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MYCOBACTERIUM TUBERCULOSIS RAPID SENSING VIA IMPROVED AND RATED MULTIPLEX POLYMER CHAIN RESPONSE

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Abstract:
This thesis serves as a showcase for the research techniques that were used to the study of the Mycobacterium tuberculosis complex. The identification of Mycobacterium tuberculosis was the primary emphasis, with the medicines pattern analysis providing a glimpse into the thesis analysis. Information was obtained at random between 2020 and 2023. A total of 158 participants across a wide age range participated in the study. High rates of first-line anti-tuberculosis medications were also seen in the study's follow-up. The case of Mycobacterium tuberculosis is summarised. Mycobacterium tuberculosis, when not linked to a host cell, takes on a thin, rod-shaped structure similar to the mycelium of a fungus. A rod between three and thirty centimetres in length, it might be either straight or slightly curved. Having a solitary, couple, or small group existence. Its final dimensions are established by the environment in which it develops. In damp places, they progressively grow into a pellicle-like mould. Mycobacterium is the term given to bacteria because of their similarity to fungus. They are oxygen-tolerant but incapable of locomotion or encapsulation. It is an obligate aerobe, a saprophyte, and an opportunistic pathogen. Evidence of TB-related degeneration in spinal column fragments from 2400 BCE Egyptian mummies shows that M. tuberculosis has been present in human populations for thousands of years. In 1650, tuberculosis (TB) was known as "consumption," and it was recognized as the leading cause of mortality.

Keywords: Multiplex Polymer Chain, Rapid Sensing, *Mycobacterium Tuberculosis*, Polymerase Chain Reaction, TB

1. INTRODUCTION:

Tuberculosis, sometimes referred to as tuberculosis TB, is an infectious illness that has been around for millennia and has since spread to almost every region of the world. Its common abbreviation is tuberculosis TB. The continued spread of tuberculosis (TB) represents a severe threat to the health of the general population. Some estimates place the number of people infected with tuberculosis somewhere in the area of one third of the total population of the world. This hope was dashed with the discovery of the infection caused by the Human Immunodeficiency Virus (HIV) and the Acquired Immunodeficiency Syndrome (AIDS), both of which contributed to the rise in the prevalence of tuberculosis throughout the globe. Both of these factors were contributors to the TB outbreak that occurred all across the world. In April of 1993, the World Health Organisation (WHO) announced

that TB was a worldwide public health emergency. This was done due to the significant effect that tuberculosis was having all over the world at the time. This step was taken as a response to the significant effect that tuberculosis was having. According to the World Health Organization's (WHO) report on global tuberculosis, which was published in 2016, there were an estimated 10.4 million new cases of TB around the world in 2015. It is unconscionable that tuberculosis claims the lives of so many individuals every single year. It is believed that is responsible for one-fourth of the total number of cases of tuberculosis that are registered each year across the whole globe. In 2015, it is projected that there will be 2.8 million new cases of tuberculosis infections. In addition, multidrug-resistant TB was found in 2.5% of newly diagnosed cases of tuberculosis in the year 2015, as well as in 16% of patients who had previously been treated for tuberculosis. M bacteria are distinguished from other types of bacteria in that they thrive in an aerobic environment, are gram-positive, do not move around, and take the shape of rods. TB. It does not have a capsule, and it does not generate spores, which are rod-like structures with widths between 1.0 and 10 micrometres (m), lengths between 0.2 and 0.6 m, and the ability to branch at any point along their length. Neither of these characteristics are present in this organism. It is characterised by a thick and convoluted cell wall that is rich in lipids and is often related with the aetiology of the illness. This is one of its distinguishing characteristics. In addition to this, the progression of it is being significantly slowed down by the mycobacteria. The infectious agent that causes tuberculosis in humans is termed tuberculosis itself, and this is the case in the vast majority of cases. It is conceivable that different types of mycobacteria might be to blame for this sickness in certain persons, as well as in some particular conditions. This could be the case in some specific people. Mycobacterium tuberculosis complex 3, which is composed of the components of Mycobacterium tuberculosis complex (MTBC), is made up of mycobacteria that are already members of the complex and hence contribute to its formation. There is another kind of mycobacteria that is referred to as nontuberculous mycobacterium (NTM), in addition to the mycobacteria that cause tuberculosis (TB). It is very necessary to bear in mind that there are two different kinds of mycobacteria known as NTM. They are present in every area of the environment, and under certain conditions, such as when a person's immune system is weakened, some of them have the potential to become pathogenic for humans and cause illnesses that are caused by opportunistic factors. This potential is only activated when the immune system of a person is compromised. There are over 200 distinct species of mycobacteria that have been discovered up to this point, with the vast majority of them consisting of bacteria that are completely safe for humans to interact with and that are found in soil. According to Kashyap et al.'s research from 2021, just a very small percentage of these species are responsible for the illness that is known as mycobacterial sickness (Wang et al., 2018|).

When a person who has pulmonary or laryngeal tuberculosis coughs, sneezes, sings, or speaks, droplet nuclei are produced. These droplet nuclei are what cause tuberculosis to spread from one person to another via the air. The nuclei of droplets are the agents that are responsible for the disease's dissemination. In addition, the utilisation of aerosol treatments, the induction of sputum, and the utilisation of aerosolization during bronchoscopy, as well as the manipulation of lesions or the processing of tissue or secretion at a medical facility or laboratory, all have the potential to contribute to the propagation of the infection. Air currents, which are generally present in any indoor environment, have the capacity to maintain droplets suspended in the air for an extraordinarily lengthy amount of time because the nuclei of droplets are so small. The nuclei of droplets, after they have been inhaled, travel down the bronchial tree and get lodged in the respiratory bronchiole or alveolus. The term "inhalation" refers to this procedure. Both the virulence of the tubercle bacillus and the innate microbicidal activity of the alveolar macrophage that ingests it play a role in the determination of whether or not an inhaled tubercle bacillus will result in the formation of an infection in the lung. Both of these aspects have a significant role in establishing the final result. In order to provide a diagnosis in a relatively short amount of time, numerous techniques, such as the enzyme-linked immunosorbent test enzyme-linked immunosorbent assay ELISA, real-time polymerase chain reaction (PCR), latex agglutination, and Gen-Probe amplified M. smegmatis, have been developed. There is now a therapy available for tuberculosis (TB). testing both directly and indirectly for the

presence of tuberculin, followed by cytometric evaluation of the findings. The death rate that is connected with tuberculosis is much higher than the mortality rate that is connected with other infectious illnesses. In order to keep up with the increased frequency of drug- and multidrug-resistant M. tuberculosis, diagnostic techniques that are not only quicker, but also more accurate, are required. There is a possibility that the plasma membrane that encases the Mycobacterium tuberculosis bacteria contains trehalose, a kind of sugar. Used in the production of a dye that may bring about a change in the colour of the finished product. It took a few minutes after the staining procedure had begun before the fluorescence could be seen, which was evidence that the bacteria were still alive. This was caused by the dye being integrated into the hydrophobic membrane of the mycobacterial cell. The fluorescence of the bacteria that had been eradicated by either heat or drugs was shown to have decreased noticeably as a result. In human sputum samples collected from TB patients, the intensity of the fluorescence was the same regardless of whether the samples were stained with trehalose analogue or Auramine O. This was the case for both types of staining. Because it does not need cleaning the samples and produces very little background fluorescence, this dye based on trehalose has the potential to be particularly advantageous for the quick detection of metabolically active M. TB in places with limited resources. This is because it does not require washing the samples. It is essential to keep this in mind, as it is one of the reasons why it has the potential to be of great assistance, and it is also one of the reasons why it may be quite useful. One person in every three is infected with tuberculosis (TB), which is caused by the acid-fast bacillus (AFB) M. TB. In 2013, 1.5 million people died as a direct result of tuberculosis-related illnesses throughout the globe. There are now 1,300,000,000 individuals living on our planet. The World Health Organisation (WHO) has presented a thorough list of objectives for a worldwide plan to eliminate tuberculosis (TB). This paper provides an overview of these top priorities. Conventional methods for detecting TB, such as the tuberculin skin test and sputum smear microscopy, are known to have a number of problems, including inadequate sensitivity or specificity, as well as false negative results. Newer diagnostic techniques, such as culture and sensitivity, have been developed to overcome these limitations. One of them is the critical need for the prompt and correct identification of TB patients in order to significantly cut down on the length of time required to get started on treatment and, as a result, avoid the further spread of sickness. TB primarily affects the respiratory system; however, the illness may spread to other sections of the body and cause complications there as well. Latent tuberculosis infections are not infectious, and as a result, individuals who have these illnesses are unable to pass the M. TB test. spread of TB to other persons. Those who are infected with tuberculosis infection but are not treated in the appropriate manner have around a 10% chance of developing active tuberculosis within the first two years following infection, which is the period during which the risk of developing active tuberculosis is at its maximum. The first two years after infection are associated with the highest levels of this risk. According to Wang et al.'s (2018) research, the risk of developing hepatitis C is highest within the first two years after infection (Kashyap et al., 2021).

