 and SYBR Green 1 dye on the reaction. Here, the study reported the difference in fluorescent intensity between SYTO 16 and SYBR Green 1 after the TB LAMP reaction. Moreover, the detailed analysis of TB RT-LAMP assay with SYTO 16 and SYBR Green 1 demonstrated that a combination of the optimal time-to-threshold (Ct) and signal-to-noise ratio (SNR) with minimal inhibition by SYTO 16 dye enabled the assay to be more efficient and with improved signal strength - a key phase of a real-time assay. Moreover, the study demonstrated the efficient sensitivity and specificity results as that of TB qPCR for the *mpt64* targeted TB RT-LAMP assay with SYTO 16 dye. The study outcome suggested that the optimized LAMP assay for TB diagnosis with SYTO-16 seems to be a precise and sensitive procedure for the rapid and early detection of MTB in sputum samples from TB patients. Hence, the optimized study was evaluated with presumptive TB patient sputum samples collected from the Tuberculosis Demonstration and Training Centre. Based on the assessed diagnostic efficacy of TB RT-LAMP assay with the Xpert MTB/RIF, culture and smear test results, the study developed a simple portable device named GeneDot. This tube scanner combined the heating block and fluorescent detection unit into a single format for TB LAMP assay. The designed portable device has the potential to hold 20 samples per test run with a highly efficient port to measure

the fluorescent intensity after the reaction. For further optimization of the TB LAMP assay for the infield diagnosis, the endpoint reading of results under a UV light chamber was performed with double blind analysis. The TB LAMP assay using SYTO 16 was demonstrated to be an accurate and sensitive method for the rapid and early detection of MTB infection in humans.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 HISTORY OF TB DISEASE

Tuberculosis (TB) disease is one of the oldest transmissible bacterial infections that disturbed humankind throughout history and human prehistory. Consequently, it is hypothesized that the Mycobacterium genus emerged more than 150 million years ago. In ancient times, TB disease is believed to have originated in animals and later transmitted to humans (Daniel, 2006). TB was prevalent in Europe during the Middle Ages and Renaissance and was called "king's evil" at the time because of the belief that it could be cured by the royal touch (Murray et al., 2016). Later, the disease was predicted as often associated with crowded living conditions and poor sanitation. The disease was stigmatized highly, and sufferers were often isolated or unaccepted. In the 18th century, the Enlightenment period brought some progress in understanding TB, with physicians being identifying it as a contagious disease (Bryder, 2002). After that, with the understanding of TB as a contagious condition, the late 19th and early 20th centuries promoted the establishment of sanatoriums or specialized TB hospitals which emphasized a regimen of fresh air, sunlight, rest, and good nutrition, which were considered essential for TB patients' recovery. The sanatorium movement, therefore, played a significant role in reducing the spread of the disease (Daniel, 2011). The 19th century marked a significant shift in understanding TB disease. In 1882, German physician Robert Koch identified the bacterium *Mycobacterium tuberculosis* as the cause of TB (Figure 1). Afterwards, the discovery transformed the understanding of the disease and flagged the way for diagnosis and treatment developments (Gradmann, 2001).

Figure 1: Discovery of *Mycobacterium Tuberculosis* as a causative agent of Tuberculosis by Robert Koch .The portrait in 1884 of Robert Koch who was honoured with the Nobel Prize for the discovery of the causative agent of Tuberculosis in 1905, an innovative work on tuberculosis disease (Kaufmann and Schaible, 2005).

The early developments in TB smear microscopic observation date back to the late 19th century by Robert Koch and laid the foundation for the development of diagnostic techniques to detect and identify the presence of the MTB in patient samples. As a result of Robert's discovery, several other researchers made further refinements to the modifications to the reagents used for the smear test. The demonstration of tubercle bacillus with alum hematoxylin stain by Paul Ehrlich was further modified by Franz Ziehl, using carbolic acid (phenol) as the fixative agent. Consequently, a pathologist named Friedrich Neelsen changed the primary stain to the basic fuchsin, allowing for the visualization of acid-fast bacilli (AFB) in sputum samples. This technique revolutionized TB diagnosis, enabling the identification of MTB under a microscope with greater accuracy. Thereafter the test was named as

Ziehl- Neelsen smear test also known as the acid-fast staining method (Kaufmann and Schaible, 2005).

The next phase of TB was the search for remedies to cure the disease condition. In 1943, the discovery of streptomycin, the first antibiotic against TB by Selman A. Waksman along with one of his graduate students named Albert Schatz, marked a turning point in TB treatment (Woodruff, 2014) (Figure 2).

Figure 2: Discovery of an antibiotic Streptomycin for TB. In 1944, Professor Selman Waksman and his graduate student Albert Schatz during experiments led to the discovery of streptomycin, an antibiotic medication, at Rutgers University. (Woodruff, 2014)

Over the following decades, there was a development of other antibiotics mainly isoniazid and rifampicin due to their shorter treatment durations compared to streptomycin, which was then promoted for the introduction of combination drug therapies known as Directly Observed Treatment Short-course (DOTS) in the 1990s. Thereafter, the DOTS became the global standard for TB treatment (Davies, 2003).

Despite these advancements in treatment, TB continued to pose challenges to the global population. The emergence of drug-resistant strains, such as multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), became a significant distress in the late 20th century and early 21st century. These forms of TB are more challenging to compact with the treatment and require extended and more complicated regimens.

There has been a renewed global focus on addressing TB. The World Health Organization (WHO) launched the End TB Strategy in 2014, intending to eliminate TB as a public health threat by 2030. The strategy emphasizes early diagnosis, prompt treatment, and prevention measures, alongside research and innovation.

2.2 WHO END TB STRATEGY

The TB end mission builds upon the goals set forth by the World Health Organization (WHO) in its End TB Strategy, which was launched in 2015 to reduce TB-related death rates by 90% and new cases by 80% between 2015 and 2030 (Uplekar et al., 2015). The strategy is associated with the Sustainable Development Goals (SDGs) of the United Nations and is focused on the three main factors, which are integrated patient-centred care and prevention, bold policies and supportive systems, and intensified research and innovation (Raviglione and Uplekar, 2006). Integrated patient-centred care and prevention are intended to ensure that every person with TB has access to prompt diagnosis, high-quality treatment with superior care, and supportive services. In order to avoid the emergence of drug-resistant TB, it also

emphasises early diagnosis through enhanced diagnostic procedures, prompt implementation of suitable treatment regimens, and adherence support. Additionally, it encourages the integration of TB services with other healthcare initiatives to improve patient-centred care and ensure TB disease management comprehensively. Bold policies and supportive systems are focused on the need for political commitment, suitable resources, and ideal healthcare systems to efficiently tackle TB by incorporating supportive policies and legal frameworks, mobilising domestic and international funding, strengthening health systems, and promoting equitable access to high-quality healthcare services for all people, particularly vulnerable and marginalised populations. Whereas the intensified research and innovation aimed to give importance to the research and innovation in driving progress towards ending TB by promoting investment in these fields to develop new strategies and technologies for prevention, diagnosis, treatment, and care (Al Abri et al., 2020). The main objective of this statement is to focus on the collaboration between researchers, policymakers, and communities that are crucial for generating evidence-based solutions and translating research findings into practice.

To achieve the objectives of the End TB Strategy, a global alliance programme is vital which involves the partnerships and collaboration between governments, international organizations, civil society, researchers, healthcare providers, and affected communities. Therefore, the efforts to control TB require a comprehensive approach, addressing not only the medical phases but also social, economic, and environmental factors that also come along with the reasons for the spread and persistence of the disease (Floyd et al., 2013) (Figure 3).

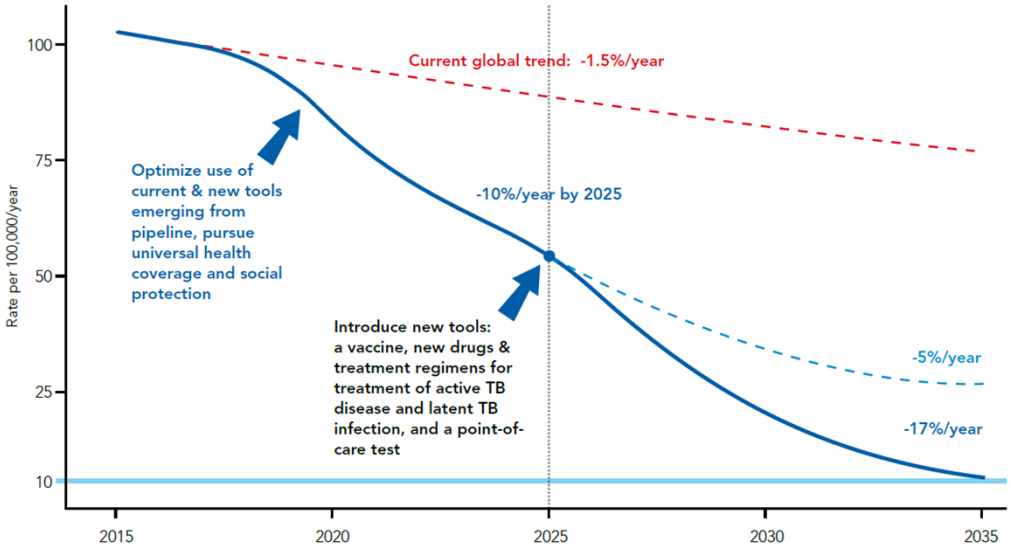


Figure 3: END TB strategy planned to decline in the TB global incidence rates by following the given principles denoted by WHO. (WHO, 2015)

2.3 EPIDEMIOLOGY

According to the WHO report, in 2021, an estimated 10.6 million people were infected with TB disease, up from 10.1 million reported in 2020, indicating an increase in incidence rate by 3.6% between 2020 and 2021. Accordingly, the global death rate in 2021 from TB infection is estimated at 1.6 million, which increased from 1.5 million in 2020 and 1.4 million in 2019, and back to the level of 2017, reversing the decline reported from 2005 to 2019. Therefore, the net reduction of reported TB cases and death rates from 2015 to 2021 was only 10% and 5.9%, respectively, which reached only about one-sixth of the way to the first milestone of the WHO End TB Strategy. The global TB incidence cases are heterogeneously distributed across particular

countries around the world. The high TB burden countries reported were ranked first to eighth in terms of the number of TB cases, with at least 100 000 incident cases, and that accounted for about two-thirds of global TB cases in 2021. The countries include India with 28% of all TB cases globally with an estimated count of 2.7 million, Indonesia with 9.2% of TB cases, China with 7.4%, the Philippines with 7.0%, Pakistan with 5.8%, Nigeria with 4.4%, Bangladesh with 3.6% and the Democratic Republic of the Congo with 2.9% of TB incidence cases (WHO, 2022) (Figure 4).

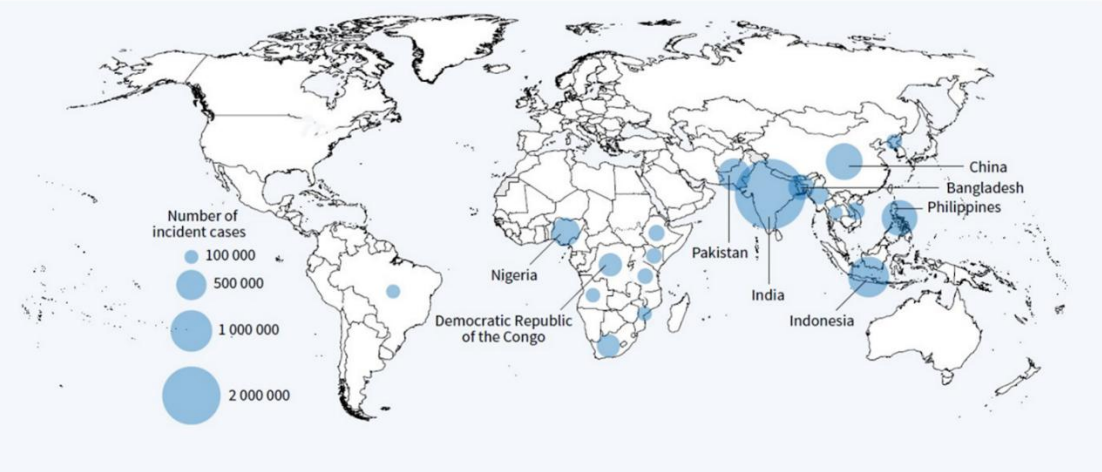


Figure 4: Global distribution of tuberculosis. Across countries which ranked first to eighth in terms of numbers of cases which remarked herewith blue labelling. (WHO Global TB Report, 2022)

The risk factors attributed to the increase in the number of TB cases are undernourishment, HIV infection, alcohol use disorders, smoking (especially among men) and diabetes, which are closely associated with TB incidence (Figure 5) (Carwile et al., 2022; Hayashi and Chandramohan, 2018; Imtiaz et al., 2017).

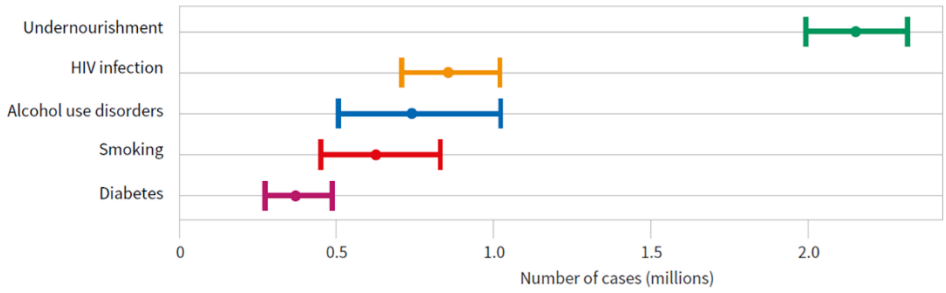


Figure 5: The global estimates of the number of TB incidence cases ascribed to five risk factors. (WHO Global TB Report, 2022)

Therefore, the detailed analysis of the TB-infected cases indicated that the distribution of TB is higher in men at 57% than in women at 33% and approximately 10% of the remaining cases are for children below 15 years of age. Despite the fact that TB disease condition is preventable and treatable, it is estimated that one-third of the global population is infected with the bacterium that causes TB.

One of the major risk factors and probability of developing TB is HIV infection which weakens the immune system, making people more defenceless and therefore becoming severe to TB infection rapidly. Therefore, the WHO denotes that HIV and TB are two interconnected epidemics that disproportionately affect people in low- and middle-income countries. From the overall TB death rates, a range of about 187,000 death cases are reported among HIV-positive individuals (Figure 6).

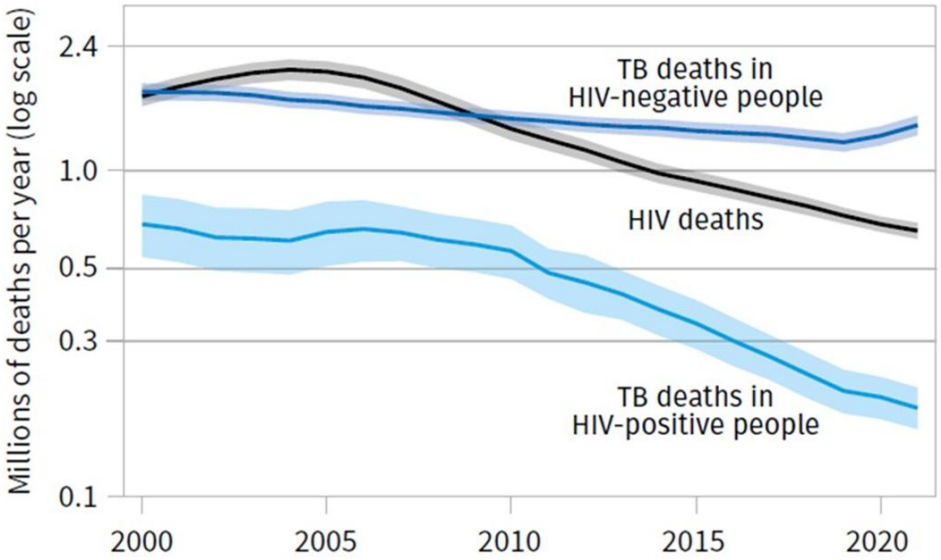


Figure 6: The global trends from the estimates of the number of TB death cases with HIV positive and HIV negative. (WHO Global TB Report, 2022)

Over half of the total HIV-related TB infection cases and death rates befall in Africa which is 30 times higher than in other regions of the world (Getahun et al., 2015). A systemic analysis revealed that TB disease was the main factor in hospitalisation for both adults (18%) and children (10%) with HIV infection where the mortality ranges from 25% in adults and 30% among children (Ford et al., 2015). Thus, to achieve efficient TB control, the social and interactive behaviour within the population need to be addressed for the innovation of the existing TB biomedical pattern (Lönnroth et al., 2010). Although there is a lack of exact precision, the other factors involved in TB infection are attributed to poor quality of life, substandard housing, overpopulation, extended crowding, scarcity of education and lack of consciousness regarding the cause and transmission of TB (Khan et al., 2019).

Despite TB being defined as an airborne disease, compared to other infectious agents like Bacillus anthracis, Rubeola measles viruses, and influenza viruses, the rate of transmission and infection is not so high for *Mycobacterium tuberculosis* to cause disease. The reason is that the chance of an individual with TB infection spreading the disease might infect an average of around only 3.2- 10 people per year (van Leth et al., 2008). However, from that, only a relatively minor proportion of infected people develop into an active TB disease condition. In the high TB burden area, as concluded from the incidence-to-prevalence ratio, the active TB disease can sustain in an infected individual for more than one year (Onozaki et al., 2015). Therefore, the rate of transmission within the population is increased and without any proper treatment, TB disease becomes fatal in which approximately 50% of active TB-infected individuals may succumb to it (Lin et al., 2014).

Active TB disease development and progress can vary between individuals. An estimation is that between 5% and 15% of TB-infected individuals can show disease symptoms within a few months to years (van der Heijden et al., 2018). In contrast, the remaining infected individuals may retain TB persistence risk throughout their lifetime (Andrews et al., 2012). Moreover, age-related and re-infection risk factors can also cause TB relapse among the population in high-burden areas (Thomas and Rajagopalan, 2001). Some reports mentioned that around 50% of active TB-positive cases confirmed with culture tests are without any prolonged coughing or other symptoms associated with TB infection which demands prompt care seeking (Hoa et al., 2010). As a result, the progression of TB disease can be clinically understated,

leading to the transfer of MTB to others nearby because of the lack of confirmation or prediction of infection during the sub-clinical period (Dowdy et al., 2013) (Figure 7).

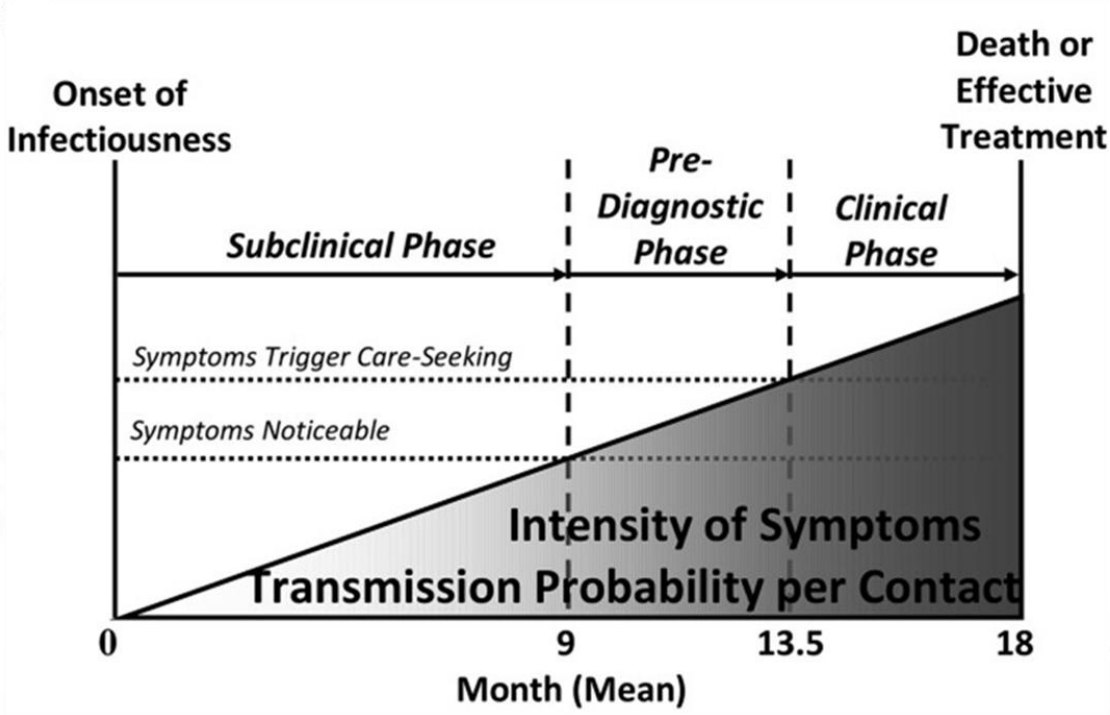


Figure 7: The Impact of Time Span on Tuberculosis: Transmission and Disease Outcome. The time span of the sub-clinical, pre-diagnostic and clinical phases from the onset of TB determines the probability of disease transmission and the final outcome of the disease condition. (Dowdy et al., 2013)

An analysis of TB epidemiology reveals noticeable inconsistencies. In certain countries, there has been a marked decline in TB infection and death rates by around 100-fold during the period between 1900 and 1980, before the discovery of proficient anti-TB drugs. The regions noted were Western Europe and the United States where the main reasons depicted are advances in hygiene and socio-economic circumstances. Whereas, most high-burden areas have seen only a slower decrease in TB infection

rates in which the current estimation showed only a 1.6% decline in TB incidence per year. The noted decline of TB disease and a lower rate of TB mortality by 80% between 1990 and 2010 was denoted in China, where more than halved the rate of TB prevalence (Wang et al., 2014). In contrast, during the same period, Africa showed a drastic increase in active TB cases and mortality rates mainly due to the effect of the HIV epidemic where 72% of global HIV-associated TB cases were noted in 2017 (Ojo et al., 2022). Although proper treatments for TB have saved more than 40 million lives between these periods, the WHO estimation of the "missing TB population" due to the remaining burden of undiagnosed or unreported TB continues to create a significant challenge in ongoing efforts to control TB (Chin and Hanson, 2017).

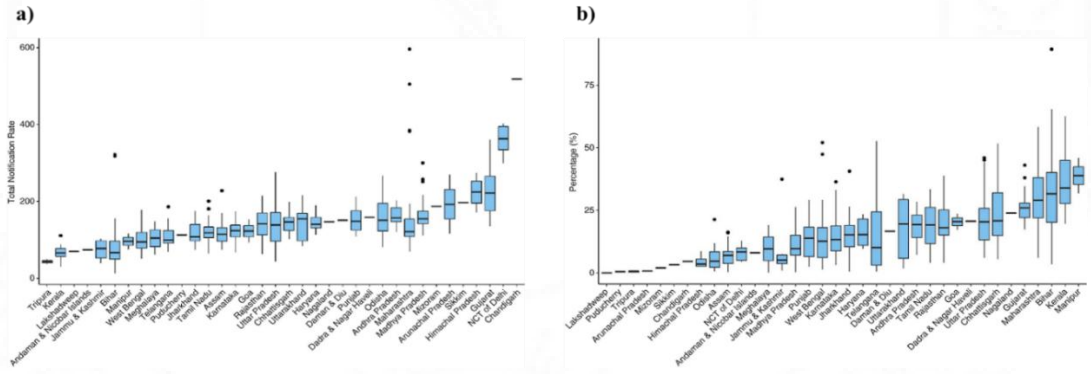
The outbreak of TB drug resistance is a significant public health concern and its spreading is mostly heterogeneous among the population. Globally, it is estimated that approximately 3.5% of newly diagnosed TB cases and 18% of previously treated cases have drug-resistant TB. However, the prevalence varies across different regions and countries, with higher rates often found in areas with limited resources and challenges in TB control (Salari et al., 2023). Several countries carry a higher burden of drug-resistant TB. These include India, China, the Russian Federation, and countries in Eastern Europe, Central Asia, and parts of Africa, in which India accounts for about one-fourth of the global burden of drug-resistant TB cases (Shivekar et al., 2020).

TB disease became a significant public health issue in India, and the country accounted for a major proportion of the global TB burden. According to the WHO Global Tuberculosis Report 2022, India reported an estimated 3 million (2.5-3.4 million) incident cases of TB in 2021, which constituted about a quarter of all TB cases

worldwide. The prevalence rate of TB in India was approximately 210 (178 -244) cases per 100,000 population (WHO, 2022). The TB prevalence and incident rates showed variance across different regions within India. According to the TB survey, the statistics report stated that Delhi holds the highest burden of all forms of TB and pulmonary TB with 747 cases per 100,000 and 534 cases per 100,000, respectively (P. Sharma et al., 2019) (India TB report, 2022). Among that, the greater risk of TB infection and disease is imposed on the vulnerable group of the population. The vulnerable group of TB infection portrayed the malnourished individuals, urban slum dwellers, people with HIV/AIDS, migrant population, prisoners, tribal and indigenous communities and health care workers. The factors contributing to transmission involved are overcrowded urban slums with poor living conditions, inadequate ventilation, and challenges in practising infection control measures with limited access to health care facilities (Figueroa-Munoz and Ramon-Pardo, 2008). Moreover, India suffers a considerable challenge in managing drug-resistant TB. The reports stated that India accounts for 27% of the rifampicin drug resistance TB in the population where the rate of cases is higher in the age group of 15–24 years with 60% from men and 34% from women and the remaining 6% from children age below 15 (Shivekar et al., 2020).

