Recent advances in diagnostic methods for multidrug-resistant tuberculosis (MDR-TB)

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Abstract -

Tuberculosis (TB) remains a global health concern, necessitating accurate and timely diagnosis for effective management. However, challenges persist in diagnosing TB and multidrug-resistant TB (MDR-TB), necessitating innovative approaches. This article explores the various methods employed in TB diagnosis. This review highlights the transformative impact of molecular methods on tuberculosis (TB) and Multi-Drug Resistant TB (MDR-TB) diagnostics, surpassing the limitations of traditional culture-based approaches. Nucleic Acid Amplification Tests (NAATs) such as Polymerase Chain Reaction (PCR) techniques, exemplified by Genedrive and GeneXpert MTB/RIF assay, offer rapid, sensitive detection of Mycobacterium tuberculosis and drug resistance. Noteworthy advancements include Truenat™ MTB assay, distinguished by its rapid isothermal DNA amplification process, and Line Probe Assay (LPA) for detecting drug resistance mutations. Whole Genome Sequencing (WGS) enables comprehensive genetic insights, vital for treatment decisions. Loop-mediated Isothermal Amplification (LAMP) shows promise, particularly in resource-limited settings. Early diagnosis is pivotal in preventing drug resistance and curbing TB spread, despite challenges of cost and complexity. This review also highlights the innovative CODE-M device, a significant advancement in MDR-TB diagnostics, developed by Team iGEM IISER Berhampur.

1. What is MDR-TB and why is it important to focus on?

Pulmonary Tuberculosis (TB), an infectious disease that has afflicted humanity for centuries, remains a formidable global health concern (*Tuberculosis*, no date). Primarily affecting the lungs, TB can lead to severe consequences if left untreated. The causative agent of TB is the bacterium *Mycobacterium tuberculosis*. Particularly prevalent in economically disadvantaged and developing nations, TB poses a significant public health risk. Each year, an estimated 10 million new cases of active TB are reported worldwide (Bloom *et al.*, no date), with approximately one-third of cases going unreported. Notably, India bears a substantial burden, accounting for around 30% of global TB cases, with an estimated 2.69 million new active TB cases annually (Bhargava and Shewade, 2020). Consequently, India is categorized as a high TB burden country (World Health Organization, 2013). A pressing challenge in TB control is the rise of drug resistance, which has been recognized by the World Health Organization (WHO) as one of the top ten global public health threats. TB

exhibits a heightened susceptibility to drug resistance, with an alarming increase in cases of Multidrug Resistant TB (MDR-TB), characterized by resistance to key antibiotics, namely Rifampicin and Isoniazid (Seung, Keshavjee and Rich, 2015). MDR-TB has an estimated mortality rate of around 40%, and India ranks second in terms of MDR-TB burden worldwide, following China (World Health Organization, 2013). MDR-TB is particularly concerning due to its challenging diagnosis and treatment which typically necessitates a prolonged two-year regimen (Chiang *et al.*, 2016). The available treatment options are limited, expensive, and often have toxic side effects, with drug therapy lasting up to two years. The cure rate for MDR-TB is low (50-60%) (Dalcolmo *et al.*, 2017), and some case studies have shown that Economical support and early nutritional intervention reduce the mortality of MDR-TB patients emphasizing the importance of early and accurate detection for improved treatment outcomes (Samuel *et al.*, 2016; Wai *et al.*, 2017; Carwile, Hochberg and Sinha, 2022).

2. Challenges in diagnosis of MDR-TB

In most developing countries where the burden of MDR-TB is high, the standard protocol for MDR-TB detection is slow and inefficient causing unnecessary stress to the patient and their family members. In most of the cases, the patient suspected to have TB is first prescribed a standard test for pulmonary TB which includes sputum smear microscopy and/or Chest X-Ray. The TB positive patients follow the normal treatment regimen for TB which includes antibiotic dose of rifampicin and isoniazid without testing for resistance against these antibiotics. In case the symptoms do not subside during the treatment, the patients are offered cascade testing to determine whether or not they are resistant to any TB drug regimen. This whole ordeal causes MDR-TB patients to lose valuable time that could have been used to implement proper treatment. This is why rapid and scalable diagnostic tests are required to help reduce the impact of TB. Some molecular diagnostic tests are gaining popularity and are slowly replacing the traditional diagnostic methods, but they come with their own drawbacks. Here we have compiled the list of all the diagnostic tests available for detecting MDR-TB.