Recent developments in the area of molecular biology have made it possible to diagnose tuberculosis more quickly using specific diagnostic methods. Several different PCR approaches have been developed throughout the years with the purpose of identifying certain sequences that are present in the M. tuberculosis organism 25. Conventional DNA-based methods, nested PCR, and real-time polymerase chain reaction (RT-PCR) are all possible foundations for these (RT-PCR). Targets consist of insertion and repetitive elements, as well as different genes that code for proteins and ribosomal ribonucleic acid (rRNA). The pace of development in this region has been fairly quick. There are a multitude of different PCR assays that have been reported that target various genes that are present in M. tuberculosis. Several gene targets, such as MPB 6430, repetitive sequences, GC repeats, devR, 38kD TRC 4, and IS 1081, have been used in a total of six separate Indian research projects. Nevertheless, since the genome of mycobacteria varies from area to region, none of the methods can be considered universally applicable. The PCR technique for detecting MTBC produces inconsistent findings, particularly with regard to the sensitivity of the test. A significant number of the molecular tests that have been described in the scientific literature are predicated on the amplification of the IS6110 gene. This gene encodes an insertion element that is thought to be unique among members of

the MTBC. If you simply target the IS6110 gene, the diagnostic accuracy may not be high enough to cover 100% of the instances. It has been discovered that the sequence is either not present at all or only present in a small number of copies in some strains that were detected in the Southeast Asian area . A significant number of clinical isolates of M. tuberculosis from South India had either a single copy (40%) or no copy (4%) of the IS6110 gene. Additionally, it has been reported that there is homology between an IS6110 derive probe and DNA isolated from potentially pathogenic mycobacterium strains (Yang et al., 2021).

2. BACKGROUND OF THE STUDY:

In today's world, nucleic acid-based technologies that are very sensitive and specific are used to identify MTB. This enables researchers to sidestep some of the limitations of the conventional, gold-standard laboratory techniques. However, culture is considered to be the gold standard due to its high sensitivity; however, it takes many weeks to generate a result, and it also has less sensitivity for the identification of EPTB than does the microscopic examination of AFB, particularly in paucibacillary specimens. As a result of the numerous issues that are associated with the performance of traditional diagnostic methods, a number of different molecular biological technologies, such as assays that are based on PCR, have been developed for the accurate detection of mycobacteria in clinical specimens of EPTB as well as for their rapid and specific identification. The polymerase chain reaction, often known as PCR, has emerged as the method of choice for diagnosing EPTB due to the fact that it is both quick and has been shown to be sensitive when it comes to the identification of bacteria in paucibacillary specimens. PCR technique is an effective diagnostic tool for mycobacterium TB because it can detect as little as 10 bacilli per millilitre in a wide range of biological materials. This makes the PCR method a useful diagnostic tool. However, the presence of PCR inhibitory compounds, which are observed to be more related with the EPTB than the pulmonary specimens, presents a significant challenge to the detection of mycobacteria using PCR methods. These compounds are observed to be more related with the EPTB than with the specimens. It is possible that substances that suppress polymerase activity are present due to the fact that the sample preparation process did not eliminate them entirely. Some studies have hypothesised that re-amplification, which occurs following inhibitor dilution, would result in an increase in yield. Variations in sensitivity are one of the obstacles that must be overcome before the PCR technology used in the laboratories of clinical analysis centres can be completely standardised. This problem might be handled by making a number of modifications to PCR so that it can recognise the low number of MTB bacilli present in the sample. Nested PCR is an alternative to the more common single PCR that uses two different PCR techniques in order to increase both sensitivity and specificity. It has been shown in a number of recently published as well as earlier studies that nested PCR is more sensitive than the conventional single PCR. In the more recent study, it was shown that nested PCR was more sensitive than the previous single PCR method when it came to diagnosing MTB. Due to the significant challenges that are associated with the diagnosis of EPTB, it is necessary to make a thoughtful selection of a large number of target genes in order to maximise the possibility of identifying MTBC in EPTB paucibacillary specimens. The purpose of this study was to do just that by testing for MTBC in samples obtained from individuals who were suspected of having EPTB. In order to improve the detection rate and identify the prevalence of EPTB, a nested PCR approach was used. This method targeted five different genes of MTBC in order to boost the detection rate.

In 2019, TB is expected to infect around 10 million individuals globally. Around 3.4% of all newly diagnosed TB patients are resistant to multiple drugs. Microbiological culture approaches, followed by species identification and drug sensitivity tests, remain the gold standard for discovering Mycobacterium tuberculosis, the aetiological cause of tuberculosis. For patients with pulmonary tuberculosis, sputum is the most often collected clinical material. Smear microscopy is popular because it is inexpensive, however its sensitivity is only 50-60%. Hence, many approaches with varying sensitivity and specificity for TB diagnosis have been developed due to the need to enhance the performance of existing microbiological tests to offer quick treatment. In this article, we compare and contrast the various approaches used over the previous two decades (Sreedeep et al., 2020).

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Successful one-step multiplex RT-qPCR needs careful modification and confirmation. This preparatory work helps to avoid the waste of time, effort, and materials that might otherwise be caused by the development of results that are below standard. In labs that consistently investigate the same targets, optimisation is particularly important since the assay parameters will be utilised in many future studies. It is abundantly clear that there is a need, both for the sake of public health and for therapeutic purposes, for laboratory tests that are both more rapid and more accurate in order to identify infections caused by Mycobacterium tuberculosis. Mycobacteria may be cultured using the radiometric BACTEC technique, which is a significant improvement in the field since it enables rapid identification and a high rate of recovery. The introduction of nucleic acid probes that are unique to certain species of mycobacteria has significantly improved researchers' capacity to rapidly verify culture data for a variety of mycobacterial species. Nonetheless, it might take anywhere from a few days to a few weeks for the condition to develop before it can be identified. Using a technique known as the polymerase chain reaction (PCR), mycobacteria, including Mycobacterium tuberculosis in particular, have been effectively detected in early BACTEC cultures. PCR techniques have been tested and shown to be reliable for the direct detection of Mycobacterium TB in clinical samples by a variety of different research groups. For the purpose of species-specific differentiation, the 16S rRNA gene, IS elements, the 32kDa and 65-kDa protein-encoding genes have all been proven to be sensitive and representative nucleic acid targets. In addition to the polymerase chain reaction (PCR), there are now also a number of other techniques of nucleic acid amplification that are used extensively. Tests such as strand displacement amplification and Q-beta-replicase probe amplification are included in this category. Many research organisations have shown that PCR-based techniques may successfully be used to directly identify Mycobacterium TB in clinical samples. For the goal of species-specific differentiation, it has been shown that the 16S rRNA gene, IS elements, and the 32-kDa and 65-kDa protein-encoding genes are all sensitive and representative nucleic acid targets. With the use of polymerase chain reaction (PCR), there has been a recent uptick in interest in several more strategies for amplifying nucleic acids. Tests such as the Q-beta-replicase probe amplification and the strand displacement amplification are two examples of the kinds of examinations that come under this category. IS6110, the most often targeted gene, may be found in the genome of Mycobacterium tuberculosis in many copies. The majority of the research that focused just on IS6110 found favourable outcomes. 7,9,10 In addition, the study conducted reveals that the IS6110 gene may be absent in around 10–15% of M. tuberculosis isolates found. Studies that have studied a variety of target genes have proved to have a higher level of both sensitivity and specificity than other types of investigations. According to a limited number of studies, the combination of the protein b gene with the MPB64 gene and the IS6110 gene has shown some potential for enhancing the sensitivity and specificity of the EPTB. It is possible to speculate that a multiple PCR employing various targets may yield a greater sensitivity for the diagnosis of GITB. This is because GITB is often a paucibacillary sickness. There have only been a few studies that have looked at the utility of multiplex PCR in GITB, and an appropriate gene target has not yet been found. For the purpose of determining whether or whether multiplex PCR using these three primers (Protein b, MPB64, and IS6110) is superior to standard bacteriological procedures (smear, histology, and culture), we conducted an experiment. When it comes to therapy, it is essential to make a prompt and accurate diagnosis of tuberculosis at an early stage .

