

RAPID DETECTION OF DRUG RESISTANCE IN *Mycobacterium tuberculosis* USING PAPER BASED METHOD IN PHOTON-MAX SYSTEM USING STORED CULTURES

PROJECT WORK

*Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology*

By

MAHALAKSHMI R (40230022)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A++" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHISALAI,

CHENNAI - 600119

APRIL – 2024



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited "A++" Grade by NAAC | 12B Status by UGC | Approved by AICTE

www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **MAHALAKSHMI R (40230022)** who carried out the Project entitled "**Rapid detection of drug resistance in *Mycobacterium tuberculosis* using Paper based method in Photon-Max system using stored cultures**" under our supervision from August 2023 to February 2024.

Internal Guide

Dr. V. Ramesh Kumar

External Guide

Dr. V. N. Azger Dusthacker

Head of the Department

Dr. V. Ramesh Kumar

Submitted for Viva voce Examination held on 05/04/2024

Internal Examiner

(Dr. S. Usha Nandhini)

External Examiner

(Dr. B. DEVI)

DECLARATION

I, Mahalakshmi R (40230022), hereby declare that the Project Report entitled **“Rapid Detection of Drug Resistance in *Mycobacterium tuberculosis* using Paper Based Method in Photon-Max System using Stored Cultures”** done by me under the guidance of Dr. V. N. Azger Dusthacker from the Department of Bacteriology, ICMR-National Institute for Research in Tuberculosis and Dr. V. Ramesh Kumar from the Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai and submitted in partial fulfilment of the requirements to be awarded the Bachelor of Technology in Biotechnology degree, is my genuine project work conducted at Department of Bacteriology, ICMR-NIRT

DATE:

PLACE: CHENNAI

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **the Board of Management of Sathyabama Institute of Science and Technology**, Chennai for their kind encouragement in doing this project and for completing its successfully. I am grateful to them.

I convey my thanks to **Dr. V. Ramesh Kumar, M. Phil., Ph.D.**, Head of the Department, Department of Biotechnology for providing me with the necessary support and details at the right time during the progressive reviews. I would also like to express my sincere and deep sense of gratitude for his constant support and encouragement during the advancement of my Project work.

I would like to express my sincere and deep sense of gratitude to my Internal Project Guide **Dr. V. Ramesh Kumar, M. Phil., Ph.D.**, Associate Professor, Department of Biotechnology, for his constant support and encouragement during the advancement of my project work.

I am extremely grateful to my External Project Guide **Dr. V. N. Azger Dusthacker, Scientist D**, for enabling me to work on my dissertation at ICMR-NIRT and for his valuable guidance, suggestions, and constant encouragement which actually paved way for the successful completion of my Dissertation/Project work.

I wish to express my thanks to all **Teaching and Non-teaching staff members of the Department of Biotechnology, Sathyabama Institute of Science and Technology** and Department of Bacteriology, ICMR-NIRT, who were helpful in many ways for the completion of my project.

ABSTRACT

Tuberculosis (TB) remains the deadliest infectious disease globally, posing a significant public health threat. However, its control is significantly hampered by two major challenges: the HIV pandemic and the rising prevalence of drug-resistant strains. Early diagnosis is crucial for effective treatment and preventing further transmission. The traditional methods for detecting the causative agent, *Mycobacterium tuberculosis* (MTB), are slow. Use of Lowenstein Jensen media (LJ) for its detection requires at least two to three weeks. Because of the bacilli's 24-hour doubling time, confirmation of the disease through visible colonies on solid media is considered the gold standard, takes around weeks for the detection of drug resistance. MGIT 960 *Mycobacterium* growth indicator tube is a for liquid culture-based technique and still requires a maximum of 7 days for the detection of drug resistance. As a result, early detection of drug resistance is critical for Tuberculosis (TB) control, and the current study is based on paper disc-based detection of drug resistance in *M. tuberculosis* using the Luciferase Reporter Phage (LRP) approach for rapid diagnosis within a day. The method utilizes a paper-based assay with Luciferase Reporter Phage (LRP) to detect the presence of MTB. LRP utilizes a genetically engineered phage that can infect MTB and the speed of the reaction will be enhanced by the use of microfluidic based approach. The paper-based format of the assay makes it potentially inexpensive, easy to use, and adaptable for use in resource-limited settings. This is the basis of the current study in which we intend to evaluate this theory. By successfully demonstrating its efficacy, paving the way for a more effective approach to combating this global health threat.

TABLE OF CONTENTS

S.NO	CONTENTS	PAGE NO.
	ABSTRACT	v
	List of Abbreviation	x
	List of Figures	xi
	List of Tables	xii
1.	INTRODUCTION	1
	1.1 Tuberculosis infection and Risk factors	4
	1.2 Symptoms of TB disease	5
	1.3 Pulmonary tuberculosis	5
	1.4 Extrapulmonary tuberculosis	5
	1.5 Drug resistance to TB	6
	1.6 Multi Drug-Resistant TB	6
	1.7 Extensively Drug-Resistant TB	7
2.	LITERATURE SURVEY	8
	2.1 History and Bacteriology of tuberculosis	8
	2.2 Morphology	9
	2.3 Pathogenesis	10
	2.4 Microfluids for tuberculosis	12
	2.5 Diagnosis	13
	2.5.1 Ziehl Neelsen Staining	13
	2.5.2 Auramine Phenol Staining	14
	2.6 Treatment	14
	2.7 Drug Susceptibility Test	15
	2.8 Macroscopic observation of Growth in Solid Media	16
	2.8.1 Solid Culture	16
	2.8.2 Agar Dilution Method/Proportion Method	17

2.8.3 The Absolute Concentration Method	17
2.8.4 The Resistance Ratio Method	18
2.9 Detection or Measurement of Metabolic Activity	18
2.9.1 MGIT 960 Method	18
2.9.2 Radiometric BACTEC 460	19
2.9.3 Resazurin Microtiter Assay (REMA)	20
2.10 Luciferase Reporter Mycobacteriophage	21
2.11 Isoniazid	23
2.12 Rifampicin	24
2.13 Luminometry	25
3. AIM AND OBJECTIVE	26
4. MATERIALS AND METHODS	27
4.1 Materials	
4.1.1 Middlebrook 7H9 Broth	27
4.1.2 Middlebrook 7H9 Agar	27
4.1.3 Soft Agar	28
4.1.4 Middlebrook 7H11 Agar	28
4.1.5 Isoniazid 7H9 Drug	29
4.1.6 Preparation of Suspension	29
4.1.7 Preparation of 0.1M Calcium Chloride	30
4.1.8 Preparation of MP Buffer	30
4.1.9 Glass Filter Paper	31
4.1.10 Preparation of Sodium citrate	32
4.1.11 Preparation of D-Luciferin	32
4.1.12 Photon-Max System	32
4.2 Procedure	34
4.2.1 Phage Propagation	34

4.2.2 Conventional Method	35
4.2.3 Paper-Based Method	35
4.2.3.1 Control	35
4.2.3.2 Isoniazid Drug	36
4.2.4 Whatman Filter Vs Glass Filter	37
4.2.5 Two Discs Vs Three Discs in Glass Filter	37
5. RESULTS AND DISCUSSIONS	39
5.1 Results	39
5.1.1 Comparison of Conventional and Paper-Based Method in Photon-Max System	39
5.1.2 Comparison of Photon-Max System with Berthold	40
5.1.3 Comparison of Whatman Filter and Glass Filter Discs in Photon-Max System	41
5.1.4 Comparison of number of discs in photon-max system	42
5.1.5 Screening the 7H9 drug method for 50 clinical Strains	44
5.1.6 Comparison of Photon-Max and Berthold system for 40 % Inhibition in INH 0.1 µg/ml	52
5.1.7 Comparison of Photon-Max and Berthold system for 50% Inhibition in INH 0.1 µg/ml	52
5.1.8 Comparison of Photon-Max and Berthold system for 40% Inhibition in INH 0.2 µg/ml	53
5.1.9 Comparison of Photon-Max and Berthold system for 50% Inhibition in INH 0.2 µg/ml	53
5.2 Discussion	54

6. SUMMARY AND CONCLUSION

57

REFERENCES

ANNEXURE

LIST OF ABBREVIATIONS

TB	Tuberculosis
MTB	<i>Mycobacterium tuberculosis</i>
MDR	Multi Drug Resistant
XDR	Extremely Drug Resistant
RMP	Rifampicin
INH	Isoniazid
OADC	Oleic Acid Albumin Dextrose Catalase
LRP	Luciferase Reporter Mycobacteriophage
μl	Microlitre
DR	Drug Resistance
DST	Drug Susceptibility Test
AST	Antimicrobial Susceptibility Testing
MIC	Minimum Inhibitory Concentration
AFB	Acid Fast Bacterium
CaCl ₂	Calcium Chloride
MgCl ₂	Magnesium Chloride
MGIT	Mycobacterial Growth Indicator Tube

LIST OF FIGURES

FIGURE NO.	FIGURE NAME	PAGE NO.
1.1	Sites of Extrapulmonary tuberculosis	6
2.1	Pathogenesis of <i>Mycobacterium tuberculosis</i>	11
2.2	LRP assay principle	22
2.3	Isoniazid structure	23
2.4	Rifampicin Structure	24
2.5	Luminometry	25
4.1	Clinical strain in LJ media	30
4.2	Glass filter paper disc	31
4.3	Photon-Max system	33
4.4	Phage propagation	34
4.5	Isoniazid Drug Method	36
5.1	Comparison between Photon-Max system and Berthold	40
5.2	Comparison between Whatman filter and Glass filter	41
5.3	Comparison between number of glass filter discs	42
5.4	Evaluation of Drug efficiency against a panel of Clinical Strains	51

LIST OF TABLES

TABLE NO.	TABLE NAME	PAGE NO.
4.1	Components of 7H9 broth	27
4.2	Components of 7H9 agar	27
4.3	Components of soft agar	28
4.4	Components of 7H11 agar	29
4.5	Components of MP buffer	31
5.1	Comparison of Conventional and Paper- Based method in Photon-max system	39
5.2	Comparison of Photon-max system with Berthold	40
5.3	Comparison of Whatmann filter and glass filter discs	41
5.4	Comparison of Conventional and Paper- Based method in Photon-max system	42
5.5	Readings for Clinical strains in Photon-Max and Berthold system	44

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) is a highly contagious disease caused by *Mycobacterium tuberculosis* that poses a significant risk to world health. This disease primarily affects the lungs but can also target other parts of the body, such as the kidneys, spine, and brain. *Mycobacterium tuberculosis*, also known as “white death” as patients' lungs collapse and they vomit white matter. It spreads when an infected person coughs or sneezes. The bacterium has a rod-like structure, with bacilli measuring 2-4 μm in length and a generation time of 15-20 hours. As a result, it spreads by the inhalation of tubercle bacilli-containing aerosol. Without treatment, TB can be fatal. Loss of appetite and weight, prolonged coughing, chest discomfort, and thick mucus are all evident indications. Treatment consists of a course of antibiotics, which may include novel medications such as bedaquiline and delamanid in drug-resistant cases.

The formation of multidrug-resistant tuberculosis in individuals treated with isoniazid and rifampicin is a significant concern in tuberculosis treatment (Tsara *et al.*, 2009). The two most common causes of medication resistance are inadequate therapy and infection by a highly resistant strain. Because drug-resistant strains are prevalent in the public, MDR-TB can transfer directly from infected to uninfected individuals. In this case, an MDR-TB infection recurs in a previously untreated person. This accounts for up to 75% of cases and is known as primary MDR-TB. According to WHO projections, there could be up to 501,000 additional cases of multidrug-resistant tuberculosis (MDR-TB).

Every year, the World Health Organization (WHO) predicts that up to half a million new cases of multidrug-resistant tuberculosis (MDR-TB) (i.e., resistant to at least rifampicin and isoniazid) arise worldwide (Chowdhury *et al.*, 2023). The intrinsic weakness of the state-run TB control system, as well as a lack of patient knowledge, are the primary causes of the growth in drug-resistant TB. People infected with TB bacteria have a 10% lifetime risk of developing tuberculosis. TB is treatable and curable; nonetheless, TB patients can die if not treated properly. Drug-resistant

tuberculosis (TB) can emerge when bacteria become resistant to the medications used to treat it. MDR-TB germs are significantly more deadly than regular TB bacteria.

Some cases of MDR-TB require extensive courses of chemotherapy, which can be expensive and cause severe adverse drug reactions in patients (McIntosh, 2017). MDR have been increasing spread of infection and extensively drug-resistant (XDR) strains. Global efforts to combat tuberculosis (TB) have saved an estimated 75 million lives since 2000. The mortality of TB has also reduced by 18% during the same period in India and globally. The World Health Organization has revised the TB death rate from 4.94 lakhs in 2021 to 3.31 lakhs in 2022, a reduction of 34%. India accounted for the highest number of TB cases in the world in 2022, with 2.8 million TB cases, representing 27% of the global burden. It was the world's second leading cause of death from a single infectious agent in 2022, following Covid-19. TB caused almost twice as many deaths as Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome stage (AIDS). More than 10 million people continue to fall ill with TB every year. 30 high burden TB countries collectively accounted for 87% of the world's TB cases in 2022. MDR have been increasing spread of infection and XDR strains. Without treatment, the death rate from TB disease is high, at about 50%. However, with treatments currently recommended by WHO (a 4–6 months course of anti-TB drugs), about 85% of people with TB can be cured. There is a positive global recovery in the number of people diagnosed with TB and treated in 2022, following two years of Covid-19-related disruptions. Countries like India, Indonesia, and the Philippines, accounted for over 60% of the global reductions.

As a top infectious disease, current estimates are that TB infects nearly two billion people or about one-third of the world population. These infected, non-disease active people are considered latent TB cases. This means that people are infected with TB bacteria, but are not yet ill or active cases. Infected people have a lifetime risk of 10 percent to falling ill to TB. A number of new methods and assays have been developed for the detection of *M. tuberculosis*, with or without detection of drug resistance. However, rapid methods are not a replacement for culture, most are not reliable when used with smear-negative specimens; conventional

Antimicrobial Susceptibility Testing is still needed to confirm cases of XDR-TB. To test for resistance to drugs other than Isoniazid and rifampin, none of the methods detect all resistant strains; and in many parts of the world, the existing infrastructure is inadequate for these assays to be used on a widespread basis (Wilson, 2011).