3. Methods for MDR-TB diagnosis

Current detection methods for MDR-TB (MDR-TB) can be broadly categorized into two groups: phenotypic and genotypic methods. The phenotypic diagnostic tools are mostly based on solid and liquid culture, whereas the genotypic diagnostic tools are mostly based on PCR reactions like qPCR, Micro RT-PCR, etc. Genotypic methods are also referred to as Molecular methods (Huang *et al.*, 2022).

3.1 Phenotypic Methods

Phenotypic methods involve isolating the bacterium *Mycobacterium tuberculosis* from clinical specimens and subsequently testing its susceptibility to various antibiotics through culture and drug-susceptibility testing (DST).

Solid culture refers to a well-established method in microbiology wherein microorganisms are cultivated on solid media for growth and analysis. However, the detection of drug-resistant bacteria using standardized drug susceptibility testing (DST) techniques or traditional approaches often entails an extensive time frame of 8 to 12 weeks.

Liquid culture refers to a cultivation technique in which microorganisms are grown in liquid media as a growth medium. Similar to solid cultures, liquid cultures require a time frame of two to twelve weeks for completion, and their implementation is generally confined to well-equipped urban laboratories that possess specialized equipment and skilled personnel. Liquid culture systems are widely recognized for their enhanced sensitivity compared to solid medium cultures, offering the advantage of reduced testing time.

However, the utilization of culture methods presents challenges in resource-constrained regions due to their significant cost implications, as the detection process typically spans a duration of two to four weeks. This prolonged duration poses significant challenges, particularly in the context of multidrug-resistant tuberculosis (MDR-TB). In cases of MDR-TB, an inaccurate treatment regimen initiated during this protracted waiting period can lead to fatal outcomes within a matter of weeks, particularly among individuals co-infected with HIV (Sachdeva and Shrivastava, 2018). Furthermore, the delayed identification of drug resistance not only hinders the timely initiation of appropriate treatment but also exacerbates the development and spread of drug-resistant strains within the community. Also, the adoption of culture methods for detecting drug-resistant microorganisms remains limited in settings with limited resources, necessitating the exploration of alternative, cost-effective diagnostic methods to ensure timely identification and management of drug resistance in microbial infections.

3.2 Molecular Methods (or Genotyping Methods)

Molecular methods have emerged as powerful tools for the diagnosis of tuberculosis (TB) and MDR-TB (MDR-TB), revolutionizing the field of TB diagnostics. Traditional culture-based methods for TB detection are time-consuming and often require several weeks for results. In contrast, molecular methods offer rapid and highly sensitive detection, enabling early diagnosis and timely initiation of treatment (Campelo *et al.*, 2021; Nandlal, Perumal and Naidoo, 2022).

Nucleic Acid Amplification Tests (NAATs) are at the forefront of molecular TB diagnostics. Techniques like Polymerase Chain Reaction (PCR) detect and amplify specific DNA or RNA sequences of Mycobacterium tuberculosis in patient samples. Kits such as the Genedrive and GeneXpert MTB/RIF assay contribute significantly to TB diagnosis. Genedrive utilizes nucleic acid amplification to identify M. tuberculosis in patient samples. In comparison, the GeneXpert MTB/RIF assay is a widely used cartridge-based NAAT that not only identifies M. tuberculosis but also simultaneously detects resistance to rifampicin, an important first-line TB drug (Zeka, Tasbakan and Cavusoglu, 2011). This integrated approach helps in the early identification of MDR-TB cases, allowing for appropriate treatment strategies. It is noteworthy, that while Genedrive serves as a valuable method for TB diagnosis, it is generally considered to be slightly less robust than the GeneXpert MTB/RIF assay in terms of sensitivity and specificity (Shenai *et al.*, 2016). Overall, molecular methods have transformed TB and MDR-TB diagnosis, providing clinicians with faster and more accurate tools to detect the disease and its drug-resistant forms.