3. PROBLEM STATEMENT:

"Tuberculosis is an infection that is spread through the air. Even though it can be treated, it's still responsible for many deaths around the world. If someone is infected with the bacterium, but don't have symptoms, the person may have inactive tuberculosis or latent tuberculosis infection (also called latent TB). It may seem like TB has gone away, but it's dormant (sleeping) inside the body."

Due to the fact that TB is an infectious disease, it may either appear as a pulmonary or extrapulmonary infection. The lungs are the organ that is most often affected, although other parts of the body, including as the spinal cord, brain, and kidneys, are also susceptible. The name of the condition originates from a Latin word that may imply either a raised bump or a nodule. The abbreviation for tuberculosis is "TB." Those who do exhibit signs of TB need to be treated with medicines, although not everyone who catches the disease will develop symptoms. Inactive tuberculosis infection, sometimes referred to as latent tuberculosis infection, takes place when the bacteria that cause tuberculosis infection are responsible for the illness yet there are no symptoms present (also called latent TB). Even though you may no longer be exhibiting symptoms of tuberculosis, the illness is still present in your body in a dormant state. When an infected individual develops symptoms of tuberculosis and is able to transfer the disease to others, they are said to have active tuberculosis or tuberculosis sickness (TB disease). TB is a potentially fatal illness that mostly affects the lungs. Bacteria are to blame for the spread of tuberculosis. Tuberculosis is an infectious disease that may be passed from person to person via the air by way of a person's vocalisations. It is possible that this will result in the dissemination of infectious droplets into the atmosphere. The infectious sickness may be passed on to further people if the droplets were breathed by those who were exposed to them. In social situations and living quarters that are too crowded, *tuberculosis* (may more easily spread. Those who have immune systems that are already impaired, such as those who are afflicted by HIV/AIDS, have an increased risk of getting TB. Antibiotics are an excellent treatment for tuberculosis. Yet, there are certain types of the bacteria that are developing immune to the treatment. The bacterium known as Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). When a person with active tuberculosis in their lungs coughs or sneezes, the droplets that come out of their mouth and nose have the potential to infect other people. Fatigue, loss of appetite, fever, and night sweats are some of the symptoms of tuberculosis (TB). There are other symptoms associated with tuberculous lung disease, including coughing, chest pain, and coughing up blood. Context has a significant role in determining how TB presents itself in various regions of the body.

The three stages of TB are:

- Primary infection.
- Latent TB infection.
- Active TB disease.

Active tuberculosis may be spread via the air when a person who has the disease coughs, sneezes, speaks, sings, or even laughs. This can also spread the disease. Only those people who are now afflicted with an active infection of the lung virus may spread it to others. The great majority of people who are exposed to tuberculosis germs via the air are able to effectively inhibit the development of the bacteria in their bodies. Some patients have a dormant infection of tuberculosis because the germs may lie dormant for a long time (Sun et al., 2011).

It is believed that there are up to 13 million people in the United States who have latent TB and are unaffected by the disease. While the bacteria are sleeping, they are still present in the body and have the potential to become active at a later date. It is possible for some people to spend their whole lives harbouring a latent tuberculosis infection inside of them without ever showing any signs or symptoms of an active case of tuberculosis. But, the illness may become active if your immune system has been weakened to the point that it is unable to battle the expansion of the TB germs. This is one of the conditions required for the disease to become active. The progression of a latent infection into an active case of TB is referred to as activation. A significant number of researchers are now working on creating countermeasures to this impact. People often need to have frequent and extended contact with a TB patient who is in an active stage of the illness in order to get infected with tuberculosis. It is beneficial to adhere to the guidelines for the prevention of infection, such as the following:

Hands should be washed with soap and water on a regular basis and thoroughly. When you cough, it's also a good idea to cough into your elbow or cover your mouth with your hand. These are also helpful strategies to avoid the transmission of germs. Attempting to avoid situations in which you would be in close closeness to other people. making sure that you take all of your medication at the proper times and in the correct amounts. Researcher shouldn't return to your job or school until doctor

has given you the go-ahead to do so. In the meanwhile, focus on getting well. To stop the spread of TB inside the medical facility, the most important preventive measures are ensuring that there is enough ventilation and wearing the right types of personal protective equipment (Majlessi et al., 2020).

4. LITERATURE REVIEW:

The majority of nations with poor incomes continue to struggle under the weight of a significant challenge to their public health brought on by tuberculosis (TB). In order to properly detect tuberculosis and prevent its spread, it is vital to have diagnostic tools that are both quick and sensitive. In addition to the traditional ways of diagnosis, a significant number of brand new, ground-breaking approaches have been published in recent times. While some methods are straightforward yet require a significant amount of manual effort, others need for highly specialised gear and extensive procedural know-how. The identification of proteins that are released by Mycobacterium TB is one method that is widely used and not difficult to get. Some of these proteins are Mpt64, the 6-kDa early secreted antigenic target, also known as Esat6, the 10-kDa culture filtrate protein, also known as Cfp10, and the antigen 85 (Ag85) complex. There is mounting molecular evidence connecting them to the pathogenicity of Mycobacterium TB, despite the fact that our understanding of the actions they engage in is now somewhat restricted. These biomarkers are currently being investigated or used in a wide variety of different methods, some of which include skin patch tests, biosensor analyses, immunochromatographic assays, immunohistochemistry assays, polymerase chain reaction-based assays, and enzyme-linked immunosorbent assays. Skin patch tests are particularly useful for determining whether a patient has an allergy to a particular substance. In this study, comparisons are made between detection techniques that are based on these proteins and other, more established tests for TB. Also, an in-depth explanation is offered on how these proteins assist in the pathogenesis of Mycobacterium tuberculosis (Babin et al., 2021) .

There is a relatively high mortality rate associated with the infectious illness known as tuberculosis (TB), which is caused by the mycobacterium tuberculosis (M. tuberculosis). In order to effectively manage tuberculosis (TB), it is necessary to detect and treat cases of the illness as soon as they arise. The typical smear microscopy method still has a poor degree of sensitivity and is unable to discover bacterial resistance to the treatments that are being used to treat the illness. This makes it impossible to determine whether or not the bacteria have developed resistance. The findings of a diagnosis based on culture may sometimes take a substantial length of time to get back, generally falling between three and four weeks. It is not feasible to discern between living and dead M. tuberculosis through the use of molecular biology methods, and active tuberculosis cannot be separated from latent tuberculosis through the use of diagnostic immunological techniques. In the most recent years, there has been an increase in the need for point-of-care processes that are straightforward, quick, accurate, and cost-effective. This is a direct result of the deficiencies that are present in the methods that are currently used for detection, as well as the ongoing spread of multidrug-resistant and extensively drug-resistant tuberculosis. Additionally, this is a direct result of the fact that the methods that are currently used for detection are outdated. In this thesis, we will look at the history of the evolution of conventional diagnostic procedures throughout the course of time, as well as the use of these methods in clinical settings. The chronic infectious illness known as tuberculosis (TB) is caused by a pathogen that is a kind of bacteria known as Mycobacterium tuberculosis. A person with tuberculosis may become a reservoir for M. tuberculosis if the diagnosis of their illness is delayed for a lengthy period of time. This raises the possibility that the infection will be passed on to other people. In order to enhance the effectiveness of therapy and lessen the likelihood that tuberculosis will spread to further people, it is essential to obtain an accurate and prompt diagnosis of the illness. Many patients who are suspected of having tuberculosis are unable to acquire a prompt diagnosis due to a number of factors, including the cost burden of transportation.

This is one of the factors that contributes to the disease's extensive distribution. Since the currently available TB diagnostic procedures are insufficient to satisfy the criteria of clinical practise, it is

required to create new approaches. These processes have to be accurate, must not take a lot of time, and must not cost a lot of money.