Mycobacterial culture can be performed on either a solid or a liquid medium. The yield of *M. tuberculosis* isolated from a liquid medium (e.g., Middle brook 7H9) is greater than that from a solid egg-based medium (e.g., Lowenstein-Jensen [LJ]) or a solid agar-based medium (e.g., Middle brook 7H1 1). Automated liquid culture systems, such as the Bactec MGIT system (BD) or the BacT/Alert (bioMerieux), provide continuous monitoring for mycobacterial growth and, in adult and paediatric studies, significantly improve the recovery of *M. tuberculosis* as well as reduce the time to detection compared to a solid medium culture (Dunn *et al.*, 2016). Integrated microfluidic system capable for airborne *Mycobacterium tuberculosis* capture, enrichment, and rapid bacteriological immunoassay was developed. These Microfluidic technologies are ideally suited to meet the challenges described for the rapid and POC detection of pathogens. An ideal POC microfluidic test for TB diagnosis in a resource limited setting should detect early infection/diseases through the use of multiple biomarkers for increased sensitivity and specificity for effective linkage to care, minimum sputum processing, single sample without the need for a follow-up sample and rapid turn, around times are essential for initiation of appropriate treatment.

The device is fabricated from polydimethylsiloxane (PDMS), simple, cheap, and disposable, perfect for field application, especially in developing countries with very limited modern instrument (Jing *et al.*, 2013). This polydimethylsiloxane is a material commonly employed in bio microfluidic systems, is permeable to air. This material property may lead to sample losses due to evaporation While the rate may be low (micro-litres per hour or lower), the sample losses cannot be neglected when the operation volume is on the order of micro-litres and the reactor is to be maintained for hours. One of the simplest and most widely used bioassay methods is a diffusion bioassay method known as disk diffusion. Because disk diffusion assays detect antibiotic activity of many classes, they are suitable for use when

there is no requirement to determine the presence of specific antibiotic types or concentrations (Driscoll *et al.*, 2012).

By referring this all methods, in this experiment, we have tried to address the Sensitivity and Specificity by using liquid disc LRP DST method with incubation time for quicker diagnosis using the paper discs and compared the results of this method those obtained by using LJ cultures. This is the first report on finding the liquid disc LRP DST method within the 4 hours incubation time in one day at star tube using Photon-Max system.

1.1 TUBERCULOSIS INFECTION AND RISK FACTORS

Tuberculosis infection distinguishes from disease as it represents a colonization of bacteria rather than an illness. Exposure to someone with TB disease and inhaling TB bacteria can result in TB infection. Typically, individuals with robust immune systems can contain the infection without developing TB disease. Those with TB infection (evidenced by a positive TB skin test but normal chest x-ray) aren't sick and aren't contagious. Treatment can eradicate bacteria and prevent TB disease. However, without preventive medication, TB infection can progress to active TB disease.

Risk factors for TB development depend on infection and its progression to disease. Once infected with TB bacteria, the likelihood of developing TB disease increases, especially for those in close contact with contagious TB cases, residing or working in high-risk settings like prisons or healthcare facilities, belonging to high-risk racial or ethnic minority groups, engaging in illicit drug use, or having HIV infection.

The risk of acquiring tuberculosis is affected by both the chance of infection and the probability of infection progressing to active illness. After obtaining the TB bacterium, an individual's probability of developing TB disease increases if the:

- Close contacts with people of contagious cases of TB
- Individuals infected with the human immunodeficiency virus (HIV)

- Individuals who inject illicit drugs
- Residents and employees of high-risk congregate settings
- People in high-risk racial or ethnic minority populations defined locally as having an increased prevalence of tuberculosis

1.2 SYMPTOMS OF TB DISEASE

These symptoms can be vague and mimic other illnesses. The symptoms of tuberculosis include persistent cough, chest pain, fatigue, night sweats, fever, loss of appetite and weight loss.

1.3 PULMONARY TUBERCULOSIS

Pulmonary tuberculosis (TB) is a contagious bacterial infection that affects the lungs. It spread to other organs. TB usually attacks the lungs (90% of cases) when it becomes active. However, the TB infection can lie dormant for years within the body, causing no symptoms. Unfortunately, some people with latent TB can reactivate the infection later in life, leading to active pulmonary TB with a persistent cough, weight loss, and fatigue.

1.4 EXTRAPULMONARY TUBERCULOSIS

Extrapulmonary tuberculosis (EPTB) refers to tuberculosis infection occurring outside the lungs. While pulmonary TB (PTB) is the most common form, EPTB affects about 15-20% of all TB cases. *M. tuberculosis* bacteria can spread through the bloodstream or lymphatic system to various organs, causing a diverse range of symptoms depending on the location. Extrapulmonary TB occurs more commonly in people with a weakened immune system and young children. In those with HIV, this occurs in more than 50% of cases. A potentially more serious, widespread form of TB is called "disseminated tuberculosis", also known as miliary tuberculosis. Miliary TB occurs when the bacteria spreads through the bloodstream and affects multiple organs throughout the body. Miliary TB currently makes up about 10% of extrapulmonary cases and requires heightened vigilance for prompt diagnosis and treatment.

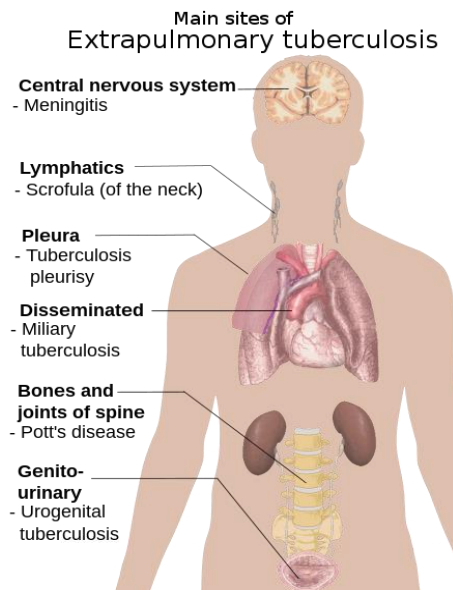


Fig 1.1: Sites of Extrapulmonary tuberculosis

1.5 DRUG RESISTANCE TO TB

A drug-resistant strain of *Mycobacterium tuberculosis* is defined as one differing from the tight distribution of wild strains that have not come into contact with the drug concerned. Drug-resistant forms of TB can develop if treatment is incorrect or incomplete. This can happen for several reasons. Because treatment for TB takes six months and can have difficult side effects, people may be tempted to stop taking their medication before they have completed treatment, particularly if they are starting to feel better. The most important way to prevent the spread of drug-resistant TB is to take all TB drugs exactly as prescribed by the health care provider.

1.6 MULTI DRUG-RESISTANT TB (MDR-TB)

Multi Drug Resistant TB is often abbreviated to MDR-TB and it is a particular type of drug resistant TB. It means that the TB bacteria that a person is infected with are resistant to two of the most important TB drugs, Isoniazid (INH) and rifampicin (RMP). If bacteria are resistant to certain TB drugs this means that the drugs don't work. Other drugs then need to be taken by the person if they are to be cured of TB.

1.7 EXTENSIVELY DRUG-RESISTANT TB (XDR-TB)

Extensively drug-resistant tuberculosis (XDR TB) is a relatively rare type of multidrug-resistant tuberculosis (MDR TB). It is resistant to almost all drugs used to treat TB, including the two best first-line drugs: Isoniazid and Rifampin. XDR TB is also resistant to the best second-line medications: fluoroquinolones and at least one of three injectable drugs (i.e., amikacin, kanamycin, or capreomycin) it is a form of tuberculosis caused by bacteria that are resistant to some of the most effective anti-TB drugs. XDR-TB strains have arisen after the mismanagement of individuals with multidrug-resistant TB (MDR-TB). Final diagnosis for TB, and especially for XDR TB, may take from 6 to 16 weeks.

This study investigates a rapid method for determining drug resistance in *Mycobacterium tuberculosis* (MTB). We are carrying out this experiment to assess the efficacy of this assay by screening clinical strains of MTB. This method uses stored MTB cultures in a paper-based, where the drugs interact with the bacteria. The Photon-Max system then analyzes the luciferase reporter molecules to determine drug resistance in Mycobacteria. This method provides faster outcomes and potentially lower costs than existing procedures.

CHAPTER 2

LITERATURE SURVEY

2.1 HISTORY AND BACTERIOLOGY OF TUBERCULOSIS

Tuberculosis (TB) is one of the oldest recorded human afflictions and is still one of the biggest killers among infectious diseases since ancient times. Tuberculosis had claimed its victims throughout much of known human history. Johann Schönlein coined the term “tuberculosis” in the 1834, though it is estimated that *Mycobacterium tuberculosis* may have been around as long as 3 million years. On March 24, 1882, Dr. Robert Koch announced the discovery of *Mycobacterium tuberculosis*, the bacteria that cause tuberculosis (Tan *et al.*, 2020). During this time, TB killed one out of every seven people living in the United States and Europe. Dr. Koch's discovery was the most important step taken toward the control and elimination of this deadly disease. A century later, March 24 was designated World TB Day: a day to educate the public about the impact of TB around the world. Consumption, phthisis, scrofula, Pott 's disease, and the White Plague are all terms used to refer to tuberculosis throughout history. It is generally accepted that *Mycobacterium tuberculosis* originated from other, more primitive organisms of the same genus *Mycobacterium*. A study by Daniel (2006) suggests that *Mycobacterium tuberculosis* may hold the grim title of deadliest microbial pathogen, having potentially caused more human deaths than any other infectious agent. The paper also proposes that the genus *Mycobacterium*, which includes the tuberculosis bacterium, has ancient origins, dating back over 150 million years (Daniel, 2006). According to Barberis *et al.*, (2017), *Mycobacterium ulcerans*, a bacterium responsible for infections dating back to ancient times, thrives under specific environmental conditions. This dependence on particular ecological factors is reflected in the bacterium's present-day global distribution (Barberis *et al.*, 2017). *Mycobacterium tuberculosis* (MTB) was the cause of the "White Plague" of the 17th and 18th centuries in Europe. During this period nearly, 100 percent of the European population was infected with MTB, and 25 percent of all adult deaths were caused by MTB (Note: The White Plague is not to be confused with the "Black Plague", which was caused by *Yersinia pestis* and occurred about 3 centuries earlier).

In a 2009 paper, Hedley-Whyte and Milamed pointed out a seemingly paradoxical situation: the growing importance of bacteriological aspects of tuberculosis diagnosis and treatment in the very era when more effective therapies are emerging. The authors contrasted this with lobar pneumonia and streptococcal infections, where the discovery of antibiotics like sulfonamides and penicillin lessened the need for such meticulous bacterial workup. Hedley-Whyte and Milamed argued that failing to recognize the unique nature of tuberculosis in this regard has significantly hampered efforts to provide the necessary resources and personnel for proper bacterial diagnosis and treatment monitoring (Hedley-Whyte & Milamed, 2009). Before the antimicrobial medication era, roentgenographic analysis and monitoring by the clinician-radiologist team dominated this sector. The clinician-bacteriologist team played a limited role.

The sluggish growth and multiplication of tubercle bacilli place a significant strain on any laboratory that provides diagnostic bacteriologic services. Additional issues arise when tubercle bacilli populations are tested for medication susceptibility by a clinical or public health laboratory. A study by Middlebrook and Cohn (n.d.) highlighted the challenges associated with establishing cost-effective tuberculosis diagnostic services. They emphasized the importance key requirements: 1. A uniform, standard method for handling and treating specimens. 2. Means for rapid cultivation of tubercle bacilli from small, as well as large, inocula. 3. A routine which involves minimal expenditure of time in examination of cultures. 4. A minimum period of incubation of cultures which permits, at the same time, the most economical utilization of incubator space and a minimum yield of false-negative cultures. 5. A technic for performing direct drug susceptibility tests which gives results that are clinically meaningful (Middlebrook & Cohn, n.d.).

2.2 MORPHOLOGY

MTB is a nonmotile, rod-shaped obligate aerobe that does not produce spores. The bacteria are rod-shaped bacilli measuring 0.8-10 μm . Because of the presence of the cord factor, they occasionally develop filamentous structures similar to fungi. Hence, they are named Mycobacteria. The cell wall of Mycobacterium is

characterized by a unique structure that is caused by chemical compositions that differ from other bacteria. Mycolic acids can be found in *M. tuberculosis*'s peptidoglycan-based cell walls. *M. tuberculosis* grows on two types of medium. Middlebrook's medium is agar-based, while Lowenstein-Jensen media is an egg-based medium. *M. tuberculosis* is an acid-fast bacterium (AFB) that is neither Gram positive nor negative. Gram staining produces the appearance of "ghost" cells. *M. tuberculosis* can be identified using the Ziehl-Neelsen stain, which appears pink on the slide (Somphavong, n.d.).

2.3 PATHOGENESIS

Tuberculosis is a highly infectious disease, and is primarily transmitted via inhalation of aerosol droplets expelled by infected hosts. Heemskerk *et al.* (2015) describe *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis, as a cunning pathogen. With an infectious dose as low as 1-200 bacilli and each inhaled droplet potentially containing hundreds, avoiding infection can be difficult. Furthermore, MTB leverages the body's natural defenses to its advantage. Following inhalation, the bacteria reach the lungs and settle in the alveoli. Here, in an immunocompetent host, they are recognized as foreign and targeted by macrophages, the body's first line of defense against invaders. However, MTB can subvert this process. The macrophages attempt to engulf and destroy the bacteria, but MTB can evade this attack and establish a latent infection, remaining dormant within the body for years before potentially reactivating as active tuberculosis (Heemskerk *et al.*, 2015).

A study by Khan *et al.* (2010) details the response of the lungs to *Mycobacterium tuberculosis*, the bacteria causing tuberculosis. When the bacteria invade the lungs, they are surrounded and contained by immune cells, forming a hardened structure called a tubercle. This tubercle is a complex structure, consisting of the bacteria itself, layers of immune cells called macrophages, and an outer layer of lymphocytes, another type of immune cell. These tubercles can be visualized on X-ray films as dense, bright shadows at the lung's periphery. They represent the body's attempt to wall off the primary infection, forming the hallmark "primary

complex" of tuberculosis. In later stages, the tubercle may enlarge. The macrophages and surrounding tissue cells can die and fuse together, forming a cheese-like necrotic center, a phenomenon known as caseation necrosis (Khan *et al.*, 2010). The tubercle then becomes calcified. If untreated several tubercles to form a large mass. This leads to the breakdown of lung tissues and the formation of large cavities. The bacteria then eat away the bronchi and blood vessels are eroded. When the patient coughs, bright red blood is coughed out. In some cases, the tubercle bacterium escapes from the lungs and enters the blood stream. It can then reach any organ of the body, especially the meninges that cover the brain. This causes a disease called tuberculosis meningitis.