3.3 Last decade in MDR-TB diagnostic:

The last decade led to several new developments in the diagnostic landscape for MDR-TB. One such notable development was Truenat. The Truenat™ MTB assay exhibits notable distinctions compared to other nucleic acid amplification tests (NAATs) for tuberculosis (TB) detection, distinguished by its rapid isothermal DNA amplification process executed on a portable chip platform. In contrast to techniques such as the culture method and the Xpert assay, Truenat exhibits heightened sensitivity and specificity (Gomathi *et al.*, 2020). Its user-friendly nature and minimal equipment prerequisites render it adaptable even in remote locales.

The NAAT based detection assays are highly sensitive and specific but they are usually bulky, requiring a PCR machine and a laboratory setup with expert supervision. Although the Truenat™ MTB assay has been successful in reducing the dependency on bulky equipment, it still requires a PCR machine.

Line Probe Assay (LPA) (GenoType MTBDR*plus*) is a kind of DNA strip-based test used to rapidly detect drug resistance in TB bacteria by the amplification of Mtb DNA isolated from a patient's sample and its hybridization with synthetic DNA probes targeting the most commonly occurring mutations. By targeting specific genetic regions, such as the rpoB gene for rifampicin resistance and the katG and inhA genes for isoniazid resistance, LPAs provide a rapid and reliable assessment of drug susceptibility and its efficiency is comparable with that of the Xpert MTB/RIF in early diagnosis of AFB smear-positive presumptive rifampicin-resistant TB patients. However, Xpert is comparatively superior to LPA in detecting *Mtb* among AFB smear-negative pulmonary TB (Yadav *et al.*, 2021).

Whole Genome Sequencing (WGS) has emerged as a cutting-edge technology that analyzes the entire genetic material of M. tuberculosis. WGS enables the detection of specific mutations associated with drug resistance and also aids in epidemiological investigations and tracking of TB outbreaks. (Meehan *et al.*, 2019) A study from Tanzania compared the WGS and clinical data in patients with drug-resistant and susceptible TB. WGS had high concordance with both phenotypic culture-based DST and Xpert® MTB/RIF assay in detecting drug resistance (kappa = 1.00). In this study, WGS revealed gene mutations linked to drug resistance in tuberculosis, emphasizing its role in treatment decisions. WGS effectively ruled out drug resistance, particularly in non-mutated cases, aligning with culture results and treatment success, suggesting its use in personalized TB treatment (Katale *et al.*, 2020).

Another research, based in Chennai, India, tackles the issue of identifying hidden drug-resistant tuberculosis (DR TB) cases. The study examined 166 DR TB samples collected between 2013 and 2016 using whole-genome sequencing (WGS) and compared the outcomes with conventional drug susceptibility tests (DSTs). WGS revealed extra mutations related to rifampicin (RIF) and isoniazid (INH) resistance, especially in cases with borderline RIF resistance. Importantly, the results from WGS highlight the need to adjust the critical concentrations for RIF DST. Additionally, previously unnoticed mutations linked to INH resistance were brought to light. This research implies that WGS could enhance the detection of DR TB and contribute to more informed treatment decisions (Tamilzhalagan *et al.*, 2021). However, the high cost of equipment, requirement of trained professionals from patient sample collection to data interpretation and its accessibility in resource-limited settings are some of the limitations of this technology.