This analysis takes into account not only the refinement and enhancement of established procedures, but also the birth and growth of brand-new diagnostic techniques (Figure 2) (Benachinmardi et al., 2019)

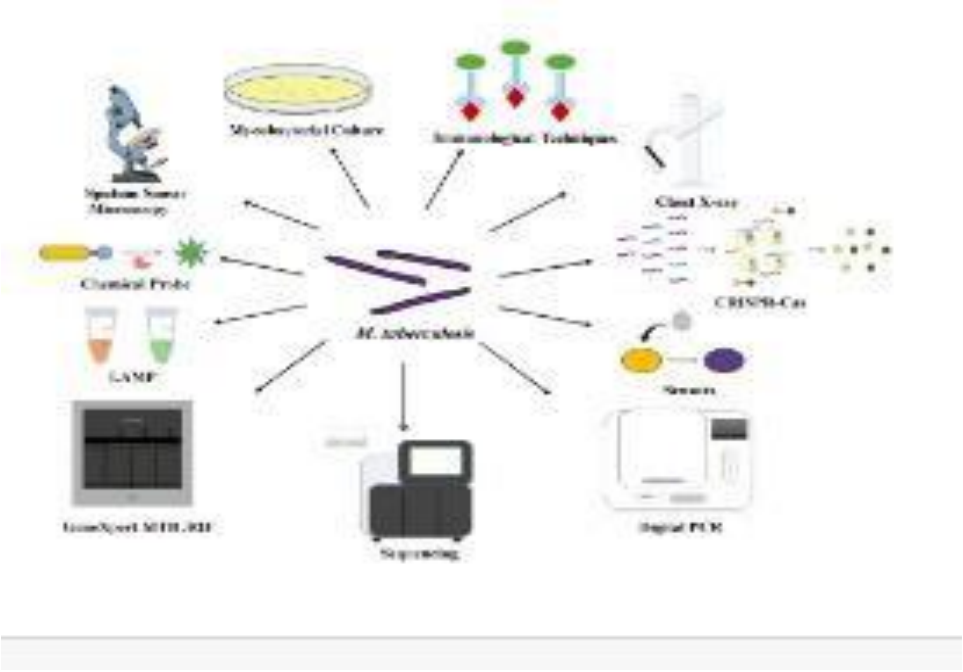


FIGURE 1 . Various diagnostic tools for TB point-of-care testing.

5. RESEARCH OBJECTIVE:

- To determine M.tuberculosis isolation and characterization from sputum samples.
- To test a monoplex PCR assay with numerous targets, including the IS6110, MPB64, and Protein b genes.
- To evaluate the Multiplex Polymerase Chain Reaction (MPCR) assay utilizing multiple targets for rapid detection of MTBC for routine diagnosis.

6. RESEARCH METHODOLOGY:

3.1 Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeats (MIRU-VNTR)

3.1.1 Isolate selection

Isolates taken from the IS6110 database were retyped using 24-MIRU if they belonged to either a 100% cluster or a 70% lineage. In addition, the MIRU typing was performed on all of the strains that had less than five copies of the IS6110 gene.

3.1.2 Isolation of genomic DNA from *M. tuberculosis*

The procedure for extracting genomic DNA was described in 2.1.5. During the processing of the IS6110 RFLP, there were times when inadequate amounts of DNA were left behind. In this particular instance, twenty microliters (uL) of PCR-grade water was poured into the empty tube before it was gently mixed.

3.1.3 MIRU Polymerase Chain Reaction

According to the findings presented by Supply, the 24 MIRU targets were amplified by using fluorescently tagged primers in a total of eight triplex polymerase chain reactions (Supply et al.). In order to set up reactions on 96-well microtitre plates (Applied Biosystems, Warrington, UK), liquid handling software called QiaSymphony was used (Qiagen, Crawley, Surrey, UK). You may discover information on PCR primer sets and master mixes in the tables that are located below.

Multiplex	Locus	Alias	Repeat unit length, bp	PCR primer pairs (5' to 3', with labeling indicated)
Mix 1	580	MIRU 4	77	GCGCGAGAGCCCGAACTGC (FAM) GCGCAGCAGAAACGCCAGC
	2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG (VIC)
	802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA
Mix 2	960	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT (FAM)
	1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC (VIC)
	3192	MIRU 31	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT (NED)
Mix 3	424	42	51	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC (FAM)
	577	43	58	CGAGAGTGGCAGTGGCGGTTATCT (VIC) AATGACTTGAACGCGCAAATTGTGA
	2165	ETR A	75	AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATTT
Mix 4	2401	47	58	CTTGAAGCCCCGGTCTCATCTGT (FAM) ACTTGAACCCCCACGCCCATTAGTA
	3690	52	58	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG
	4156	53	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT (NED)
Mix 5	2163b	QUB-11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT (FAM)
	1955		57	AGATCCCAGTTGTCGTCGTC (VIC) CAACATCGCCTGGTTCTGTA
	4052	QUB-26	111	AACGCTCAGCTGTCGGAT (NED) CGGCCGTGCCGGCCAGGTCCTTCCCGAT
Mix 6	154	MIRU 2	53	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT (FAM)
	2531	MIRU 23	53	CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGTTCGCCCTTTTGTC
	4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT (NED)
Mix 7	2059	MIRU 20	77	TCGGAGAGATGCCCTTCGAGTTAG (FAM) GGAGACCGCGACCAGGTACTTGTA
	2687	MIRU 24	54	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA (VIC)
	3007	MIRU 27	53	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA (NED)
Mix 8	2347	46	57	GCCAGCCGCCGTGCATAAACCT (FAM) AGCCACCCGGTGTGCCTTGTATGAC
	2461	48	57	ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC
	3171	49	54	GGTGCGCACCTGCTCCAGATAA (NED) GGCTCTCATTGCTGGAGGGTTGTAC

mix	1	2	3	4	5
Loci	4-26-40	10-16-31	0424- 0577-2165	2401-3690-4156	2163b- 1955-4052
$MgCl_2$ final concentration	3mM	2mM	1,5 mM	3mM	1,5 mM
H2O	7,5	8,3	8,7	7,5	8,7
Buffer 10 X	2	2	2	2	2
Q Solution 5x	4	4	4	4	4
$MgCl_2$ 25 mM	1,2	0,4	0	1,2	0
DNTP 5mM	0,8	0,8	0,8	0,8	0,8
Primers EACH[a]	0,4	0,4	0,4	0,4	0,4
Hotstart DNA pol	0,08	0,08	0,08	0,08	0,08
Total premix	18	18	18	18	18

mix	6	7	8
Loci	2-23-39	20-24-27	2347-2461-3171
$MgCl_2$ final concentration	2.5 mM	1,5 mM	2mM
H2O	7,9	8,7	8,3
Buffer 10 X	2	2	2
Q Solution 5x	4	4	4
$MgCl_2$ 25 mM	0.8	0	0,4
DNTP 5mM	0,8	0,8	0,8
Primers EACH[a]	0,4	0,4	0,4
Hotstart DNA pol	0,08	0,08	0,08
Total premix	18	18	18

Table 2. MIRU Polymerase Chain Reaction

3.1.4 Fragment analysis of the MIRU PCR products

The ROX-labelled 1000bp DNA marker (MapMarker 1000, 8fmol/band/L, Bioventures, Tennessee, USA) was dispensed into each of the 96 wells of the microtiter plate that had 96 wells.

The Fragment Analysis tool was used to assign amplicon sizes in order to calculate the number of MIRU copies that were present in each well (Applied Biosystems, Warrington, UK). For each well, the fluorescent signal was visually inspected in order to check that the programme had correctly identified the fragments, as well as to search for stutter-peaks, which are caused by strand slippage during primer annealing, as well as huge fluorescent flares, which are caused by high amplicon concentrations. There is a possibility that large-flare PCR results will need to be diluted and retested.

3.1.5 Entry of MIRU Data into BioNumerics

We have included the MIRU type, which is a 24-digit number, for each individual isolate in the BioNumerics database. For determining whether or not the IS6110 clusters and lineages were

supported by evidence of relatedness, only the MIRU data was employed. After that, we utilised MIRU to build clades and subclades by calculating the genetic distance between the several IS6110 low copy number strains and coming to those conclusions.

ESAT-6 and the Host Response

2.5 Patient selection

Isolates grown from a group of TB patients who had been reported before (Breen et al 2008a) were selected for the purpose of this study. Using IS6110 and MIRU-VNTR in the same way as was detailed in sections 2.1 and 2.2, respectively, it was established whether or not the isolates were connected. ***esx*A PCR and Sequencing**

The author was the one who originally carried out, optimised, and thought of the polymerase chain reaction (PCR). Amplification of a product with a length of 228 base pairs might be used to assess whether or not the gene is present. The following are the conditions that were agreed upon.

Target	Primer	Sequence
*esx*A	Forward	3' CCAGGGAAATGTCACGTCCATTCA 5'
	Reverse	3' AACATCCCAGTGACGTTGCCTT 5'

Table 3. *esx*A PCR and Sequencing

The following optimal amounts of reagents were used in the production of the PCR master mix: 1x PCR buffer, 2mM MgCl2, 0.2mM dNTPs, 0.4M of each primer, and 0.5 units of Taq per litre (Invitrogen, Paisley, UK). The quantity of DNA that was provided was 1 litre, and its concentration was no less than 0.5 nanograms per millilitre. A starting temperature of 95 degrees Celsius is maintained for five minutes, and then there are thirty cycles in which temperatures are maintained at those levels for thirty seconds at a time (95 degrees, 50 degrees, and 72 degrees). A last elongation step of 72 degrees Celsius for five minutes was utilised.