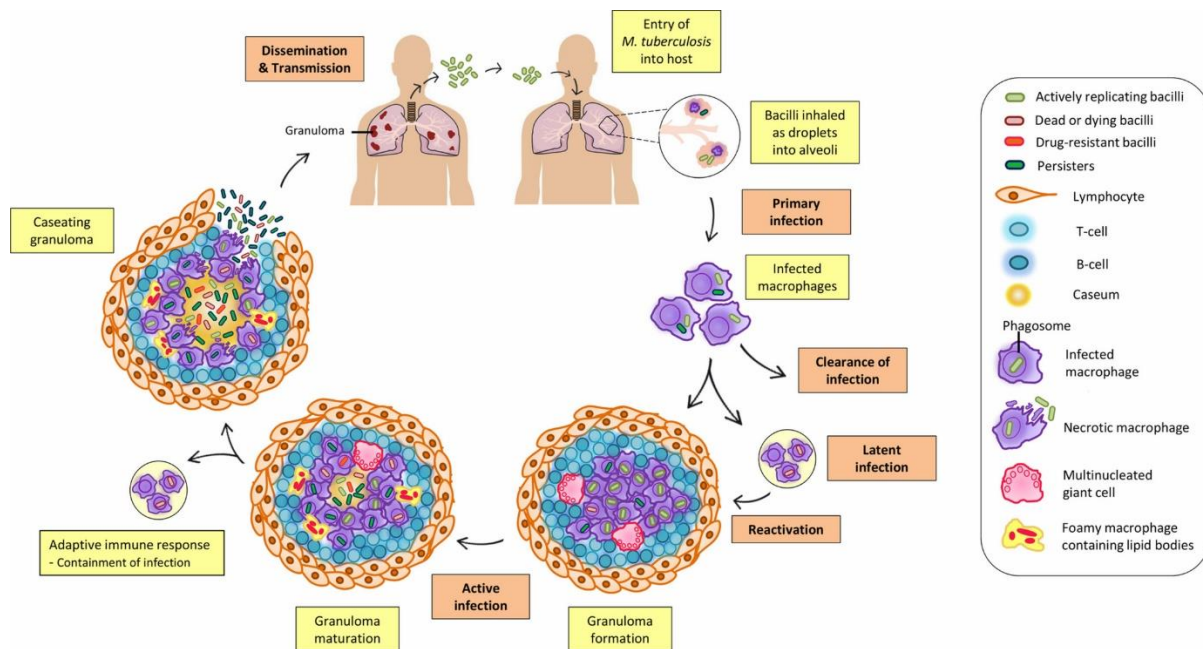


Fig 2.1: Pathogenesis of *Mycobacterium tuberculosis*

(https://doi.org/10.1007/978-981-16-8957-4_12)

Sia and Rengarajan (2019) outlined the progression of a *Mycobacterium tuberculosis* infection. The initial stages of infection are characterized by innate immune responses that involve the recruitment of inflammatory cells to the lung. Following bacterial dissemination to the draining lymph node, dendritic cell presentation of bacterial antigens leads to T cell priming and triggers an expansion of antigen-specific T cells, which are recruited to the lung. The recruitment of T cells, B cells, activated macrophages and other leukocytes leads to the establishment of

granulomas, which can contain *Mycobacterium tuberculosis*. Most infected individuals will remain in a 'latent' state of infection, in which no clinical symptoms are present. A small percentage of these people will eventually progress and develop active disease, which can lead to the release of *M. tuberculosis* from granulomas that have eroded into the airways. When individuals with active tuberculosis (TB) cough, they can generate infectious droplets that transmit the infection (Sia & Rengarajan, 2019).

2.4 MICROFLUIDICS FOR TUBERCULOSIS

Whitesides (2006) describes microfluidics as a "laboratory on a chip" technology. This technology allows miniaturization and integration of complex functions that facilitate their use for high throughput applications. The microfluidic device comprises a microchannel network that has been moulded to allow fluids to pass through different channels of different diameter, usually ranging from 5 to 500 μm (Whitesides, 2006). WHO goal of eliminating TB globally by 2050, effective POC TB diagnostics must be implemented to prevent delayed and or misdiagnosis of TB which presently fuel the epidemic with high annual mortality rates. Miniaturization of TB diagnosis by using on chip microfluidics technologies or by integrating novel nanotechnologies could be strategy for developing inexpensive and effective diagnostics for resources constrained countries. An ideal POC microfluidic test for TB diagnosis in a resource limited setting should detect early infection/diseases through the use of multiple biomarkers for increased sensitivity and specificity for effective linkage to care, minimum sputum processing, single sample without the need for a follow-up sample]and rapid turnaround times are essential for initiation of appropriate treatment. Lee *et al.* (2010) highlight the promise of nano/microfluidic technologies for diagnosing and monitoring infectious diseases in both resource-rich and resource-limited settings. Miniaturized Nano/microfluidic platforms that precisely manipulate small fluid volumes can be used to enable medical diagnosis in a more rapid and accurate manner. In particular, these Nano/microfluidic diagnostic technologies are potentially applicable to global health applications, because they are disposable, inexpensive, portable, and easy-to-use for detection of infectious diseases (Lee *et al.*, 2010).

2.5 DIAGNOSIS

Tuberculosis is diagnosed by identifying *Mycobacterium tuberculosis* bacteria in a clinical specimen obtained from the patient. Gastric washings, laryngeal swabs, bronchoscopy (with bronchoalveolar lavage, bronchial washings, and/or transbronchial biopsy), and fine needle aspiration are common alternative sample sources for diagnosing pulmonary tuberculosis in patients who cannot produce a sputum sample. Many different cultures are available. Traditionally, cultures employed Lowenstein-Jensen (LJ), Kirchner, or Middlebrook media (7H9, 7H10, and 7H11). Mycobacteria can be identified using AFB culture, although results may take four to eight weeks to confirm. More automated systems like MB/BacT, BACTEC 9000, Versa TREK, and the Mycobacterial Growth Indicator Tube (MGIT). McNerney *et al.* (2015) suggest that Microscopic Observation Drug Susceptibility Assay (MODS) Culture could be a more rapid and accurate method for diagnosing tuberculosis compared to the low-cost smear microscopy currently used in many TB-endemic countries. A key limitation of smear microscopy is its insensitivity, often missing cases due to the low numbers of bacteria present in clinical specimens (McNerney *et al.*, 2015).

2.5.1 Ziehl Neelson Staining

Dr. Franz Ziehl (1857–1926) was a German bacteriologist from Lubek. In 1882, he introduced the Carbol fuchsin stain for tubercle bacillus. Mycobacteria's acid-fastness is determined by the presence of Mycolic acid in their cell walls. Fuchsin, the primary stain, binds to mycolic acids in the cell wall. Intense decolourization (strong acid) does not remove the primary stain from the cell wall, and AFB preserves the red colour of fuchsin. Counterstain (Methylene blue) creates a contrasting background. A standard AFB staining process entails placing the cells in suspension onto a slide, air drying the liquid, and then heat fixing the cells. The slide is saturated with Carbol Fuchsin, which is followed by heating till it's dry and rinsed with tap water. The slide is next flooded with a 1% solution of hydrochloric acid in isopropyl alcohol (or methanol) to remove the carbol fuchsin, eliminating the stain from cells that are not covered by a waxy lipid coating. The cells are then stained with methylene blue and examined under a microscope while immersed in

oil. Studies have found that an AFB stain without a culture has a low negative predictive value. An AFB culture should be performed in association with an AFB stain, as it has a considerably larger negative predictive value.

2.5.2 Auramine – Phenol Staining

Caulfield and Wengenack (2016) described a diagnostic technique for identifying acid-fast organisms, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The method involves staining air-dried and heat-fixed smears with a fluorescent dye called Auramine-O-Phenol for seven minutes. After removing excess dye with water, they are decolourized using an acid-alcohol mixture and counterstained with potassium permanganate. A fluorescent stain containing auramine O and rhodamine B dyes binds to nucleic acids in acid-fast organisms (Caulfield & Wengenack, 2016). Under a fluorescent microscope, Mycobacteria show slender yellow luminous rods that stand out against the dark background. This strategy allows for a broader perspective of the smear using a single field. This reduces the number of fields needed to scan a whole slide.

2.6 TREATMENT

When TB bacteria become active (multiplying in the body) and the immune system can't stop the bacteria from growing, this is called TB disease. TB disease can be treated by taking several drugs for 6 to 12 months. A study by Babiarz *et al.* (2014) emphasizes the critical importance of completing the full course of tuberculosis medication precisely as prescribed. Stopping treatment prematurely increases the risk of relapse, while improper medication use can lead to drug resistance in the remaining bacteria. This drug resistance makes subsequent treatment of tuberculosis significantly more challenging and expensive (Babiarz *et al.*, 2014). Zimmer *et al.* (2021) discuss directly observed therapy (DOT) as a strategy to ensure treatment adherence for tuberculosis patients. In some cases, local health department staff may meet with patients at designated times to directly observe them taking their medications. This intervention helps ensure patients

complete the full course of treatment in the shortest possible timeframe, reducing the risk of relapse and drug resistance (Zimmer *et al.*, 2021).

For new patients with drug sensitive TB the World Health Organization (WHO) recommends that they should have six months of TB drug treatment. This should consist of a two month "intensive" treatment phase followed by a four month "continuation" phase. For the two month "intensive" TB drug treatment phase they should receive:

Isoniazid (H/Inh)

Rifampicin (R/Rif)

Pyrazinamide (Z/Pza)

Ethambutol (E/Emb)

Followed by, Isoniazid (H/Inh) with rifampicin (R/Rif)

For the "continuation" TB drug treatment phase. It is essential to take several TB drugs together. If only one TB drug is taken on its own, then the patient will very quickly become resistant to that drug it is recommended that patients take the TB drugs every day for the six months, although taking them three times a week is possible in some circumstances. It is extremely important that all the recommended TB drugs are taken for the entire time. The amount of any drug that a patient needs to take, depends on the patient's weight. The treatment probably won't work if only one or two of the TB drugs are taken, or if the treatment is interrupted or stopped early. This is because the TB bacteria develop resistance to the TB drugs. The patient is then still ill, and to be cured they have to take drugs for the treatment of drug resistant TB.

2.7 DRUG SUSCEPTIBILITY TEST

A study by Jang and Chung (2020) highlights the importance of drug susceptibility testing (DST) in guiding tuberculosis treatment. DST determines a specific strain's susceptibility to various medications. A "sensitive" result means that the patient with that strain has a high probability of treatment success, while a "resistant" result means that there is a high possibility of treatment failure and that

the therapy should be changed. Thus, standardized and reliable DST of *M. tuberculosis* provides guidance for the treatment of the patient. A false resistance result may prompt the physician to replace an effective regimen with a less effective one, with a consequent detrimental effect on the patient's health (Jang & Chung, 2020). Moreover, therapy for drug-resistant TB lasts longer and uses more expensive drugs, meaning a waste of drugs and money for the health system and a high probability of side-effects for the patient. A false sensitivity result prompts clinician to continue with ineffective treatment and may result in the spread of drug-resistant strains in the community. Carrying out DST also provides an assessment of the drug resistance rates and trends in a country (or in different areas of a country); standardization is therefore essential to allow data from different laboratories to be compared. Standardization of DST is difficult: at each step there are certain variables that are not simple to control. For example, the potency and stability of drugs vary with conditions of sterilization and storage. Human factors may influence DST results: incorrect drug concentrations and/or inocula for the particular test may be used. Moreover, a deep understanding of resistance criteria is essential for reliable results. Two principles form the basis of DST proportion method - critical concentration and critical proportion.

The critical concentration is the level of drug in the medium that inhibits the growth of wildtype organisms but not that of resistant mutants; the critical proportion is the proportion of resistant mutants compatible with therapeutic success. As a result, the treatment time extends up to 2 years which involves second line drugs people may get MDR-TB in two ways and then DST takes another 4 weeks due the lower generation time of MTB. Generally, we can classify DST in 4 ways as follows

2.8 MACROSCOPIC OBSERVATION OF GROWTH IN SOLID MEDIA

2.8.1. Solid Culture

This is one of the conventional methods that demands a significant amount of time for growth. Tubercle bacilli have a 24-hour doubling time, thus it takes around 6-8 weeks for them to appear as the well-known 'yellow - rough-buff-tough' colonies on solid Lowenstein Jensen (LJ) slopes. Currently, LJ slopes are the gold standard

for disease diagnosis and drug sensitivity pattern identification. The Lowenstein-Jensen (LJ) medium is most commonly used for tuberculosis cultivation. LJ medium containing glycerol promotes the growth of *M. tuberculosis*, whereas LJ medium lacking glycerol but containing Pyruvate promotes the growth of *M. bovis* and drug-resistant strains of *M. TB*. Malachite green inhibits the growth of non-acid-fast organisms (L-Asparagine for nitrogen source).

2.8.2 Agar Dilution Method/ Proportion Method

Birinci *et al.* (2002) point out a limitation of the commonly used agar proportion method for mycobacterial susceptibility testing. This method requires a lengthy incubation period of three weeks before an isolate can be definitively classified as either drug-susceptible or drug-resistant (Birinci *et al.*, 2002). This method makes use of the agar plating process. A set of drug-containing and drug-free 7H10 or 7H11 agar plates is created. Tenfold dilutions of the processed sample are spotted on these plates. When the colonies appear, the number of colonies on both the control and test plates is recorded. This number is multiplied by the appropriate dilution factor to get the number of viable colonies. Hanson and Martin (1978) acknowledged that while both the conventional broth dilution method and the agar dilution technique with Steers replication were acceptable for mycobacterial susceptibility testing, they presented limitations for routine use. The broth dilution method, though accurate, was considered too time-consuming for everyday clinical applications. The agar dilution technique, while faster, could be wasteful when testing only a small number of organisms due to the use of Steers replicator, a device that replicates inoculation patterns (Hanson & Martin, 1978).