The recent study to estimate the TB burden of India by parliamentary constituency (PC) revealed that India accounted for 26% of the total estimated global gap between incidence and notifications, assessed mainly due to incomplete diagnosis and underreported issues. The reason is the difference in the notification of TB cases between public and private sector notified cases (Figure 8). For example, Although

two states, Tripura and Lakshadweep, have shown low TB estimates from both sectors of notification, the other two states, Kerala and Bihar, showed lower TB notification in the public sector and higher in the private sector. However, the reasons depicted between the documentation of Kerala and Bihar for these TB cases are different. The report stated that Kerala accomplished the incorporation of TB services with primary healthcare facilities, effective private sector management and leadership from local self-government whereas Bihar had a shortage of ground-level healthcare staff and facilities which led to low TB notification and a decrease in TB treatment rates. Therefore, improved community awareness and enhanced public-private sector engagement are necessary to close the gap between incidence and notifications of TB cases (Pardeshi et al., 2021).



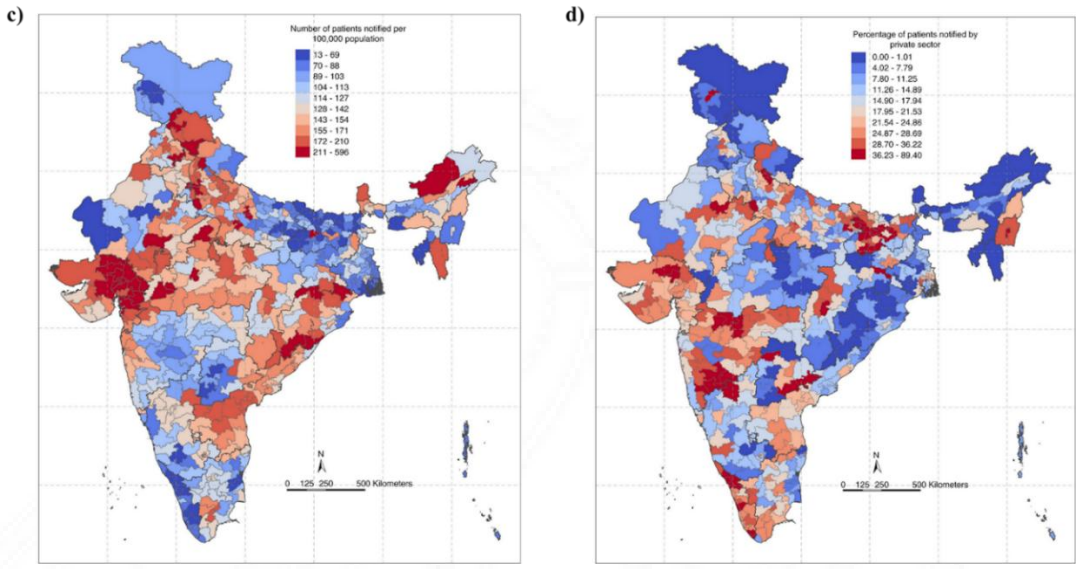


Figure 8: TB notification rates across India. a) Total state TB notification rate denoted in per 100,000. b) Private sector state TB notification in percentage. c) Total state TB notification rate denoted in per 100, 000 in geographical distribution. d) Private sector state TB notification in percentage in geographical distribution. (Pardeshi et al., 2021)

The efforts needed to control TB disease worldwide include improving diagnosis and treatment accessibility, reinforcement of healthcare systems, employing infection control measures, addressing drug resistance, and endorsing public awareness and education. Although progress has been made in controlling TB, continued efforts, resources, and collaboration are essential to eliminate TB as a global public health threat.

2.4 MYCOBACTERIUM TUBERCULOSIS

The discovery of *Mycobacterium tuberculosis* (MTB) as a causative of TB disease in 1882 was a significant achievement in the history of medicine and is credited to Robert Koch, a German physician and microbiologist and a founder of the science

of bacteriology. A monthly evening gathering of the Berlin Physiology Society held on 24 March 1882 was where Koch announced the discovery of tubercle bacillus. Thereafter, Koch was awarded the Noble Prize in Physiology and Medicine in 1905 for his work on TB and his contributions to the field of microbiology. He developed a methodology known as Koch's postulates, a set of four criteria to identify infectious disease causative agent that remains a fundamental tool for microbiologists today. As the first postulate stated, Koch was able to identify a bacteria in the sputum of every TB patient he examined, but not in healthy individuals. Using the second postulate, he was able to isolate the bacteria from the sputum of TB patients and grow it in a laboratory culture. The third postulate stated that the isolated bacterial organism must cause disease when inoculated into the healthy host. Therefore, the injection of isolated bacteria into the bloodstream of laboratory animals, and guinea pigs, showed TB-like symptoms such as swollen lymph nodes, weight loss and development of lesions. Finally, the fourth postulate stated and proved that the re-isolation of the bacterium from the diseased host was identical to the isolate collected from the sputum of TB patients. All these explanations, thus, concluded that a bacteria called *Mycobacterium tuberculosis* present in the tuberculous lesions are the major causative agent of TB (Kaufmann and Schaible, 2005) (Figure 9).

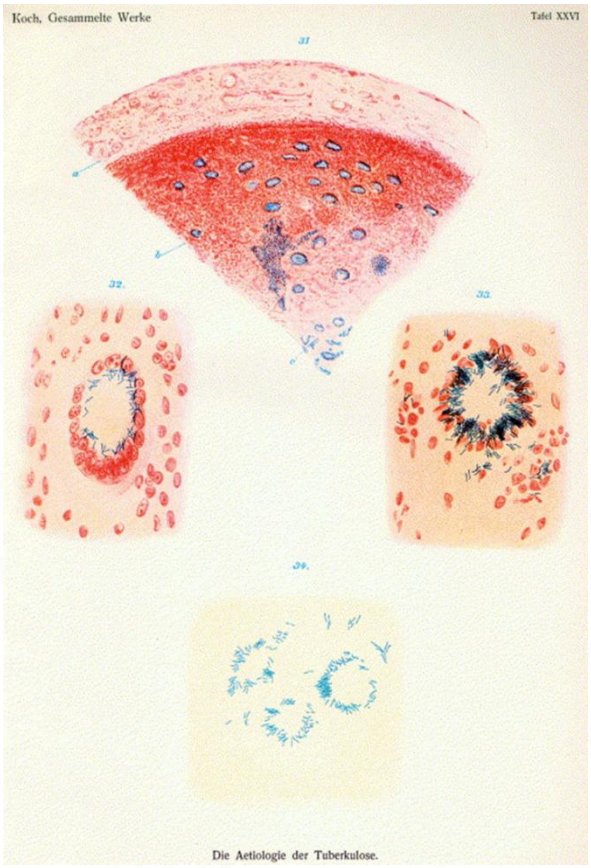


Figure 9: The original drawing of *Mycobacterium tuberculosis* bacteria within infected tissue by Robert Koch on his aetiology of tuberculosis (Kaufmann and Schaible, 2005)

Further study on *Mycobacterium tuberculosis* (MTB) depicted that it is a slow-growing bacterial species with a replication time ranging from 12 to 24 hours under optimum conditions, an obligately aerobic, with a rod-shaped structure which is approximately 2–4 μm in length. The high lipid content of the cell wall of MTB makes it unique, and the genomic sequencing of the organism showed that around 30% of the genes were involved in lipid production and metabolism (Russell, 2001). The waxy coating of mycolic acids and other complex lipopolysaccharides constituting the asymmetric lipid bilayer comprising the MTB cell wall is made of lipids (Jankute et

al., 2015). The inner leaflet mycolic acids are long-branched-chain fatty acids that are covalently bound to the outer layer of glycolipids and waxy components and form a hydrophobic layer around the cell. The outer and inner membranes form a periplasmic space, with a thin peptidoglycan layer on the innermost side. This layer is covalently linked to arabinogalactan and lipoarabinomannan, which are bound to mycolic acids (Kleinnijenhuis et al., 2011) (Figure 10).

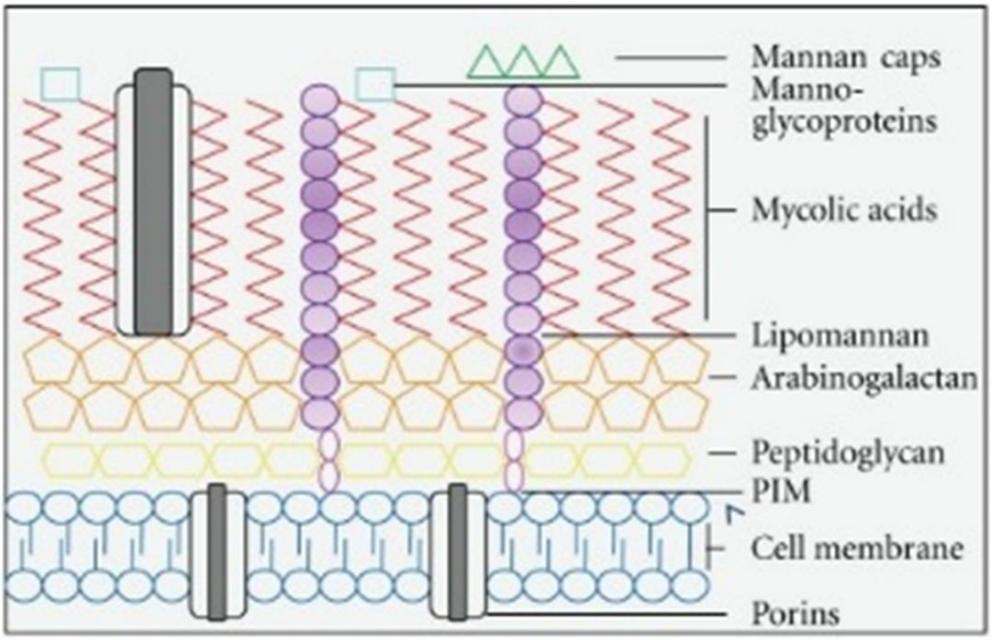


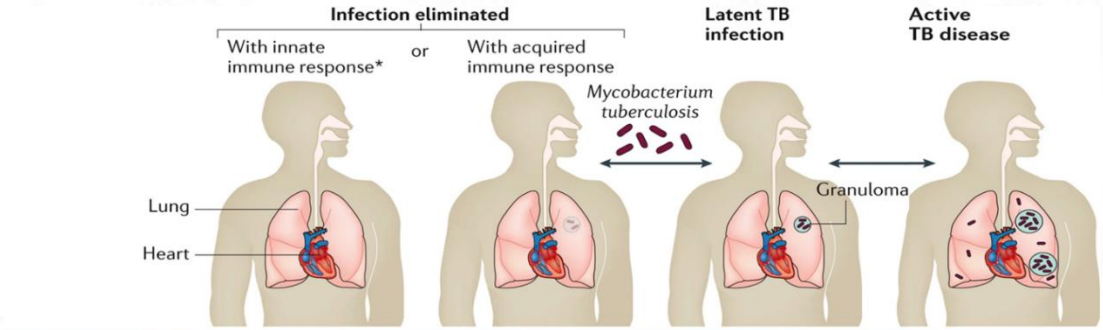
Figure 10: The schematic representation of *Mycobacterium tuberculosis* bacteria cell wall with the major components and its distribution. (Kleinnijenhuis et al., 2011)

This complex lipid layer provides several functions for MTB such as non-permeability, protection against host immune response by preventing fusion with lysosomes, thus avoiding the normal progression of the phagosome with MTB into an acidic, hydrolytically active compartment, and virulence which prevents the

development of progressive immune response required to activate the host cell (Kalscheuer et al., 2019). Moreover, the identified MTB-secreted proteins denoted as early secretory antigenic target ESX, are also a type VII secretion system vital for mycobacterial virulence and viability. Besides that, the virulence depends on the ability of MTB to survive the immunological response from the host, their potential to cause damage to the lungs and to be transferred effectively to infect a nearby individual (Coscolla and Gagneux, 2014). This virulence has been explained as the secretion of effector proteins and manipulation of macrophage-mediated death pathways by MTB that promote the bacteria to evade the host defence mechanism, thereby ensuring intracellular survival with the formation of a protective layer called granuloma that can persist in normal healthy people during the latency period of MTB infection (Barry et al., 2009). Therefore, the MTB is considered as conditional pathogenic bacteria because for the infection to upgrade into a disease stage it requires the compromisation of the immunological state of the host. The interplay between the MTB and the host cell determines the progress of TB control. The containment of MTB inside macrophage may fail when there is a change in the immune status of the host due to the consequence of malnutrition, old age, HIV co-infection or any treatment with TNFα blockers (Shim, 2014). The exact mechanism and mutual regulation by which how the bacteria reside inside the macrophages of the host is still unknown. Such detailed analysis and knowledge of such mechanisms would be feasible for the development of proper treatment to control TB infection.

2.5 DIFFERENT STAGES OF TB

A person who is initially exposed to or inhaling MTB from a nearby individual could lead to two broad outcomes for the body. This includes the elimination of TB infection which can be accomplished either with the innate immune response or with the acquired T-cell immune response of the host. However, the retaining of MTB infection within the body occurs either in two stages: a quiescent or inactive stage without any symptoms known as Latent Tuberculosis Infection and an active stage which could progress with symptoms and could spread to nearby individuals, referred to as Active TB disease (Figure 11).



TST	Negative	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Positive
Sputum smear	Negative	Negative	Negative	Positive or negative
Infectious	No	No	No	Yes
Symptoms	None	None	None	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy

Figure 11: The detailed spectrum of tuberculosis from infection to active disease condition (Pai et al., 2016).

2.5.1 Latent Tuberculosis Infection (LTBI)

LTBI is an inactive stage of TB disease residing in an individual after exposure to MTB bacteria that are controlled by the immune system and therefore remain at a dormant stage, preventing them from spreading and causing disease. Though the infected individual carries MTB in the quiescent stage, the disease is non-contagious, asymptomatic, and can either remain dormant throughout their lives or shift to active TB due to many factors. The experimental studies on mice, guinea pigs, rabbits and non-human primates aided to recognise the immunological response stages that happen inside the body to control the infection with MTB bacteria (Orme et al., 2015). The access route of MTB through the respiratory tract after inhalation results in the translocation of bacteria to the lower respiratory tract of the infected individual where it comes across the macrophages present in the alveoli of the lungs which are the dominant cell type that bacteria target as a primary route of entry. Other immune cell types involved in the inactivation of MTB include dendritic cells and monocytes. The bacteria are internalized into the cells with numerous different receptors on the macrophages. The internalized MTB then blocks the active fusion of phagosome with lysosome thereby ensuring its survival inside the macrophage (Russell, 2011). Thereafter, the MTB intended to gain access to the lung parenchyma where the development of infection progresses. There are two possible estimated mechanisms involved in the migration of MTB into the lung parenchyma. One of them is the direct infection of epithelial cells and reaching the parenchyma; the other involves the transmigration of macrophages with MTB across the epithelium cell layer. In either pathway, MTB accesses the parenchyma, which results in an increased number of cells

being recruited to the infection site, leading to a multicellular host response called granuloma formation. The granuloma structure is, therefore, a cluster of immune cells, primarily the macrophages surrounded by other immune cells such as lymphocytes, which are formed as defence mechanisms to contain MTB infection, preventing the bacteria from spreading throughout the lungs or to other parts of the body. Therefore, the containment of bacteria within granuloma limits the initiation of replication and promotes the persistence of the MTB inside the cell (Pl et al., 2014). Hence, the survival of MTB inside the granuloma is attributed to host-microbe interactions where when the host immune responses are predominant, the MTB remains dormant. Such a stage of TB infection is termed latent or inactive (LTBI) (Figure 12).

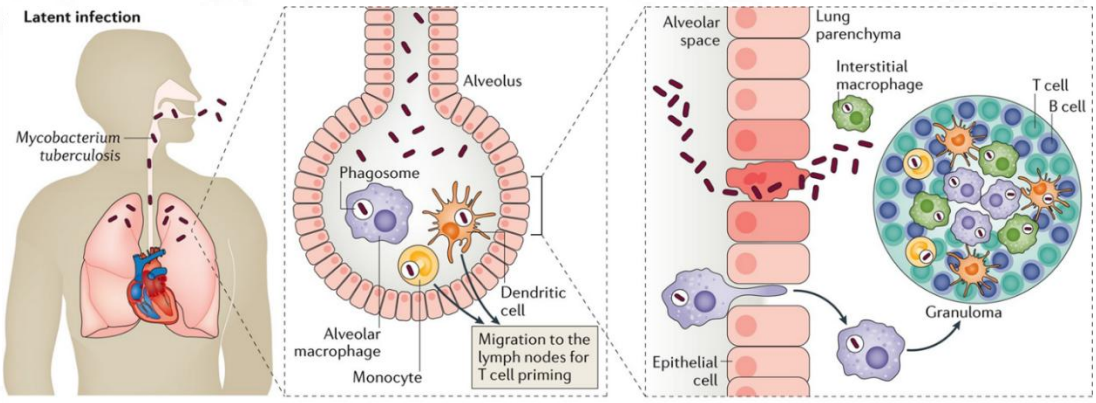


Figure 12: The granuloma formation during Latent TB Infection (LTBI) (Pai et al., 2016).

The diagnosis of patients with LTBI typically involves a combination of medical history assessment, tuberculin skin testing (TST), and interferon-gamma release assays (IGRAs). These tests evaluate the cell-mediated immune response to

MTB antigens that occurs when the dormant or quiescent state of MTB persists within the body without any symptoms of TB infection (Carranza et al., 2020).

The TST test, also known as the Mantoux test, involves the intradermal injection of purified protein derivative (PPD) tuberculin into the forearm, beneath the skin of the patient. The delayed-type hypersensitivity (DTH) response on the site of injection is examined after a waiting period of 48-72 hours by measuring the diameter of swelling due to inflammation called induration in millimetres by a healthcare professional (Yang et al., 2012). The reaction observed in a positive TST is a delayed-type hypersensitivity response which involves the interaction between the PPD tuberculin and specific immune cells, called T lymphocytes or memory T cells developed from the immune response in individuals who have been infected with the MTB. The swelling due to inflammation of 15 mm or larger measured using a ruler, read after 48 or 72 h, is therefore considered as an indication of past or current MTB infection. The administration of TST data reading and interpretation, it requires trained healthcare personnel who can interpret the risk factors associated with determining the positive results (Yang et al., 2012). Moreover, the TST can yield false positive results, particularly in individuals who have received the BCG vaccine and those who have exposure to environmental non-TB mycobacteria, which can cause a cross-reaction leading to a positive TST result even in the absence of TB infection (Burl et al., 2010).

An alternative test called interferon (IFN)-gamma release assays (IGRAs) are, therefore, developed to improve the specificity of the TB diagnosis and to overcome some of the limitations associated with TST (Esmail et al., 2014). The IGRA is a blood test to measure the release of interferon-gamma by the specific immune response when

exposed to MTB antigens called an early secretory antigen (ESAT-6) and culture filtrate protein 10 (CFP-10). The amount of IFNγ level in the plasma or serum is quantified using various detection methods such as ELISA or ELISPOT assay (Pourakbari et al., 2019). By focusing on the specific immune response to TB antigens, IGRAs aim to provide a more targeted and specific assessment of latent TB infection compared to TST.

Though these tests would probably benefit patients with LTBI for treatment and therapy, the prediction of positive TST or IGRA results does not provide the confirmed results of the LTBI condition. The reason is mainly due to the persistence of memory CD4+ T cells even after the elimination of infection successfully with their immunological responses (Mx et al., 2012).

Moreover, several studies have mentioned that there is a chance of developing active tuberculosis in 5 to 15% of persons with latent infection during their lifetime and the level of percentage can increase if the patient has co-infection with HIV, tumour necrosis factor α inhibitors, diabetes, and organ or hematologic transplantation. Thus, the estimated 2 billion latently infected humans are considered an extensive carriers of TB disease (Getahun et al., 2015).

2.5.2 Active TB Disease

A combination of immunological cells such as macrophages, dendritic cells and T cells with MTB forming granuloma is sufficient to control asymptomatic LTBI. Whereas, when the bacteria residing inside the granuloma cells overcome the defence

mechanism of the immune system, a replication initiation of MTB results in the progression from LTBI to active TB disease (Figure 13).

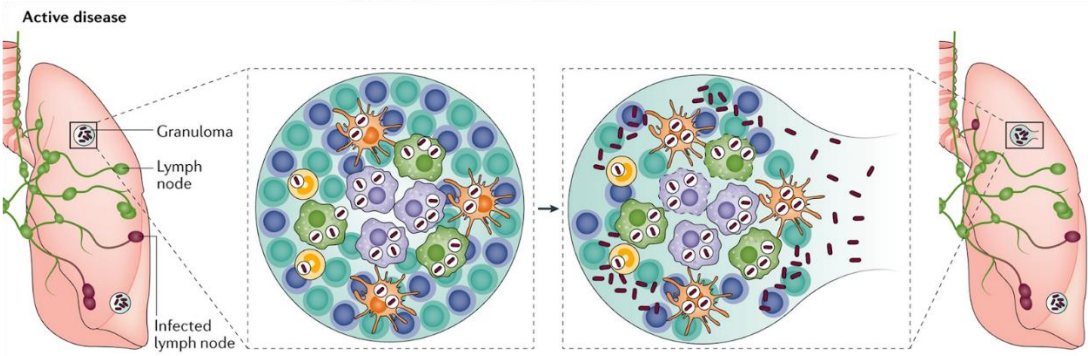


Figure 13: TB bacterial replication within granuloma and dissemination of bacteria during Active TB disease (Pai et al., 2016).

The factors that trigger active TB conditions include HIV infection, other immunosuppressive conditions or medications during organ transplantation, ageing, malnutrition and chronic illnesses such as diabetes or kidney diseases (Ai et al., 2016). As the TB become active the person starts to experience symptoms such as persistent cough, fever, night sweats, weight loss and fatigue. Therefore, active TB is a contagious disease which can spread to others through the air when an infected person nearby coughs or sneezes (Chandra et al., 2022). Accordingly, the early detection and prompt treatment of active TB are critical to prevent severe complications, the transmission of disease to others nearby and the development of drug-resistant strains of TB.

Though the active TB infection primarily affects the lungs, it can also target other parts of the body, mainly the kidneys, spine or brain organs. Due to that, the active TB disease condition is categorised into two types based on the clinical manifestations: pulmonary TB and extra-pulmonary TB.

Pulmonary TB is a common form of active TB disease that mainly affects the lungs, accounting for approximately 80-85% of all TB cases, in which respiratory symptoms are predominant (Campbell and Bah-Sow, 2006). On the other hand, extra-pulmonary TB occurs when TB bacteria spread from the lungs to other body parts through the bloodstream or lymphatic system, accounting for the remaining 15-20% of cases (Musellim et al., 2005). The symptoms of extra-pulmonary TB mainly depend on the site of the infection. The common types of extra-pulmonary TB comprise:

1. **Lymphatic TB**: The most prevalent kind of extra-pulmonary TB, which affects the lymph nodes and makes up roughly 35% of all TB cases outside the lungs. The lymph nodes act as filters and trap the bacteria, causing inflammation and swelling. The symptoms of lymphatic TB include a painless swelling of the lymph nodes, which can gradually increase in size and become tender or painful (Mohapatra and Janmeja, 2009).

2. **Bone and joint TB**: A relatively rare form of TB infection, accounting for only about 1 to 3% of all cases of TB. This can spread through the bloodstream and affects the spine, hips, knees, and ankles, leading to pain, swelling, stiffness, and deformity due to inflammation (Pigrau-Serrallach and Rodríguez-Pardo, 2013).

3. **Genitourinary TB**: The disease mainly disturbs the urinary tract and reproductive organs of patients through the hematogenous spread of the bacilli. This accounts for 15% of extra-pulmonary TB cases and is more frequent in patients undergoing kidney transplantation than considering the general population. This disease can cause inflammation and damage to the affected area, leading to symptoms such as pain, urinary frequency or urgency, and blood in the urine (Zajaczkowski, 2012).

4. **Meningeal TB**: A rare but serious form of extra-pulmonary TB that affects the membranes surrounding the brain and spinal cords. The disease predominantly affects children. This condition can cause inflammation and damage to the meninges, leading to symptoms such as headache, fever and neck stiffness (Garg, 2010).

5. **Abdominal TB**: The disease accounts for 11% of patients with extra-pulmonary TB, mainly affecting the digestive system, including the liver, spleen and intestines. The symptoms include abdominal pain, diarrhoea and weight loss (Das and Shukla, 1976; Uzunkoy et al., 2004).