Loop-mediated Isothermal Amplification (LAMP) is a promising molecular technique for TB diagnosis, particularly in resource-limited settings. LAMP is an isothermal amplification method that targets specific TB DNA sequences, offering simplicity and minimal equipment requirements. The diagnostic value and sensitivity of LAMP have been compared with that of several other diagnostic methods including the GeneXpert MTB/RIF assay. A Chinese study compared Loop-Mediated Isothermal Amplification (LAMP) to GeneXpert, mycobacterial culture, smear microscopy, TSPOT.TB, and the ratio of TB-specific antigen to phytohemagglutinin (TBAg/PHA ratio) for tuberculosis diagnosis. GeneXpert showed the highest sensitivity, followed closely by LAMP. LAMP had similar sensitivity and diagnostic value as GeneXpert and culture. Smear microscopy had lower sensitivity, while TSPOT.TB had low specificity. Using TBAg/PHA ratio > 0.2 improved the specificity but maintained low sensitivity (Deng *et al.*, 2021). Another Gambian study compared TB-LAMP, GeneXpert MTB/RIF, smear microscopy, and culture. TB-LAMP exhibited 99% sensitivity and 94% specificity while GeneXpert showed highest sensitivity (99.1%) but lower specificity (96%), which suggests that although both show diagnostic utility, LAMP is more suitable for new TB cases screening in

peripheral clinics with less infrastructure as compared to GeneXpert, especially in developing countries (Bojang *et al.*, 2016).

Early diagnosis facilitated by these methods plays a crucial role in preventing further drug resistance, improving patient outcomes, and curbing the spread of TB in the community. However, most of these tests are not mainstream yet because of their relatively high cost and complicated operations.
One such notable development in MDR-TB diagnostic was the CODE-M device envisioned by Team iGEM IISER Berhampur.

3.4 CODE M: Cas Optimized DEvice for MDR-TB detection

The rising threat of drug resistance in healthcare systems necessitates proactive measures to combat diseases like tuberculosis (TB). In response to this challenge, the 2021 iGEM team - IISER Berhampur initiated the CODE M project (Cas Optimized DEvice for MDR-TB detection) with the objective of developing a molecular diagnostic kit for early TB and MDR-TB detection. Sputum samples collected from patients will undergo genomic DNA extraction to detect the presence of Mycobacterium tuberculosis (mtb). Due to the typically low quantity of TB bacteria in sputum specimens, Loop-mediated Isothermal Amplification (LAMP) technique will be employed to specifically target and amplify mtb DNA sequences, ensuring higher DNA amplification yield with minimal setup requirements compared to conventional PCR.

The primer design focuses on conserved regions within the rpoB and katG genes of *Mycobacterium tuberculosis*. One set of primers features a phosphorothioate modification, preserving the integrity of the modified strand during subsequent treatment with T7 exonuclease. This modification inhibits T7 exonuclease cleavage, resulting in single-stranded (ssDNA) LAMP fragments corresponding to the rpoB and katG target regions. Successful amplification triggers a colorimetric change mediated by phenol red, confirming the presence of TB in the sample.

Next, the ssDNA is assessed for MDR-TB mutations using the CRISPR/Cas14 system. To evaluate Cas14a's detection capability, single guide RNA (sgRNA) sequences were designed specific to the rpoB and katG target sequences. These complexes are introduced to the ssDNA fragments. To streamline the detection process and enhance portability, using a mobile phone camera within a compact device, similar to its successful application in SARS-CoV-2 detection was proposed. The fluorescence image captured by the mobile phone camera will be analyzed using a MATLAB program implemented in the setup.

In summary, specific mutations enable perfect base-pairing between sgRNAs and MDR-TB target sequences, activating Cas14a and causing reporter DNA cleavage with subsequent fluorescence emission. Mismatches between the guide RNAs and the target DNA will fail to activate Cas14a, resulting in the absence of fluorescence. This approach allows for the differential detection of different mutant forms of MDR-TB.