2.8.3 The Absolute Concentration Method

Van Klingerren *et al.* (2007) describe a particular test primarily used to determine the most accurate drug dosage for a patient, rather than simply diagnose the presence of a disease (Van Klingerren *et al.*, 2007). An inoculum of a known turbidity, usually McFarland standard 1.0 is cultured on media with varying concentration of the same drug. Subsequently, when sufficient growth is achieved,

the MIC (Minimal Inhibitory Concentration) is measured. A dosage above the MIC is generally prescribed. This method is greatly affected by bacterial load and the viability of the bacilli (Training Manual M Tuberculosis C DST: Ministry of Health and Family Welfare, n.d.). A study by Bert van Klingeren *et al.*, (2007) showed 100% and 96% sensitivity, efficiency and reproducibility, for Isoniazid and Rifampin respectively.

2.8.4 The Resistance Ratio Method

This method is a comparison of number of colonies on solid media for a standard wild type laboratory stain, H37RV, and the sample strain. 10-fold dilutions of H37RV and the sample strain are inoculated into a set of drug-containing and drug free media (Acharya *et al.*, 2010). Resistance is expressed as the proportion of the minimal inhibitory concentration (MIC) of the test stain divided by the MIC for the wild laboratory strain in the same set (Training Manual M Tuberculosis C DST: Ministry of Health and Family Welfare, n.d.). Acharya S *et al* (2008) performed a comparative study using proportion and resistant ratio method. In that study, resistant ratio method failed to identify 0.9% of the resistant strains when compared with proportion method.

2.9 DETECTION OR MEASUREMENT OF METABOLIC ACTIVITY OR PRODUCT

2.9.1 MGIT 960 Method

MGIT stands for Mycobacterial Growth Indicator Tube. This test is based on growth of the *M. tuberculosis* strain in a drug containing tube compared to the drug free tube (Growth control). It is one of the liquid culture-based semi-automated systems available for TB diagnosis and also for subsequent Drug Sensitivity Testing. The samples are suspended in ADC enriched Middle brook 7H9 broth in commercially available MGIT tubes that can be fed into the automated machine for incubation as well as diagnosis of the disease. A study by Enrico Tortoli *et al.*, 2002 showed BACTEC MGIT 960 appears to be a suitable replacement for the

radiometric method of anti-microbial susceptibility testing of *M. tuberculosis*. The problem of frequent contamination of BACTEC MGIT 960 tests needs to be quickly resolved; in fact, 14 strains had to be reprocessed because of contamination (Tortoli *et al.*, 2002).

This assay works on the simple procedure of quenching. The tubes have silicone at the bottom with a fluorescing compound. The fluorescence is quenched by the presence of oxygen. When all the oxygen in the tube is consumed, maximum fluorescence is observed and recorded by the MGIT system.

Subramanyam *et al.* (2012) describe a specific culture technique for mycobacteria that incorporates a head space, to provide aeration, which some bacteria require for growth. To prevent overgrowth by unwanted microbes, the culture medium is supplemented with a commercially available antibiotic mixture called PANTA. This PANTA mixture contains five antibiotics: polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and Azlocillin. These antibiotics act to suppress the growth of most common bacteria, allowing for the targeted cultivation of the mycobacteria of interest (Subramanyam *et al.*, 2012). As a result, only MTB grow and their growth can be measured by the oxygen consumption from the air trapped in the tubes. As oxygen gets consumed, the fluorescence increases. This method is more sensitive and more efficient than radiometric BACTEC 460.

2.9.2 Radiometric BACTEC 460

A study by Harvell *et al.* (2000) demonstrated that the BACTEC 460 radiometric mycobacterial broth culture system, though no longer in use, consistently yielded faster and higher recovery rates of *Mycobacterium tuberculosis* from respiratory specimens in patients with pulmonary tuberculosis compared to conventional culture methods (Harvell *et al.*, 2000). This technique exploits the use of radioactive. The carbon atoms in palmitic acid in the Middlebrook 7H12 media are radio-labelled. On consumption, the lipid is metabolized to $^{14}\text{CO}_2$, during growth of MTB. The BACTEC 460 TB system has been reported to yield 15-20% increased culture positivity of clinical specimens as compared to conventional solid media such as LJ medium, with an average time-to-detection of positive growth from 8 to 14

days as compared to 3 to 5 weeks on solid media. The introduction of the BACTEC 460 TB system revolutionized laboratory testing for *Mycobacteria* and has established itself as the gold standard for culture and susceptibility testing. One of the disadvantages of the BACTEC 460 TB system is the use of ¹⁴C-Labeled radioactive substrate. Because of the strict regulations of handling and waste disposal of radioactive material it became necessary to develop a non-radiometric technique for mycobacterial culture and susceptibility testing.

2.9.3 Resazurin Microtiter Assay (REMA)

Resazurin dye, is an oxidation reduction indicator, has been used to appraise viability and bacterial contamination and to assess drug sensitivity pattern. This assay is essentially colorimetric and exploits the colour variation of dyes in the reduced and oxidized states. Resazurin is a blue dye in its oxidized state. Once reduced, the dye turns pink.

Hence, it is an oxidation-reduction indicator. Khalifa *et al.*, (2013) described the Resazurin Microtiter Assay (REMA) plate method for determining the minimum inhibitory concentration (MIC) of drugs against *Mycobacterium tuberculosis* clinical isolates. In REMA a variety of drug concentration in Middlebrook 7H9 broth are dispensed into 96-well microtiter plates and incubated for a week, after treatment with Resazurin dye and observed for colour change. On cellular oxidation, the dye gets reduced to resorufin, which is red as well as fluorescent. The intensity of fluorescence is a direct measure of the bacterial load in the well. The 96-well plates are fed onto a plate reader and recorded for fluorescence at the appropriate λ_{max} . The REMA plate method has been described for MIC determination of *M. tuberculosis* clinical isolates and has been tested successfully against INH and RIF for the detection of MDR-TB (Khalifa *et al.*, 2013).

Kumar *et al.*, (2018) investigated the Resazurin assay (RA) performed in microtiter plates as a method for determining the minimum inhibitory concentration (MIC) of drugs against *Mycobacterium tuberculosis* clinical isolates. Similar to the REMA plate method described previously, the Resazurin assay appears effective. The study successfully tested this method against Isoniazid (INH) and Rifampicin

(RIF) for the detection of Multidrug-resistant Tuberculosis (MDR-TB) (Kumar *et al.*, 2018). In their 2002 study, Palomino *et al.*, acknowledged a limitation of the Resazurin Microtiter Assay (REMA) method. While effective, REMA lacks specificity for *Mycobacterium tuberculosis*. Contamination by other bacteria during the week-long incubation period can interfere with the accuracy of measurements by affecting the resazurin reduction and fluorescence, potentially leading to misinterpretations of the actual *M. tuberculosis* load (Palomino *et al.*, 2002).

2.10 LUMINOMETRIC ANALYSIS BY LYSIS WITH MYCOBACTERIOPHAGE

2.10.1 Luciferase Reporter Mycobacteriophage (LRP ASSAY)

Branchini *et al.*, (1997) describe a technique called the Luciferase Reporter Phage (LRP) assay. This method utilizes reporter phages, viruses that infect bacteria, engineered to express the firefly luciferase gene within viable Mycobacteria. Firefly luciferase is a popular reporter molecule widely used in molecular biology and biochemistry. It offers a convenient way to monitor the activity of promoters in various systems, including bacteria, cultured cells, transgenic plants, and animals. The key function of firefly luciferase is to catalyze a reaction that produces yellow-green light from a molecule called luciferin. This light-generating reaction requires ATP, magnesium ions (Mg^{2+}), oxygen (O_2), and a slightly alkaline environment (pH 7.8). By measuring the emitted light intensity, scientists can indirectly assess promoter activity in the Mycobacteria infected with the reporter phages (Branchini *et al.*, 1997). Luciferase reporter phage is a Mycobacteriophage which is a subdivision of bacteriophage which is capable of infecting Mycobacteria. Firefly luciferase gene is incorporated in the mycobacteriophage when inoculated along with *M. Tuberculosis*; these Mycobacteriophage infect them and inject the firefly luciferase gene into the bacterium. Being a lysogenic phage, its DNA gets incorporated in the host DNA. This gene is transcribed and translated to produce Firefly luciferase. When D-luciferase is added, it produces fluorescence. This is only possible when the cell is viable. The LRP assay is used for both diagnosis and the testing of drug susceptibility pattern.

Dusthacker *et al.*, (2008) built upon the work of Branchini *et al.* (1997) by demonstrating that the Luciferase Reporter Phage (LRP) assay can be a powerful tool not only for studying gene expression in Mycobacteria but also for identifying dormant bacilli, those that are not actively growing. This makes LRP a valuable technique for investigating persistent tuberculosis infections (Dusthacker *et al.*, 2008). When introduced into MTB suspension, these Mycobacteriophage infect them and incorporate the firefly luciferase gene into the bacterium. For fluorescence; D-luciferin is converted to oxy-Luciferin and light, and requires Mg^{2+} , O_2 , and ATP. This conversion is catalyzed by luciferase enzyme.

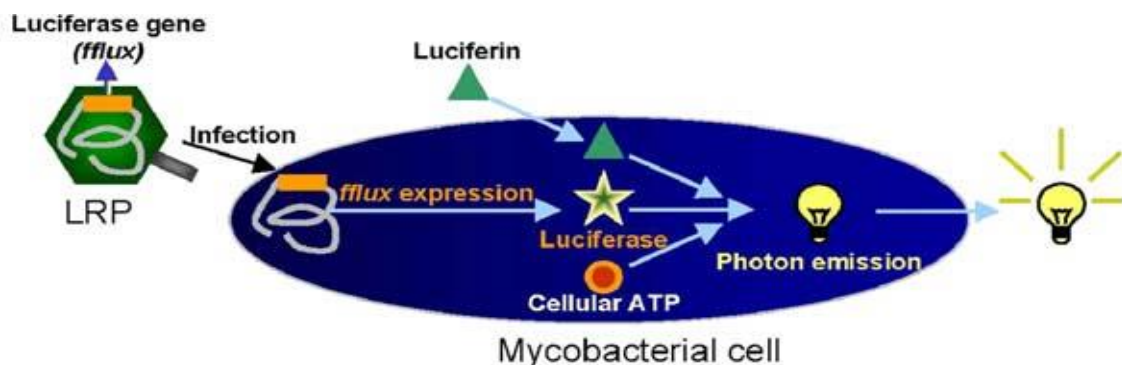
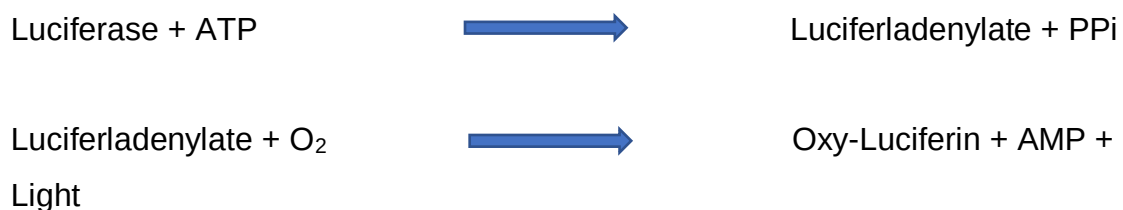


Fig 2.2: LRP assay principle

(<https://pubmed.ncbi.nlm.nih.gov/15495583/>)

This release of light is due to the excited Oxy-Luciferin returning to its ground state. TM4-based reporter phages expressing luciferase gene driven by promoters of Mycobacterial persistence genes were found to exhibit enhanced photon production in both dormant and actively growing *M. tuberculosis* cultures (Dusthacker *et al.*, 2008). Unlike other DST methods, LRP doesn't require much instrumentation and complicated protocols for the assay. Also, results can be generated rapidly and economically. Majority of the aforementioned assays can be performed only in highly equipped laboratories, but for LRP, small laboratories with containment facilities are enough. In our study we have focused on the Disc LRP result, with equal volume of D-Luciferin and reading were taken in luminometry to

determine the resistance and sensitivity of the culture with the comparison of the LJ culture. Once it is compared it can give a quicker result in one day and reduce the cost of DST furthermore. Kalaiselvi *et al.* (2017) explain the principles behind using the Luciferase Reporter Phage (LRP) assay for drug susceptibility testing in *Mycobacterium tuberculosis* (MTB). Light production in this assay relies on metabolically active MTB cells. When these cells are infected with LRPs, the reporter phages replicate, and the firefly luciferase gene they carry is expressed. This expression results in the emission of light. However, if the MTB strain is susceptible to a specific anti-TB drug being tested, the drug will inhibit its metabolic activity. Consequently, the reporter phages won't replicate within the bacteria, and no luciferase will be produced, leading to the absence of light. In contrast, drug-resistant MTB strains remain unaffected by the drugs and continue their metabolic processes. This allows the reporter phages to replicate normally, leading to luciferase production and light emission, similar to the control group that wasn't exposed to any drugs. By measuring the presence or absence of light, researchers can effectively determine the drug susceptibility of MTB strains using the LRP assay (Kalaiselvi *et al.*, 2017).

2.10.2 Isoniazid

Isoniazid, also known as iso-nicotinoyl hydrazide (INH), is an antibiotic used for the treatment of tuberculosis. It was discovered in 1952 and has since been approved for treatment of latent and active tuberculosis infections. For the active tuberculosis, it must be used in combination with other tuberculosis medications to prevent the development of drug resistance. Isoniazid is bactericidal to rapidly dividing Mycobacteria, but is bacteriostatic if the Mycobacteria are slow-growing. It inhibits the cytochrome P450 system and hence acts as a source of free radicals.



Fig 2.3: Isoniazid Structure

2.10.3 Rifampicin

Campbell *et al.* (2001) highlight the significance of Rifampicin, an antibiotic belonging to the rifamycin class. It is used to combat various bacterial infections, including tuberculosis, leprosy, and Legionnaire's disease. Rifampicin works by inhibiting RNA production in bacteria, effectively crippling their ability to function. Discovered in 1965, it was first marketed in Italy in 1968 and later approved for use in the United States in 1971. Recognizing its crucial role, the World Health Organization has included Rifampicin on its List of Essential Medicines, underlining its importance as a cornerstone of effective and safe healthcare practices. Prized for its potency and broad spectrum of activity against bacterial pathogens, Rifampicin stands as a key component in tuberculosis treatment regimens (Campbell *et al.*, 2001). Rifampicin is made by the soil bacterium *Ammycolatopsis rifamycinica*.