DNA amplification was detected by agarose gel electrophoresis with ethidium bromide staining.

The *esxA* gene was sequenced from PCR positive isolates, using a second PCR protocol

Target	Primer	Sequence
*esx*A plus flanking region	Forward	3' CCAAGAAGCAGCCAATAAGC 5'
	Reverse	3' GGAGCTTCCATACCTTCGTG 5'

The following optimal amounts of reagents were used in the production of the PCR master mix: 1x PCR buffer, 2mM MgCl2, 0.2mM dNTPs, 0.4M of each primer, and 0.5 units of Taq per litre (Invitrogen, Paisley, UK). The quantity of DNA that was provided was 1 litre, and its concentration was no less than 0.5 nanograms per millilitre. A starting temperature of 95 degrees Celsius is maintained for five minutes, and then there are thirty cycles in which temperatures are maintained at those levels for thirty seconds at a time (95 degrees, 50 degrees, and 72 degrees). A last elongation step of 72 degrees Celsius for five minutes was utilised. Using the use of ethidium bromide staining of agarose gel electrophoresis, we discovered that the gene had been amplified. Purification of PCR products was carried out using the QIAGEN QIAquick PCR Purification Kit in accordance with the methodology that was specified by the manufacturer (Qiagen, Crawley, UK).

A cycle sequencing reaction was set up using the purified PCR product. Four tubes were set up for each PCR product, consisting of two forward and two reverse reactions. A final concentration of

1.05μM of one primer (2μL), 2μL Big Dye RRM V3.1 (Applied) Biosystems, Warrington, UK), 10μL water and 5μL purified PCR product. Thermocycling conditions of 96°C for 1 minute, followed by 40 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 72°C for 4 minutes.
Each 19 mL cycle sequencing product was precipitated by adding 3 millilitres of 3 millimetres of sodium chloride at a pH of 4.65, 62.5 millilitres of
95% ethanol, and 14.5 millilitres of water. A vigorous vortex was applied to the mixture just before it was subjected to centrifugation at >15,000g for 15 minutes at 4°C. It was possible to discharge the supernatant without affecting the particle in any way. In order to clean the pellet, we flipped each tube upside down and poured 200 litres of ethanol with a concentration of 70 percent into it. The tubes were centrifuged at over 15,000 g for a period of 5 minutes at a temperature of 4 degrees Celsius. When the supernatant was removed, the particle was completely dehydrated and ready for use.
Before being put in the automated plate sequencer, 15 litres of formamide were added to each tube individually (Applied Biosystems, Warrington, UK).

3.1.6 Sequence Analysis

In order to conduct an analysis of the obtained sequences, the author used BioNumerics (version 3.5). (Applied Maths, Sint-Martens-Latem, Belgium). The researcher checked all of the base calls in the consensus sequences by hand to ensure that they were accurate. After that, the sequences were contrasted with the one from the Mycobacterium tuberculosis esxA gene.

3.1.7 esxA mRNA Reverse Transcriptase PCR 3.1.7.1 Evaluation of optimal *esx*A expression

After 7, 14, and 28 days of incubation at 37 degrees Celsius in Kirshner's medium, the lag, log, and stationary phases of the development of M. tuberculosis H37Rv were respectively exhibited (E&O Laboratories, Scotland). This is the technique that was followed in order to extract RNA at the periods specified. The reverse transcription and quantitative polymerase chain reaction (RT-qPCR) method was applied to each RNA extract in the manner that will be detailed further down.

3.1.8 RNA Extraction

In order to propagate cultures of the selected isolates, Kirshner's broth from E&O Laboratories in Scotland was utilised. These cultures were then incubated at 37 degrees Celsius for ten days. After that time period, the turbidity of the broth culture was used as the primary method for determining the amount of development. Macfarland 0.5 was the minimum acceptable amount of turbidity in the water. In order to achieve the level of transparency that was specified for Macfarland 1, any broths that were much darker than this one were diluted with fresh Kirshner's broth (E&O Labs, Scotland). After that, the broths were heated to 37 degrees Celsius and left there for as long as was necessary to obtain the required amount of esxA transcription.

Each 10 mL broth had 35 mL of 5M guanine thiocyanate (GTC) and 245 mL of -mercaptoethanol (0.7% final content) added to it to stabilise the RNA. For thirty minutes at room temperature, this solution was centrifuged at 2,000 g. After careful pipetting, the deposit was resuspended in 1 mL of RNApro (MP Biomedicals, Illkirch, France), and the supernatant was discarded.
After that, we poured one millilitre of this solution into an RNA extraction tube that included lysing matrix beads (MP Biomedicals, Illkirch, France). These tubes were ribolyzed in a FastPrep 24 ribolyser at the 6.0 setting for a period of forty seconds (MP Biomedicals, Illkirch, France). Following that, we subjected the tubes to a centrifuge run of more than 12,000g for five minutes at room temperature.

The supernatant, which was about 750 L, was carefully transferred from the old microcentrifuge tube into the new one without disturbing the matrix contact. In order to get the most amount of RNA out of the transferred samples, we let them sit at room temperature for five minutes.
After adding 300 litres of chloroform to each sample, vortexing it for ten seconds, and letting it sit at room temperature for five minutes, the nucleoproteins were able to be dissociated, which helped

increase the purity of the RNA. After subjecting the test tubes to a centrifugation at 12,000g for five minutes at room temperature, the top phase was delicately transferred to a fresh microcentrifuge tube in an effort to maintain the integrity of the interface. The sterilised tube was then given 500 mL of ice-cold 100% ethanol, and it was turned upside down five times. Following that, the samples were cooled to a temperature of 80 degrees Celsius for a period of 15 minutes.

The supernatant was discarded after the samples were centrifuged at 12,000g for 20 minutes at a temperature of 4 degrees Celsius. The nucleic acid pellet was washed after the addition of 500 litres of ice-cold, 70 percent ethanol (produced with water that had been treated with diethylpyrocarbonate, or DEPC), and inversion of the pellet. After centrifuging the material for 15 minutes at a temperature of 4 degrees Celsius and 12,000g. When all of the supernatant was drained, the pellet was subjected to air drying at a temperature of 50 degrees Celsius. After being rehydrated with 100 ul of RNAase-free water, the pellet was either put to use immediately away or frozen at -80 degrees Celsius for subsequent use.

3.1.9 RNA clean-up

The RNA that had been extracted was mixed with 1 litre of Turbo DNase (1 unit) and 5 litres of 10 times Turbo DNase buffer in a volume of 43 litres (Ambion, Turbo DNA-free kit, Applied Biosystems, Warrington, UK). This mixture was maintained in an incubator at a temperature of 37 degrees Celsius for thirty minutes. With the addition of one more microliter of Turbo DNase, the mixture was let to continue developing in the incubator for a further half an hour. The mixture was allowed to settle at room temperature for 5 minutes after 10 L of DNase Inactivation Reagent from the Ambion, Turbo DNA-free kit that was purchased from Applied Biosystems in Warrington, United Kingdom, was added to it. During this time, the mixture was stirred every so often. After that, the mixture was centrifuged at 12,000g for one minute and ninety seconds, and the supernatant was poured into a fresh tube after the procedure was finished. After being treated with DNase, this RNA is either immediately usable or may be stored at a temperature of -80 degrees Celsius for later use.

3.1.10 Isoniazid Resistance Gene Sequencing

Genomic DNA was obtained by first inoculating the sample into Kirshner's medium (developed by E&O Laboratories in Scotland), and then cultivating the sample until detectable growth was achieved. After being centrifuged into a pellet, the cells were washed twice in 1x TBE and then transferred to a 2 mL screw-cap tube. After being exposed to temperatures of more than 95 degrees Celsius for thirty minutes, the organisms were killed. After being treated in a Ribolyser for 45 seconds, glass beads purchased from Becton Dickinson in Oxford, United Kingdom, were placed inside of the tubes (FastPrep 24, MP Biomedicals in Illkirch, France). After centrifuging the tube for 5 minutes at 12,000g, the supernatant was separated from the remaining liquid. Dr. Alastair McGregor, who was doing research for his MSc degree and collaborating with the author, was the one who retrieved the DNA.