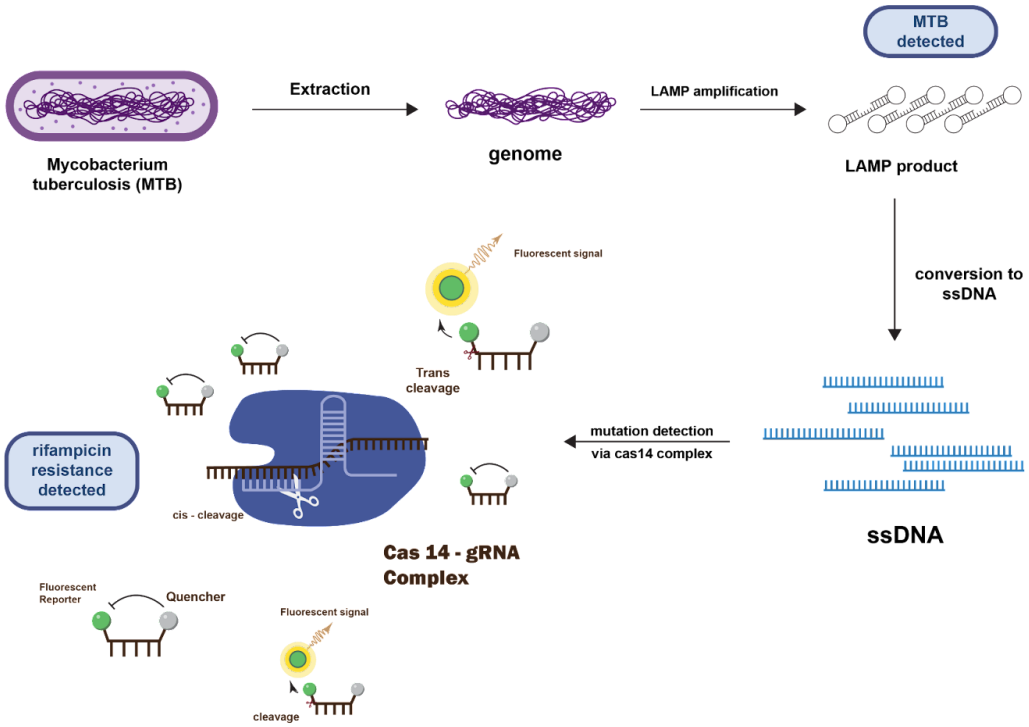


Figure 1. Schematic representation of the stepwise processes involved in MDR-TB detection using the CODE-M kit. The concluding illustration in the process shows the cleavage of the fluorescent quencher pair via the Cas14a1 sgRNA complex to detect the presence of Mycobacterium tuberculosis mutated KatG and RpoB genes (MDR-TB).

4.2 Mathematical Models and Bioinformatic analysis

In-silico methods were used to gather a preliminary understanding of the disease using epidemiological models and phylogenetic analysis. This data was later incorporated in designing the kit.

Epidemiological Model

The modified SIR model emphasizes the importance of early detection in reducing the spread of MDR-TB (MDR-TB) and facilitating the rapid recovery of MDR-TB patients, ultimately leading to a decline in active MDR-TB cases. This model assumes that mortality and infection rates are higher in cases of no detection and late detection, and it does not include background birth and mortality rates. A well-mixed population was considered, where new infections originate only from interacting susceptibles with the infected. All parameters in the model are constant, and the recovery time is assumed to be the same for late, early, and undiagnosed cases. Additionally, it is assumed that all susceptibles are equally susceptible, meaning everyone has the same immune system strength. The methodology involves a simplified model with compartments representing susceptible individuals (S), infected MDR-TB cases with no detection (I_n), infected MDR-TB cases with late detection (I_l), infected MDR-TB cases with early detection (I_e), recovered individuals (R), and deceased individuals (D). In this model, we assume that if S individuals in a population are susceptible to TB, and I_n, I_l, and I_e represent the number of individuals infected by MDR-TB with no detection, late detection, and early detection respectively. R represents the number of individuals who have recovered, while D represents the number of deceased individuals (Sharma *et al.*, 2017; Yu, Shi and Yao, 2018).