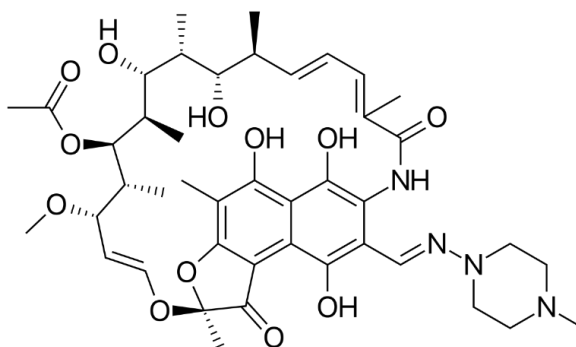


Fig 2.4: Rifampicin Structure

2.10.5 Luminometry

Luminometry is a technique by which the amount of light produced in a chemiluminescence or bioluminescence reaction can be determined by the help of luminometer, in which, the number of photons emitted were converted into electronic signal and measured in terms of Relative Light Units (RLU).



Fig 2.5: Luminometry

CHAPTER 3

AIM AND OBJECTIVE

AIM

To standardize and assess the methodology for rapid detection of *Mycobacterium tuberculosis* using the Photon-Max system, as well as to compare the standardized photon system's performance to existing methods.

OBJECTIVE

1. To develop an efficient, rapid detection of *Mycobacterium* using paper-based method.
2. To assess the methodology for the detection of various sensitive and resistant strains of *Mycobacterium tuberculosis*.
3. To compare the performance of the standardized photon system to existing *Mycobacterium* detection methods.
4. To assess the drug inhibition on *Mycobacterium tuberculosis*.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Middlebrook 7H9 Broth

Middlebrook 7H9 Broth with Glycerol is a supplemented medium which supports the growth of mycobacteria, including *M. tuberculosis*.

Table 4.1: Components of 7H9 broth

Ingredients	For 1000 ml	For 400 ml
Middlebrook 7H9 Broth	4.7 g	1.88 g
Distilled water	900 ml	360 ml
Glycerol	5 ml	2 ml

Mix the ingredients, autoclave and allow to cool. Add OADC to a final concentration of 10% to the broth.

4.1.2 Middlebrook 7H9 Agar

Table 4.2: Components of 7H9 agar

Ingredients	For 1000 ml	For 400 ml
Middlebrook 7H9 Broth	4.7 g	1.88 g
Agar (1%)	10g	4g
1M CaCl ₂	1ml	400µl
Glycerol	5 ml	2 ml
Distilled water	900 ml	360 ml

Mix the ingredients, autoclave and allow to cool. Pour the media onto a sterile plate and seal it with parafilm.

4.1.3 Soft Agar

The soft agar overlay method is a technique used to assess the presence of inhibitory compounds produced by bacteria.

Table 4.3: Components of soft agar

Ingredients	For 1000 ml	For 400 ml
Middlebrook 7H9 Broth	4.7 g	1.88 g
Agar (0.7%)	10g	4g
1M CaCl ₂	1ml	400µl
Glycerol	5 ml	2 ml
Distilled water	900 ml	360

4.1.4 Middlebrook 7H11 Agar

7H11 agar is a specialized culture medium used in microbiology laboratories for the isolation and cultivation of *Mycobacterium tuberculosis* (MTB). Mix the ingredients, autoclave at 121° C and 15psi pressure for 30-60 minutes. Once the broth has cooled down completely, place the media in the biosafety cabinet and add 10% OADC growth supplement (30ml for 300ml media). Pour the agar medium into petri plates (300ml gives approx. 20 plates) and store the sealed petri plates at 4° C.

Table 4.4: Components of 7H11 agar

Ingredients	For 1000 ml	For 400 ml
Middlebrook 7H9 Broth	4.7 g	1.88 g
Agar (1%)	10g	4g
1M CaCl ₂	1ml	400µl
Glycerol	5 ml	2 ml
Distilled water	900 ml	360 ml

4.1.5 Preparation of Isoniazid Drug in 7H9

Measure 0.01g isoniazid in a vial and add 1ml distilled water to attain 0.01g/ml concentration of isoniazid. To prepare working standard 1 (100 µg/ml), dissolve 10 µl of standard stock in 990 µl of complete 7H9. To attain 1 µg/ml concentration of isoniazid, dissolve 10 µl of working standard 1 in 990 µl of complete 7H9. Final concentration of Isoniazid 1 µg/ml is used for further experiments.

4.1.6 Preparation of Suspension

Take 2/3 loop of Mycobacterial colonies from the LJ medium. Transfer the bacterial inoculum from the loop into the sterile screw-cap glass tube (Bijou bottle). Gently vortex the suspension for 2 minutes after closing the bottle to ensure a homogenous suspension. Then add 600 µl of sterile, complete 7H9 and vortex again.

Finally, the suspension is allowed to settle for 5 minutes before proceeding with the experiments. This process ensures a well-mixed and standardized bacterial suspension for use in subsequent analyses.



Fig 4.1: Clinical Strains in LJ media

4.1.7 Preparation of 0.1M Calcium Chloride

Calcium chloride is an inorganic compound, a salt with the chemical formula CaCl_2 . It is a colourless crystalline solid at room temperature, highly soluble in water.

14.7g of CaCl_2 was dissolved in 100ml of distilled water. Then the contents were mixed and sterilized by autoclaving at 15lbs for 15 minutes. Dissolve 2ml of 1M CaCl_2 in 18ml of distilled water.

4.1.8 Preparation of MP Buffer

- Tris base
 - Concentrated HCL
 - Magnesium Chloride
 - calcium chloride
- **1M Tris:** 121 grams of Tris base was dissolved in 800ml of distilled water. The PH was adjusted using concentrated HCL and made up to 1000ml
 - **5M NaCl:** 292 grams of sodium chloride was dissolved in 1000ml of distilled water.
 - **1M MgCl_2 :** 203.3 grams of Magnesium Chloride was dissolved in 1000ml distilled water.
 - **1M CaCl_2 :** 147.02 grams of Calcium chloride in 1000ml of distilled water.

Table 4.5: Components of MP buffer

Ingredients	For 1000 ml
1M Tris	50ML
5M NaCl	30ml
1M MgCl ₂	10ml
1M CaCl ₂	2ml
Distilled water	8ml
Made up to	1000ml

4.1.9 Glass Filter Paper

Filter paper has various properties. The important parameters are wet strength, porosity, particle retention, volumetric flow rate, compatibility, efficiency and capacity. There are two mechanisms of filtration with paper; volume and surface. By volume filtration the particles are caught in the bulk of the filter paper. By surface filtration the particles are caught on the paper surface. Filter paper is mostly used because even a small piece of filter paper will absorb a significant volume of liquid. In my study we were used the surface filtration mechanism to absorb the reagents and sputum into paper with incubation time. Glass filter paper is made into micro volume disc with the help of metal hand paper punch machine.



Fig 4.2: Glass filter paper disc

4.1.10 Preparation of Sodium citrate (0.05M-pH: 4.5)

About 1.47g of tri-sodium citrate was dissolved in 100ml of distilled water with boiling if necessary. The PH was adjusted using citric acid crystals. Then it was autoclaved and stored at 4°C.

4.1.11 Preparation of D-Luciferin (0.33M)

One ml of sterile distilled water was added to 5mg of D-Luciferin and mixed properly. 400ml of D-Luciferin was dissolved in 19ml of 0.05M sodium citrate (PH 4.5) in sterile Mc Cartney bottle and 1ml of dimethyl sulfoxide (DMSO) was added. It was stored at 40°C. The solution will be stable for 1 week if properly refrigerated.

Note: D-luciferin is a light sensitive chemical. Hence, the Mc Cartney bottle should be covered fully with aluminium foil during preparations as well as storage.

4.1.12 Photon-Max System

The Photon-Max system detects and measures the emitted light from the paper platform. Photon-Max system is used for detecting viable bacteria. This utilizes the principle of LRP assay by detecting the photons and analyze the response of *Mycobacterium tuberculosis* (MTB) to drugs in the drug susceptibility testing (DST). Luciferase reporter phage (LRP) assay involves viable Mycobacteria infected with reporter phages expressing firefly luciferase gene. Luciferase is one of the popular reporter molecules used in molecular biology and biochemistry. Luciferase can be used to monitor promoter response activity in bacteria, cultured cells and transgenic plants or animals. Firefly luciferase catalyzes the production of yellow-green light from Luciferin in the presence of ATP, Mg²⁺, and O₂ at pH 7.8.

For luminometry, the phage cell mixture of 100 µl D-luciferin was taken in a star tube for conventional method and equal volume of D-luciferin were added for disc and drug method of 50 µl D-luciferin was added and mixed well. Then it was put into the photon-max system to measure LRP.



Fig 4.3: Photon-Max system

4.2 METHODS

4.2.1 Phage propagation

- Dispense 250 µl of MP buffer in cryovials
- Add 25 µl of the phage in the first vial. Mix and transfer 50 µl to the next
- Similarly, dilute the phage up to the desired dilution in the same buffer (select dilutions that is one above and one below the seed phage titre)
- Add 250 µl of bacterial suspension (*M. smegmatis*) equivalent to 2-3 Mc Farlands standard to the last three dilutions and incubate at 37°C for 30 minutes
- After incubation, take 200 µl from each dilution, mix with 5 ml of soft agar (should be at 45°C) separately and pour on to the 7H9 base medium
- Incubate the plates at 37°C for 18-24 hours
- Choose the plates showing lacey pattern of plaque formation to harvest the phages
- Pour 5 ml of MP buffer to the plates, shake for 2 hours at room temperature in rotatory shaker (-80RPM)
- Aspirate and filter the buffer using 0.45µm membrane filter
- Label the bottle containing the lysate with the name of the phage, dilution at which phage was harvested, date and store at 4°C

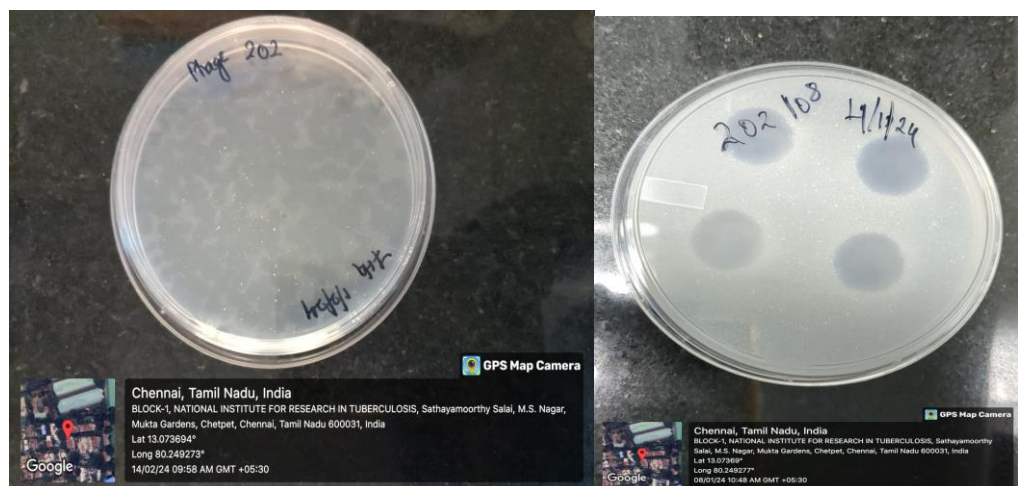


Fig 4 .4: Phage Propagation

4.2.2 Conventional Method

- Add 400µl of bacterial suspension, 50 µl of phage 202, and 20 µl of 0.1M CaCl₂ to a star tube.
- Then the star tube was plugged with non-absorbent cotton plug and kept in incubator for 3 hours for the growth of the *Mycobacterium tuberculosis*.
- After 3 hours of incubation, 100µl of this mixture is transferred to another star tube using 200ml pipette.
- Then add 100µl D-Luciferin in the same star tube.
- Finally reading is taken in photon-max system.

4.2.3 Paper Based Method

4.2.3.1 Control

- Glass filter paper disc were added to star tube inside the biosafety cabinet and labelled as control.
- Add 38µl of suspension with complete 7H9 to the disc.
- Then the star tubes were plugged with non-absorbent cotton plug and kept in incubator for two hours for the growth of the *Mycobacterium tuberculosis*.
- After two hours incubation, add 8µl phage 202 and 4µl 0.1M CaCl₂.
- The star tubes were plugged with non-absorbent cotton plug and incubated for 2 hours.
- After the incubation, 50µl D-luciferin was added to the disc.
- Finally, the reading is taken in photon-max system.

4.2.3.2 Procedure for DST of Isoniazid

- Glass filter paper disc were added to star tubes inside the biosafety cabinet and labelled as control.
- Label the star tubes as control, INH 0.1 µg/ml and INH 0.2 µg/ml.
- Add 3.8µl and 7.6µl of isoniazid from working stock 1 µg/ml to INH 0.1 µg/ml and INH 0.2 µg/ml tubes, respectively.
- Then 38µl and 30.5µl of mycobacterial suspension was added to discs, control and both the INH tubes respectively.
- The star tubes were plugged with non-absorbent cotton plugs and incubated for two hours to allow *Mycobacterium tuberculosis* to interact with isoniazid drug.
- After two hours incubation, add 8µl phage 202 and 4µl 0.1M CaCl₂.
- The star tubes were plugged with non-absorbent cotton plug and incubated for 2 hours.
- After the incubation, 50µl D-luciferin was added to the disc.
- Finally, the reading is taken in photon-max system.

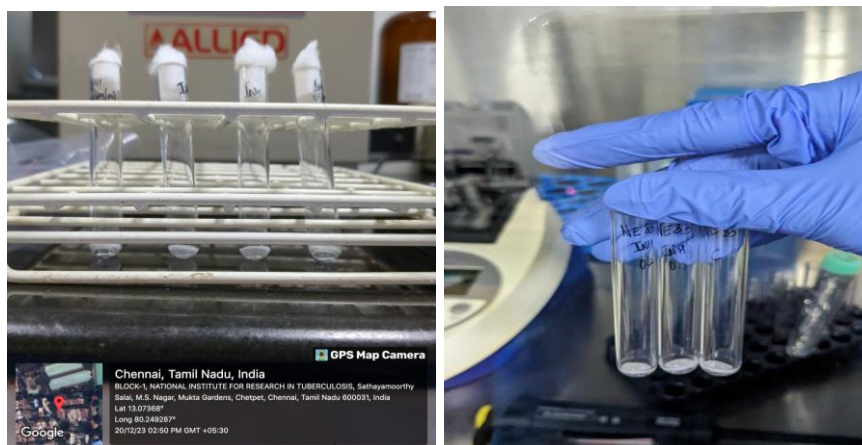


Fig 4.5: Isoniazid drug method

4.2.4 Whatman filter Vs Glass Filter

- Label two sets of star tubes as control, INH 0.1 µg/ml and INH 0.2 µg/ml for Whatman filter and glass filter.
- Glass filter and Whatman filter paper disc were added to star tubes inside the biosafety cabinet.
- To the control tubes, add 38µl of mycobacterial suspension on the disc.
- Then add 3.8µl and 7.6µl of isoniazid from working stock 1 µg/ml to INH 0.1 µg/ml and INH 0.2 µg/ml tubes, respectively.
- 30.5µl of mycobacterial suspension was added to isoniazid drug tubes.
- The star tubes were plugged with non-absorbent cotton plugs and incubated for two hours to allow *Mycobacterium tuberculosis* to interact with isoniazid drug.
- After two hours incubation, add 8µl phage 202 and 4µl 0.1M CaCl₂.
- The star tubes were plugged with non-absorbent cotton plug and incubated for 2 hours.
- After the incubation, 50µl D-luciferin was added to the disc.
- Finally, the reading is taken in photon-max system.