The following PCR parameters were used in order to amplify the inhA and katG genes from particular regions of each and every strain. In the course of his studies for his Master of Science degree, Dr. Alastair McGregor amplified DNA while collaborating with the author. In order to produce the ideal PCR master mix in our laboratory, we made use of the following components: 1x KCl buffer (Bioline, London, UK), 0.04M of each primer, 0.15mM dNTP, and 2 units of Taq polymerase (Bioline, London, UK). The amount of water that was added brought the total capacity up to 90 litres. Following that, ten litres of the DNA that had been purified were added. The method contains thirty cycles with temperatures of 95 degrees Celsius for one minute, 56 degrees Celsius (katG) or 65 degrees Celsius (inhA) for two minutes, and 72 degrees Celsius (katG) or 72 degrees Fahrenheit (inhA) for three minutes. During the last seven minutes, we decided to maintain a temperature of 72 degrees Fahrenheit.

Target	Primer	Sequence	Reference
katG (185bp fragment targeting codon 315)	Forward	5' GTC ACA CTT TCG GTA AGA CC 3'	(O'Sullivan 2007)
	Reverse	5' TTG TCC CAT TTC GTC GGG 3'	(O'Sullivan 2007)
katG (296bp fragment targeting codon 463)	Forward	5' GCG AAG CCG AGA TTG CCA GC 3'	(O'Sullivan 2007)
	Reverse	5' ACA GCC ACC GAG CAC GAC GA 3'	(O'Sullivan 2007)
inhA (248bp fragment of the promoter region)	Forward	5' CCTCGCTGCCCAGAAAGGGA 3'	(Telenti et al. 1997)
	Reverse	5' ATCCCCCGGTTTCCTCCGGT 3'	(Telenti et al. 1997)

Table 4. Isoniazid Resistance Gene Sequencing

Where a PCR product was detected by agarose gel electrophoresis with ethidium bromide staining, it was purified using the QIAGEN QIAquick PCR Purification Kit Protocol (Qiagen, Crawley, UK) following manufacturer‘s instructions.

The final result of the purified PCR was used in the construction of the cycle sequencing reactions. Each PCR product needed four tubes, with two forward and two reverse reactions for each gene to be carried out in their respective tubes. The forward and reverse reactions for each gene were carried out in the relevant tubes. A final concentration of 1.05 M of one primer (two millilitres), two millilitres of Big Dye RRM V3.1 (Applied Biosystems, Warrington, UK), ten millilitres of water, and five millilitres of purified PCR product were used in this reaction. Temperature conditions of 96 degrees Celsius for one minute, followed by forty cycles of 96 degrees Celsius for ten seconds, fifty degrees Celsius for five seconds, and seventy-two degrees Celsius for four minutes. Conditions were maintained at these temperatures for the duration of the experiment.

Each 19 microliter cycle sequencing product was precipitated by adding 14.5 microliters of water, 62.5 microliters of ethanol with a concentration of 95%, and 3 microliters of sodium chloride with a pH of 4.6. The mixture was given a brief swirl using a vortex, and then it was centrifuged at >15,000g for 15 minutes at a temperature of 4 degrees Celsius. The supernatant may be removed without disturbing the particle if one used the skimming method. Once each tube had 200 litres of ethanol with a concentration of 70 percent added to it, the pellet was cleaned by turning the tubes upside down. After centrifuging the test tubes at a temperature of 4 degrees Celsius for five minutes at over 15,000g, the results were recorded. After the removal of the supernatant, the particle was allowed to completely dry out before it was put to use. A total of 15 L of formamide was poured into each of the tubes before putting them into the automated plate sequencer (Applied Biosystems, Warrington, UK). As part of the research that he was carrying out in order to get his MSc degree, Dr. Alastair McGregor was the one who sequenced the data. He did so while working under the guidance of the author.

3.2 Sequence

After loading the sequences into BioNumerics, version 3.5 (Applied Mathematics, Sint-Martens-Latem, Belgium), which was utilised to carry out the research, the author conducted analysis on the sequences. The investigator relied on his or her eyes to determine whether or not the consensus sequences included correct base-calling. After that, the Clustal W software was used to align them with the gene sequences of Mycobacterium tuberculosis H37Rv that were a match.

3.3 Fitness Assays

Isolates were cultivated in Kirshner's medium, which was produced by E&O Laboratories in Scotland, over a length of time that lasted until 0.5 Macfarland of growth was visible. This process

took place over the course of many weeks. In order to inoculate a growth supplement that consisted of bovine albumin, catalase, oleic acid, dextrose, and polyoxyethylene stearate, 0.5 millilitres of each broth was utilised. This was carried out in a tube known as a mycobacterial growth indicator tube (MGIT), which was made by Becton Dickinson in Oxford, England, in the United Kingdom. The incubation of the MGIT tubes was carried out with the assistance of the MGIT 960 system. When these cultures had shown promising results over a period of twenty-four hours, dilutions of 1:10 (which were given the designation dilution A) and 1:10,000 (which were given the designation dilution B) were manufactured. An aliquot of each dilution with a capacity of 0.5 millilitres was transferred in triplicate into brand new MGIT tubes that already contained growth supplement. Via the use of the MGIT 960, the incubation procedure was carried out on these tubes. The method was repeated thrice for each individual isolate that was tested. Calculating the growth rate constant, k, required a modified version of the Youmans and Youmans method (Youmans and Youmans 1949): k = (logA – logB / t, where A is the largest inoculum (dilution A), B is the smallest inoculum (dilution B), and t is equal to the difference of the three replicates of the time to positivity (TTP) between dilution A and B. The equation G = log 2/k was used in order to facilitate the calculation of the generation time G. This method was repeated a total of three times for each of the different strains.

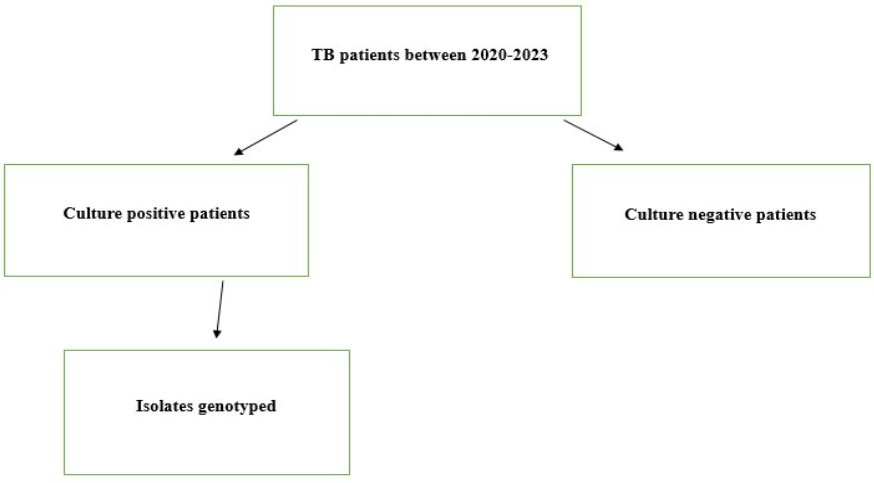
3.4 Homogenization and Decontamination

Homogenization of clinical samples is essential to release the mycobacteria from the sputum in which they are contained. Sputum samples for mycobacterial culture are heavily contaminated with commensal flora. It is important to remove these, as they may grow more rapidly than the mycobacteria and overgrow the culture. Destruction of contaminants is dependent on exposure of the sample to a chemical that will selectively kill them without inhibiting the growth of mycobacteria. Homogenization and decontamination can be combined in one operation. N-acetyl-L-cystine uses as mucolytic agent to assist liquefaction of sputum samples.

3.5 NALC-NaOH Method

Sodium hydroxide (NaOH), a decontaminating agent, also acts as an emulsifier. Because of its potential toxicity, NaOH should be used at the lowest concentration that effectively digests and decontaminates the specimen. The addition of a mucolytic agent, N-acetyl-Lcysteine (NALC), reduces the concentration of NaOH required and also shortens the time required for decontamination, thus aiding the optimal recovery of acid fast bacilli. • **DNA Extraction of Mycobacterium Tuberculosis**

7. CONCEPTUAL FRAMEWORK:



8. RESULTS:

Molecular Characteristics

The median number of IS6110 copies was determined to be 9, while the mean was found to be 9.2 across all 158 strains in the database. There were
90 (57%) strains that were considered to have a low copy number because they had less than five copies of IS6110. A maximum of 21 copies of IS6110 were found in a single strain.