$$\frac{dS}{dt} = -aSI_n - bSI_l - cSI_e$$

$$\frac{dI_n}{dt} = d(aI_n + bI_l + cI_e)S - eI_n$$

$$\frac{dI_l}{dt} = f(aIn + bI_l + cI_e)S - eI_l$$

$$\frac{dI_e}{dt} = (1 - d - f)(aI_n + bI_l + cI_e)S - eI_e$$

$$\frac{dR}{dt} = (1 - m_n)eI_n + (1 - m_l)gI_l + (1 - m_e)hI_e$$

$$\frac{dD}{dt} = m_n eI_n + m_l gI_l + m_e hI_e$$

where,

a,b, c = Transmission rates of I_n, I_l, I_e respectively

d, f = ratio of population no and late diagnosed respectively

e = inverse of recovery period

m_l, m_e, m_n = Mortality rate of late, early and not diagnosed respectively

The relevant ordinary differential equations (ODEs) are solved using the ODEint function in the Python programming language, employing appropriate initial conditions. To establish a benchmark, the simulation results are presented with the following initial conditions

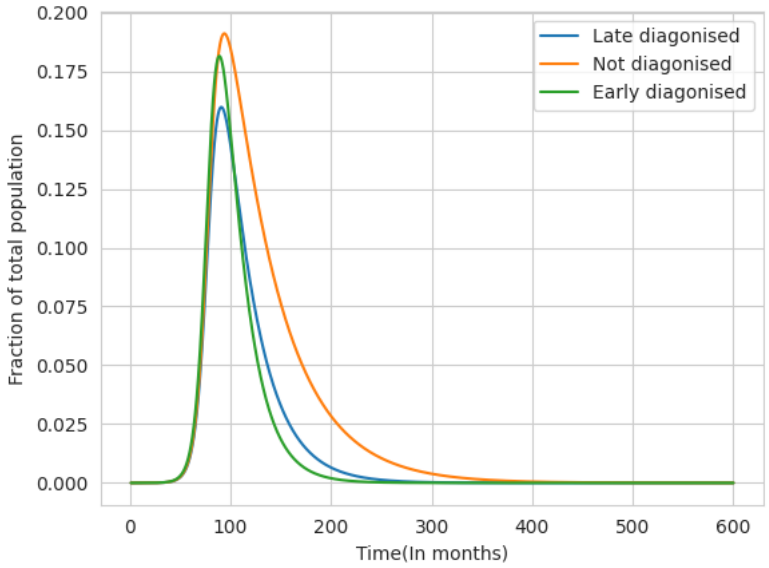
$S|t=0=1-3\times10-6,$

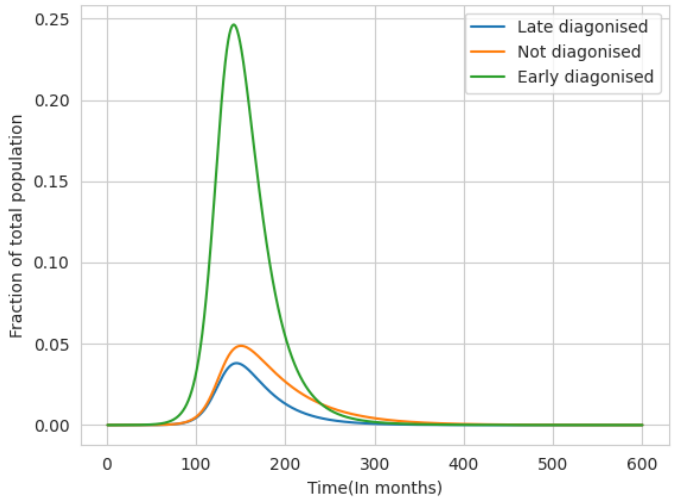
$In|t=0=10-6,$

$Il|t=0=10-6,$

$Ie|t=0=10-6,$

$RT|t=0=0, D|t=0=0.$





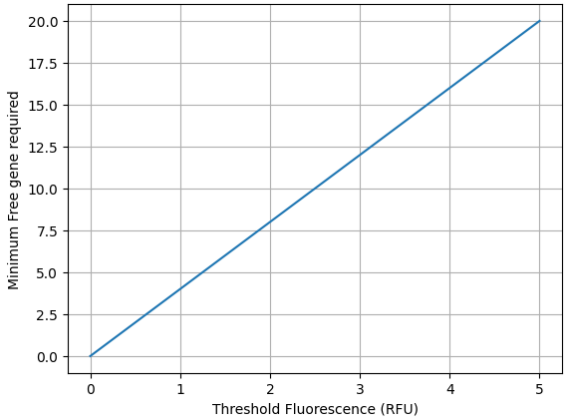


Figure 3:
a. A plot showing the dynamics of the evolution of the fraction of late-diagnosed, not-diagnosed, and Early-diagnosed TB patients with time in India when the fraction of I_N,I_L,I_E is 0.3, 0.3, 0.4, respectively. b. plot showing the dynamics of the evolution of the fraction of late-diagnosed, not-diagnosed, and Early-diagnosed TB patients with time in India when the fraction of I_N,I_L,I_E is 0.1, 0.1, 0.8 respectively. c. This is a plot between Threshold Fluorescence (Relative Fluorescent Units (RFU).) Vs Minimum amount of Free Gene required. The plot has a positive slope which indicates direct proportionality between the two.

The analysis of figure 3 reveals a clear relationship between the rate of no and late detection and the total number of infection cases. As the rate of no and late detection increases, there is a corresponding increase in the total fraction of the infected population. For instance, when the no and late detection rate is 0.1 (as shown in Figure 4), the total fraction of the infected population is approximately 0.35. However, when the no and late detection rate increases to 0.3 (as depicted in Figure 3), the total fraction of the infected population rises to approximately 0.55. These findings strongly indicate a clear and direct correlation between diagnostic delays in individuals and an increase in the incidence of Multidrug Resistant Tuberculosis (MDR-TB) cases. Conversely, this correlation also suggests that prompt and early diagnosis of MDR-TB is of paramount

importance in curtailing the infection rate. Such timely diagnosis plays a pivotal role in reducing both the overall number of active cases and the subsequent transmission of the disease.