4.2.5 Two Discs Vs Three Discs in Glass Filter

- Label two sets of star tubes as control, INH 0.1 µg/ml and INH 0.2 µg/ml for 2 discs and 3 discs in glass filter.
- Glass filter paper disc were added to star tubes inside the biosafety cabinet.
- To the control tubes, add 38µl of mycobacterial suspension on the disc.
- Then add 3.8µl and 7.6µl of isoniazid from working stock 1 µg/ml to INH 0.1 µg/ml and INH 0.2 µg/ml tubes, respectively.
- 30.5µl of mycobacterial suspension was added to isoniazid drug tubes.
- The star tubes were plugged with non-absorbent cotton plugs and incubated for two hours to allow *Mycobacterium tuberculosis* to interact with isoniazid drug.
- After two hours incubation, add 8µl phage 202 and 4µl 0.1M CaCl₂.

- The star tubes were plugged with non-absorbent cotton plug and incubated for 2 hours.
- After the incubation, 50µl D-luciferin was added to the disc.
- Finally, the reading is taken in photon-max system.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 RESULTS

5.1.1 Comparison of Conventional and Paper- Based method in Photon-Max system

Table 5.1: Comparison of Conventional and Paper- Based method in Photon-max system

Method	Sample	Photon-Max Value RLU
Conventional	Control	28372
	INH 0.1 µg/ml	20118
	INH 0.2 µg/ml	16282
Paper-Based	Control	11228
	INH 0.1 µg/ml	648
	INH 0.2 µg/ml	393

In this experiment, both the conventional and paper-based methods utilized Rv suspension for readings. Interestingly, the paper-based method showed a significantly higher percentage of inhibition. This translates to more efficient drug interaction within the test, potentially leading to faster and more accurate results. Therefore, the paper-based method emerges as a more efficient and sustainable alternative to the conventional approach.

5.1.2 Comparison of Photon-max system with Berthold

Table 5.2: Comparison of Photon-max system with Berthold

Sample	Photon-Max Value RLU	Berthold
Blank	614	160
Control	11228	688
INH 0.1 µg/ml	648	344
INH 0.2 µg/ml	393	193

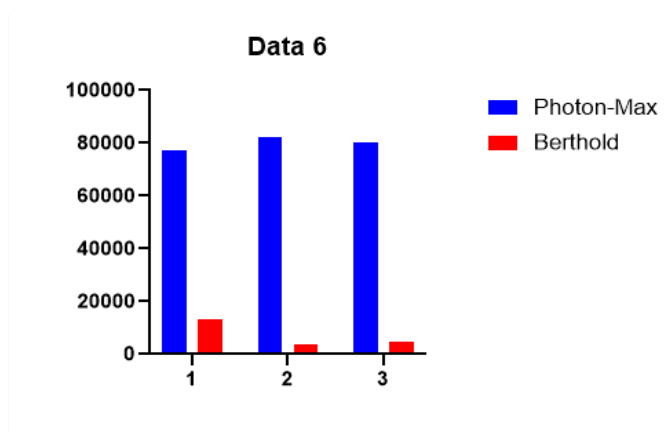


Fig 5.1: Comparison between Photon-Max system and Berthold

In this experiment, by utilizing Rv suspension readings were recorded in both Photon-max and Berthold system. Using a paired T-test with a significance level 0.05 (95% confidence), the differences between the Photon-max and Berthold system were evaluated, p-value of 0.0035 indicating statistically significant difference. The Photon-max system provided a good reading compared to Berthold system. This translates to more efficient drug interaction within the test, potentially leading to faster and more accurate results. Therefore, the photon-max system emerges as a more efficient alternative to Berthold.

5.1.3 Comparison of Whatman filter and glass filter discs in photon-max system

Table 5.3: Comparison of Whatmann filter and glass filter discs

Type of Disc	Sample	Photon-Max Value RLU	Berthold Value RLU
Whatman Filter	Control	9458	509
	INH 0.1 µg/ml	647	411
	INH 0.2 µg/ml	409	462
Glass Filter	Control	22284	1794
	INH 0.1 µg/ml	15476	1250
	INH 0.2 µg/ml	12594	877

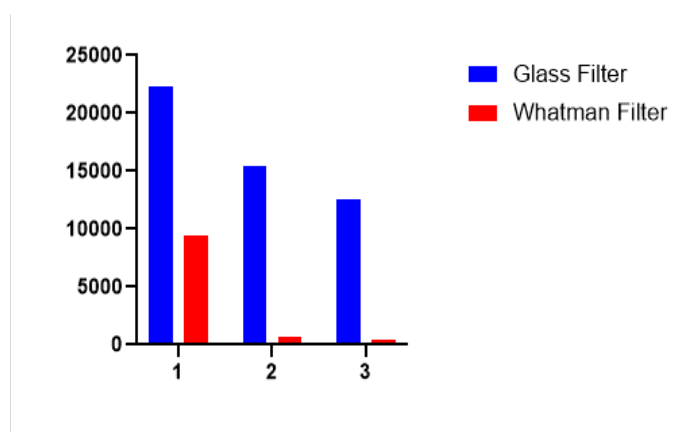


Fig 5.2: Comparison between Whatman Filter and Glass Filter

In this experiment, both the Whatman filter and the Glass filter paper used Rv suspension to take readings. Using a paired T-test with a significance level 0.05 (95% confidence), the differences between the Whatman and Glass Filter were evaluated, p-value of 0.0036 indicating statistically significant difference. In comparison to the Whatman Filter, the glass filter paper demonstrated a greater percentage of inhibition. As a result, Glass filter paper outperforms Whatman Filter paper in terms of efficiency and sustainability.

5.1.4 Comparison of number of discs in photon-max system

Table 5.4: Comparison of Conventional and Paper- Based method in Photon-max system

Sample	Photon-Max Value RLU
Blank	614
Conventional	9164
Control 2 discs	22284
Control 3 discs	713

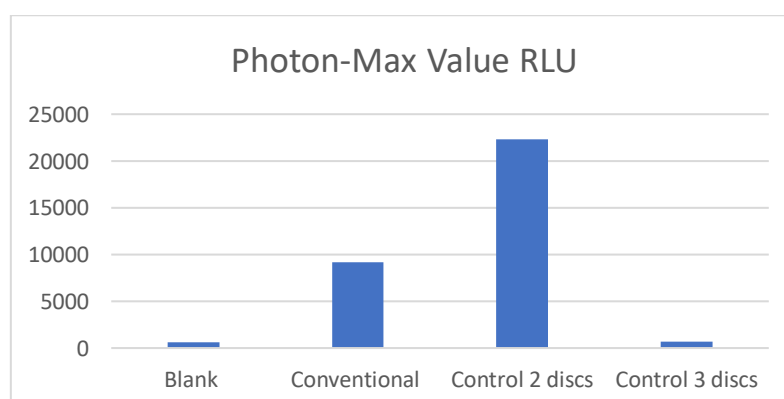


Fig 5.3: Comparison between number of Glass Filter disc

In this experiment, a number of discs made of glass filter paper were used for reading using Rv suspension. The readings from the two discs provided a better value for the control than the three discs. As a result, the experiments can be conducted using two discs from a glass filter.

5.1.5 Screening the 7H9 drug method for 50 clinical strains

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
Rv	Control	11228	688
	INH 0.1 µg/ml	648	344
	INH 0.2 µg/ml	393	193
HK 191	Control	11290	1205
	INH 0.1 µg/ml	12786	1004
	INH 0.2 µg/ml	9714	629
HK 056	Control	10832	736
	INH 0.1 µg/ml	655	299
	INH 0.2 µg/ml	680	266
DE 8407	Control	16838	2888
	INH 0.1 µg/ml	14760	1055
	INH 0.2 µg/ml	23890	2941
DE 7890	Control	10130	566
	INH 0.1 µg/ml	10480	774
	INH 0.2 µg/ml	9548	592
HS 051	Control	24422	2419
	INH 0.1 µg/ml	12968	713
	INH 0.2 µg/ml	699	163
DE 7320	Control	21116	1411
	INH 0.1 µg/ml	17076	978
	INH 0.2 µg/ml	14050	595

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system
(Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
DE 14989	Control	14280	1320
	INH 0.1 µg/ml	13948	619
	INH 0.2 µg/ml	657	469
HS 089	Control	12136	545
	INH 0.1 µg/ml	9092	237
	INH 0.2 µg/ml	10540	408
HK 184	Control	114562	6108
	INH 0.1 µg/ml	11776	518
	INH 0.2 µg/ml	39650	-
LL 32963	Control	9810	261
	INH 0.1 µg/ml	9516	335
	INH 0.2 µg/ml	663	135
DE 008	Control	36724	2481
	INH 0.1 µg/ml	23872	1312
	INH 0.2 µg/ml	13392	1566
LMT 061	Control	51468	4879
	INH 0.1 µg/ml	18938	1960
	INH 0.2 µg/ml	25490	1270
DE 010	Control	19958	1242
	INH 0.1 µg/ml	11728	998
	INH 0.2 µg/ml	15636	901

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system
(Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
LMT 066	Control	66036	4723
	INH 0.1 µg/ml	19522	2161
	INH 0.2 µg/ml	14080	1702
LMT 068	Control	71704	4248
	INH 0.1 µg/ml	41016	5767
	INH 0.2 µg/ml	16310	2144
LL28918	Control	34316	2374
	INH 0.1 µg/ml	15694	1528
	INH 0.2 µg/ml	16456	1277
DE R-58	Control	11160	682
	INH 0.1 µg/ml	9952	1076
	INH 0.2 µg/ml	6201	559
LMT 026	Control	9834	714
	INH 0.1 µg/ml	587	689
	INH 0.2 µg/ml	13746	839
DE 016	Control	18746	1135
	INH 0.1 µg/ml	15318	1522
	INH 0.2 µg/ml	12070	1311
NE 85	Control	12694	1224
	INH 0.1 µg/ml	11550	1150
	INH 0.2 µg/ml	12054	1388

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system
(Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
NP 33	Control	29216	2299
	INH 0.1 µg/ml	17750	1811
	INH 0.2 µg/ml	13432	933
NP 40	Control	28632	1994
	INH 0.1 µg/ml	17824	1841
	INH 0.2 µg/ml	29378	2598
NE 69	Control	27246	2108
	INH 0.1 µg/ml	13926	796
	INH 0.2 µg/ml	11744	1135
NP 37	Control	24288	1869
	INH 0.1 µg/ml	15014	755
	INH 0.2 µg/ml	13092	691
LL 7869	Control	24504	2104
	INH 0.1 µg/ml	18942	1061
	INH 0.2 µg/ml	11502	803
NP 28	Control	20112	2080
	INH 0.1 µg/ml	11316	1662
	INH 0.2 µg/ml	9484	759
NP 29	Control	30214	2489
	INH 0.1 µg/ml	16258	1193
	INH 0.2 µg/ml	10034	786

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system
(Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
NE 75	Control	37286	2813
	INH 0.1 µg/ml	24052	2102
	INH 0.2 µg/ml	19054	1025
LL 32963	Control	40184	2622
	INH 0.1 µg/ml	19872	1126
	INH 0.2 µg/ml	15830	838
LMT 71	Control	17742	922
	INH 0.1 µg/ml	13442	679
	INH 0.2 µg/ml	9134	500
LL 9372	Control	24886	1425
	INH 0.1 µg/ml	15512	1389
	INH 0.2 µg/ml	13724	834
LL26175	Control	14078	1817
	INH 0.1 µg/ml	23396	2819
	INH 0.2 µg/ml	20958	1304
NE 003	Control	23216	1688
	INH 0.1 µg/ml	15170	1102
	INH 0.2 µg/ml	20622	1536
NP 44	Control	43784	4170
	INH 0.1 µg/ml	30598	3811
	INH 0.2 µg/ml	21652	1499

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system
(Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
NE 015	Control	51294	3776
	INH 0.1 µg/ml	40118	2629
	INH 0.2 µg/ml	35028	1924
DE 011	Control	26488	1930
	INH 0.1 µg/ml	13328	1069
	INH 0.2 µg/ml	10224	839
NE 83	Control	20536	1623
	INH 0.1 µg/ml	13234	814
	INH 0.2 µg/ml	9364	602
NE 87	Control	16132	560
	INH 0.1 µg/ml	11180	373
	INH 0.2 µg/ml	9820	314
DE 15871	Control	41994	4005
	INH 0.1 µg/ml	38954	3007
	INH 0.2 µg/ml	28414	2103
LL 27085	Control	12664	753
	INH 0.1 µg/ml	10922	636
	INH 0.2 µg/ml	8902	486
HS 035	Control	47084	4228
	INH 0.1 µg/ml	43894	3990
	INH 0.2 µg/ml	30476	2334

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system
(Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
NE 002	Control	16276	879
	INH 0.1 µg/ml	12548	534
	INH 0.2 µg/ml	10952	484
DE 15490	Control	48218	3124
	INH 0.1 µg/ml	39046	2534
	INH 0.2 µg/ml	37964	1408
NE 70	Control	17218	880
	INH 0.1 µg/ml	15286	727
	INH 0.2 µg/ml	9124	332
NE 012	Control	14862	648
	INH 0.1 µg/ml	11332	562
	INH 0.2 µg/ml	10036	445
NP 35	Control	9578	745
	INH 0.1 µg/ml	336	263
	INH 0.2 µg/ml	749	188
NE 001	Control	25918	1722
	INH 0.1 µg/ml	21148	1470
	INH 0.2 µg/ml	15836	1102
LL 26046	Control	15701	951
	INH 0.1 µg/ml	12048	866
	INH 0.2 µg/ml	10818	501

Table 5.5: Readings for Clinical strains in Photon-Max and Berthold system (Cont.)