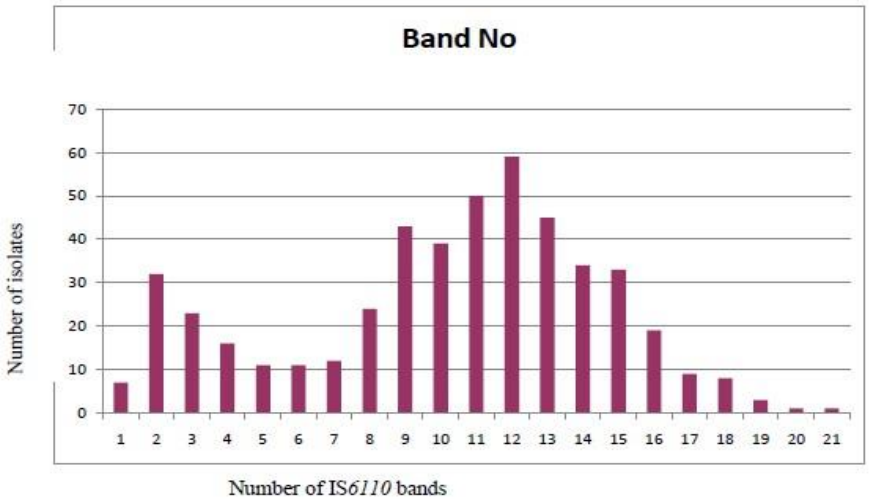


Figure 10 : Distribution of strains with varying numbers of IS*6110*. These data show a bimodal distribution, comprising approximately 20% of strains with fewer than 5 *IS6110* copies. The mode number of copies was 12 and the maximum 21.

There were 28 groups of 101 strains that were 100% identical to one another. Strains with five or more copies of IS6110 were present in just 20 of these clusters. The vast majority of these groups consisted of just two (13 clusters) or three (five clusters) separate isolates. There were four isolates in one group, and eight in another. These groups, along with their respective IS6110 fingerprints, are summarised in table

100% Cluster	Number of IS*6110* copies	No. of isolates per cluster	IS*6110* fingerprint
			MT14323
1	4	2	07:182
2	2	11	07:147
3	1	3	03:495
4	3	3	02:204
5	2	2	03:442
6	1	7	07:132
7	8	2	02:230
8	8	2	04:511
9	11	2	02:109
10	12	3	03:303
11	13	3	02:292
12	17	3	04:053
13	9	2	03:188
14	8	2	03:220
15	5	3	04:612
16	7	2	02:011
17	12	8	03:014
18	9	2	04:599
19	8	2	04:468
20	13	2	04:419
21	14	3	02:093
22	15	2	03:138
23	11	6	04:473
24	12	3	02:023
25	13	2	02:129
26	8	2	03:468
27	1	3	03:251
28	1	16	02:017
Total No. of clustered isolates		101	
No. clustered isolates with ≥5 copies IS*6110*		56	

Table 6: Summary of 28 100% clusters. The given cluster number is listed in the first column. The number of IS*6110* copies is listed in column two. The number of isolates in each cluster is shown in column three whilst the fingerprint of a named member of each cluster is shown in columns for and five.

There were 70 clusters of 158 isolates each with 70% similarity to other clusters. However, 39 isolates from three of these lineages had just five or fewer copies of IS6110. Most of the remaining 49 lineages (consisting of 279 strains) only had one or two members (33 out of 67). Table below shows where the remaining lineages are dispersed. In addition, a chart displaying the six most common lineages and an example IS6110 fingerprint are provided.

Number of isolates in a 70% lineage	Number of lineages
2	33
3	9
4	7
5	5
6	6
8	1
10	1
13	2
15	1
16	1
21	1

Number and size of lineages. Most lineages (33) contained only two isolates. Six lineages contained or more isolates.

Analysis of the 100% clusters was performed to establish two important pieces of information; the level of laboratory contamination and the rate of transmission in the community.

Laboratory Contamination Rate and Transmission Events

After conducting an investigation with the multidisciplinary TB team at OJPS, it was determined that six clustered isolates were caused by laboratory contamination incidents. This conclusion was reached after the study. This results in a laboratory contamination rate of 1.3% when using the entire number of strains that were typed during the course of this investigation, which was n = 158.

After removing from the total number of clustered strains (56) the clustered strains that were likely the result of contamination in the laboratory, which amounted to six, the total number of truly clustered strains was found to be fifty. A rate of transmission that was found to be 6.3% was determined by using the formula that was developed by Small and colleagues (equation 3.1; Small 1994).

Secondary Typing: MIRU-VNTR Data

Conventional PCR and agarose gel electrophoresis were used to assess the isolates from the first twelve months of this investigation utilising twelve loci of the MIRU-VNTR. After that, a 24-loci MIRU-VNTR was used in an automated capillary DNA sequencer to determine the genotype of the succeeding isolates. After analysing the IS6110 genotypes, we chose isolates to type using 24 MIRU-VNTR loci. Analyses were conducted on isolates belonging to one of the six biggest 70% lineages and having less than five copies of IS6110.

The optimisation, implementation, and execution of all 12 loci of MIRU-VNTR genotyping were all carried out by the author. Additionally, the author optimised and implemented the automated 24-loci

genotyping approach. Research assistants Laura Wright and Selina Bannoo analysed the bulk of the isolates in this chapter using the latter approach.

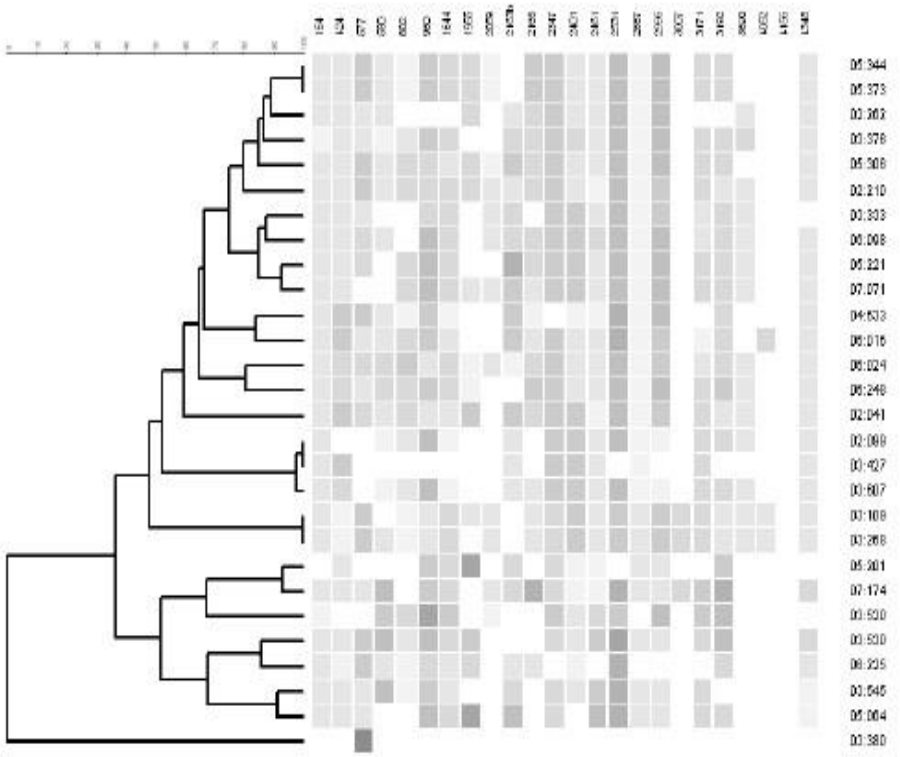
Secondary MIRU-VNTR typing of IS6110 low copy number strains

MIRU-VNTR typing identified a single potential transmission event involving an IS6110-deficient strain. There were 18 MIRU-VNTR loci that were shared between isolates 03:109 and 03:268. However, the assessment of the health records did not reveal any evidence of an epidemiological connection between the individuals. Figure shows a MIRU-VNTR dendrogram for all strains with low IS6110 copy numbers.

	154	424	577	580	802	960	1644	1955	2059	2163	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
03;109	2	1	4		1	2	3	2	2		2	3	4	2	4	2	4	3	3	2	2	2		2
03;268	2	1	4	2	1	2	3		2		2	3	4	2	4	2	4	3	3	2	2	2		2

Table 8: MIRU-VNTR genotypes of two low-copy IS*6110* isolates. The MIRU-VNTR loci are listed across the top of the table. The copy number of each of the two isolates are listed below. The isolates are indistinguishable.

Displays a perfect match between isolates 02:099 and 03:427. Patient records analysis revealed no evidence of an epidemiological connection between the cases. However, only 9 MIRU-VNTR regions were shared amongst these samples. Similarly, MIRU-VNTR genotyping showed that isolates 05:344 and 05:373, which were both found in the North London isoniazid epidemic, were genetically indistinguishable from one another.