6. **Pleural TB**: A form of extra-pulmonary TB that affects the lining of the lungs and the chest cavity, accounting for approximately 20% of all cases of TB outside the lungs. When TB bacteria enter the body, they can spread through the bloodstream to the pleura, which is the membrane that lines the lungs and the chest cavity. This can cause inflammation and damage to the pleura, accumulating fluid or pus in the space between the lungs and the chest wall known as a pleural effusion. The symptoms of pleural TB include chest pain, difficulty in breathing, cough, high fever and fatigue (Vorster et al., 2015).

7. **Miliary TB**: The disease is a rare and serious form of TB that accounts for less than 2% of all TB cases, occurring when the bacteria that cause TB spread throughout the body via the bloodstream. The name ‘miliary’ denoted for the TB disease comes from the tiny millet seed-sized lesions that appear on the X-rays of the affected organs, resembling grains of millet. The miliary TB can affect any organ in the body including the lungs, liver, spleen and bone marrow. The symptoms usually observed in patients

include weight loss, hematologic abnormalities, fatigue, fever, night sweats, cough and difficulty in breathing (Mert et al., 2001).

The diagnosis of extra-pulmonary TB remains a challenging factor because of the inaccessible site for detection and non-specific symptoms, which are difficult for the clinical evaluation and interpretation of the type of extra-pulmonary TB. Therefore, the proportion of extra-pulmonary TB cases can vary depending on several factors, such as the patient population and the prevalence of different types of TB in a given region (Kulchavenya, 2014).

2.5.3 Drug-Resistant TB

The preferred drug regimen for active drug-sensitive TB disease is the standard treatment with a combination of four antibiotics with a minimum of 6 months of therapy. The most commonly used drug regimens are rifampicin, isoniazid, pyrazinamide and ethambutol. This four-drug combination is typically administered for an initial intensive phase lasting two months, followed by a continuation phase of four months with rifampicin and isoniazid (Nahid et al., 2016). Even though this drug regimen showed a higher success rate in TB treatment efficiency, there are some limitations to consider in the medical field. The medications used in the standard regimen such as isoniazid and rifampicin can have potential toxicity which can include liver toxicity, gastrointestinal disturbances and allergic reactions. Moreover, an effective TB treatment requires strict adherence to the prescribed medication regimen to achieve successful outcomes that could be challenging for some patients due to various reasons, including socioeconomic factors, lack of access to healthcare or drug

side effects. These problem results can therefore lead to treatment failure, relapse or the development of drug resistance (Saukkonen et al., 2006).

The phenomenon of drug resistance in TB infectious disease was first described during the first human TB therapy trial in 1948 (Kerantzas and Jacobs, 2017). Drug resistance to TB can occur due to several mechanisms that allow the MTB to develop resistance to the antibiotics used for the treatment of the disease. One of the reasons for the resistance is due to the mutations in the genetic material of the TB bacteria that can lead to changes in the structure or function of the target proteins that antibiotics normally react with. As a result, the antibiotics become less effective and the bacteria develop resistance. For example, the gene coding for the beta subunit of TB RNA polymerase mutation results in resistance towards rifampicin primary anti-TB drug because of the change in their binding site (Nebenzahl-Guimaraes et al., 2014). Another drug-resistant mechanism includes the inactivation of certain antibiotics by the MTB-produced enzymes which modify the structure of the antibiotic molecules, rendering them ineffective in inhibiting bacterial growth. For instance, the mutation in the bacterial catalase enzyme gene, the katG gene, fails to activate the antibiotic isoniazid (Cade et al., 2010).

Other most common TB drug resistance associated with specific gene mutations are on embB, gyrA and gyrB genes. An enzyme arabinosyl transferase encoded by the embB gene is mainly involved in the cell wall arabinan biosynthesis pathway, which is a target of ethambutol drug. Any kind of mutation in this gene can cause a structural difference in the enzyme which reduces the affinity of ethambutol, thereby resulting in resistance towards that first-line anti-TB drug (Plinke et al., 2011).

One of the second-line TB drugs, fluoroquinolones, inhibits the mycobacterial DNA gyrase enzyme activity which has a role in MTB DNA replication and repair. The mutations in the gene that encodes the subunits of DNA gyrase, gyrA and gyrB, result in structural changes in the enzyme thereby reducing the binding affinity of the anti-TB drug fluoroquinolones, leading to resistance (Chien et al., 2016).

There are two main categories of drug-resistant TB disease in which the combination of such mutations in different genes can lead to other levels of resistance including multidrug-resistant TB (MDR-TB) and extensive drug-resistant TB (XDR-TB). The MDR-TB is resistant to two of the first-line antibiotics, isoniazid and rifampicin and XDR-TB is a more severe form in which along with isoniazid and rifampicin, there is resistance towards a second-line drug called fluoroquinolones and at least one of the three injectable second-line drugs named as amikacin, kanamycin and capreomycin under the group of aminoglycosides (Sd et al., 2012). To minimize drug-resistant disease transmission and to reduce further resistance to provided drugs, it is necessary to consider the early and rapid detection of active drug-resistant TB in patients. Otherwise, the chance of MDR-TB and XDR-TB threat may reach a higher grade which would lead to a rapid incidence of TB cases that would be difficult to control. Therefore, one of the estimations of the rise in TB disease and the rapid spread of global TB is mainly a result of the continuous evolution of drug resistance in TB disease (Seung et al., 2015).

The major problem involved in the treatment of active drug resistance is the use of second-line drugs which are often less effective, more toxic and expensive. Since the treatment regimens are usually longer often ranging from 9 to 12 months,

when compared to drug-susceptible TB. If there is an increase in the number of patients reported with drug resistance TB, the effective control of the disease and appropriate drug regimens would be inconvenient. Such an incidence of spreading drug resistance cases has been reported in countries India, China, South Africa, Russia and other countries in Eastern Europe (Dheda et al., 2014). The proper diagnosis of MDR-TB and XDR-TB requires access to specialized laboratory facilities for drug susceptibility testing. Drug Susceptibility Testing (DST) is one of the gold standard methods for diagnosing TB drug resistance. It involves phenotypic testing by using culture agar proportion methods and genotypic testing by using molecular testing methods either LPA or whole genome sequencing (WGS). The use of WGS on clinical isolates to identify the resistance-associated mutations and as a diagnostic method for the detection of drug resistance would be a faster method for proper treatment and for controlling TB spread by understanding the transmission dynamics of strains (Gygli et al., 2019). Though the method is feasible for the diagnosis of drug-resistant TB, the culture test is considered the test for clinical care due to the high cost and imperfect sensitivity for the identification of causal mutation by the WGS method (Walker et al., 2015).

2.5.4 HIV- Associated TB

HIV- associated tuberculosis represents a significant global health challenge and TB control due to the synergistic interaction between the human immunodeficiency virus (HIV) and MTB, which could simply be defined as the co-infection of TB with HIV. When an individual with HIV becomes infected with TB bacteria, the progression of the disease is accelerated and the risk of developing active

TB increases significantly. Therefore, the progression and activation of latent TB due to the compromised immune system lead to the active TB disease condition with symptoms similar to that of regular TB disease such as persistent cough, fever, night sweats, weight loss, fatigue and chest pain (Habib, 2009). A recorded HIV test result was notified in 76% of TB patients in 2021, which is an increase from 73% reported in 2020. The highest prevalence of HIV-associated TB is found in the WHO African Region (WHO, 2021). Even so, there is a geographical difference, the current estimation of above 25% chance of developing active TB positive mainly for HIV-infected individuals when compared to HIV negative. The reason for that risk is mainly due to a decrease in the immunological T-cell counts in the infected patient which can be directed to failure in controlling TB disease condition (Havlir et al., 2008). Though HIV patients are not definitely TB infectious or spreader for others, they have a higher risk chance of progression to active TB disease.

The management of HIV-associated TB involves a comprehensive approach that includes antiretroviral therapy (ART) to suppress HIV replication and strengthen the immune system, along with anti-tuberculosis treatment to target the TB infection (Suthar et al., 2012). Several studies reported that even if the count of CD4+ T-cells is within the normal range by ART, there are chances of developing TB risk about two-fold in HIV-positive patients (Lawn et al., 2009).

The HIV infection shows different staging of active TB disease in smear-negative patients from the usual slow progress of TB with low bacterial load to one with reduced pulmonary cavity formation and sputum bacillary load which frequently affects the lower lobes of the lungs (Getahun et al., 2007). Therefore, it is mandatory

to screen the HIV positive patients for the confirmation of active TB if they are experiencing any symptoms such as night sweat, coughing or weight loss thereafter need to be treated and evaluated without any delay and those without any symptoms should be provided with LTBI preventive treatment depending on the epidemiology and TB burden (Pai et al., 2016).

The mortality rate of HIV-associated TB is higher when compared to TB in individuals without HIV, mainly due to the susceptibility of patients to severe forms of TB, including extrapulmonary TB. This can also be influenced by the HIV infection stage, timely diagnosis and treatment initiation, availability of ART and drug resistance. The co-infection with drug-resistant TB strains further increases the risk of mortality, as treatment options become limited. Effective treatment for both HIV and TB is essential for improving the outcomes of TB. Adherence to treatment is critical, as interrupted or incomplete treatment can lead to treatment failure, drug resistance and poor outcomes (Mollel et al., 2020). The close monitoring of treatment response, regular follow-ups and support for treatment adherence are essential components of disease management. Therefore, integrated HIV and TB procedures along with a strong healthcare infrastructure are crucial for effectively managing co-infection and mortality rates.

2.6 CURRENT DIAGNOSTIC METHODS FOR TB DETECTION

2.6.1 Smear Microscopy Test

Tuberculosis is a major global health concern, so it necessitates accurate and timely diagnostic methods to prevent further spreading among the population. The smear microscopy test for TB is preferred as the primary diagnostic method due to its simplicity, cost-effectiveness and wide availability of feasible equipment for TB tests (Schmid et al., 2014). The method requires only a set of microscopic equipment which can provide visual evidence of tuberculosis and the bacterial burden by observing the MTB and in some cases mainly in high-burden countries, the test is more specific without any more additional confirmatory methods (Yassin and Cuevas, 2003).

The smear test conducted on patients suspected of pulmonary TB symptoms is performed by collecting respiratory tract-originating specimens in the form of sputum or induced sputum or broncho-alveolar lavage or a lung biopsy. Under the guidance of a healthcare professional, the patient is provided with a sterile, leak-proof container for sputum collection. The recommendation endorsed by WHO and the International Union against Tuberculosis and Lung Disease (IUATLD) involves the collection of three early morning sputum samples from deep coughing, which is usually more representative of lower respiratory tract secretions, obtained by holding it momentarily and then forcefully coughing to produce sputum without or with minimized saliva content (Singhal and Myneedu, 2015). However, the third collected sputum for the diagnosis of tuberculosis has been demonstrated in numerous studies to be insignificant because almost all cases can be identified from the first and/or

second collected sputum specimen. Therefore, reducing the number of sample collection would be favourable as it reduces the workflow of high-burden laboratories with reduced cost and makes the process more accessible to the population (Ozkutuk et al., 2007). For the efficiency of the TB test, it is crucial to collect an adequate volume of sputum, typically about 5-10 ml which would be sufficient for analysis (Yassin and Cuevas, 2003).

The direct smear microscopic results have limitations mainly due to the lower sensitivity, especially for HIV co-infected patients, and unequal test quality under programmatic conditions. Therefore, a study depicted that the sputum sample processing by centrifugation along with chemical treatment (including bleach or sodium hydroxide) before observing under a microscope reported leads to a higher yield of bacilli concentration with similar specificity as that of direct smear test (Steingart et al., 2006). Therefore, the studies suggested evaluating and implementing the bleach method for smear tests in settings where MTB culture tests are not routinely performed would be favourable. After that, the simplified processing of sputum samples has shown higher sensitivity in TB detection compared to direct sputum smear tests under microscopy.

Besides such a collection of sputum samples, the preparation of smear and staining with high-quality reagents is also vital for accurate TB detection with a microscope. The phases include the spreading of collected sputum samples onto a sterile slide using a loop or a spreading device to ensure the smear is not thick or clumped. Thereafter, the prepared smear is left to air dry completely. The dried smear is then fixed by passing through the flame of a Bunsen burner or using a heat-fixed

procedure. This procedure helps to ensure that the smear adheres firmly to the slide and prevents the loss of bacteria during subsequent staining steps. The Ziehl-Neelsen smear tests are performed by flooding of smear with 1% carbol fuchsin, a red-coloured primary stain containing fuchsin and phenol. The gentle heating of the slide thereafter facilitates the penetration of the stain into the cell wall of MTB. The washing of the slide with acid alcohol which is the solution of hydrochloric acid and ethanol removes the primary stain from non-MTB bacteria but retains it in acid-fast bacilli. This step is critical as it differentiates acid-fast bacilli from other bacteria. Thereafter the slide is counterstained with a contrasting dye, such as methylene blue, which stains non-acid fast bacteria and background debris. Finally, the slide is washed, air-dried and examined under a microscope using oil immersion lenses. The observed MTB appear as red rods against a blue background (Bhandari, 2021) (Figure 14).

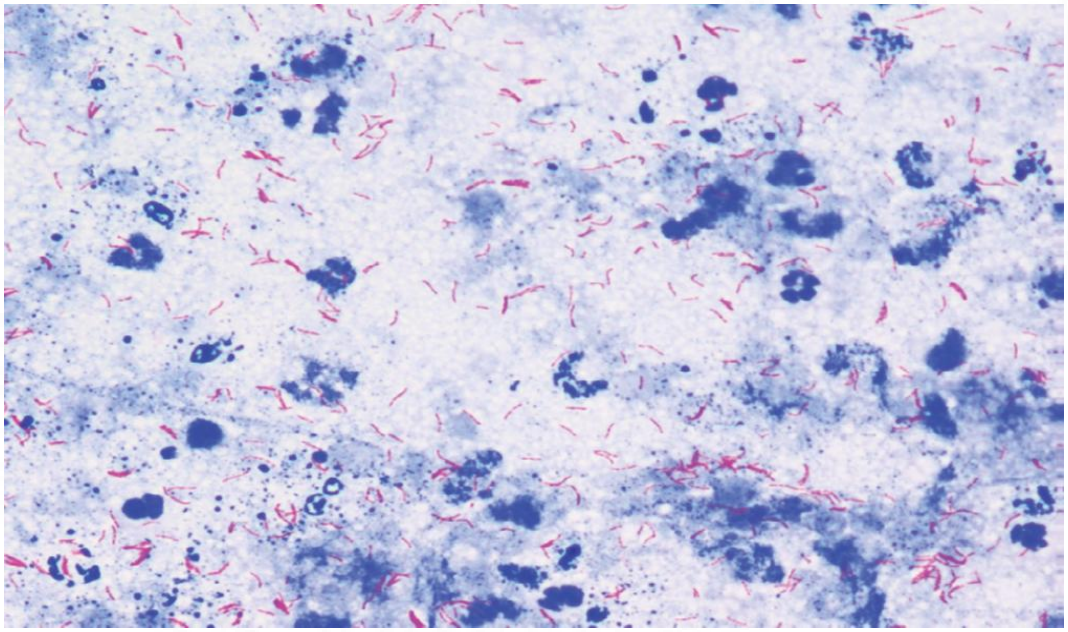


Figure 14. Ziehl-Neelsen stain TB test results: The observation at 100X oil immersion microscopic view as red rods against a blue background. (Bhandari, R., 2021)

This staining method for smear tests is generally considered as having lower sensitivity compared to more advanced diagnostic procedures, such as culture or molecular techniques. The primary reason is that the detectability of the smear test is compromised when the number of bacteria in a sputum sample is less than 10,000 bacteria/ml (Rasool et al., 2019).

The Auramine- Rhodamine test is a fluorescent staining technique, an alternative method to the traditional Ziehl- Neelsen smear test which offers enhanced sensitivity for the detection of MTB. The procedure involves a covering of the slide with a solution of Auramine-Rhodamine, a fluorescent dye which selectively binds to the mycolic acids in the cell wall of MTB. Thereafter the slides are illuminated with UV light, causing the MTB stained with Auramine- Rhodamine to fluoresce with a yellow-green colour (Driver et al., 2012). To visualize the fluorescent bacilli, the microscope is equipped with a specific filter set that allows the yellow-green fluorescence to be observed while blocking the remaining wavelengths of light. Therefore, the MTB as fluorescent bacilli can be observed and identified under the microscope, appearing as bright yellow-green rods against a dark background which enhances the visibility and contrast resulting in the identification and differentiation of bacteria from background debris or other non-acid fast bacteria (Figure 15). Therefore, this staining is more sensitive than Ziehl-Neelsen staining where it can detect a lower number of AFB in the sputum or respiratory samples (Dzodanu et al., 2019).

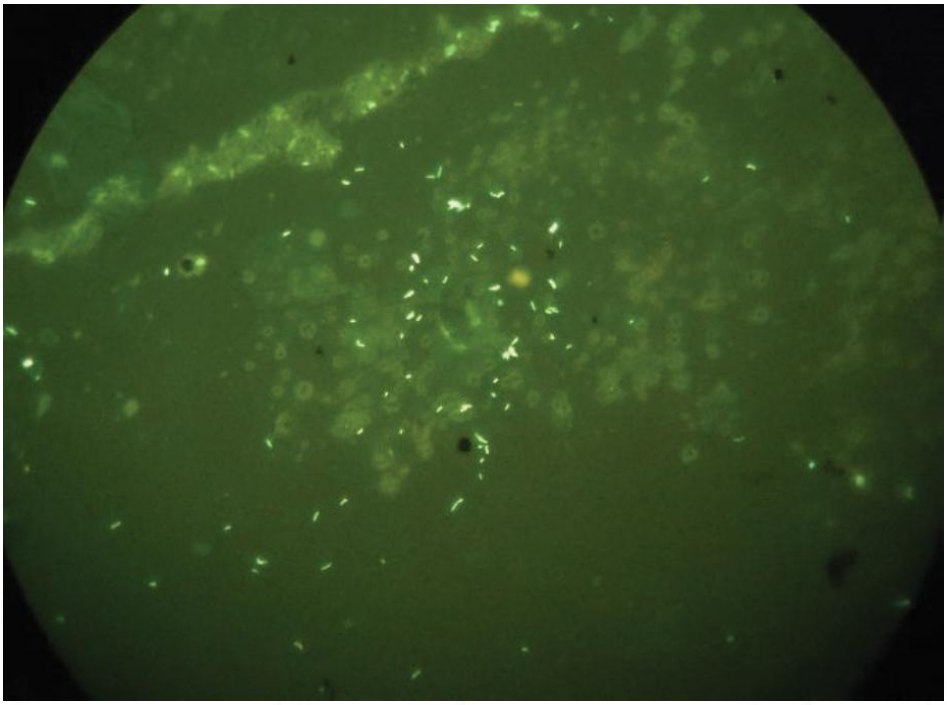


Figure 15. Auramine- Rhodamine staining TB test. The observation at 40X high power microscopic view as bright yellow-green rods against a dark background (Bhandari, R., 2021).

Many studies have revealed that the smear-positive sensitivity rate of Auramine-Rhodamine stained smears (12.5%, 16.56%, 19.1%, 12.47%) was better than Ziehl-Neelsen stained smears (11.5%, 10.41%, 12.4%, 9.89%). The comparison study further revealed the sensitivity difference between these two smear microscopic TB tests where the sensitivity of the Auramine-Rhodamine test was denoted as 95.83%, which is higher than the Ziehl-Neelsen test (91.67%). Moreover, the average time taken for the detection of TB differences showed between these two tests were significant (4.32 mins for the Ziehl-Neelsen test and 2.28 mins for the Auramine-Rhodamine test) (Bhandari, 2021). Due to that higher sensitivity and faster detection of the Auramine-Rhodamine smear test, the staining allows for higher sample

throughput which enables the laboratory technicians to process and examine a large number of samples in a shorter period that would be beneficial in high-burden settings where the timely diagnosis of TB is critical (Laifangbam et al., 2009). Though the Auramine-Rhodamine smear test offers these merits, the conventional Ziehl-Neelsen staining remains a valuable diagnostic method as it is more widely available and cost-effective.

The smear test is routinely described as simple and affordable, however, it requires quality training and precision of clinical staff to handle microscopy as it requires multiple examinations and programmatic evaluations that may take days rather than hours. Consequently, patients may discontinue the diagnostic process if they are not provided with assured approval and confirmation of TB disease (Squire et al., 2005).

2.6.2 Chest X-Ray

The entrance pathway of TB infection in humans is mainly through the respiratory tract, which targets the pulmonary parenchyma in the lungs, the initial site of infection in the body. Therefore, the chest X-ray is used as one of the primary diagnostic tool for the detection of pulmonary tuberculosis (TB). Even though the method is not specific to TB disease condition, the WHO guidelines are recommending to use the chest X-ray as a supplementary diagnostic tool for patients with TB symptoms and smear negative results (van Cleeff et al., 2005). The observation of infiltration in the upper region of lungs, which often appear as hazy opacities which can either be localized or widespread on both lungs and enlargement of the lymph nodes in the chest, termed as lymphadenopathy, depicted as enlarged, round opacities

on the X-ray. An important required to interpret the results of chest x-ray for TB diagnosis is the experience and interpretation ability of the reader. The lack of training and experience, therefore, leads to intra reader and inter reader variability in the confirmation of results (Candemir and Antani, 2019).

2.6.3 Molecular Diagnostic Methods

Due to several limitations mentioned about the culture and smear microscopic diagnostic methods, some bio-molecular tests are recommended by WHO for the initial diagnosis of TB-suspected patients.

2.6.3.1. WHO Endorsed Tests

a. Xpert MTB/RIF

One of the molecular tests endorsed by WHO is the Xpert MTB/RIF or MTB/RIF Ultra as the diagnostic test for suspected pulmonary TB (WHO 2022). They are known as cartridge-based nucleic acid amplification tests (CB-NAAT) that can detect the TB DNA present in the collected patient sample as well as mutation associated with the rpoB gene which codes for the beta subunit of RNA polymerase in MTB, which is the target of an anti-tuberculosis drug called rifampicin (Nurwidya et al., 2018).

The Xpert MTB/RIF assay uses the GeneXpert Instrument System in which a hemi- nested real-time PCR method is employed to detect DNA sequences specific to the MTB in sputum samples within two hours (Friedrich et al., 2013). Moreover, the processing of the sample and detection of infection is fully automated, therefore the

method and instrumentation are suitable for the rapid evaluation of TB disease. The real-time PCR amplification of the 81bp region of the rpoB gene, a rifampicin binding site, with five-set probe primers. Any amplification failure from one of the primer sets denotes the mutation in the rpoB gene depicting the resistance towards the rifampicin drug. Therefore, the following patient result can be interpreted as MTB positive with rifampicin drug resistance. The meta-analysis of the study showed the sensitivity and specificity range of Xpert MTB/RIF at 89% and 99% respectively compared to the gold standard culture test for the diagnosis of pulmonary TB from adult patient samples (Lawn and Nicol, 2011; Steingart et al., 2014) (Figure 16).

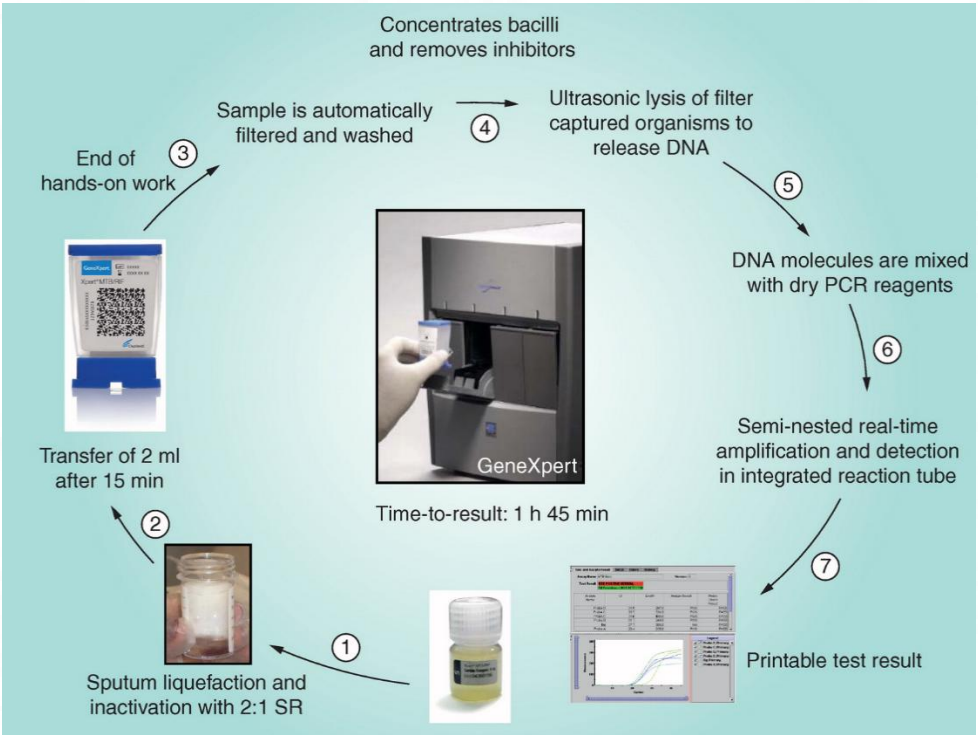


Figure 16. A step-by-step description of the Xpert MTB/RIF assay procedure for diagnosing TB and rifampicin resistance. (Lawn and Nicol., 2011)

An updated version of the Xpert MTB/RIF assay known as Xpert MTB/RIF Ultra was developed with the purpose to increase the sensitivity of tests for patients with paucibacillary disease or HIV where the number of bacterial counts are lower (Horne et al., 2019). The Xpert MTB/RIF Ultra primers are designed for 81 base pair regions of the rpoB gene and the portions of the multi-copy IS1081 and IS6110 insertion elements target sequences. The additional primer probe sequence for the target region of IS1081 and IS6110 insertion sites enhanced the detection of MTB due to the presence of multiple copies of these genes in most TB strains (Chakravorty et al., 2017). Therefore, the studies revealed that Xpert MTB/RIF Ultra has improved sensitivity compared to Xpert MTB/RIF, especially in detecting low bacterial loads and the reported sensitivity levels range from approximately 90% to 98% for TB detection. This enhanced sensitivity is particularly advantageous in populations with higher rates of HIV co-infection, pediatric and extrapulmonary TB cases. The specificity reported was lower than Xpert MTB/RIF in participants with a history of tuberculosis (Zifodya et al., 2021). But for the detection of rifampicin resistance from patient samples, both Xpert Ultra and Xpert MTB/RIF showed similar sensitivity and specificity (Chakravorty et al., 2017). The evaluated sensitivity and specificity can vary depending on the study population, sample quality, TB prevalence, and drug resistance in the tested population. Moreover, the recent Xpert MTB/XDR launched in 2020 is for pulmonary tuberculosis and resistance to isoniazid, fluoroquinolones, ethionamide, and amikacin in patients with presumptive pulmonary tuberculosis. A recent evaluation of multicentre studies revealed that the Xpert MTB/XDR provides accurate results for the detection of isoniazid and fluoroquinolone resistance, therefore, can provide an optimised treatment regimen based on the results but mentioned that

due to the limited number of the target region for the amplification of other drug-specific genes, it might cause alteration in the prediction of the resistance test evaluation in different settings (Pillay[a] et al., 2022).