Sample	Drug conc.	Photon-Max RLU	Berthold RLU
NP 34	Control	12180	458
	INH 0.1 µg/ml	10534	363
	INH 0.2 µg/ml	9134	330

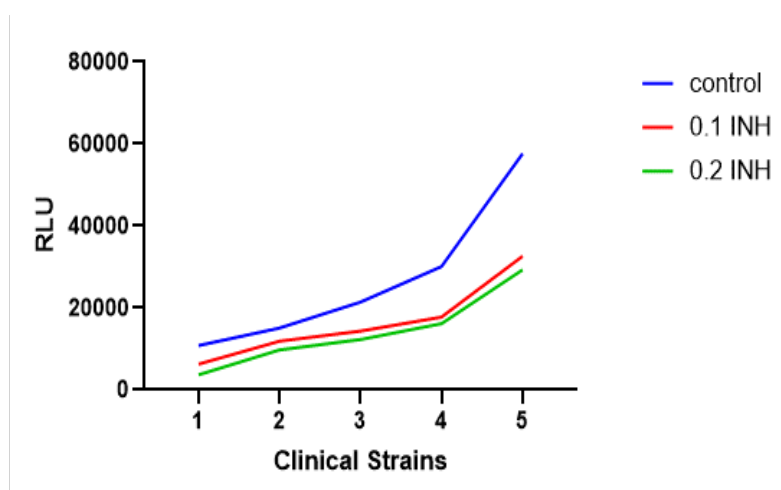


Fig 6.4: Evaluation of Drug Efficiency against a panel of Clinical TB Strains

In this experiment, a panel of clinical strains were used for reading using Glass filter paper. This experiment tests the effect of isoniazid drug on the final *Mycobacterium Tuberculosis*. Using ANOVA test with a significance level 0.05 (95% confidence), the differences between the control and drug concentration were evaluated, p-value of 0.0036 indicating statistically significant difference. The readings for the isoniazid test tube have shown a reduction compared to the control. As a result, the standardizations made in this experiment are working and more efficient compared to the conventional method.

5.1.6 Comparison of Photon-Max and Berthold system for 40% Inhibition in INH 0.1 µg/ml

		Photon-Max	
		S	R
Berthold	S	10	5
	R	5	30

Sensitivity in Photon-Max: 33%

Specificity in Photon-Max: 86%

5.1.7 Comparison of Photon-Max and Berthold system for 50% Inhibition in INH 0.1 µg/ml

		Photon-Max	
		S	R
Berthold	S	7	9
	R	3	31

Sensitivity in Photon-Max: 70%

Specificity in Photon-Max: 77%

5.1.8 Comparison of Photon-Max and Berthold system for 40% Inhibition in INH 0.2 µg/ml

		Photon-Max	
		S	R
Berthold	S	24	8
	R	4	14

Sensitivity in Photon-Max: 86%

Specificity in Photon-Max: 67%

5.1.9 Comparison of Photon-Max and Berthold system for 50% Inhibition in INH 0.2 µg/ml

		Photon-Max	
		S	R
Berthold	S	15	5
	R	6	24

Sensitivity in Photon-Max: 28%

Specificity in Photon-Max: 83%

5.2 DISCUSSIONS

The fight against the spread of Multidrug-Resistant Tuberculosis (MDR-TB) in resource-limited settings requires a simple, rapid, and affordable drug susceptibility test (Article et al., 2007). To control the spread of this disease, the most crucial facet of treatment is its early diagnosis. Whereas the existing WHO recommended assays such as solid and liquid based assays are either time consuming or very expensive (Alsayed & Gunosewoyo, 2023). In this context a paper based LRP assay using microfluidic approach of using a paper matrix has been developed for conducting chemical reactions which requires least possible volume of reagents and at least 6 to 8 times of less time to the detection of drug susceptibility of MTB against the important first-line drugs namely Isoniazid and Rifampicin. However, it required optimization of filter disc employed, time required for the incubation steps so as to make the assay to have a good sensitivity and specificity in comparison with the gold standard. So, in our study we have received a total of 50 cultures from pulmonary specimens from National Institute for Research in Tuberculosis (ICMR), Chennai. And we had compared DST results performed using Lowenstein Jensen (LJ) with the disc-based Luciferase reporter assay. LJ slopes are the solid culture-based media which requires 4 weeks of time for DST until then patients remains untreated or being put on a regimen without knowing the Drug Resistance pattern for the isolate with which he or she is being infected. LRP assay is one of the rapid assays which has been accepted for screening of newer antimicrobial compounds and is still in a research-scale when determining drug susceptibility testing (DST) for existing anti-TB drugs. There are some of the factors still needs to be standardized before it can be commercialized for determination of DST. This was evident in one of the concluded multicenter studies of the assay at NIRT. One of the factors which could be playing a vital role leading to discrepant results in the multicenter study will be the bacillary load and the age of the culture (cells in log phase are preferable). Bacillary load is measured in terms of the level of turbidity measured using McFarland turbidity standard. Hence it was aimed to optimize the turbidity required for DST using LRP assay.

Moreover, there were two LRP constructs used at NIRT namely phAE129 and phAETRC202. The former is a construct developed by Bill Jacobs's lab at Albert Einstein College of Medicine, New York and later at NIRT (Dusthacker et al., 2008). LRP construct PhAETRC202 as developed using a temperature sensitive mutant of Mycobacteriophage TM4, with the promoter of "act" gene to drive the luciferase gene, whereas phAE129 was developed from a lytic Mycobacteriophage D29. The Photon-Max has a sensor at the bottom of the slot allotted for the star-tubes. The sensor detects the number of photons released and converts it into an arbitrary scale of measurement called RLU (Relative Light Units). Since such a deviation from control vials occurs only in phAETRC202 infected samples, it can be hypothesized that, in a sample specific manner, there happens an up regulation or down-regulation of gene upon infection by phAETRC202, in such a way that either the doubling time is shortened or the Luciferase expressed is increased.

In addition to all of these, LRP assays are less time consuming and more economical. It can help the physicians to detect drug resistance earlier and initiate the correct treatment well before the infection becomes more severe. In this study various time point optimization was carried out for the bacterial exposure to the drug and for the phage cell mixture and the primary aim was to standardize the best reaction time combination which can achieve the maximum sensitivity, specificity, positive and negative predictive value.

The critical concentration of isoniazid, a primary anti-TB medicine, varies according to the method used. The World Health Organization (WHO) has recently modified the critical concentration for isoniazid susceptibility testing. The medium used includes Lowenstein-Jensen (0.2 µg/ml), Middlebrook 7H10 (0.2 µg/ml), Middlebrook 7H11 (0.2 µg/ml), and BACTEC™ Mycobacterial Growth Indicator Tube™ 960 (MGIT, 0.1 µg/ml). The WHO updated the isoniazid critical concentrations in inhA promoter mutations resulting in a small MIC increase (0.25-2 mg/L in MGIT).

We evaluated a standardized method for *Mycobacterium tuberculosis* (MTB) drug susceptibility testing using a 4-hour incubation period. Using 0.1 µg/ml INH with 50% inhibition demonstrated higher sensitivity (70%) and specificity (77%), compared to the gold standard. Increasing the INH concentration to 0.2 µg/ml

increased specificity (83%) but reduced sensitivity (28%). Lowering the inhibition threshold to 40% caused a trade-off between sensitivity and specificity for INH 0.1 µg/ml and 0.2 µg/ml. The results for 0.1 µg/ml were 33% sensitivity and 86% specificity, while for 0.2 µg/ml it was 86% sensitivity and 67% specificity.

The strain is stated to be sensitive when the percentage inhibition exceeds 50%, and resistant when the percentage inhibition is less than 50%. The strains that exhibited more than 40% were likewise classified as sensitive. Standardized parameters such as bacterial load, disc type, medication concentration, and culture age can yield varying outcomes. The 40% inhibition threshold in INH 0.2 µg/ml resulted in the highest sensitivity and specificity. There are some of the factors still needs to be standardized before it can be commercialized for determination of DST. Further optimization is crucial to improve the sensitivity and specificity of drugs and to achieve results comparable to established methods.

The study addressed the critical need for a rapid and affordable drug susceptibility test (DST) for *Mycobacterium tuberculosis*, particularly in resource-limited settings. The paper-based LRP assay using a microfluidic approach offers promise for faster detection of drug resistance to first-line drugs like Isoniazid and Rifampicin compared to traditional methods. While the research demonstrates promising results with a standardized protocol achieving good sensitivity and specificity, further optimization is required, particularly regarding factors like turbidity, incubation times, and LRP construct selection. Once these refinements are made, this paper-based LRP assay has the potential to revolutionize tuberculosis diagnosis by enabling faster treatment initiation and improved patient outcomes, especially in combating the spread of Multidrug-Resistant Tuberculosis. Overall, the paper-based LRP assay represents a significant step forward for both environmental and healthcare aspects of tuberculosis control. By enabling faster diagnosis and treatment initiation, it can contribute to breaking the cycle of TB transmission and improving public health outcomes, particularly in vulnerable populations.

CHAPTER 6

SUMMARY AND CONCLUSION

In conclusion, tuberculosis (TB) remains a significant global health threat, particularly the emergence of multidrug-resistant (MDR-TB) strains. While TB is treatable and curable, improper treatment or infection by a resistant strain can lead to MDR-TB, posing a greater challenge. Despite progress in reducing TB mortality rates, millions are still infected annually. Resource-limited settings often face the highest burden, highlighting the need for improved diagnostic tools and treatment strategies. The ongoing fight against TB requires a multi-pronged approach, including early diagnosis, adherence to treatment regimens, and the development of new methods to combat drug resistance. This introduction effectively sets the stage for further discussion on potential solutions to address the challenges posed by TB.

In our study, we performed LRP for around 50 strains, and they gave considerable RLU values. From that RLU values the percentage inhibition was calculated. The strain is stated to be sensitive when the percentage inhibition is greater than 50% and as resistance, the percentage inhibition is lesser than 50%. Then we compared the results of the LRP, with an incubation time of 4 hours for the Photon-Max and Berthold systems. From comparing the results obtained, we concluded that Isoniazid 0.2 µg/ml with 40 % inhibition results is better than results obtained from the 50% inhibition threshold.

Luciferase Reporter Mycobacteriophage assay is one of the most rapid methods for tuberculosis diagnosis. After successful standardization of this method, this can be introduced in real-time fields such as hospitals and early detection of DST to control the spread of MDR-TB. An LRP assay only requires a mere capital investment in the form of a luminometer. This approach holds promise for faster diagnosis and treatment initiation in the fight against drug-resistant TB. However, further optimization is crucial to improve the sensitivity and specificity of the assay and to achieve results comparable to established methods.

REFERENCES

Acharya, S., Ghimire, P., Khadka, D., & Nepali, S. (2010). Comparison of Proportion and Resistance Ratio Methods for Drug Susceptibility Testing of *Mycobacterium tuberculosis* Isolated from Patients Attending National Tuberculosis Centre, Nepal. *SAARC Journal of Tuberculosis, Lung Diseases and HIV/AIDS*, 5(1), 13–20. <https://doi.org/10.3126/SAARCTB.V5I1.3078>

Article, O., Mathew, S., Nalini, S. M., Rahman, F., Dastageer, A., Sundaram, V., & Paramasivan, C. N. (2007). SIMPLE DIRECT DRUG SUSCEPTIBILITY TESTS ON SPUTUM SAMPLES FOR EARLY DETECTION OF RESISTANCE IN TUBERCLE BACILLI. *Indian J Tuberc*, 54, 184–189.

Babiarz, K. S., Suen, S. C., & Goldhaber-Fiebert, J. D. (2014). Tuberculosis treatment discontinuation and symptom persistence: an observational study of Bihar, India's public care system covering >100,000,000 inhabitants. *BMC Public Health*, 14(1), 418. <https://doi.org/10.1186/1471-2458-14-418>

Barberis, I., Bragazzi, N. L., Galluzzo, L., & Martini, M. (2017). The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. *Journal of Preventive Medicine and Hygiene*, 58(1), E9. [/pmc/articles/PMC5432783/](https://doi.org/10.1007/s10086-017-1583-1)

Birinci, A., Coban, A. Y., Ekin, B., & Durupinar, B. (2002). Comparison of the proportion method with mycobacteria growth indicator tube and E-test for susceptibility testing of *Mycobacterium tuberculosis*. *Memorias Do Instituto Oswaldo Cruz*, 97(3), 351–352. <https://doi.org/10.1590/S0074-02762002000300013>

Branchini, B. R., Magyar, R. A., Marcantonio, K. M., Newberry, K. J., Stroth, J. G., Hinz, L. K., & Murtiashaw, M. H. (1997). Identification of a firefly luciferase active site peptide using a benzophenone-based photooxidation reagent. *Journal of Biological Chemistry*, 272(31), 19359–19364. <https://doi.org/10.1074/jbc.272.31.19359>

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., & Darst, S. A. (2001). Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell*, 104(6), 901–912. [https://doi.org/10.1016/S0092-8674\(01\)00286-0](https://doi.org/10.1016/S0092-8674(01)00286-0)

Caulfield, A. J., & Wengenack, N. L. (2016). Diagnosis of active tuberculosis disease: From microscopy to molecular techniques. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases*, 4, 33–43. <https://doi.org/10.1016/J.JCTUBE.2016.05.005>

Chowdhury, K., Ahmad, R., Sinha, S., Dutta, S., & Haque, M. (2023). Multidrug-Resistant TB (MDR-TB) and Extensively Drug-Resistant TB (XDR-TB) Among Children: Where We Stand Now. *Cureus*, 15(2). <https://doi.org/10.7759/CUREUS.35154>

Daniel, T. M. (2006). The history of tuberculosis. *Respiratory Medicine*, 100(11), 1862–1870. <https://doi.org/10.1016/j.rmed.2006.08.006>

Driscoll, A. J., Bhat, N., Karron, R. A., O'Brien, K. L., & Murdoch, D. R. (2012). Disk diffusion bioassays for the detection of antibiotic activity in body fluids: applications for the Pneumonia Etiology Research for Child Health project. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, 54 Suppl 2(SUPPL. 2). <https://doi.org/10.1093/CID/CIR1061>