MIRU-VNTR dendrogram of all low IS*6110* copy number strains. The level of relatedness (%) is on the left hand side. The MIRU-VNTR loci names are listed at the top and isolate numbers are listed on the right.

Identification of mycobacterium tuberculosis and culture sensitivity

Table no. 6.5 show anti- tuberculin drugs pattern in *M. tuberculosis* isolated organism.

Number of sample for antibiotic susceptibility	25	
Drugs	sensitivity	Resistant
Streptomycin	19%	6%
Isoniazid	11%	14%
Rifampin	16%	9%
Ethambutol	21%	4%
Pyrazinamide	21%	4%
Capreomycin	24%	1%
Kanamycin	24%	1%
Amikacin	24%	1%
Ciprofloxacin	24%	1%
Ethionamide	24%	1%
Levofloxacin	21%	4%
Para amino salicylic acid	23%	2%
Rifabutin	23%	2%
Ofloxacin	20%	5%

IDENTIFICATION OF MYCOBACTERIUM TUBERCULOSIS AND CULTURE SENSITIVITY

Today, tuberculosis presents a worldwide problem since it continues to spread rapidly and infect a wide cross-section of the human population. Therefore, early detection is crucial for managing this condition. This innovative method, used by BACTEC MGIT960, is excellent for the quick diagnosis of tuberculosis-associated microorganisms. Fluorescent dye is the foundation of this method.

Reasons for the epidemic's persistence include a lack of preventative measures, government policies that have failed in their fight against tuberculosis (such as the distribution of subpar anti-TB medications), a shortage of qualified medical professionals, and an absence of public education and awareness. Uncleanliness, exposure to crowds, socioeconomic status, lifestyle, malnutrition, immunological variables, societal incorrect concepts, etc. all have important roles. These microbes are so intricate that they can survive in hostile environments and the host body, despite the existence of an immune response. Due of their methods, they are able to sustain a nonspecific immune response.

I. The inhibition of phagosome – lysosomes fusion.

II. The inhibition of phagosome inhibition.

III. The recruitment and retention of tryptophan aspartate containing coat protein phagosome to prevent their delivery to lysosome and IV. The expression of membrane of the host- induced repetitive glycine- rich family of protein.

Tuberculosis chemotherapy has a totally different strategy than that used for other bacterial illnesses. Due to its slow metabolic activity and extended generation period, this organism represents a novel therapeutic target. Tuberculosis may be found in places where antibiotics have a hard time penetrating, such as the lung cavity, the empyema pus, or the substance.

Different populations of bacteria are present inside the host, a theory supported by a number of therapeutic experiments on animals and humans. It is hypothesised that organisms in the pulmonary cavity are multiplying in an aerobic environment, and as a result, their behaviour may be duplicated in in vitro testing. The organism is in a pH-low environment, which is known to reduce the efficacy of agents like aminoglycosides. Isoniazid, for instance, is crucial early in treatment because its bactericidal action immediately decreased the sputum, but each of the anti-tuberculosis medications plays a vital role in dealing with one of these populations. Countable as viable since its primary activity is against. the organism growing aerobically in pulmonary cavity.

Due of its insensitivity to high pH, pyrazinamide is well suited for eliminating microorganisms within necrotic foci. This includes the observation that pyrazinamide seems to be of little benefit after the second month of treatment. Rifampin is crucial for eradicating persisters and sterilising patients because it eliminates bacteria that metabolise slowly. Sputum, as shown by animal experiments and human trials. The treatment for this microorganism involves a cocktail of anti-tuberculosis medications to combat drug resistance. Eventually, even microbes develop resistance. Firstline anti-tuberculosis medicines, in particular isoniazid and rifampin, increase sensitivity from 44% to 76%, but resistance rates from 56% to 24%. Mycobacterium TB has a limited permeability for numerous compounds, including antibiotics and chemotherapeutic drugs, due to the peculiar structure of its mycolic acid-containing cell wall. Mycobacteria have long had a natural resistance to antibiotics including tetracycline, fluoroquinolones, and aminoglycosides; nevertheless, it was only recently discovered that the efflux mechanism plays a significant part in this resistance.

During suboptimal medication treatment, M.tuberculosis becomes resistant to drugs due to spontaneous mutation in a chromosomal gene, leading to the selection of resistant strains. A complicated link between classical mutations associated with resistance to one medication might be connected to other medicines, but no single pleiotropic mutation has been shown to create a multiple drug resistance phenotype in M. tuberculosis. The rate of spontaneous mutation per round of replication is just 0.0033 in prokaryotes. The larger the genome, the slower the mutation rate per base pair. Previous research has revealed that the mutation rate varies depending on the kind of treatment being used, but for the majority of the most important anti-tuberculosis drugs, it is about 1 in 10100 mutations per cell cycle. Due of the very low odds (1/1000000000000000000), anti-TB medications are typically used in combination to maximise their effectiveness.

Development of multiple drugs resistance *tuberculosis* (MDR-TB)

I. Inadequate or rushed care: - MDR-TB, which may infect even those who have never had tuberculosis before and have never been exposed to any of the medications used to treat it. Medical mistakes (in terms of medicine, dosage interval, or treatment length) cause a substantial number of new cases of multidrug-resistant TB each year.

II. Lack of Treatment Compliance: Noncompliance with medical advice is a common problem. tough to forecast and is often under-estimated by doctors. Mental health issues, alcoholism, drug addiction, and living on the streets are all strong predictors of treatment noncompliance.

III. Poor administrative control on acquisition and distribution of the medications without sufficient mechanism on quality control and bioavailability test contributes significantly to the emergence of MDR-TB.

9. CONCLUSION:

It is my hope that the findings of my study will be able to assist me in achieving a more in-depth grasp of a selection of the fundamental issues that are linked with the decision-making process for TB patients. The fact that tuberculosis (TB) strikes persons most regularly between the ages of 20 and 34, and that this strikes patients of both sexes, is indicative of the disease's propensity to both escape and triumph inside the immune systems of people who are within this age range. The findings of these exams indicate that there is a significant prevalence of MDR-TB not only in male but also in female case patients. This finding applies to both cases where patients were diagnosed with tuberculosis. In order to stop the spread of multidrug-resistant tuberculosis (MDR-TB) among the

human population, it would seem to be an imperative need to do DST on these patients before re-treating them. In order to put a stop to the spread of MDR-TB, this would need to be the case. The results of my study indicate that the organism has already developed an early resistance to the medicines that make up the first line of defence. If treatment is not carried out in an appropriate manner, it is conceivable that the organism may acquire resistance to these medicines in the future, despite the fact that some of the drugs in the second line of defence are effective. Because of this, putting an end to the spread of multidrug-resistant tuberculosis in human populations is an issue of the highest significance in today's world. The RNTCP was successful in providing treatment to around eighty percent of patients throughout twenty districts and fifteen states in India. The proportion of patients who are able to successfully finish their treatments is now higher than it was under the prior plan by more than double, and the number of patients who die away as a result of the condition is now lower than it was by less than an eighth of what it was under the previous programme.

Because to the effective efforts of the RNTCP, almost eighty percent of patients in twenty districts and fifteen states in India were able to obtain treatment. These districts and states are located in India. The percentage of patients who are able to successfully complete their treatments is now higher than it was under the previous plan by more than double, and the number of patients who pass away as a result of the condition is now lower than it was under the previous plan by a little less than an eighth of what it was under the previous programme. Both of these statistics are significantly improved in comparison to what they were under the previous plan.

To summarise, the identification of the genotype of M. tuberculosis is an incredibly important component of the holistic approach to the investigation of TB. The discovery of breakouts and over-represented lineages of strains, on the other hand, may further add to an understanding of the organism's evolutionary success. This is in spite of the fact that there is a lot of difficulty in establishing a connection between genotype and phenotype. The use of prospective genotyping in real time has the potential to not only make the prevention of diseases feasible, but also to allow for the monitoring and treatment of diseases that already exist. It is of the utmost need to close the informational chasm that exists between the laboratory and clinical practises in order to eradicate this worldwide illness once and for all.

10. LIMITATION:

Microbiological culture approaches, followed by species identification and drug sensitivity tests, remain the gold standard for discovering Mycobacterium tuberculosis, the aetiological cause of tuberculosis. Patients with pulmonary tuberculosis often have sputum collected as a clinical specimen. Despite its usefulness, multiplex PCR has several drawbacks that can't be overlooked. Primers inhibiting themselves, inefficient amplification, and inconsistent results across templates are the three main problems with PCR.

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