Although the Xpert MTB/RIF assay has proven to be a valuable tool in TB diagnosis, the major limitations for resource-limited settings and health care are the requirement of expensive equipment and reliable electrical supply, making it relatively costly compared to other preliminary tests. Moreover, the limited testing capacity of machinery, where the currently ongoing instruments for TB testing showed the maximum capability of running up to 16 cartridges at a time, suggests the test is not well suited for high-throughput screening of a large number of TB samples, especially in settings with a high prevalence of the disease.

b. Line Probe Assay

Another molecular-based diagnosis of TB with drug resistance is line probe assay (LPA). The assay is a DNA strip-based test that uses nucleic acid amplification and reverse hybridization methods to rapidly detect TB and drug resistance mutations, which can be performed directly on clinical specimens, such as sputum (Ling et al., 2008). The assay is designed to detect genetic mutations associated with resistance to rifampicin and isoniazid, two key first-line anti-TB drugs. The genes employed for testing the mutations include rpoB for rifampicin and katG for isoniazid. The assay provides the results in the form of visible bands on a strip, which correspond to specific genetic targets. This test delivers results within 6 hours and a comparison of sensitivity and specificity with conventional culture-based drug tests showed 78.5% and 100% respectively for detecting rifampicin and isoniazid resistance (Somoskovi et al., 2008).

In addition to the rapidity of TB diagnosis, the NAAT based testing has additional advantages such as the possibility to be applied directly to collected patient sputum samples without isolating the bacteria from culture methods and to samples containing non-viable bacteria due to the inactivation of bacteria by heating or chemical treatment, the ability for high throughput and the decrease in the demand for laboratory biosafety requirements. Though the real-time DNA amplification method is observed as a favourable tool for the rapid detection of TB infection, the difficulty to launch in several resource-limited settings is mainly due to the high cost of the specialized machinery, the requirement of laboratory infrastructure facilities with continuous access of power and specialized laboratory expertise for operation and interpretation of results.

c. Truenat MTB

A point-of-care molecular assay called Truenat MTB by Molbio Diagnostics endorsed by WHO in 2020 is a chip-based real-time PCR method developed for detecting TB and rifampicin (RIF) resistance. A rapid and sensitive TB diagnosis closer to patients is a key global priority for TB control. With that mission, this portable point-of-care system with single testing capability using automated reporting and data transfer without sophisticated infrastructure requirements was found as a feasible technique for diagnostic purposes (Ngangue et al., 2022). The components used for the assay are an automated battery-operated Trueprep Auto device for the bead-based DNA extraction from patient sputum samples, a chip-based real-time micro PCR performed with TrueNat MTB chip and a fully automated real-time quantitative micro PCR analyser to read the presence of specific amplified genomic

sequences by using TrueLab PCR analyzer device, available in 1-, 2-, or 4-module configurations (Lee et al., 2019). The time taken for the TB DNA extraction from sputum samples was estimated as 25 minutes and another 35 minutes for the diagnostic result and an additional one hour for the testing of rifampicin resistance by running the Truenat MTB-RIF Dx test. The reported studies illustrated that the device could function at up to 40°C ambient temperature and can sustain up to 80% relative humidity (Nikam et al., 2014).

The Truenat MTB and MTB Plus assays are quantitative and semi-quantitative TB detection methods respectively showed comparable accuracy to the Xpert MTB/RIF assay. Using the bacteriological culture method as a standard reference, the reported overall sensitivity of the Truenat MTB assay was 83% and that of the Truenat MTBPlus assay showed 89%. The reported specificity was 99% for the Truenat MTB and 98% for the Truenat MTBPlus assay (Penn-Nicholson et al., 2021). Compared to the sequential detection of rifampicin resistance using phenotypic drug susceptibility testing as a standard reference, the Truenat MTB-RIF Dx test depicted a sensitivity of 93% and specificity of 95% as in the report of WHO 2022. Therefore, the performance Truenat MTB assay compared to the reference standard of TB culture reported was similar to that of the Xpert MTB/RIF assay. The Truenat test, thus, proved to be a feasible method for the detection of TB and drug-resistant TB in primary healthcare centres with minimal resources (Ngangue et al., 2022).

d. TB-LAMP

The Loop-mediated Isothermal Amplification (LAMP) assay, developed by Eiken Chemical Co., Ltd., is a molecular diagnostic approach designed to specifically

detect Mycobacterium tuberculosis complex (MTBC) DNA. Unlike conventional polymerase chain reaction (PCR) - based assays, LAMP operates at a constant temperature, typically between 60-65°C, facilitating DNA amplification without the need for sophisticated thermal cycling equipment (Notomi et al., 2000). It targets two specific genomic regions, IS6110 and gyrB, to enhance sensitivity and specificity. The assay allows for the simultaneous processing of up to 16 tests per run, including 14 patient samples, along with one positive and one negative control, which is essential for each run to ensure accuracy. The test utilizes a fluorescence-based detection system, where the reaction mix contains calcein, a fluorescent dye initially quenched by its interaction with manganese ions (Mn^{2+}). During amplification, the release of pyrophosphate ions (PPi) leads to the precipitation of Mn^{2+}, thereby unquenching calcein and producing fluorescence. This fluorescence is further enhanced through the interaction of calcein with magnesium ions (Mg^{2+}). The fluorescence signal can then be visually detected as an endpoint reading using a UV light source within the fluorescence detection unit. This method ensures a simple yet effective means of confirming the presence of M. tuberculosis DNA (Human Diagnostics Worldwide, 2020).

The World Health Organization (WHO) endorsed TB-LAMP in 2016 as a replacement for smear microscopy for diagnosing pulmonary TB in adults. This recommendation was based on a systematic review and meta-analysis, which demonstrated that TB-LAMP offers higher sensitivity than smear microscopy while maintaining comparable specificity (WHO, 2016). The assay is designed for use in

resource-limited settings, offering a simple, visual detection method where amplified products can be identified using ultraviolet light (Boehme et al., 2007).

Several studies have assessed the diagnostic accuracy of TB-LAMP. A meta-analysis by Shete, P.B et al. (2019) evaluated 13 studies with 4,760 participants across multiple countries and reported a pooled sensitivity of approximately 80% and specificity of 98% when compared with culture as the gold standard (Shete,P B et al. (2019). In comparison to smear microscopy, TB-LAMP demonstrated higher sensitivity while maintaining similar specificity. Further, a study conducted in India reported a sensitivity of 82% and specificity of 96.8%, reinforcing its potential for TB diagnosis in high-burden settings (Nair et al., 2020).

The advantages of TB-LAMP include its rapid turnaround time, minimal infrastructure requirements, and ease of use. The assay is designed to be performed manually, reducing reliance on complex automated equipment. Additionally, its robustness under field conditions makes it well-suited for peripheral laboratories (WHO, 2016). Unlike smear microscopy, which has a lower sensitivity, TB-LAMP provides a more reliable means of detecting TB, particularly in paucibacillary cases (Iwamoto et al., 2003).

Despite its advantages, TB-LAMP has several limitations regarding the screening of TB disease. One key drawback is the requirement for proprietary instruments and reagents, including the HumaLoop T device, which accommodates a maximum of 14-16 samples per run. This limitation can hinder scalability in high-throughput settings (Human Diagnostics Worldwide, 2020). Additionally, the LAMP

method is susceptible to non-specific amplification, which can lead to false-positive results if proper precautions are not taken (Njiru, 2012). The assay also does not provide information on drug resistance, necessitating additional testing for comprehensive patient management (WHO, 2016).

2.6.4 Culture Test

The culture test is considered the gold standard for TB diagnosis, as it has high sensitivity and specificity in detecting MTB from collected sputum or blood samples of patients. The culturing of MTB is an essential test for TB diagnosis as it can also be used to examine drug susceptibility and for the confirmation of smear-negative AFB with any clinical TB suspicion.

The patient specimens used to grow the bacteria are usually from the sputum collected after spontaneous coughing, coughing induction using saline inhalation, bronchoscopy, and gastric aspiration (Susilawati and Larasati, 2019). After the decontamination of the collected clinical samples, the MTB is capable of adapting to the conditions given on a medium containing a source of carbon and nitrogen with buffer salts and trace elements (Chatterjee et al., 2013). The culturing media that allow for the abundant growth of MTB bacteria that are mainly followed for diagnostic purposes are Löwenstein-Jensen (LJ) solid medium and Mycobacteria Growth Indicator Tube (MGIT) liquid medium.

The LJ medium is a type of solid medium developed by two scientists named Löwenstein, and Jensen in the early 20th century and has since become one of the most commonly used methods for culturing of MTB. Basically, it is a selective egg-based medium containing several factors essential for the growth of MTB, including egg

yolks, the malachite green and glycerol, which promote the growth of MTB and suppress the growth of other bacteria. The components in the media called egg yolk serves as a nutrient source for MTB, mainly the presence of lecithin, a phospholipid that helps to transport the essential factors for the growth across the bacterial cell membrane, and cholesterol which is optimum for the growth and replication of MTB. The important content of the LJ medium, glycerol, as carbon and energy source for the growth of MTB, providing the bacterium with the necessary nutrients to proliferate which enhances the survival and recovery from clinical specimens. Whereas, the malachite green, an antimicrobial agent, serves as a selective agent that inhibits the growth of other bacteria and fungi, while promoting the growth of MTB due to the presence of the thick cell wall. This content is, therefore, essential for culturing slow-growing bacteria because of the possibility of overgrown contamination by other microorganisms during culturing (Allen, 1998). Other components such as asparagine which acts as a nitrogen source, and a pH-maintaining buffer, potassium dihydrogen phosphate, are also present in LJ media to promote the growth of MTB in culture tests (Joloba et al., 2014). The incubation of inoculated petri plates at 37ºC for 4- 8 weeks shows the growth of MTB as small, cream-coloured colonies with rough and dry textures (Figure 17).

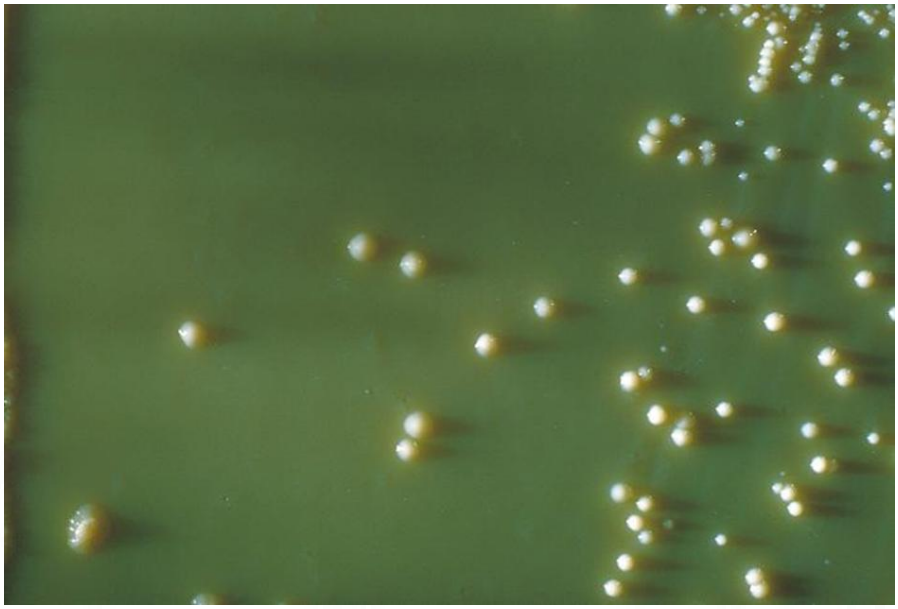


Figure 17. Lowenstein-Jensen plate culture with colonies of MTB growth as small, cream-coloured colonies with rough and dry texture (cdc.gov, PHIL ID#2904).

The results are interpreted as positive if the colonies exhibit the characteristics of colony formation of MTB, and negative if there is no growth or colonies that have not resembled the features of MTB growth. The growth rate of MTB is relatively slow on the LJ medium, taking 4 to 8 weeks to form visible colonies on culture plates for confirmation (Nambiar et al., 2017).

Another method called the MGIT culture test for TB diagnosis is a liquid culture technique with a 16- by the 100-mm tube that uses a specialized broth containing a fluorescent indicator and a modified Middlebrook7H9 broth to identify the growth of MTB (Ganeswrie et al., 2004). Moreover, it contains a range of components, including OADC and PANTA supplements, which play important roles in supporting mycobacterial growth and inhibition of contaminants. The OADC codes for oleic acid, albumin, dextrose and catalase whereas the PANTA stands for

polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin. The OADC is required for the growth and survival of MTB by providing the essential composition of fatty acids, protein, carbohydrates and enzymes. The PANTA supplement is a combination of antibiotics and antifungal agents to inhibit the growth of contaminants and other bacteria that may be present in the clinical specimen (Peres et al., 2011). The fluorescent detection method for MTB growth is a silicon rubber disk impregnated with ruthenium-based dye (ruthenium pentahydrate) that is quenched in the non-inoculated medium due to the presence of oxygen (Goosen et al., 2022). The actively growing MTB consumes the dissolved oxygen in the media, which is indirectly measured by the amount of fluorescence emitted by the dye. The oxygen sensor in the MGIT system consists of an LED light source that emits a 365-nm wavelength of light, a photodetector that detects the amount of fluorescence emitted by the dye, and a microprocessor that calculates the concentration of oxygen based on the amount of fluorescence detected (Essawy et al., 2014) (Figure 18).

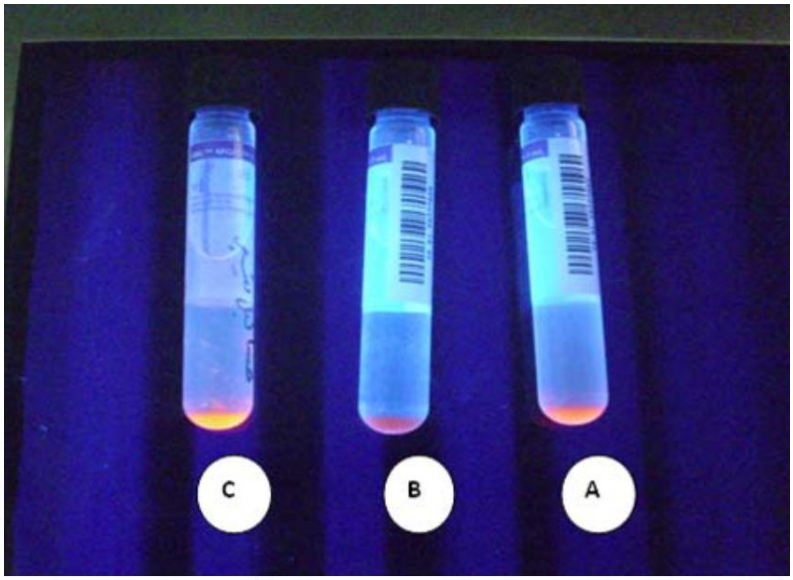


Figure 18. Mycobacteria Growth Indicator Tube (MGIT) culture test for the detection of MTB by fluorescent orange colour A) Positive TB result B) Negative Control C) Positive Control (Essawy *et al*., 2014)

Therefore, the method enables continuous monitoring of oxygen levels in MGIT tubes after inoculating with TB-infected clinical samples at 37ºC, which is essential for constant analysis of MTB growth. The MGIT culture method can detect the MTB growth more rapidly than LJ culture because it requires only 1 to 2 weeks for confirmation, with higher sensitivity (Huang et al., 2004). However, the sub-optimal laboratory infrastructure and lack of skilled staff in resource-limited conditions often limit the practical use of the culture method for TB diagnosis compared to other diagnostic methods (Lawn, 2015). Even though the culture test is not recommended to use as a first-line test, it remains a crucial part of TB diagnostics, where the persistent positive results from patients can predict the possible relapse of TB (Horne et al., 2010).

MATERIALS

AND

METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS:

- GENOMIC DNA:

  a) The genomic DNA of the *Mycobacterium tuberculosis* H37Rv strain was obtained from the Department of Pathogen Biology at Rajiv Gandhi Centre for Biotechnology (RGCB).

  b) The non-mycobacterial strains such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Acinetobacter baumanii* and *Enterococcus facalis* were acquired from the Division of Microbial Technology at Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST).

- TB PATIENT SAMPLES:

  The early morning Pulmonary TB patient sputum samples were acquired from the State Tuberculosis Demonstration and Training Centre (State TB Cell), Directorate of Health Services, Kerala.

- REAGENTS:

  a) All the reagents required for the reactions mentioned were purchased from New England Biolabs (NEB), except as otherwise noted.

  b) The DNA intercalating Fluorescent dyes, SYTO 16 and SYBR Green 1 and PCR *Taq* Polymerase (DyNAzyme II Polymerase) for Gibson assembly cloning and dNTPs Mix were purchased from ThermoFisher Scientific.

  c) Primers purchased from Eurofins Genomics.

- TB LAMP PRIMERS

  F3 – 5’ TGCTCAAGGTCTACCAGAA 3’

  B3 – 5’GTGACTGCGAAGTTCTGAT 3’

  FIP – 5’ CCTGCCACAGCGTGTCATCCAACGACCACGTACAAG 3’

BIP – 5’ ATCCGCTGCCAGTCGTCTGCTATCGATACCTGTTGTCC 3’

LF – 5’ AGGTGATTGGCTTGCGAT 3’

LB – 5’ AAGGTGAACTGAGCAAGCA 3’

- TB qPCR PRIMERS

  Forward sequence – 5’ ATGACACGCTGTGGCAGGCTGA 3’

  Reverse sequence – 5’ AAGCCGGCATTCGGCGCTAT 3’

  Probe sequence – 5’ ATCCGCTGCCAGTCGTCTTCCCCAT 3’

- GIBSON ASSEMBLY CLONING PCR PRIMERS

  Forward sequence - 5’ CGAGGTCGACGGTATCGATATCGTTTTGCTCTGTTGTTC 3’

  Reverse sequence - 5’ CCACCGCGGTGGCGGCCGCTACGTGGGACCAATACCTG 3’

- BUFFERS AND SOLUTIONS

  - Lysis Buffer:
    - NaCl - 0.4 M,
    - EDTA - 2mM,
    - Tris-Cl - 10mM
    - Triton X-100 - 0.5%

    pH 8 and made up to 500ml with distilled water.

  - Neutralizing Buffer:
    - Tris-Cl - 10mM

    pH adjusted to 7.5 and made up to 500ml with distilled water.

  - Gibson Assembly Cloning Isothermal Buffer (2X):

    - Tris-Cl – 200mM
    - $MgCl_2$ – 20mM
    - Dithotritol (DTT) – 20mM
    - dNTPs – 0.4mM
    - NAD^+ - 2mM

- Polyethylene Glycol (PEG) 8000 – 10%

pH adjusted to 7.5 and volume made up to 1.1ml (1100µl) with nuclease-free water , Stored at -20ºC.

- Gibson Assembly Cloning Reagent Master Mix:
    - Gibson Assembly Cloning Isothermal Buffer – 1.3X
    - Phusion DNA Polymerase – 0.033U/µl
    - Taq DNA ligase – 5.3U/µl
    - T5 Exonuclease – 0.005U/µl

  Volume made up to 200µl with nuclease-free water and stored at -20ºC.

- Tris-acetic acid- EDTA Buffer (50X):
    - Tris-Cl – 2M
    - Glacial acetic acid – 1M
    - EDTA disodium salt – 50mM

  Made up to 500ml with distilled water.

3.2 METHODS:

- **TB LAMP ASSAY:** The genomic DNA of *Mycobacterium tuberculosis* H37Rv maintained at -20ºC was used as a template for the TB LAMP assay standardization by selecting the *mpt64* gene sequence specific to the strain as the target for the amplification. The details of the *mpt64* gene were attained from GenBank on the NCBI website with an accession number NC_000962, which is depicted as a sequence code for an MTB-specific immunogenic protein Mpt64.

Based on the detailed analysis of suitable conditions required for the LAMP assay, specific primers for the experiment were designed along with Loop forward and Loop backward primers to enhance the reaction by using a primer designing tool, LAMP designer 1.16 version (Premier Biosoft Interpairs) and were synthesized with HPLC purification grade. The specificity of primers was verified using the basic local alignment search tool (BLAST) against non-tuberculosis *Mycobacterium* species DNA sequences in the non-redundant GenBank database.

To perform the TB LAMP assay, the tubes containing 1.6µM inner LAMP primers (FIP and BIP), 0.4µM outer LAMP Primers (F3 and B3) and 0.2µM loop primers, along with 1X WarmStart LAMP Master Mix (NEB, MA, USA) made up to 25µl by adding 5µl of target DNA template were used. As a non-template control (NTC), 5µl of autoclaved distilled water was added to the LAMP reaction instead of the target template DNA.

The TB LAMP assay was carried out at 65ºC for 40 minutes using Thermomixer comfort machinery (Eppendorf SE). After running 1% agarose gel electrophoresis with 2µM ethidium bromide stain and along with GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific), the amplified products were imaged under a Gel documentation imaging system (E-Box CX5; VILBER).

- **TB LAMP DETECTION METHOD:** To evaluate a suitable dye for the detection of the TB LAMP assay, SYTO 16 and SYBR Green 1 fluorescent dye were compared at an optimum concentration of 2µM (Eischeid, 2011). After the TB LAMP assays with SYTO 16 and SYBR Green 1 respectively, the fluorescent intensity of the amplified products with volume 5µl was measured using a

fluorescent plate reader (Infinite Mplex, Multimode microplate reader; Tecan, Switzerland) by setting excitation at 480nm and emission at 520nm. The intensity of the fluorescence value of each reaction tube after the assay measured at 520nm emission was graphically plotted by using GraphPad Prism.

- **TB REAL-TIME LAMP (TB RT-LAMP) ASSAY:** The real-time amplification of DNA with SYTO 16 and SYBR Green1 dyes in the TB LAMP reaction was monitored by using the instrument Applied Biosystem QuantStudioTM5 Real-Time PCR instrument (ThermoFisher Scientific). In this experiment, the procedure program assigned a 45-minute reaction with 65˚C by measuring the data of fluorescence signal produced every 1 minute.

- **TB quantitative PCR (TB qPCR) ASSAY:** A TaqMan-based TB quantitative PCR (TB qPCR) assay was also optimized by targeting the *mpt64* gene. Primers and a probe sequence were designed using Primer3plus software (Primer3Plus version 3.2.6). The TB qPCR reaction mix comprised of 0.4µM TB qPCR primer sets (forward and reversal respectively), 0.2µM probe tagged with FAM and BHQ1, 1X Luna Universal Probe qPCR Master mix (Luna Universal Probe qPCR Kit, NEB, MA, USA), and distilled water along with 5µl DNA template made up to volume 20µl. The thermocycling program was designed at 95ºC for 60 seconds, followed by 40 cycles of 95ºC for 15 seconds, and 60ºC for 30 seconds along with a fluorescent data reader at the final stage of each cycle.

- **POSITIVE CONTROL RECOMBINANT PLASMID DNA CONSTRUCTION:** To evaluate the analytical sensitivity of the TB RT-LAMP assay, a plasmid cloned with *mpt64* was generated to use as the positive control.