Dunn, J. J., Starke, J. R., & Revell, P. A. (2016). Laboratory Diagnosis of Mycobacterium tuberculosis Infection and Disease in Children. *Journal of Clinical Microbiology*, 54(6), 1434–1441. <https://doi.org/10.1128/JCM.03043-15>

Dusthacker, A., Kumar, V., Subbian, S., Sivaramakrishnan, G., Zhu, G., Subramanyam, B., Hassan, S., Nagamaiah, S., Chan, J., & Paranj Rama, N. (2008). Construction and evaluation of luciferase reporter phages for the detection of active and non-replicating tubercle bacilli. *Journal of Microbiological Methods*, 73(1), 18–25. <https://doi.org/10.1016/J.MIMET.2008.01.005>

Hanson, C. W., & Martin, W. J. (1978). Modified agar dilution method for rapid antibiotic susceptibility testing of anaerobic bacteria. *Antimicrobial Agents and Chemotherapy*, 13(3), 383–388. <https://doi.org/10.1128/AAC.13.3.383>

Harvell, J. D., Hadley, W. K., & Ng, V. L. (2000). Increased sensitivity of the BACTEC 460 mycobacterial radiometric broth culture system does not decrease the number of respiratory specimens required for a definitive diagnosis of pulmonary tuberculosis. *Journal of Clinical Microbiology*, 38(10), 3608–3611. <https://doi.org/10.1128/JCM.38.10.3608-3611.2000>

Hedley-Whyte, J., & Milamed, D. R. (2009). Lobar pneumonia treated by Musgrave Park physicians. *The Ulster Medical Journal*, 78(2), 119. [/pmc/articles/PMC2699200/](https://pubmed.ncbi.nlm.nih.gov/2699200/)

Heemskerk, D., Caws, M., Marais, B., & Farrar, J. (2015). *Tuberculosis in Adults and Children*. 2. <https://doi.org/10.1007/978-3-319-19132-4>

Jang, J. G., & Chung, J. H. (2020). Diagnosis and treatment of multidrug-resistant tuberculosis. *Yeungnam University Journal of Medicine*, 37(4), 277. <https://doi.org/10.12701/YUJM.2020.00626>

Jing, W., Zhao, W., Liu, S., Li, L., Tsai, C. T., Fan, X., Wu, W., Li, J., Yang, X., & Sui, G. (2013). Microfluidic device for efficient airborne bacteria capture and enrichment. *Analytical Chemistry*, 85(10), 5255–5262. <https://doi.org/10.1021/AC400590C>

Kalaiselvi, K., Mangayarkarasi, V., Gomathi, N. S., Balaji, S., Mane, S. R., & Shunmugam, R. (2017). LUCIFERASE REPORTER MYCOBACTERIOPHAGES FOR EVALUATING NORBORNENE-BASED ANTITUBERCULOSIS DRUG SUSCEPTIBILITY TESTING ON MYCOBACTERIUM TUBERCULOSIS. *Asian Journal of Pharmaceutical and Clinical Research*, 10(9), 406–408. <https://doi.org/10.22159/AJPCR.2017.V10I9.19660>

Khalifa, R. A., Nasser, M. S., Gomaa, A. A., Osman, N. M., & Salem, H. M. (2013). Resazurin Microtiter Assay Plate method for detection of susceptibility of multidrug resistant Mycobacterium tuberculosis to second-line anti-tuberculous drugs. *Egyptian Journal of Chest Diseases and Tuberculosis*, 62(2), 241–247. <https://doi.org/10.1016/J.EJCDT.2013.05.008>

Khan, A. N., Al-Jahdali, H. H., Allen, C. M., Irion, K. L., Al Ghanem, S., & Koteyar, S. S. (2010). The calcified lung nodule: What does it mean? *Annals of Thoracic Medicine*, 5(2), 67. <https://doi.org/10.4103/1817-1737.62469>

Kumar, K., Giribhattanavar, P., Sagar, C., & Patil, S. (2018). A rapid and simple resazurin assay to detect minimum inhibitory concentrations of first-line drugs for Mycobacterium tuberculosis isolated from cerebrospinal fluid. *Journal of Global Antimicrobial Resistance*, 12, 157–161. <https://doi.org/10.1016/J.JGAR.2017.09.012>

Lee, W. G., Kim, Y. G., Chung, B. G., Demirci, U., & Khademhosseini, A. (2010). Nano/Microfluidics for diagnosis of infectious diseases in developing countries. *Advanced Drug Delivery Reviews*, 62(4–5), 449–457. <https://doi.org/10.1016/J.ADDR.2009.11.016>

McIntosh, J. (2017). *Tuberculosis (TB): Symptoms, treatment, diagnosis, and more*. Medical News Today. <https://www.medicalnewstoday.com/articles/8856>

McNerney, R., Cunningham, J., Hepple, P., & Zumla, A. (2015). New tuberculosis diagnostics and rollout. *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases*, 32, 81–86. <https://doi.org/10.1016/J.IJID.2015.01.012>

Middlebrook, G., & Cohn, M. L. (n.d.). *BACTERIOLOGY OF TUBERCULOSIS: LABORATORY METHODS*.

Palomino, J. C., Martin, A., Camacho, M., Guerra, H., Swings, J., & Portaels, F. (2002). Resazurin Microtiter Assay Plate: Simple and Inexpensive Method for Detection of Drug Resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, 46(8), 2720. <https://doi.org/10.1128/AAC.46.8.2720-2722.2002>

Sia, J. K., & Rengarajan, J. (2019). Immunology of Mycobacterium tuberculosis infections. *Microbiology Spectrum*, 7(4). <https://doi.org/10.1128/MICROBIOLSPEC.GPP3-0022-2018>

Somphavong, S. (n.d.). *Molecular epidemiology of Mycobacterium tuberculosis and antibiotic resistance in Lao PDR*. Retrieved March 26, 2024, from <https://theses.hal.science/tel-02100060>

Subramanyam, B., Sivaramakrishnan, G. N., Dusthacker, A., Nagamiah, S., & Kumar, V. (2012). Phage lysin as a substitute for antibiotics to detect Mycobacterium tuberculosis from sputum samples with the BACTEC MGIT 960 system. *Clinical Microbiology and Infection*, 18(5), 497–501. <https://doi.org/10.1111/J.1469-0691.2011.03601.X>

Tan, Z. M., Lai, G. P., Pandey, M., Srichana, T., Pichika, M. R., Gorain, B., Bhattamishra, S. K., & Choudhury, H. (2020). Novel Approaches for the Treatment of Pulmonary Tuberculosis. *Pharmaceutics*, 12(12), 1–54. <https://doi.org/10.3390/PHARMACEUTICS12121196>

Tortoli, E., Benedetti, M., Fontanelli, A., & Simonetti, M. T. (2002). Evaluation of automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to four major antituberculous drugs: comparison with the radiometric BACTEC 460TB method and the agar plate method of proportion. *Journal of Clinical Microbiology*, 40(2), 607–610. <https://doi.org/10.1128/JCM.40.2.607-610.2002>

Training manual M tuberculosis C DST:: Ministry of Health and Family Welfare. (n.d.). Retrieved March 22, 2024, from <https://tbcindia.gov.in/showfile.php?lid=2991>

Tsara, V., Serasli, E., & Christaki, P. (2009). Problems in diagnosis and treatment of tuberculosis infection. *Hippokratia*, 13(1), 20. [/pmc/articles/PMC2633248/](https://pubmed.ncbi.nlm.nih.gov/19404448/)

Van Klinger, B., Dessens-Kroon, M., Van Der Laan, T., Kremer, K., & Van Soolingen, D. (2007). Drug susceptibility testing of *Mycobacterium tuberculosis* complex by use of a high-throughput, reproducible, absolute concentration method. *Journal of Clinical Microbiology*, 45(8), 2662–2668. <https://doi.org/10.1128/JCM.00244-07>

Whitesides, G. M. (2006). The origins and the future of microfluidics. *Nature* 2006 442:7101, 442(7101), 368–373. <https://doi.org/10.1038/nature05058>

Wilson, M. L. (2011). Recent advances in the laboratory detection of *Mycobacterium tuberculosis* complex and drug resistance. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 52(11), 1350–1355. <https://doi.org/10.1093/CID/CIR146>

Zimmer, A. J., Heitkamp, P., Malar, J., Dantas, C., O'Brien, K., Pandita, A., & Waite, R. C. (2021). Facility-based directly observed therapy (DOT) for tuberculosis during COVID-19: A community perspective. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases*, 24, 100248. <https://doi.org/10.1016/J.JCTUBE.2021.100248>

ANNEXURE



GRADUATE APTITUDE TEST IN ENGINEERING 2024

अभियांत्रिकी स्नातक अभिक्षमता परीक्षा २०२४

ORGANISING INSTITUTE: INDIAN INSTITUTE OF SCIENCE, BENGALURU

SCORE CARD

Name of the Candidate

MAHALAKSHMI RAJAN

Name of the Parent/Guardian

RAJAN K

Registration No.

BT24S57401464

Test Paper

Biotechnology (BT)

Date of Examination

February 10, 2024

GATE Score

188

Marks out of 100

28.0

All India Rank (AIR)
in the test paper

6943

Qualifying Marks

General

38.9

Number of candidates
appeared for the test paper

17078

EWS/OBC-NCL

35.0

SC/ST/PwD

25.9



R. Malli

Prof. Chandra Sekhar Seelamantula

Prof. Chandra Sekhar Seelamantula
Organising Chairperson, GATE 2024
On behalf of NCB-GATE
Ministry of Education (MoE)



86c9881c677fe63d72ca2cf4675b487

A candidate is considered **qualified** if the marks secured are greater than or equal to the qualifying marks mentioned for the category, for which a valid category certificate, if applicable, must be produced along with this Score Card.

This Score Card is valid
up to 31st March 2027.

GATE SCORE COMPUTATION

The GATE 2024 score is calculated using the formula

$$\text{GATE Score} = S_q + (S_t - S_q) \frac{(M - M_q)}{(M_t - M_q)}$$

where

M is the marks obtained by the candidate in the paper mentioned on the GATE 2024 Score Card

M_q is the qualifying marks for general category candidates in the paper

M_t is the mean of marks of top 0.1% or top 10 (whichever is larger) of all the candidates who appeared for the test paper

$S_q = 350$, is the score assigned to M_q

$S_t = 900$, is the score assigned to M_t

M_q is 25 marks (out of 100) or $\mu + \sigma$, whichever is greater. Here, μ is the mean and σ is the standard deviation of marks of all the candidates who appeared for the test paper.

Qualifying in GATE 2024 does not guarantee admission to a postgraduate program or scholarship/financial assistance. Admitting institutes may conduct additional tests or interviews for final selection of candidates.

Graduate Aptitude Test in Engineering (GATE) 2024 was organised by Indian Institute of Science, Bengaluru, on behalf of National Coordination Board (NCB) - GATE for the Department of Higher Education, Ministry of Education (MoE), Government of India.



Georgia Institute of Technology

March 08, 2024

Mahalakshmi Rajan
gtID: 904046616

Dear Mahalakshmi,

Congratulations on being accepted into the prestigious Masters in Bioinformatics (Biology) program at Georgia Tech. We are pleased to offer you admission for the Fall 2024 semester on the Atlanta campus.

Admission to this program is highly selective and your qualifications and achievements have set you apart from the other applicants. We are confident that you will not only excel academically but also thrive within our dynamic community.

At Georgia Tech, you will become a part of a diverse and inclusive student body where each member provides a unique perspective to our collaborative ecosystem. Tech has proudly led the way in research and innovation for over 135 years, and we believe that your contributions here will be a catalyst for positive change. We invite you to [discover all that our Atlanta campus](#) has to offer. Located in the heart of a diverse and vibrant metropolitan neighborhood, you will find a wealth of opportunities for education, entertainment, and professional experience.

Once again, congratulations on your remarkable achievement. Please indicate your enrollment intentions by logging into your [Application Status Portal](#) and completing the Reply to Offer of Admission form as soon as possible.

If you have any questions regarding courses, financial assistance, or orientation, please [contact your admitting program](#).

We are committed to your success and look forward to welcoming you to the Georgia Tech graduate community. Please refer to the second page for the terms and conditions of your admission.

Regards,

A handwritten signature in black ink that reads "Joy O. Olabisi".

Dr. Joy Olabisi
Assistant Vice Provost for Graduate Education
Executive Director of Graduate Admissions

Dear Mahalakshmi,

Congratulations! It is our pleasure to offer you admission to the Master of Science in Bioinformatics program in the College of Science. We are excited for you to begin your studies in the Fall 2024 term at the Boston campus.

The Admissions Committee was impressed with your academic and personal achievements and believes you will make an excellent contribution to the program. We have confidence in your future success and look forward to embarking on this journey with you.

To secure your place, please visit your [applicant portal](#) to complete your enrollment form and submit your tuition deposit as indicated by **July 15, 2024**.

ABOUT THE COLLEGE OF SCIENCE

Now more than ever, science and technology are essential skills. The College of Science's physical sciences, life sciences, and mathematics programs challenge you to think in ways you may not have considered before. Our unique programs include an industry-based curriculum and the technical and soft skills needed to pursue careers in business, data, and regulatory fields. You will stand at the forefront of discovery, invention, and innovation, enabling you to pursue your desired career path in academia or industry. If you have questions regarding this admission offer, registration, curriculum, or other aspects of your enrollment, we are here to help. You will find our direct contact information listed on your [applicant portal](#).

We look forward to welcoming you to the Northeastern University community.

Sincerely,



Carla Mattos, Ph.D.
Associate Dean for Graduate Affairs and Professional Programs
College of Science

Decision on submission to Journal of Biotechnology Inbox x



em@editorialmanager.com

to me ▾



Manuscript Number: JBIOTEC-D-23-00737

Phage Therapy for antibiotic-resistant infections and strategies to overcome phage resistance

Dear Phage therapy and resistance R,

Thank you for submitting your manuscript to Journal of Biotechnology. After careful evaluation, I regret to inform you that your manuscript does not fit within the scope of the journal, and I must therefore reject it.

I hope you are successful in finding an alternative publication for your work. For alternative journals that may be more suitable for your manuscript, please refer to our Journal Finder (http://journalfinder.elsevier.com?dgcid=eman-jf-editor-reject-norev_email).

We appreciate you submitting your manuscript to Journal of Biotechnology and thank you for giving us the opportunity to consider your work.

Kind Regards,

Christoph Wilhelm Sensen, Dr. rer. nat.

Chief Editor

Journal of Biotechnology