Each set of *mpt64*-specific Gibson assembly Cloning PCR primers overlapped with the 20bp region of a plasmid called pL4440 to be cloned was designed using the software NEBuilder Assembly tool, version 2.7.1 (NEB, MA, USA). The plasmid called pL4440 used for cloning was digested with Xba1 and Hind111 for Gibson assembly cloning. The digested pL4440 was purified from an agarose gel after electrophoresis by using GeneJet Gel Extraction Kit (Thermo Fisher scientific). On the other hand, the *mpt64* sequence flanked with a 20bp segment of pL4440 PCR product was amplified by using MTB H37Rv genomic DNA as a template. This was performed by following a 40-cycle PCR procedure in which the initial 5 cycle stages were carried out at 95ºC denaturation, 50ºC annealing and 72ºC extension temperature and the remaining 35 cycles with 60ºC annealing temperature. The PCR reaction was carried out with *Taq* Polymerase (DyNAzyme II Polymerase) enzyme. After the reaction, the amplified PCR products were confirmed by running 2% agarose gel electrophoresis. Purified the resulting PCR product from the gel by using GeneJet Gel Extraction Kit (Thermoscientific) for cloning.

The cloning reaction mixtures were prepared in 20µl volume containing the purified Gibson assembly PCR amplified *mpt64* gene sequence and digested pL4440 plasmid in a 1:2 ratio with volume 5µl and remaining 15µl of Gibson assembly cloning master mix. Flicked the tube several times and centrifuged to collect the mixtures to the bottom of the tube. Incubated the tube at 50ºC for 1 hour. Thereafter the transformation of *E.coli* DH5α with the cloned plasmid was completed by following standard procedure with antibiotic ampicillin added LB

culture plates as the selective medium for the incubation at 37ºC overnight. The resulting colonies obtained were inoculated into a tube with 5ml LB broth containing 50µg/ml antibiotic ampicillin which were incubated at 37ºC overnight by using a shaking incubator adjusted with 200 rpm. The plasmid DNA extraction from cultured LB broth was done with GeneJET Plasmid Miniprep Kit (Thermoscientific). The recombinant plasmid extracted was confirmed by PCR amplification and double digestion of cloned plasmid (pL-mpt64) with Sal1 and EcoR1. After the confirmation, the recombinant plasmids (pL-mpt64) were isolated from the cultured transformed *E.coli* DH5α cells and were used as standard plasmids for the assays mentioned for the study.

- **DETECTION LIMIT AND SPECIFICITY OF TB RT-LAMP ASSAY:** To assess and compare the quantitative sensitivity of TB RT-LAMP assay with the TB qPCR method, 10-fold serial dilutions of plasmid cloned with mpt64 (pL-mpt64) were prepared as the template. The pL-mpt64 plasmid concentration measured was converted into copy numbers by following the equation:

  The number of pL-mpt64 copies/µl = (M x 6.02x1023x10-9)/ (n x660) in which M depicts the amount of pL-mpt64 in nanogram/microlitre (ng/µl) and n is the total base pair of pL-mpt64 (3334bp) plasmid and 660 is denoted as the average weight per base pair.

  The comparison of the analytical sensitivity of TB RT-LAMP and TB qPCR was verified by using the pL-mpt64 in a range from 10^8 to 10^0 copies per reaction. The amplification time of TB RT-LAMP and the threshold time of TB qPCR were plotted against the log values of the serially diluted pL-mpt64 amplified products

detected to plot a standard amplification curve. The experiments in this study were conducted in three replicates to confirm the results.

The analytical specificity of the TB RT-LAMP assay was examined by using DNA extracted from *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Acinetobacter baumanii*, *Enterococcus faecalis* and *Escherichia coli*.

- **EVALUATION OF TB RT LAMP WITH CLINICAL SAMPLES:** A cross-sectional study was implemented at the State Tuberculosis Demonstration and Training Centre (State TB Cell), Directorate of Health Services, Thiruvananthapuram, Kerala. Ethical clearance for the research study was obtained from the SCTIMST National Ethics Committee to conduct this study. During the study period, a total of 350 pulmonary presumptive TB patient samples were collected and tested for smear microscopy, Xpert MTB/RIF and Culture tests and concurrently performed with TB RT-LAMP tests after taking written informed consent to collect the epidemiological, demographical and clinical data by following World Health Organization (WHO) national guidelines. Patients above 15 years of age suspected of having pulmonary TB disease were included in this study by collecting early morning sputum samples as the State TB centre recommended. The obtained patient sputum samples were decontaminated with NALC-NaOH solution. The NALC-NaOH solution was freshly prepared, due to the shelf life of NALC being 24 hours, by taking 4% NaOH and 2.95% Tri-Sodium citrate (Himedia) in 25ml, into that added 250mg NALC (N- Acetyl-L-Cysteine) powder (Himedia).

- **DNA ISOLATION FROM CLINICAL SAMPLES FOR TB RT-LAMP ASSAY:** For the diagnostic purpose, the MTB DNA was extracted from decontaminated sputum samples using the procedure followed for the GenoLyze Kit which provided a rapid extraction of MTB DNA with modification to ensemble our test (Crudu et al., 2012). For that, a 5 ml sputum sample was treated with an equal volume of NALC-NaOH, which was mixed, vortexed, and placed at room temperature for 15 minutes. Following centrifugation at 3000 rpm for 10 minutes, the supernatant was discarded and added 100µl Lysis buffer (0.4 M NaCl, 2mM EDTA, 10mM Tris-Cl, and 0.5% Triton X-100, pH 8). Homogenized the pellet and incubated the tubes at 95˚C for 20 minutes to break down the cell walls of MTB and then added 100µl Neutralization buffer (10mM Tris-Cl, pH 7.5). To conduct further experiments, the tubes were sealed and maintained at 4˚C.

- **GENEDOT END-POINT TB LAMP ASSAY FOR IN-FIELD DIAGNOSIS:** To evaluate the feasibility of the TB LAMP assay for the blind diagnosis of TB patient samples collected, a prototype device was designed to be easy to handle with the simplest configuration for performing a test. The prototype device named GeneDot was built with a heating block to amplify DNA under isothermal (65ºC) conditions. The instrument is designed with 20 samples loading block and a specific wavelength filter (˜480nm excitation and 520nm emission) which moves in a linear stage from one tube position to another to measure the fluorescent intensity after LAMP assay. A touch screen and a serial port to connect to the desktop were made available to display the wavelength intensity parameters and data analysis after the TB LAMP assay (see Figure 37). The measurement of the

GeneDot scanning fluorescent intensity was designed as the values above 25000 were depicted as positive and below 25000 as negative.

- **TB LAMP END POINT READING USING UV LIGHT CHAMBER**: To evaluate the visual determination of TB positives and negatives by double blind analyses, a UV light Chamber with excitation at 480nm and emission at 520nm wavelength using the instrument E-Box CX5; VILBER was used. The tubes observed as bright white colours under dark background predicted as TB positive and colourless as TB negative.

- **STATISTICAL DATA ANALYSIS:** The diagnostic performance of the TB RT-LAMP assay was compared to smear microscopy, Xpert MTB/RIF, and the gold standard Mycobacterial Culture test. The data were initially recorded in a Hospital logbook. The values were entered into Microsoft Excel with descriptive analysis to determine the demographic, epidemiologic, and clinical characteristics of the study-reported patients. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratio, disease prevalence and accuracy with percentage (%) and 95% Confidence intervals between these three diagnostic methods were determined using MedCalc Software, Belgium. The agreement between tests was measured by calculating Cohen's kappa value (κ) and 95% CI for κ was calculated by following the interpretations which range from 0 to 1 in which 1 depicts perfect agreement and 0 as no agreement (GraphPad Software, LA Jolla CA, USA).

RESULTS

4. RESULTS

4.1 AMPLIFICATION OF mpt64 GENE USING TB LAMP ASSAY

An MTB-specific gene, *mpt64*, was used as the target for the LAMP assay. Six gene-specific primers were designed as shown in Table 1, with Forward and Backward outer primers (F3 and B3, respectively), Forward and Backward Inner primers (FIP and BIP, respectively), and Loop forward and backward primers (LF and LB, respectively). The primers were designed by following the factors necessary for an optimum LAMP reaction, including a segment of inner FIP/BIP primers called F2/B2 designed with a melting temperature suitable for the Bst. Polymerase enzyme. The remaining portion of the FIP/BIP region, called F1c/B1c, was designed with a slightly higher melting temperature than F2/B2 so that the primers facilitate the amplified product to form a loop structure immediately after the formation of single-stranded DNA from the target. The outer primers F3/B3 were designed with a lower melting temperature than FIP/BIP, thereby promoting the inner primers to initiate the polymerase reaction. Figure 19 shows here the primer sequences with their binding sites designed with suitable melting temperatures required for the LAMP assay to amplify the *mpt64* gene sequence.

Table 1. The *mpt64* gene-specific primers design for the LAMP assay by following the parameters necessary for the reaction.

Primers	Sequence (5'-3')	Base pair	GC%	Tm (˚C)	Fragment length
F3 MTB	TGCTCAAGGTCTACCAGAA	19	47	60	242bp
B3 MTB	GTGACTGCGAAGTTCTGAT	19	47		
FIP MTB	CCTGCCACAGCGTGTCATCCAACGACCACGTACAAG	36	58	F2/B2 - 64 F1c/B1c - 69	
BIP MTB	ATCCGCTGCCAGTCGTCTGCTATCGATACCTGTTGTCC	38	55		
LoopF MTB	AGGTGATTGGCTTGCGAT	18	50	60	
LoopB MTB	AAGGTGAACTGAGCAAGCA	19	47		

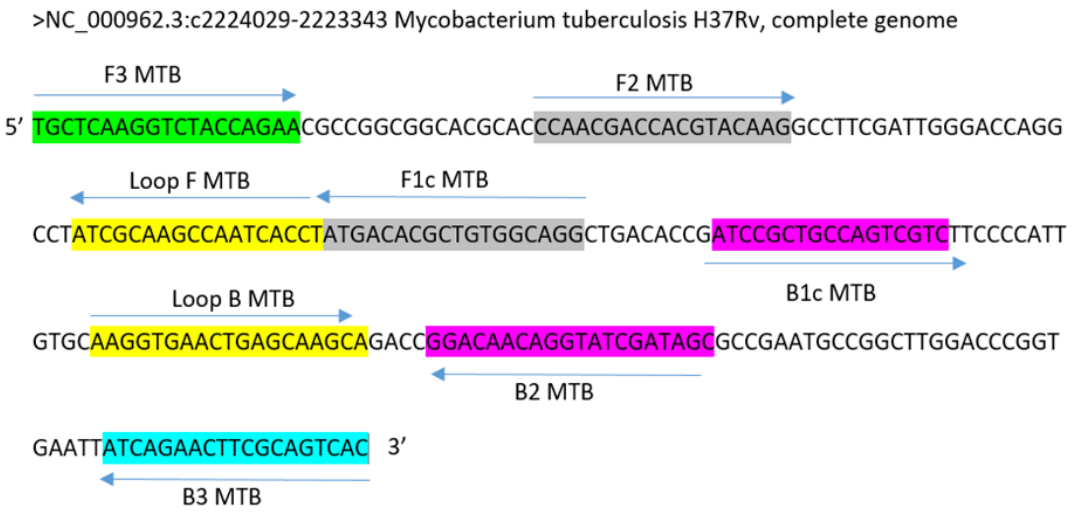


Figure 19. The primers binding site in the *mpt64* gene for the TB LAMP assay.

After the designing of LAMP primers specific to the *mpt64* gene in MTB, the genomic DNA of the *Mycobacterium tuberculosis* H37Rv strain was used as a template for the standardization of the LAMP assay. The amplified *mpt64* sequence after LAMP assay at 65ºC for 40 minutes was verified by running 1% agarose gel electrophoresis. As a control, the LAMP assay was performed without template DNA, named as Non-Template Control (NTC). The *mpt64* amplified products were observed

as LAMP-specific ladder-like structures after running gel electrophoresis for 30 minutes as shown in Figure 20.

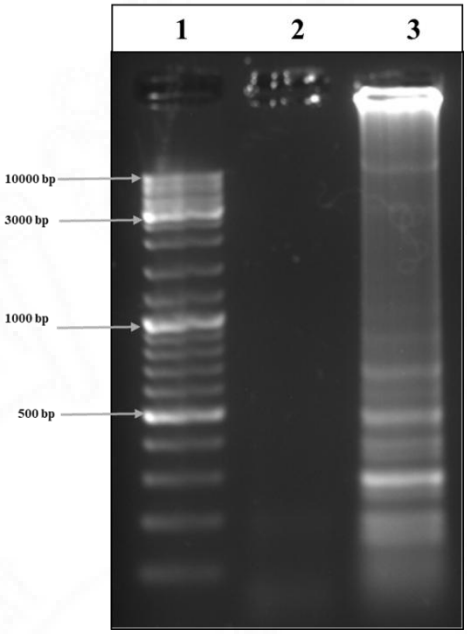


Figure 20. Agarose gel electrophoretic analysis *mpt64* amplified product after TB LAMP reaction. 1) Non-template Control (NTC) 2) TB LAMP amplified *mpt64* gene product of *Mycobacterium tuberculosis* H37Rv.

4.2 AN OPTIMAL FLUORESCENT DYE FOR TB LAMP ASSAY

To determine an optimal DNA-binding fluorescent dye for the confirmation and interpretation of results from the LAMP assay, two DNA intercalating dyes named SYTO 16 and SYBR Green 1, were used. Initially, the study focused on the effect of these two dyes on the LAMP reaction. To verify that, the LAMP assay of the *mpt64* gene with an optimal 2µM concentration of SYTO 16 and SYBR Green 1, respectively, was used. The observation of LAMP amplified products after 40 minutes by running the gel electrophoresis showed ladder-like structures for both SYTO 16

and SYBR Green 1 mediated LAMP reaction. Therefore, the results concluded as no significant difference or no inhibitory effect was observed between the LAMP assay with SYTO 16 and SYBR Green 1 fluorescent DNA intercalating dyes as shown in Figure 21.

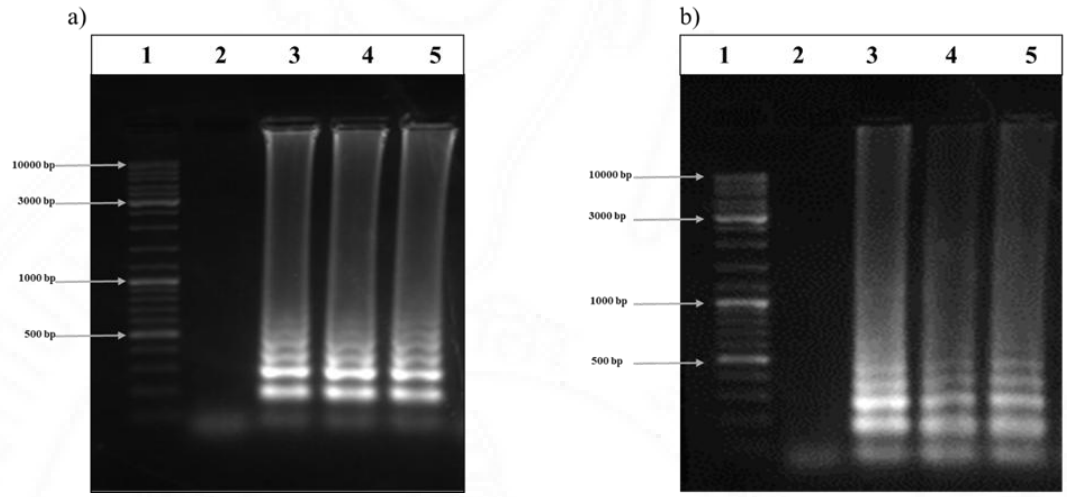


Figure 21. Amplification of *mpt64* gene by TB LAMP assay. a) With SYTO 16. b) With SYBR Green1. The wells marked as 1. Marker, 2. Non-Template Control (NTC), 3 to 5 are three trials of *mpt64* LAMP amplified products of MTB H37Rv genomic DNA template.

Next, the study focused on finding the difference in fluorescent intensity of these two dyes after the LAMP reaction by comparing the amplified product and NTC. For that, 5µl of LAMP amplified solution with SYTO 16 and SYBR Green 1, respectively, were loaded and analysed separately by using a fluorescent plate reader (Infinite Mplex, Multimode microplate reader; Tecan, Switzerland) by setting excitation at 480nm and emission at 520nm wavelength. The results showed that the LAMP assay with SYTO 16 dye has a significantly higher fluorescent intensity compared to SYBR Green 1 (Figure 22).

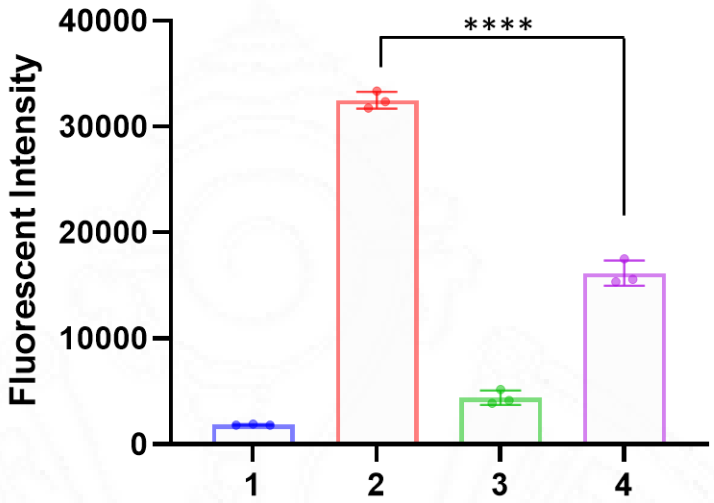


Figure 22. Comparison of fluorescent intensity of TB LAMP assay. The plot on 1 and 2 represent the NTC and amplified *mpt64* gene product with SYTO 16, respectively. The plot on 3 and 4 illustrate the NTC and amplified *mpt64* gene product with SYBR Green 1, respectively ($p<0.0001$).

4.3 REAL-TIME LAMP ASSAY WITH SYTO 16 and SYBR GREEN 1

The real-time monitoring of the LAMP assay with SYTO 16 and SYBR Green 1 dyes was performed to further evaluate and measure the time scale required, thereby the result can confirm the perfect dye for the LAMP assay. Real-time LAMP assay (RT-LAMP) with SYTO 16 and SYBR Green 1, therefore, was performed by using the instrument Applied Biosystem QuantStudioTM5 Real-Time PCR by programming temperature at 65ºC for 45 minutes as shown in Figure 23.

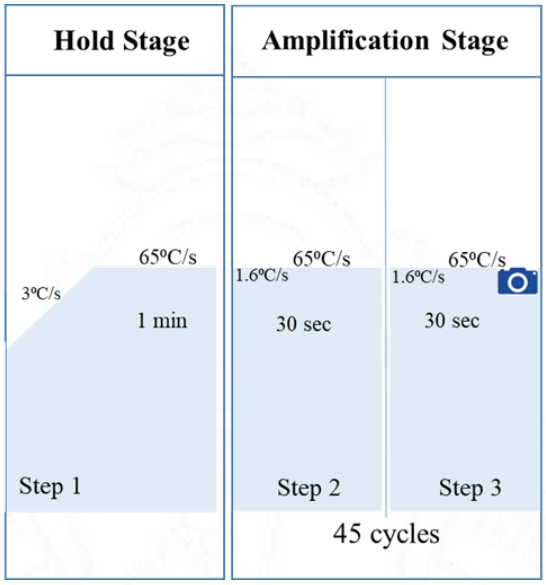


Figure 23. The amplification stages of RT-LAMP assay. The programmed temperature and time scale used in the experimental method of the Quant Studio Design and Analysis software for the Real-time LAMP assay.

The results from the assay showed that the SYTO 16 dye enabled a shorter amplification time (T_{amp}) by initiating the LAMP reaction at approximately 9 minutes. On the other hand, SYBR Green 1 showed a delay in T_{amp} which took around 20 minutes to initiate the amplification of *mpt64* DNA. Moreover, the signal magnitude (ΔRn) observed in the amplification plot showed approximately 3.5 fold increase for SYTO 16 dye compared to SYBR Green 1 as shown in Figure 24. Therefore, the overall results confirmed that the LAMP reaction with SYTO 16 dye at concentration 2µM would be more favourable than compared to SYBR Green 1 dye. For these reasons, SYTO16 was selected as the dye of choice for the LAMP assay in this study.

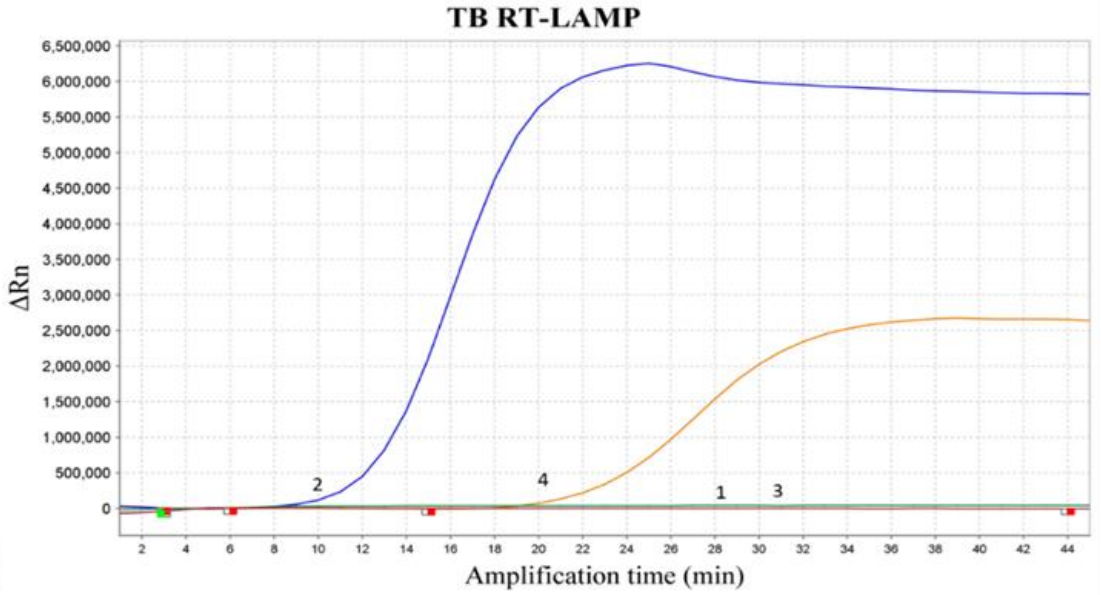


Figure 24. Real-time TB LAMP (TB RT-LAMP) assay using SYTO 16 and SYBR Green 1. 1 and 3 represent the NTC of TB RT-LAMP with SYTO 16 and SYBR Green 1 respectively. 2 and 4 illustrated the TB RT- LAMP amplified *mpt64* gene segment with SYTO 16 and SYBR Green 1 respectively.

4.4 LIMIT OF DETECTION OF RT-LAMP ASSAY WITH SYTO 16.

4.4.1 Recombinant pL-mpt64 as a reference template

To accurately evaluate the limit of detection RT-LAMP with SYTO 16, a plasmid (pL4440) cloned with *mpt64* was used as a standard reference template. The cloning of pL4440 with the *mpt64* gene segment was performed by following a protocol called Gibson Assembly cloning. The Gibson assembly primers were designed by following the methods described above (see method section for details). The primers designed for Gibson assembly cloning and the amplification of 666bp of the *mpt64* gene segment overlapped with 20 bp of pL4440 on both sides are illustrated in Figure 25 and Figure 26.

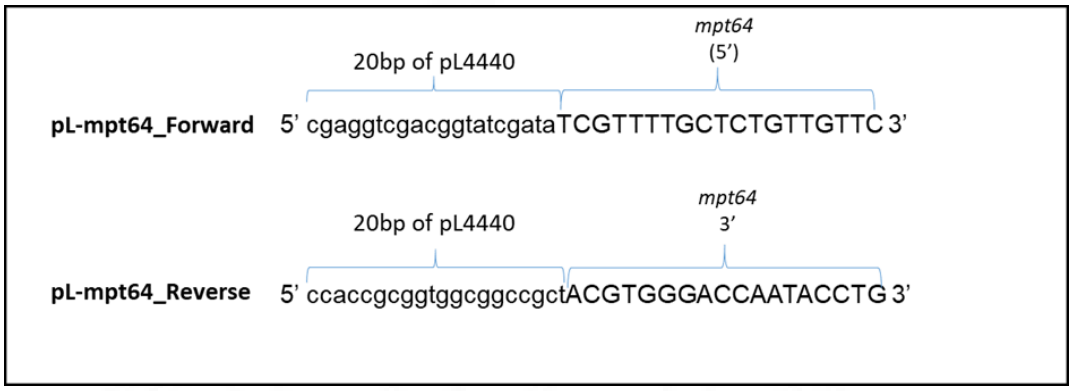


Figure 25. Gibson assembly cloning primers. The designed primers with 20bp of pL4440 sequence at the 5' end region of forward and reverse primers for *mpt64* sequence, respectively.

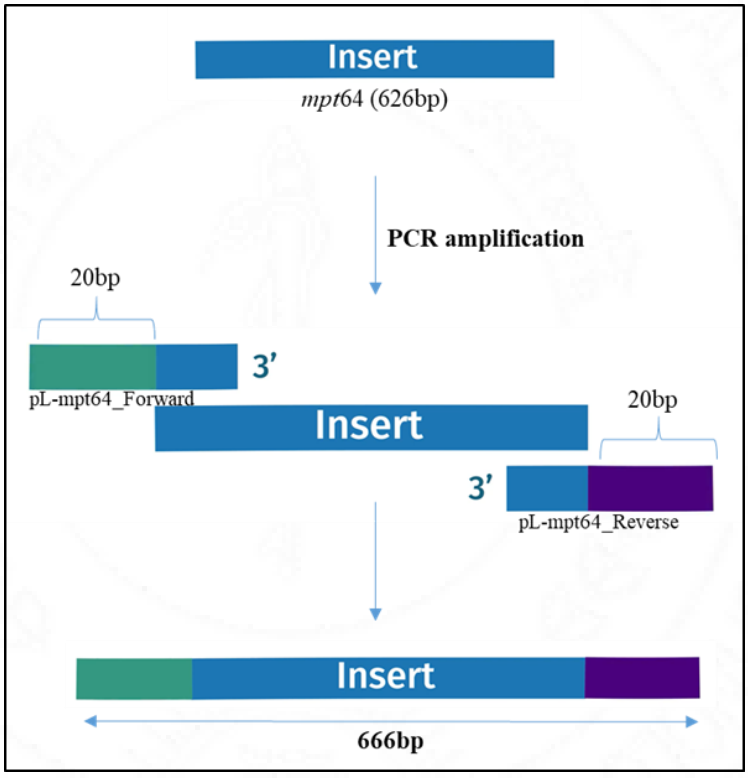


Figure 26: The diagrammatic representation of amplification of the *mpt64* gene segment overlapped with 20 bp of pL4440 on both sides with 666bp product size for Gibson assembly cloning. (Refer to the Method section for details)

The pL4440 vector was digested with Xba 1 and HindIII to obtain the linear plasmid (2712bp product size) for the cloning of the amplified product with size 666bp (Figure 27).

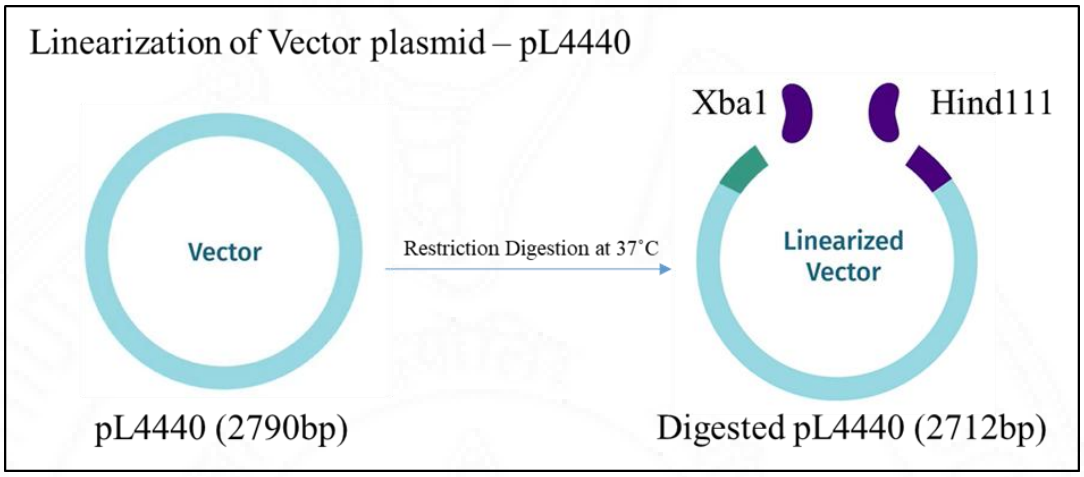


Figure 27: Restriction digestion of pL4440 (2790bp) with Xba1 and Hind111 provided a segment of plasmid with 2712bp in size.

The confirmation of the amplification of the *mpt64* gene segment and restriction digestion of pL4440 with Xba1 and Hind111 was obtained from the observation of the resulting DNA bands from agarose gel electrophoresis (Figure 28).

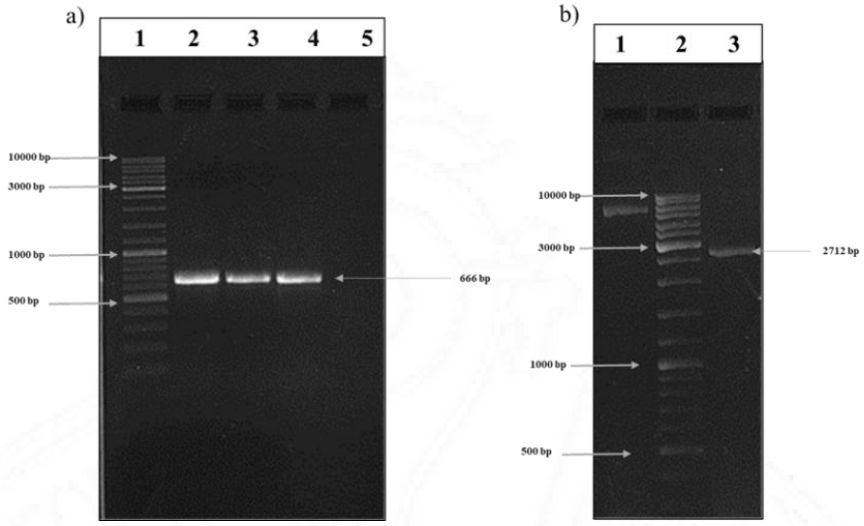


Figure 28: Cloning of pL4440 with *mpt64* gene segment. a) PCR amplified Gibson assembly *mpt64* gene product after running 2% agarose gel electrophoresis: 1. DNA ladder. 2 to 4 are 666bp of amplified *mpt64* gene segment and 5. NTC of PCR. b) The restriction digestion of pL4440 with Xba1 and Hind111 after running 1% agarose gel electrophoresis: 1. undigested pL4440. 2. DNA ladder 3. Xba1 and Hind111 digested pL4440 with 2712 bp size.

The gel-purified mixture of digested pL4440 and amplified *mpt64* gene products with a short stretch of homology on both ends was taken in a 1:2 ratio which was then mixed with 15µl Gibson assembly cloning Master mix (refer to the material section for the Master mix composition). After the reaction steps with endonuclease, DNA polymerase and DNA ligase for 1 hour under 50ºC incubation results in the cloning of the *mpt64* gene segment with digested pL4440 plasmid (Figure 29). To ascertain the cloned pL-mpt64 fragments, the transformation of DH5α cells was performed with 10µl of Gibson assembly reaction products.

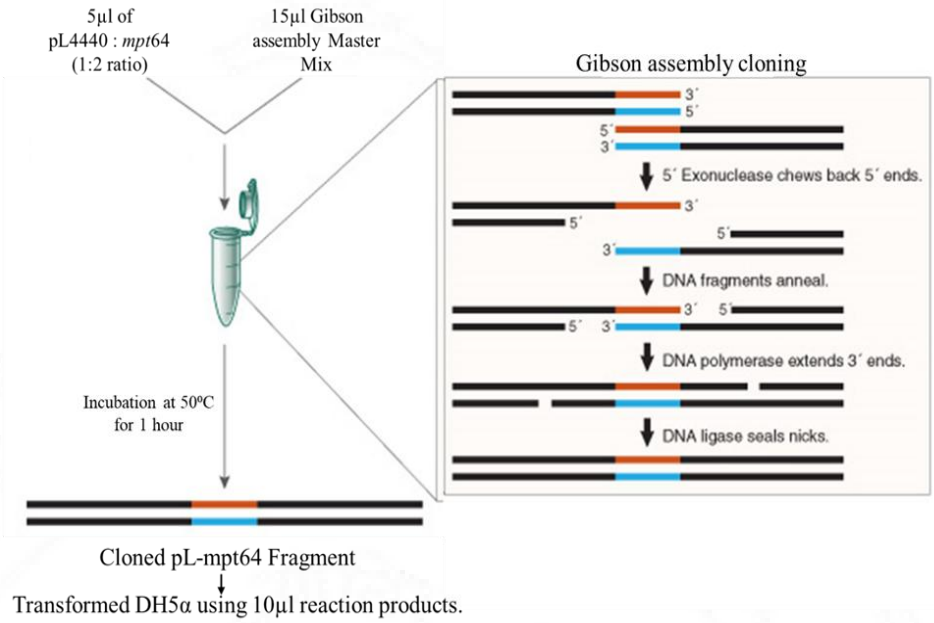


Figure 29: Gibson assembly cloning process of pL4440 with *mpt64* gene segment during incubation. (Gibson et al., 2009)

The resulting cloned plasmids were isolated from transformed colonies on cultured LB plates with ampicillin (Figure 30 a). The cloned plasmid (pL-mpt64) was confirmed in colony number 3 after obtaining 2667 bp and 667 base pairs of completely digested DNA fragments from Sal1 and EcoR1 restriction digestion (Figures 30 b).

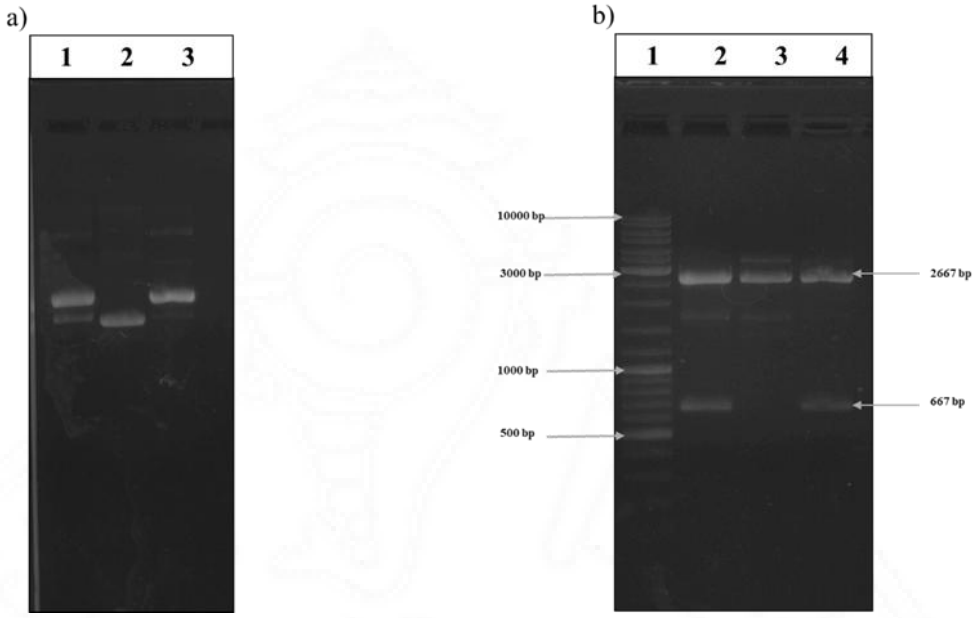


Figure 30: Agarose gel electrophoresis with a) The isolated plasmids from three colonies on the LB culture plates with ampicillin, respectively. b) The Sal1 and EcoR1 restriction-digested plasmids from colonies: 2 to 4 denote colonies 1 to 3 respectively and 1. DNA ladder.

In addition, the results from RT-LAMP and TB qPCR tests with pL-mpt64 plasmid showed the efficiency of the plasmid as a reference to decipher the sensitivity of RT-LAMP assay with SYTO 16 (Figure 31).

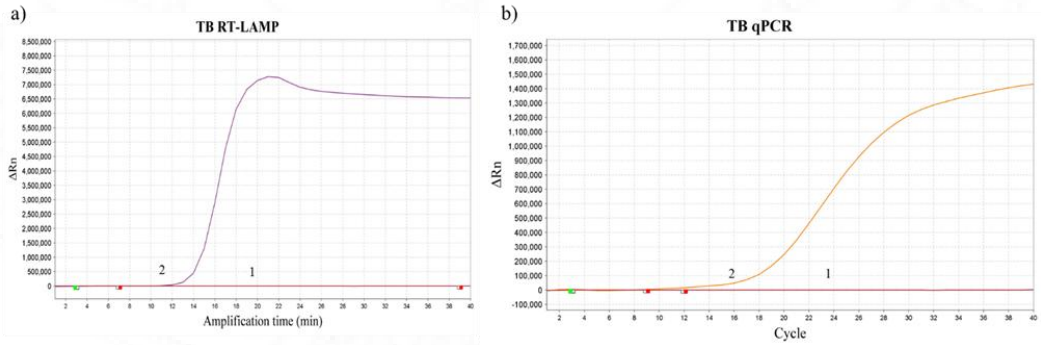


Figure 31: The plasmid cloned with the *mpt64* gene segment (pL-mpt64) with the target region for a) TB RT-LAMP assay and b) TB qPCR assay.

The constructed recombinant plasmid, named as pL-mpt64, therefore, confirmed the presence of target regions for TB RT-LAMP assay and TB qPCR assay for further experiments (Figure 32).

Figure 32: Recombinant plasmid pLmpt64 as a standard reference for the study to detect the sensitivity of TB RT-LAMP assay with SYTO 16.

4.4.2 Limit of Detection of RT-LAMP Assay with SYTO 16

To determine the limit of detection of the TB RT-LAMP assay with SYTO 16, the reference plasmid DNA pL-mpt64 was serially diluted with autoclaved distilled water about ten times. A series of 1.91 x 10^8 to 1.91 copies/µl of plasmids, calculated from the concentration of plasmid in nanogram/µl as stated in the Methods section, was added to the tubes in parallel as the template for the reactions. The experiment was repeated three times to ensure the detection limit. To validate the consistency of TB RT-LAMP assay results, a TB qPCR assay was used as a reference. The results indicated that the TB RT-LAMP assay with SYTO16 could detect DNA with a lower

detection limit, similar to the TB qPCR method (Figure 33). The standard curve obtained depicted a significant linear correlation between TB RT-LAMP amplification time and the concentration of the *mpt64* target with R^2= 0.97 value, which ensured the reliable diagnostic performance of the assay with SYTO 16 (Figure 34).

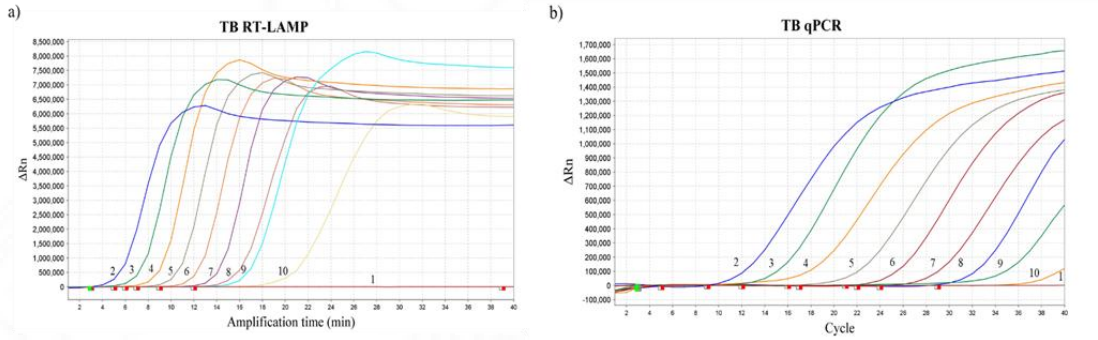


Figure 33: The limit of detection of TB RT-LAMP and TB qPCR assay for the amplification of *mpt64* gene in MTB with serially diluted pLmpt64, **2 to10**: 1.91 x 10^8, 1.91 x 10^7, 1.91 x 10^6, 1.91 x 10^5, 1.91 x 10^4, 1.91 x 10^3, 1.91 x 10^2, 1.91 x 10^1, 1.91 x 10^0 and **1**: NTC.

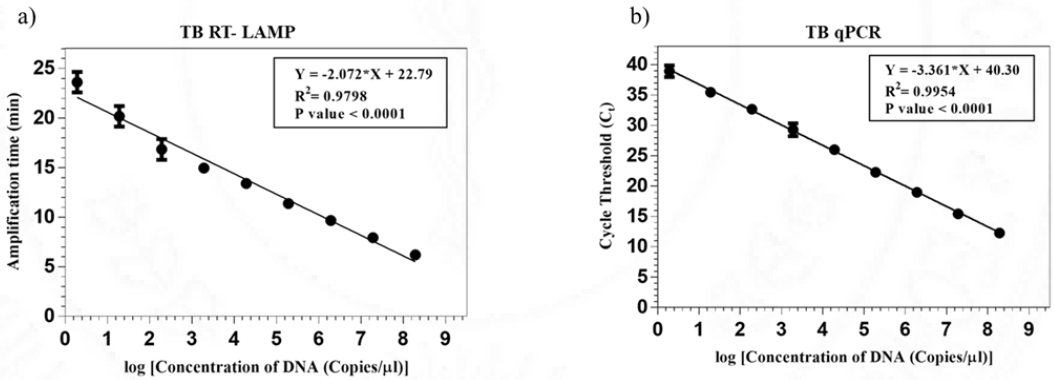


Figure 34: The standard curve analysis between the amplification time and the template concentration: a) TB RT-LAMP assay. b) TB qPCR assay.

4.5 SPECIFICITY OF RT-LAMP ASSAY

To verify the specificity of TB LAMP primers designed, the genomic DNA of other non-mycobacterial strains such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Acinetobacter baumanii*, *Enterococcus facalis* and *Escherichia coli* were tested using the protocol of the TB RT-LAMP assay. The specificity of the TB LAMP primers designed with the *mpt64* gene showed an accurate amplification of the sample-specific with MTB H37Rv genomic DNA, with significant changes in the rate of fluorescence after the reaction (Figure 35). As a result, the TB LAMP assay can identify patients without TB as negative using the ability to specifically detect MTB DNA from commonly present bacteria in clinical samples, thus avoiding false positives that could otherwise lead to unnecessary treatment.

The in silico specificity analysis of the TB-LAMP primers demonstrated 100% specificity in BLAST results and showed amplification exclusively for the Mycobacterium tuberculosis complex (MTBC) in in silico PCR, with no cross-reactivity to non-tuberculous bacteria, as shown in Table 2 (Bikandi J 2004).

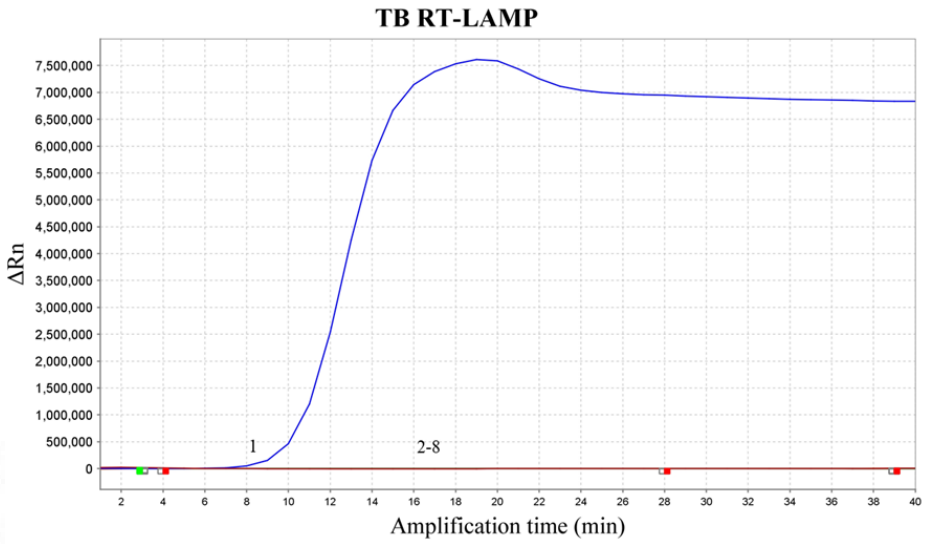


Figure 35: Specificity of the TB RT-LAMP assay. 1-7 *Mycobacterium tuberculosis* H37Rv, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Acinetobacter baumanii*, *Enterococcus faecalis* and *Escherichia coli*. 8: NTC.

Table 2: In-silico Specificity analysis of designed mpt64 TB RT-LAMP primers

		BLASTN Similarity	Insilico PCR
1.	*Mycobacterium tuberculosis complex*	100% Significant	Amplification
Non-Tuberculous Mycobacterium			
1.	*Mycobacterium marinum*	Non- Significant	No Amplification
2.	*Mycobacterium avium complex*	Non-Significant	No Amplification
3.	*Mycobacterium ulcerans*	Non-Significant	No Amplification
4.	*Mycobacterium abscessus*	Non-Significant	No Amplification
5.	*Mycobacterium smegmatis*	Non-Significant	No Amplification
6.	*Mycobacterium intracellulare*	Non-Significant	No Amplification
7.	*Mycobacterium leprae*	Non-Significant	No Amplification
8.	*Mycobacterium kansaii*	Non- Significant	-
9.	*Mycobacterium simiae*	Non- Significant	-
10.	*Mycobacterium scrofulaceum*	Non-Significant	-
11.	*Mycobacterium szulgai*	Non-Significant	-
12.	*Mycobacterium gordonae*	Non-Significant	-
13.	*Mycobacterium simiae*	Non- Significant	-
14.	*Mycobacterium xenopei*	Non-Significant	-
15.	*Mycobacterium malmoense*	Non-Significant	-
16.	*Mycobacterium terrae complex*	Non-Significant	-

17.	*Mycobacterium haemophilum*	Non-Significant	-
18.	*Mycobacterium genavense*	Non-Significant	-
19.	*Mycobacterium xenopei*	Non-Significant	-
20.	*Mycobacterium chelonae*	Non-Significant	-
21.	*Mycobacterium fortuitum*	Non-Significant	-
22.	*Mycobacterium peregrinum*	Non-Significant	-
23.	*Mycobacterium vaccae*	Non-Significant	-
24.	*Mycobacterium chimaera*	Non-Significant	-
25.	*Mycobacterium kansaii*	Non- Significant	-
26.	*Mycobacterium simiae*	Non- Significant	-

4.6 CLINICAL EVALUATION OF TB LAMP ASSAY

4.6.1 Patients Characteristics

The performance of the TB RT-LAMP assay was evaluated using 350 presumptive TB clinical patient samples. Of these patient samples, there were 219 (63%) males and 131 (47%) females, which suggests that the female population represented was slightly less compared to males in the study. The age of the patients collected for the analysis was between 15 to 85 years, with a median of 53 years, comprising the population with an economically active group. Among these, 16 (4.6%) patients had contact with known TB patients. Out of these samples for the study, only 1(0.3%) had shown HIV-positive status. The information collected from patients indicated that about 178 patients had a history of alcohol and tobacco consumption which comes to around 50.8% among the collected patient samples. Moreover, the two (0.6%) migrants and the urban slum of 7 (1.8%) in the study were also noted in the demographical data. In our study group, eight patients (2.3%) were healthcare workers, and their clinical work might have contributed to their health problems.

Among the presumptive TB patient samples the most common clinical signs were cough in 313 (89.42%), asthma in 21 (6%), fever in 14 (4%), weight loss in 11 (3.14%) and loss of appetite in 4 (1.14%). The other symptoms depicted among the individuals were Hemoptysis in 5(1.43%), Chronic Obstructive Pulmonary Disease (COPD) and wheezing in 7 (2%) as shown in Table 3.

Table 3. The demographical, epidemiological and clinical data characteristics of presumptive TB patients.

Demographical and Epidemiological Data	Total Number of Reported Patient samples (N= 350)
Mean of Age (± SD)	53.5 (±15.72)
Male, N (%)	219 (63%)
Female, N (%)	131 (47%)
Contact of known TB patients, N (%)	16 (4.6%)
HIV Positives, N (%)	1 (0.3%)
Alcohol Consumption, N (%)	90 (26%)
Tobacco Consumption, N (%)	88 (25%)
Migrant, N (%)	2 (0.6%)
Urban Slum, N (%)	7 (1.8%)
Health Care Worker, N (%)	8 (2.3%)

Clinical Data	Total Number of Reported Patient samples (N = 350)
Cough, N (%)	313 (89.42%)
Asthma, N (%)	21 (6%)
Fever, N (%)	14 (4%)
Hemoptysis, N (%)	5 (1.43%)
Weight loss, N (%)	11 (3.14%)
COPD*, N (%)	10 (2.8%)
Loss of Appetite, N (%)	4 (1.14%)
Wheezing, N (%)	7 (2%)

*Chronic Obstructive Pulmonary Disease

4.6.2 Comparison of TB RT-LAMP Assay with Xpert MTB/RIF.

After a total of 350 patient sputum samples were processed by following decontamination and the DNA extraction procedure mentioned in the Methods section, the performance of TB RT-LAMP assay results was compared with Xpert MTB/RIF

test. Of these, 60 patient samples showed TB RT-LAMP assay positive and the remaining 290 showed TB RT-LAMP assay results as negative. For the Xpert MTB/RIF also, a total of 60 patient samples were positive whereas the remaining 290 samples were negative. Though numerically the same, only 56 positive samples out of 60 positives from Xpert MTB/RIF showed positive for TB RT-LAMP assay. The remaining four Xpert MTB/RIF positive patient samples were negative for TB RT-LAMP assay. Therefore, the overall sensitivity and specificity comparison of TB RT-LAMP with Xpert MTB/RIF showed 93.33% (95% CI: 83.80% to 98.15%) and 98.62% (95% CI: 96.51% to 99.62%), respectively, equivalent to a PPV of 93.33% (95% CI: 84.07% to 97.38%) and NPV of 98.62% (95% CI: 96.52% to 99.46%). Cohen's kappa value observation showed a 0.92 (95% CI: 0.865 to 0.975) range of almost perfect agreement between the results of the Xpert MTB/RIF and the TB RT-LAMP assay as shown in Table 4.

Table 4. Comparison of TB RT-LAMP assay with Xpert MTB/RIF for diagnosing pulmonary tuberculosis.

	Xpert MTB/RIF Positive	Xpert MTB/RIF Negative	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
TB RT-LAMP											
Positive	56	4	93.33	98.62	67.67	0.07	17.14	93.33	98.62	97.71	0.92
Negative	4	286									
95% CI (N =350)			83.80 to 98.15	96.51 to 99.62	25.51 to 179.50	0.03 to 0.17	13.34 to 21.51	83.80 to 98.15	96.51 to 99.62	95.55 to 99.01	0.865 to 0.975

Based on the detailed analysis of four patient samples which were negative for TB RT-LAMP assay but positive for Xpert MTB/RIF, in which the Ct values for Xpert MTB/RIF were defined as Medium, Low, and Two Very Low results. Among the four patients, one was female, and the other three were male, ranging from 64 to 75 years

of age. All of these patients complained of having coughing conditions, in which one was suffering from a blood pressure (BP) issue and the other had asthma as a symptom.

On the other hand, the results in which Xpert MTB/RIF was negative whereas positive for four samples in the TB RT-LAMP assay revealed that one among them had contact with a patient confirmed with TB disease. Among the four patients, one was female, and the other three were males, ranging from 43 to 64 years of age. Moreover, considering the symptoms, one among them showed hemoptysis while others had a cough.

4.6.3 Comparison of TB RT-LAMP and Xpert MTB/RIF with Smear test.

A primary diagnostic method called a smear test showed positive for 42 (11.7%) and negative for 308 (88.28%) patient samples. The comparative analysis of TB RT-LAMP assay with the smear test showed a sensitivity of 80.95% (95% CI: 65.88% to 91.40%) and a specificity of 91.56% (95% CI: 87.88% to 94.41%), corresponding to the PPV of 56.67% (95% CI: 46.81% to 66.02%) and the NPV of 97.24% (95% CI: 94.97% to 98.50%). The overall sensitivity and specificity of the Xpert MTB/RIF revealed similar values as that of TB RT-LAMP (80.95% sensitivity and 91.56% specificity). The kappa value observation between the TB RT-LAMP assay and smear method showed a 0.61 (95% CI: 0.49 to 0.73) range of substantial agreement in results, similar to Xpert MTB/RIF (Table 5).

Table 5. Comparison of TB RT-LAMP assay and Xpert MTB/RIF with Smear test for the diagnosis of pulmonary tuberculosis.

	Smear Positive	Smear Negative	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
TB RT-LAMP											
Positive	34	26	80.95	91.56	9.59	0.21	12.00	56.67	97.24	90.29	0.61
Negative	8	282									
95% CI (N= 350)			65.88 to 91.40	87.88 to 94.41	6.45 to 14.25	0.11 to 0.39	8.79 to 15.87	43.24 to 69.41	94.64 to 98.80	86.69 to 93.18	0.49 to 0.73
Xpert MTB/RIF											
Positive	34	26	80.95	91.56	9.59	0.21	12.00	56.67	97.24	90.29	0.61
Negative	8	282									
95% CI (N= 350)			65.88 to 91.40	87.88 to 94.41	6.45 to 14.25	0.11 to 0.39	8.79 to 15.87	43.24 to 69.41	94.64 to 98.80	86.69 to 93.18	0.49 to 0.73

The categorisation or grading of the smear test was verified for the detailed analysis of false negatives in TB RT-LAMP assay and Xpert MTB/RIF on patient samples. The grading of sputum smears in tuberculosis diagnosis follows a scale that includes the categories of scanty, 1+, 2+, and 3+. The scanty specifies the presence of 1–9 acid-fast bacilli (AFB) observed in 100 fields of the sputum sample under a microscope. When 10–99 AFB are detected in 100 fields, the grade is assigned as 1+ whereas the 2+ is denoted when there are 1–10 AFB per field, based on the examination of around 50 fields (Kassa et al., 2021). The smear grade 3+ is represented for the observation of more than 10 AFB per field, by examining a minimum of 20 fields. Based on the grading of the smear test, 8 scanty positive samples were shown as negative in TB RT-LAMP assay and Xpert MTB/RIF. The other positive smear test grades, 3+, 2+ and 1+, were positive for both TB RT-LAMP assay and Xpert MTB/RIF (Table 6).

Table 6. Comparison of TB RT-LAMP assay and Xpert MTB/RIF with 3+, 2+, 1+ and Scanty smear positives for the diagnosis of pulmonary tuberculosis.

	Smear Positive				Smear Negative	Total
	3+	2+	1+	Scanty		
TB RT-LAMP						
Positive	5	10	9	10	26	**60 (17.14%)**
Negative	0	0	0	8	282	**290 (82.86%)**
Total	**5 (1.42%)**	**10 (2.8%)**	**9 (2.6%)**	**18 (5.14%)**	**308 (88%)**	**350**
Xpert MTB/RIF						
Positive	5	10	9	10	26	**60 (17.14%)**
Negative	0	0	0	8	282	**290 (82.86%)**
Total	**5 (1.42%)**	**10 (2.8%)**	**9 (2.6%)**	**18 (5.14%)**	**308 (88%)**	**350**

A detailed analysis of these false negative results of TB RT-LAMP and Xpert MTB/RIF showed that 7 out of the 8 negatives were from the same patient samples, and the remaining one was from two other patients. A patient sample that showed negative for the TB RT-LAMP assay was smear scanty and very low Xpert MTB/RIF positive.

The detailed analysis of 26 smear negatives that are positive for TB RT-LAMP and Xpert MTB/RIF assays showed that the 23/26 are positives for both TB RT-LAMP and Xpert MTB/RIF assay along with symptoms associated with TB infections such as cough, asthma, COPD and wheezing. The remaining three samples of TB RT-LAMP assay positives are negative for Xpert MTB/RIF which showed the symptoms related to TB such as cough and hempysis. Whereas, the three patient samples reported in which TB RT-LAMP assay was negative and Xpert MTB/RIF test was positive with the cough and asthma as the symptoms reported during the diagnosis.

4.6.4 Comparison of TB RT-LAMP, Xpert MTB/RIF and smear test with Culture as the reference test.

Among the 350 patient samples, 47 (13.42%) samples were positive for the standard reference Culture test. In that, 42 samples were positive for TB RT-LAMP

assay and, for Xpert MTB/RIF, 41 patient samples showed positive. The overall sensitivity and specificity of the TB RT-LAMP assay, when compared to the Culture test, were 89.36% (95% CI: 76.90% to 96.45%) and 94.06% (95% CI: 90.77% to 96.44%), respectively corresponding to a PPV of 70.00% (95% CI: 59.59% to 78.69%) and NPV of 98.28% (95% CI: 96.14% to 99.24%). The comparison of the Xpert MTB/RIF assay with the Culture test denoted a sensitivity and specificity of 87.23% (95% CI: 74.26% to 95.17%) and 93.73% (95% CI: 90.38% to 96.18%), respectively equivalent to a PPV of 68.33% (95% CI: 57.94% to 77.17%) and NPV of 97.93% (95% CI: 95.73% to 99.01%). The kappa value between the TB RT-LAMP assay and culture test was 0.75 (95% CI: 0.649 to 0.845) which showed a substantial agreement between the tests. Substantial agreement between Xpert MTB/RIF and culture result was also observed as 0.725 (95% CI: 0.624 to 0.826). Therefore, compared to the culture reference test, the TB RT-LAMP assay showed a slightly higher rate of positive results confirmation than the Xpert MTB/RIF test. Moreover, the comparison of smear test specificity, PPV and NPV with reference test denoted 95.38% (95% CI: 92.37% to 97.45%), 66.67% (95% CI: 50.45% to 80.43%) and 93.83% (95% CI: 90.53% to 96.25%), respectively. Though the smear test showed higher specificity on the TB patient analysis, the sensitivity was lower compared to the Xpert MTB/RIF and TB RT-LAMP assay. The kappa value between the smear test and culture was 0.575 (95% CI: 0.445 to 0.705) denoted only a moderate agreement between tests (Table 7).

Table 7. Comparison of TB RT-LAMP assay, Xpert MTB/RIF and Smear test with Culture as a standard reference for the diagnosis of pulmonary tuberculosis.

	Culture Positive	Culture Negative	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
TB RT-LAMP											
Positive	42	18	89.36	94.06	15.04	0.11	13.43	70	98.28	93.43	0.75
Negative	5	285									
95% CI (N= 350)			76.90 to 96.45	90.77 to 96.44	9.51 to 23.80	0.05 to 0.26	10.04 to 17.45	59.59 to 78.69	96.14 to 99.24	90.30 to 95.79	0.649 to 0.845
Xpert MTB/RIF											
Positive	41	19	87.23	93.73	13.91	0.14	13.43	68.33	97.93	92.86	0.725
Negative	6	284									
95% CI (N= 350)			74.26 to 95.17	90.38 to 96.18	8.88 to 21.79	0.06 to 0.29	10.04 to 17.45	55.04 to 79.74	95.55 to 99.24	89.64 to 95.32	0.624 to 0.826
Smear											
Positive	28	14	59.57	95.38	12.89	0.42	13.43	66.67	93.83	90.57	0.575
Negative	19	289									
95% CI (N= 350)			44.27 to 73.63	92.37 to 97.45	7.34 to 22.64	0.30 to 0.60	10.04 to 17.45	50.45 to 80.43	90.53 to 96.25	87.01 to 93.42	0.445 to 0.705

Further analysis of the resulting data revealed that 18 samples were positive for the TB RT-LAMP assay but negative for the culture test. Likewise, the Xpert MTB/RIF assay also showed 19 positives for the negative culture results. Among 18 TB RT LAMP positives, the Xpert MTB/RIF showed similar results for 15 patient samples. The remaining three positive patient samples of TB RT-LAMP assay were Xpert MTB/RIF negative, where two patients depicted cough and one with hemoptysis as symptoms in the clinical analysis. The additional three patient samples showed Xpert MTB/RIF positives denoted the results as 2 very low positives and the other as medium positive. The predominant symptoms of these patient samples were cough, fluctuation in BP and asthma.

4.6.5 Comparison of TB RT-LAMP and Xpert MTB/RIF with Culture Positive Smear Negative results.

To elucidate the role of TB RT-LAMP assay as a primary test in detecting TB with efficient accuracy, a comparison of TB RT-LAMP assay and Xpert MTB/RIF assay with culture-positive and smear-negative samples was performed. The

sensitivity of TB RT-LAMP and Xpert MTB/RIF stated as 73.68% (95% CI: 48.80% to 90.85%) and 68.42% (95% CI: 43.45% to 87.42%), respectively. The specificity, PPV and NPV of the TB RT-LAMP assay was stated as 95.80% (95% CI: 92.79% to 97.81%), 53.85% (95% CI: 33.37% to 73.41%) and 98.21% (95% CI: 95.87% to 99.42%), respectively. Whereas, the Xpert MTB/RIF stated the specificity, PPV and NPV as 95.47% (95% CI: 92.38% to 97.57%), 50% (95% CI: 29.93% to 70.07%) and 97.86% (95% CI: 95.39% to 99.21%), respectively. The kappa value between the TB RT-LAMP assay and Culture positive and Smear negative results showed 0.803 (95% CI: 0.696 to 0.910) which denoted almost perfect agreement between the tests. Whereas, Xpert MTB/RIF showed a kappa value of 0.789 (95% CI: 0.680 to 0.899) showing substantial agreement with culture-positive and smear-negative analysis. These results indicated that the TB RT-LAMP assay had shown better efficiency than the smear test for the primary TB diagnostic test with similar accuracy level as that of Xpert MTB/RIF (Table 8).

Table 8. Comparison of TB RT-LAMP assay and Xpert MTB/RIF with Culture Positive and Smear Negative patient samples.

	Culture +ve Smear -ve	Culture -ve Smear -ve	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
TB RT-LAMP											
Positive	14	12	73.68	95.80	17.56	0.27	6.23	53.85	98.21	94.43	0.593
Negative	5	274									
95% CI (N= 305)			48.80 to 90.85	92.79 to 97.81	9.49 to 32.50	0.13 to 0.58	3.79 to 9.56	33.37 to 73.41	95.87 to 99.42	91.23 to 96.72	0.418 to 0.768
Xpert MTB/RIF											
Positive	13	13	68.42	95.47	15.11	0.33	6.21	50.00	97.86	93.79	0.545
Negative	6	274									
95% CI (N= 306)			43.45 to 87.42	92.38 to 97.57	8.19 to 27.88	0.17 to 0.64	3.78 to 9.53	29.93 to 70.07	95.39 to 99.21	90.47 to 96.22	0.364 to 0.726

4.6.6 Comparison of TB RT-LAMP assay with Positives in Xpert MTB/RIF, Culture and Smear test.

To further determine the efficiency of the TB RT-LAMP assay, the results were compared with the total positives and negatives of the Xpert MTB/RIF, Culture and smear test which comprised 304/350 patient samples. The total Xpert MTB/RIF, Culture and smear test positives and negatives noted were 28 and 276 samples, respectively. Within that, the TB RT-LAMP assay showed a sensitivity of 100% (95% CI: 87.66% to 100%) and a specificity of 99.28% (95% CI: 97.41 to 99.91%), corresponding with the PPV of 93.33% (95% CI: 77.93% to 99.18%) and NPV of 100% (95% CI: 98.66% to 100%). The kappa value analysis of TB RT-LAMP between these positives in all three tests denoted a 0.96 (95% CI: 0.909 to 100) perfect agreement with the study. According to the data mentioned above, the TB RT-LAMP assay could be used to validate first-line tests for TB disease diagnosis mainly in resource-limited regions (Table 9).

Table 9. Comparison of TB RT-LAMP assay with Positives in Xpert MTB/RIF, Culture and Smear test.

	Culture +ve Smear +ve Xpert +ve	Culture - Smear - Xpert -	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
TB RT-LAMP											
Positive	28	2	100	99.28	138.00	0.00	9.21	93.33	100	99.34	0.96
Negative	0	274									
95% CI (N= 304)			87.66 to 100	97.41 to 99.91	34.69 to 549.03		6.21 to 13.04	77.93 to 99.18	98.66 to 100.00	97.64 to 99.92	0.909 to 1.000

4.6.7 Comparison of TB RT-LAMP assay and Xpert MTB/RIF with TB qPCR test.

The protocol followed for the extraction of DNA from sputum samples and the target gene selected for the TB RT-LAMP assay were different compared to the Xpert MTB/RIF. Therefore, the analysis of TB qPCR results on the 350 patient samples was

compared with the obtained results from the TB RT-LAMP assay to evaluate the efficiency of the DNA extraction method and the specificity of the *mpt64* target gene. Out of 60 positive TB RT-LAMP assay results, TB qPCR showed positive results for 58 patient samples. Therefore, the sensitivity and specificity of the TB qPCR assay were 96.67% (95% CI: 88.47% to 99.59%) and 100% (95% CI: 98.74% to 100%), respectively, equivalent to the PPV of 100% (95% CI: 93.84% to 100%) and NPV of 99.32% (95% CI: 97.55% to 99.62%). The findings of this evaluation were similar compared to the sensitivity obtained from the Xpert MTB/RIF assay. The kappa value between TB qPCR and TB RT-LAMP assay showed was 0.980 (95% CI: 0.951 to 1.00) as a perfect agreement between studies. The specificity of TB qPCR compared to Xpert MTB/RIF was 99.31% (95% CI: 97.53% to 99.92%) corresponding to the PPV of 96.55% (95% CI: 87.54% to 99.11%) and NPV of 98.63% (95% CI: 96.54% to 99.46%). A perfect agreement between TB qPCR and Xpert MTB/RIF was observed as 0.939 (95% CI: 0.890 to 0.987) (Table 10).

Table 10. Comparison of TB RT-LAMP assay and Xpert MTB/RIF with TB qPCR test.

	TB RT-LAMP Positive	TB RT-LAMP Negative	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
TB qPCR											
Positive	58	0	96.67%	100.00%	-	0.03	17.14	100.00	99.32	99.43	0.980
Negative	2	290									
95% CI (N =350)			88.47% to 99.59%	98.74% to 100.00%	-	0.01 to 0.13	13.34 to 21.51	93.84 to 100.00	97.55 to 99.92	97.95 to 99.93	0.951 to 1.000
	Xpert MTB/RIF Positive	**Xpert MTB/RIF Negative**	**Sensitivity (%)**	**Specificity (%)**	**Positive Likelihood Ratio**	**Negative Likelihood Ratio**	**Disease Prevalence (%)**	**Positive Predictive Value (%)**	**Negative Predictive Value (%)**	**Accuracy (%)**	**Kappa Value (κ)**
TB qPCR											
Positive	56	0	93.33	100.00	-	0.07	17.14	100.00	98.64	98.86	0.959
Negative	4	290			-						
95% CI (N =350)			83.80 to 98.15	98.74 to 100.00		0.03 to 0.17	13.34 to 21.51	93.62 to 100.00	96.55 to 99.63	97.10 to 99.69	0.918 to 0.999

The comparison of TB qPCR Ct (Cycles of threshold) values with Xpert MTB/RIF semi quantitative classification of Ct values (High for Ct value below 14,

Medium for Ct between 14 and 19, Low for Ct value between 19 and 27 and very low for Ct value above 27) revealed that the process of DNA extraction and the target gene specified for amplification could be the reason for the variation in a grouping of results (Geleta et al., 2015) (Figure 36).

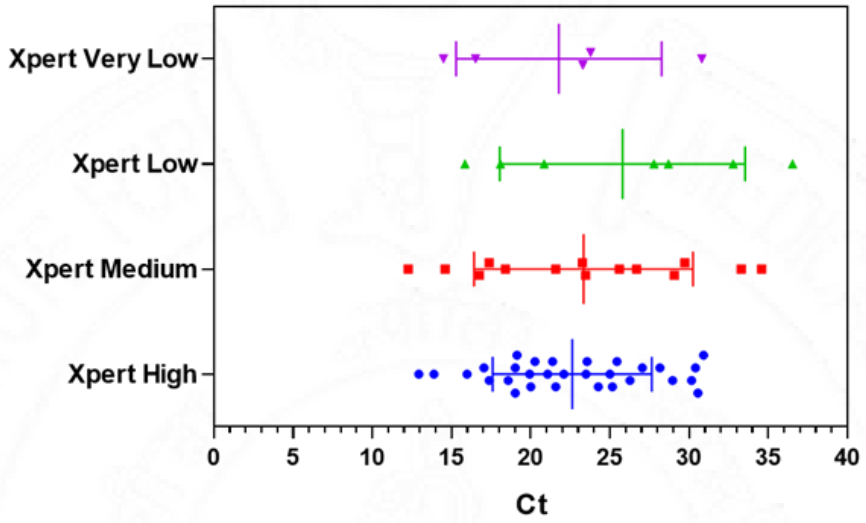


Figure 36. Comparison of Ct values between Xpert MTB/RIF and TB qPCR test.

Further analysis in which two patient samples were exclusively positive for TB RT-LAMP assay was denoted as false positive since these samples were negative for TB qPCR, Xpert MTB/RIF, and Culture and Smear tests. A detailed analysis of these patients revealed TB symptoms, including coughing in one patient and hemoptysis in the other that are compatible with the suspicion in TB patients.

4.6.8 Evaluation of GeneDot TB LAMP assay with TB RT-LAMP assay.

A prototype device designed called GeneDot TB LAMP assay was tested for the amplification of the *mpt64* gene in patient samples. The prototype designed with a

heating block to amplify DNA under isothermal (65ºC) conditions with 20 samples loading block and a specific wavelength filter (˜480nm excitation and 520nm emission) which moves in a linear stage from one tube position to another for the measurement of the fluorescent intensity after the LAMP assay. A touch screen and a serial port to connect to the desktop were made available to display the wavelength intensity parameters and data analysis after the TB LAMP assay. The measurement of the GeneDot scanning fluorescent intensity was designed as the values above 25000 were confirmed as positive and below 25000 as negative (Figure 37).

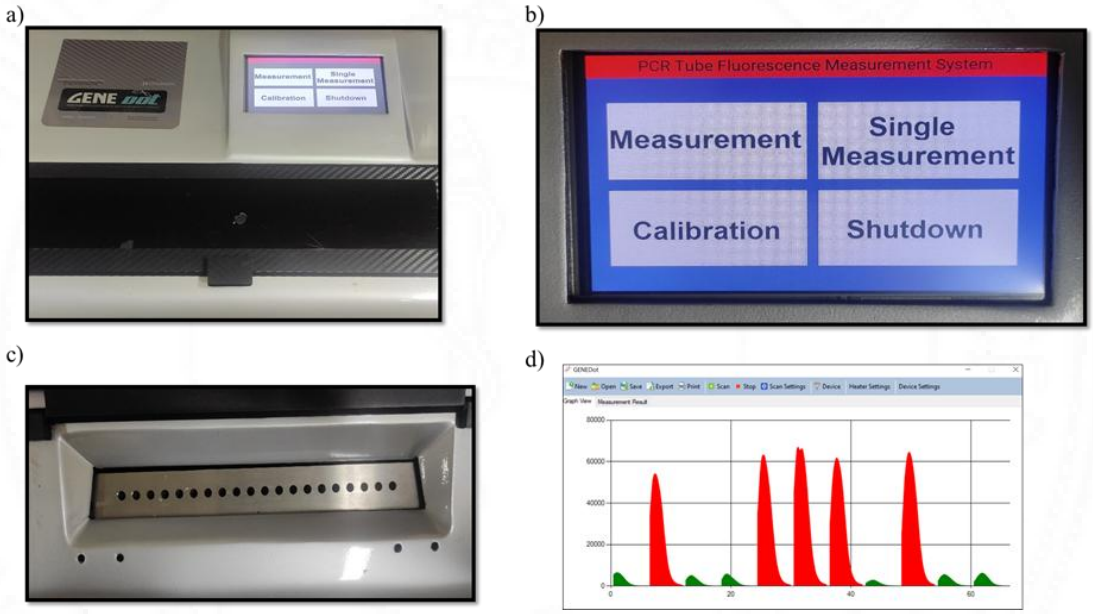


Figure 37: GeneDot TB LAMP assay. a) The prototype device for TB detection is named as GeneDot. b) A touch screen for the display of the measurement fluorescent intensity for the analysis of results data. c) The heating block at 65ºC with the capacity to hold 20 samples underneath with a specific wavelength filter having ~ 480nm excitation and ~520nm emission. d) The illustration of scanning intensity in GeneDot software, in which above 25000 is denoted as TB Positive (red) and below 25000 as TB Negative (green).

Based on the designed scanning fluorescent intensity measurement parameters, the results depicted 60 positive samples and 290 negative samples as obtained from

TB RT-LAMP assay without any false positives or false negatives in the data (Figure 38).

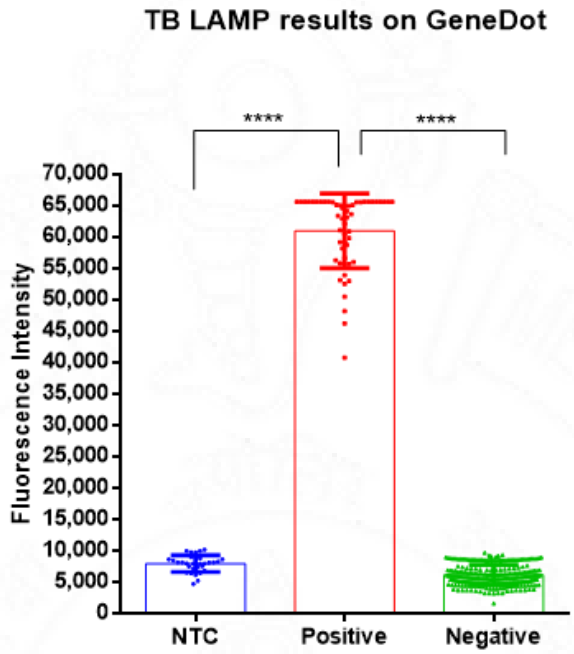


Figure 38: The fluorescent intensity plot on the Genedot readings of TB LAMP assay on 350 MTB patient samples. ($p < 0.0001$)

The overall sensitivity and specificity of GeneDot TB LAMP with TB RT-LAMP showed 100% (95% CI: 94.04% to 100%) and 100% (95% CI: 98.74% to 100%), respectively, corresponding to the PPV of 100% (95% CI: 94.04% to 100%) and NPV of 100% (95% CI: 98.74% to 100%) with a kappa value of 1depicting completely perfect agreement with the TB RT-LAMP assay results. The evaluation of the results from GeneDot TB LAMP showed that the prototype used could confirm positive within 30 minutes with an easy-to-handle procedure (Table 11).

Table 11. Comparison of GeneDot TB LAMP with TB RT-LAMP assay.

	TB RT-LAMP Positive	TB RT-LAMP Negative	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	Disease Prevalence	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)	Kappa Value (κ)
GeneDot TB LAMP											
Positive	60	0	100	100		0.00	17.14%	100	100.00%	100	1
Negative	0	290									
95% CI (N =350)			94.04 to 100	98.74 to 100			13.34% to 21.51%	94.04% to 100.00%	98.74% to 100.00%	98.95 to 100	1.0

4.6.9 TB LAMP endpoint reading using UV light chamber.

Using the UV light chambers with 520nm wavelength emission, the endpoint results of TB LAMP assay with MTB patient samples could be predicted based on the difference in intensity of fluorescence observed on tubes. The bright white colour in tubes under dark background predicted as positive and colourless as negative (Figure 39).

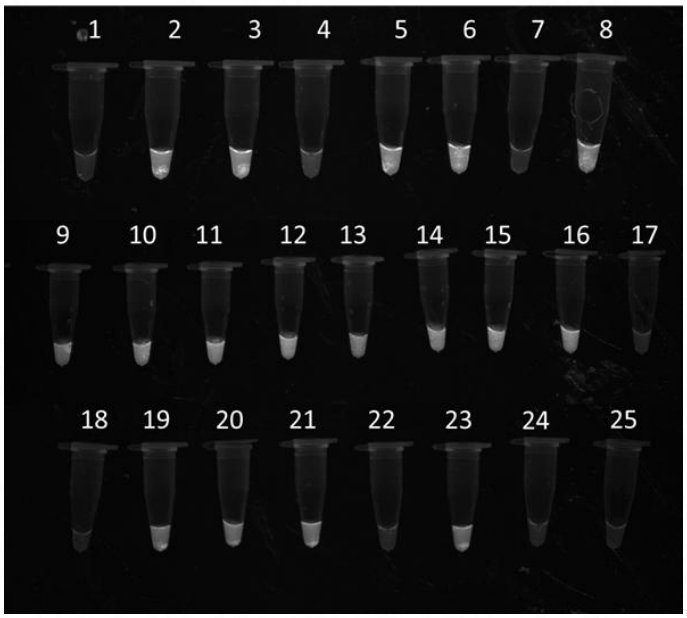


Figure 39. The endpoint analysis of TB LAMP assay under UV light illumination. The tubes showing bright white colour under dark background are depicted as Positive and colourless as Negative for TB LAMP assay.

The evaluation and comparison of the results from the TB LAMP assay endpoint reading under UV light with TB RT-LAMP assay showed that 33 positives could be predicted as positive (33/60) and the remaining 27 positives as unpredictable. Though the method was affordable for TB primary diagnosis, there was a possibility of obtaining false negative results from the blind test evaluation (Table 11).

Table 12. Comparison of TB LAMP endpoints with TB RT-LAMP assay

	TB RT-LAMP Positive	TB RT-LAMP Negative	Total Number of samples(%)
UV End Point Positive	33	0	**33 (9.42%)**
UV End Point Negative	27	290	**317 (90.57%)**
Total Number samples (%)	**60 (17.14%)**	**290 (82.857%)**	**350**

DISCUSSION

5. DISCUSSION

The detection of TB infection at an early stage is one of the crucial factors necessary for proper disease management, mainly among the highly burdened resource-limited areas. The lack of rapid, affordable, and accurate diagnostic methods in such settings has detained the control of TB disease among the population. Despite its lower sensitivity, the smear microscopy test has remained one of the primary diagnostic tools for evaluating TB disease conditions among patients. To achieve the "End TB" strategy of the WHO, a rapid method of TB detection with higher sensitivity has been demanded in the peripheral health regions as a primary test. The development in the field of the nucleic acid amplification test (NAAT) has promoted a way to generate a highly sensitive rapid detection method for diagnosing TB. However, the currently operating NAAT is marked as a limited method due to its demand for complex and sophisticated instrumentation, mainly in low and middle-income countries. A NAAT method called LAMP assay, the amplification of DNA under isothermal conditions, is an alternative to the current NAAT based diagnostic tool (Notomi et al., 2000). Compared to the existing test, the main factors reflected as advantages of LAMP assay are the rapid reaction and detection, a simple and reliable equipment requirement for the diagnostic purpose with higher sensitivity.

Therefore, the major features that need to consider for an adequate LAMP assay for diagnostic purposes are the designing of the LAMP primers specific for a target region of interest, suitable conditions that would be manageable for the operator to predict the results precisely, and the outcome with higher sensitivity level compared to the existing primary TB diagnostic test mainly used in peripheral laboratories.

In this study, the *mpt64* gene region was selected as a target sequence because of its prevalence in Indian MTB isolates than the IS6110 gene, and thus it was assumed that the LAMP assay targeting this gene would be more effective for the detection of TB in such high TB burden countries (Balne et al., 2013). The LAMP primers designed for the *mpt64* gene were specific and optimum for the TB LAMP reaction after considering the factors necessary to amplify the target template under an isothermal condition.

The study focused on developing the TB LAMP assay with an apt method to detect the amplification with maximum sensitivity. The fluorescent dyes SYTO 16 and SYBR Green 1 were mainly considered for the study by focusing on the factors suitable for a cost-effective diagnostic test (Abbasi et al., 2016; Gudnason et al., 2007).

According to the results from the study, SYTO 16 was depicted as a suitable fluorescent dye for detecting amplified products by showing higher fluorescent intensity with lower inhibition without delaying the reaction compared to SYBR Green1 fluorescent dye. Therefore, these performance characteristics of SYTO 16 made this dye more convenient in TB LAMP assay than SYBR Green 1 for TB diagnostic purposes.

In the study, the evaluation of the sensitivity limit of the TB LAMP assay by real time monitoring (TB RT-LAMP) with *mpt64*-specific primers and SYTO 16 dye explained the ability of the method to detect below 10 copies of DNA, as that of the TB qPCR assay. Moreover, the *mpt64* primer set has shown specificity in detecting *Mycobacterium tuberculosis* strain compared to the TB RT-LAMP assay on the non-tuberculosis strains used for the study as a reference.

Based on these detailed observations of TB RT-LAMP assay, a study on the patient samples was conducted concurrently, along with Xpert MTB/RIF, smear test, and culture obtained from a clinical laboratory. In the cross-sectional study to evaluate the performance of the TB RT-LAMP assay, the results of the assay were compared with Xpert MTB/RIF and smear test in the diagnosis of TB using culture as a reference test.

From the analysis, the overall sensitivity of the TB RT-LAMP assay was 89.36% (95% CI: 76.90% to 96.45%), followed by 87.23% (95% CI: 74.26% to 95.17%) of Xpert MTB/RIF and 59.57% (95% CI: 44.27% to 73.63%) of the smear test compared to the culture test. The consideration of specificity revealed a higher level for the smear test with 95.38% (95% CI: 92.37% to 97.45%) than TB RT-LAMP with 94.06% (95% CI: 90.77% to 96.44%) and Xpert MTB/RIF with 93.73% (95% CI: 90.38 to 96.18) from the analysis. The eighteen patient samples were positive for TB RT-LAMP assay but were negative for the culture test; similarly, Xpert MTB/RIF showed 19 samples as positive, for which the culture test were negative as observed in the study. A possible explanation for those results is that the culture pre-test sample was treated with NaOH before the test to decontaminate by eliminating other microorganisms, which might have delayed MTB growth or caused bacterial death, which could lead to detecting false negative results despite the sample being TB positive (Pang et al., 2016). Conversely, NAAT tests could detect dead bacteria, while culture tests specifically detect live bacteria (Agrawal et al., 2016).

Comparing the molecular-based detection methods, the sensitivity and specificity of TB RT-LAMP with Xpert MTB/RIF were 93.33% (95% CI: 83.80% to 98.15%) and 98.62% (95% CI: 96.51% to 99.62%), respectively. Likewise, several

studies have shown that LAMP and Xpert MTB/RIF provide a comparatively similar level of sensitivity and specificity for diagnosing tuberculosis (Nliwasa et al., 2016; Shete et al., 2019).

Both the TB RT-LAMP and Xpert MTB/RIF assays showed 80.95% (95% CI: 65.88% to 91.40%) sensitivity and 91.56% (95% CI: 87.88% to 94.41%) specificity compared to the smear microscopy. Therefore, the performance of the TB RT-LAMP assay was denoted similarly to the level of the Xpert MTB/RIF test, indicating the assay has an efficiency of Xpert MTB/RIF for the rapid detection of TB in resource-poor settings.

Moreover, the comparison of results from the TB RT-LAMP assay and Xpert MTB/RIF test with culture-positive and smear-negative samples revealed a sensitivity of 73.68% (95% CI: 48.80% to 90.85%) and 68.42% (95% CI: 43.45% to 87.42%), respectively. The results indicated that the TB RT-LAMP assay with similar detection accuracy as that of Xpert MTB/RIF could be a better option for a primary test or an add-on confirmation method for smear-negative samples with TB symptoms.

The difference in the procedure of DNA extraction used for the standardization of TB LAMP assay compared to Xpert MTB/RIF was confirmed by evaluating the Ct values observed from the TB qPCR method. The semi-quantitative results of Xpert MTB/RIF defined by the manufacturer are denoted as high for Ct values below 14, medium for Ct values from 14 to 19, low for Ct values from 19 to 27, and very low for Ct values above 27. The comparison of Ct values from TB qPCR with Xpert MTB/RIF revealed the differences in the DNA extraction process and the target gene specified for the LAMP amplification method.

The comparison of sensitivity and specificity of TB qPCR with TB RT LAMP showed 96.67% (95% CI: 88.47% to 99.59%) and 100% (95% CI: 98.74% to 100.00%), respectively. The two patient samples were positive for TB RT-LAMP assay and were negative for all other tests, including TB qPCR. Therefore, the proportional rate of indeterminate or false positive results found in TB RT-LAMP assay was 0.57% compared to the results data on 350 presumptive TB samples.

The evaluation of the results from the TB RT-LAMP assay of this study revealed that the objectives of the WHO's End TB Strategy for developing rapid diagnostic methods for TB detection were almost clearly could defined. The comparison of the diagnostic accuracy of TB RT-LAMP assay with culture test in detecting TB disease indicated that it could be used as a replacement or as an add-on test for smear microscopy. Moreover, the difference in the accuracy of detection between TB RT-LAMP assay with SYTO 16 and Xpert MTB/RIF was noted as 2.13 %, which indicated the eligibility of TB RT-LAMP assay for testing patient sputum samples with signs and symptoms are consistent for TB testing (WHO, 2016).

The TB RT-LAMP assay has been developed as an open platform system that can run in an isothermal fluorescent reader or any qPCR machine programmed to run under isothermal cycles. During the COVID-19 pandemic, qPCR equipment has become an integral component of most healthcare and diagnostic testing centres which can be used to run the TB RT-LAMP assay. We compared the TB RT-LAMP assay with the recommendations by the Treatment Action Group for TB point of care test (TB_event_POC_fullsurveyreport_ENG_2008.pdf, 2009) and found it to meet the requirements for all major points, including high throughput screening capabilities (Table 13).

Table 13. Comparison of TB RT-LAMP with recommendations of TAG group 2009

Test Specification	Recommendations of Treatment action group 2009 from TAG report	TB RT-LAMP
Medical decision	Treatment initiation	Treatment initiation
Sensitivity- Adults (for pulmonary TB)	95% smear postive, MRS positive 60-80% smear negative, MRS positive	100% 73.66%
Specificity- Adults	95% compared to MRS	94.06%
Time to results	3 hours maximimum	1.5 hours
Throughput	20 tests/day	48 or 96 or 384 tests/run - possible to conduct more than 4 runs per day. LAMP-PCR machines can run 16 tests/run - possible to conduct more than 4 runs per day.
Specimen type	Urine, oral, breath, venous blood, Sputum	Sputum
Sample preperation	-3 steps max -Safe- biosafety 1 -Ability to use -approximate volume -Preparation that is not highly time sensitive	- 2 steps - Biosafety 1 - No need of accurate pipetting - Not highly time sensitive
Number of samples	One sample per test	One sample for test
Readout	Easy to read, unambigous	Exponential amplification of positive samples, easy to read
Waste disposal	-Simple burning -Environmentallly acceptable disposal	- Environmentally acceptable disposal
Controls	-Positive control included in the test kit -Quality control simpler and easier	- Positive control included in the kit - Quality control is with positive and negative controls - easy to perform
Reagents	-All reagents in self-contained kit -Kit contains sample collection device	- All reagents in self-contained kit - Sample collection tube included in the kit
Storage/stability	-Shelf life of 24 months, including reagents -Stable at 30^0 C and at higher temperature for shorter period of time -Stable in high humidity environment	- Accelerated shelf like shows >24 months - Stable at room temperature for 1-2 hours - Kit worked at 80-90% humidity
Instrumentation	-Instrument works in tropical conditions	- Utlizes open qPCR machines. These machines have been used in tropical

		conditions
Power requirement	-Can work on battery	Depends on qPCR machine models - battery operated qPCR machines, if available, can be used
Training	1 day max. Training time Can be performed by any health worker	Half a day training
Cost	<US$10 per test after scale-up	< US$5 per test after scale-up

When utilizing a quantitative polymerase chain reaction (qPCR) machine for the TB RT-LAMP assay, it is possible to process either 48, 96, or 384 tests in a single run. This throughput allows for the completion of more than four runs per day, depending on the specific machine configuration and operational efficiency. This capacity significantly increases testing efficiency and facilitates timely diagnosis and treatment for patients. The flexibility and efficiency of the open TB RT-LAMP assay present a notable advancement over traditional pooled testing methods, such as those using the Xpert MTB/RIF or Xpert Ultra systems. This improvement is crucial for enhancing the speed and reliability of TB diagnostics, ultimately contributing to better patient outcomes.

The study demonstrated the TB LAMP assay has great potential for clinical diagnosis of TB peripheral laboratories with limited equipment, such as those in developing countries; the test can be considered a promising diagnostic tool. Thus, the study could be concluded that the development of new and improved technologies in the detection of TB has been an important step toward improving TB diagnosis and treatment. After the standardization and analysis of TB RT-LAMP assay with the currently ongoing TB diagnostic methods, a prototype device, the GeneDot TB LAMP assay, was found to be an excellent tool for diagnosing tuberculosis with a maximum

turnaround time of 40 minutes which the device could handle up to 20 samples at a time. These prototype advances have enabled the detection and diagnosis of tuberculosis (TB) with accuracy and rapidity, resulting in a more favourable outcome.

Another simplest configuration made for the TB LAMP assay was the endpoint analysis of results under UV illumination, on which the study showed a bias between the prediction of positive and negative results. As a result, the method predicted false negatives from the blind test. Even though the method is easily deployable and affordable for field testing, the ability to distinguish between the results has been observed as a drawback for rapid TB detection.

From this feedback, the study on patient samples considered TB LAMP assay as a useful tool for the rapid diagnosis of TB. Therefore, the portable constant temperature fluorescence detecting instrument made during the study would be helpful for the in-field diagnosis of the rapidly spreading TB disease. Thus, it can be concluded that the development of new and improved technologies in the detection of TB has been an important step toward improving TB diagnosis and treatment.

SUMMARY

AND

CONCLUSION

6. SUMMARY AND CONCLUSIONS

The study revealed the LAMP assay with SYTO 16 fluorescent dye for the specific detection of the *mpt64* gene in *Mycobacterium tuberculosis* under optimized conditions at 65ºC within 40 minutes as a simple and effective method for rapidly diagnosing TB infection. The optimization of the LAMP primers with suitable melting temperatures and favourable conditions enhanced the efficiency of the *mpt64* gene amplification under isothermal conditions. Moreover, the addition of loop primers resulted in rapid amplification with increased efficiency, which enhanced the detection of amplified products within a short time. The SYTO 16 fluorescent dye at an optimized concentration showed a better combination of signal-to-noise ratio and the time-to-threshold for DNA amplification during the LAMP assay.

The standardized TB RT-LAMP assay with SYTO 16 showed the ability to detect DNA templates within a lower detection limit. The sensitivity test with the *mpt64* gene sequence cloned plasmid showed a detection limit lower than 10 copies with a significant correlation between TB RT-LAMP amplification time and the concentration of the *mpt64* target with R^2= 0.97 value, similar to the TB qPCR method, which is an important characteristic required for TB diagnosis. Moreover, the specificity of TB RT-LAMP was confirmed with the observation of no cross-amplification from the genomic DNA of other non-mycobacterial strains.

The applicability of TB RT-LAMP assay was evaluated with patient sputum samples, and the results were compared with those obtained from smear, culture, and Xpert MTB/RIF tests. Following the decontamination and a rapid DNA extraction method from 350 blind patient sputum samples, the TB RT-LAMP assay comparison

with Xpert MTB/RIF test showed sensitivity and specificity of 93% and 98.62%, respectively, and with culture test showed 89.36% and 94.06%, respectively, which were in agreement with the aim of WHO TB END strategy (Al Abri et al., 2020).

Moreover, the 73.68% sensitivity of TB RT-LAMP assay with culture-positive and smear-negative patient samples and 100 % sensitivity and 99.28% specificity with total Xpert MTB/RIF, smear, and culture test results denoted the efficiency of the LAMP assay as a primary diagnostic tool for TB or as an add-on confirmation method after a smear test, particularly in resource-limited areas where the rapid and accurate detection of TB is crucial for the effective management and control of the disease.

As the TB RT-LAMP assay showed high sensitivity, specificity, and accuracy without sophisticated laboratory settings, the TB LAMP assay with a device named GeneDot is a portable constant temperature along with fluorescence intensity reading capacity and was found as an eligible prototype for in-field TB diagnosis.

The TB LAMP assay could be useful for on-site TB detection within favourable laboratory settings in conjunction with a simple and rapid DNA extraction method such as chelex-based or magnetic nanoparticle-based DNA extraction. Thus, it can be concluded that this study proves that LAMP assay is a promising technology for the detection of TB that would be an important stepping stone toward improving TB diagnosis and treatment in high-burden countries.

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ANNEXURES

APPENDIX A- COPY RIGHT PERMISSION

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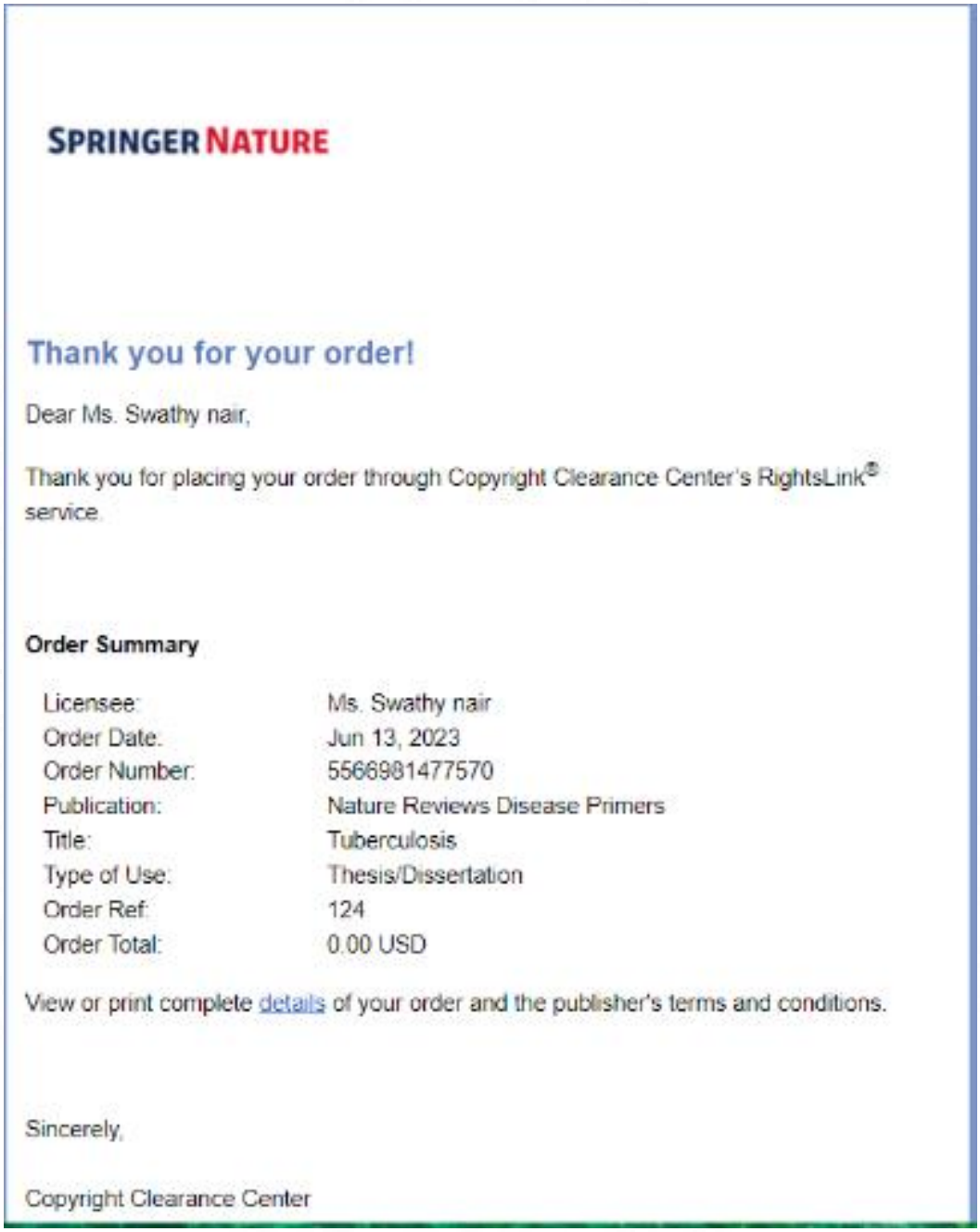

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APPENDIX C – Ethics committee approval

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तिरुवनन्तपुरम - ६९५०११, केरल, इंडिया
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Institutional Ethics Committee

(IEC Regn No. ECR/189/Inst/KL/2013/RR-16)

11.07.2018

SCT/IEC/ 1230/JUNE-2018

Dr. Anoopkumar Thekkuveettil
Scientist G
Division of Molecular Medicine
SCTIMST, Thiruvananthapuram

Dear Dr. Anoopkumar,

The Institutional Ethics Committee reviewed your application to conduct the study entitled "NUCLEIC ACID BASED DIAGNOSTIC KIT FOR DETECTION OF TUBERCULOSIS (IEC/ 1230)" on 10th July, 2018.

The following documents were reviewed:

1. *Covering letter addressed to the Chairperson, IEC, SCTIMST dated 28.06.2018*
2. *IEC Application Form*
3. *Copy of an email reply received from Dr Sanjeev Nair on 25.06.2018*
4. *Copy of an email reply received from Dr Anitha Kumari on 25.06.2018*
5. *Copy of an email reply received from Dr Neena PS on 26.06.2018*
6. *TAC Approval Letter*
7. *Project Proposal*
8. *Patient Information sheet and Consent Form in English and Malayalam*
9. *CV of Principal Investigator and Co-Principal Investigators*

The IEC Review Criteria
The study fulfils the expedited criteria from ethics review criteria vide section 9.1 of the Standard Operating Procedures (April 2017) of the SCTIMST-IEC.

IEC Decision
The IEC approved the conduct of the study in the present form.

Remarks:

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

There was no member of the study team who participated in voting / decision making process. The ethics committee is organized and operated according to the requirements of Good Clinical Practice and the requirements of the Indian Council of Medical Research (ICMR).

Sincerely,

Mala Ramanathan
Member Secretary, IEC

Ouriginal

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Fig 1
Discovery of Mycobacterium Tuberculosis as a causative agent of Tuberculosis by Robert Koch.
Fig 2 Discovery of an antibiotic Streptomycin for TB.
Fig 3 END TB strategy planned to decline in the TB global incidence rates by following the given principles denoted by WHO. FFig 4 Global distribution of tuberculosis. Fig 5 The global estimates of the number of TB incidence cases ascribed to five risk factors. Fig 6 The global trends from the estimates of the number of TB death cases with HIV-positive and HIV-negative cases Fig 7 The Impact of Time Span on Tuberculosis: Transmission and Disease Outcome. Fig 8 TB notification rates across India. Fig 9 The original drawing of Mycobacterium tuberculosis bacteria within infected tissue by Robert Koch on his aetiology of tuberculosis Fig 10 The schematic representation of Mycobacterium tuberculosis bacteria cell wall with the major components and its distribution. Fig 11 The detailed spectrum of tuberculosis from infection to active disease condition Fig 12 The granuloma formation during Latent TB Infection (LTBI) Fig 13 TB bacterial replication within granuloma and dissemination of bacteria during Active TB disease Fig 14 Ziehl-Neelsen stain TB test results. Fig 15 Auramine- Rhodamine staining TB test. Fig 16 A step-by-step description of the Xpert MTB/RIF assay procedure for diagnosing TB and rifampicin resistance. Fig 17 Lowenstein-Jensen plate culture with colonies of MTB growth as small, cream-coloured colonies with a rough and dry texture Fig 18 Mycobacteria Growth Indicator Tube (MGIT) culture test for the detection of MTB by the fluorescent orange colour Fig 19 The primers binding site in the mpt64 gene for the TB LAMP assay.