Phylogenetic Analysis

Tracing the evolutionary lineage of these strains serves two main purposes. Firstly, it allows us to understand the patterns of genetic mutations that have enhanced the fitness of these strains over time that facilitated their resistance to multiple drugs. Secondly, it provides a foundation for predicting whether additional strains are likely to develop multidrug resistance, thereby increasing their virulence and prevalence in the future.

To achieve these objectives, the model also aimed at examining the evolution of MDR-TB (MDR TB) strains, focusing on the KatG and RpoB genes Building upon recent research, the analysis expanded to include GyrA, as mutations in this gene have been associated with the emergence of extensively drug-resistant TB (XDR TB), which is resistant to fluoroquinolones and second-line injectable drugs.

By conducting multiple sequence alignments of KatG, RpoB, and GyrA proteins, tree topologies were generated that revealed interesting insights. The RpoB gene exhibited the lowest number of substitutions per site (0.008), whereas KatG and GyrA had higher rates of substitutions, with 0.05 and 0.2 substitutions per site, respectively. Most sequences displayed shorter branch lengths, indicating a slower rate of evolution, although a few sequences with longer branches suggested potential rapid evolution. It is worth noting that while some sequences were truncated at the C-terminal ends, many were full-length, indicating that longer branch lengths were not solely artifacts of the tree construction.

The tree topologies based on the three genes displayed distinct patterns. As anticipated from the substitutions per site analysis, RpoB produced a single cluster with longer branches, while KatG and GyrA yielded more than one cluster, also characterized by longer branches. Notably, Mycobacterium tuberculosis strain TKK-01-0051 exhibited the highest percentage of substitutions per site in the KatG tree, alongside other sequences for which strain information was unavailable. Similarly, in the GyrA and RpoB trees, we observed instances of fast-evolving sequences.

While it is not guaranteed that these rapidly evolving sequences or strains will develop multidrug resistance in the future, it is plausible that some of the mutations they harbor may play a crucial role in conferring resistance to the first and second-line drugs used in treatment. Therefore, it is possible that some of the mentioned strains may acquire mutations associated with multidrug resistance (MDR) in the future, even though they are currently not drug-resistant, such as *Mycobacterium tuberculosis* strain TKK-01-0051.

Limitations and scope for future studies

While the phylogenetic analysis can inform us of the fast-evolving strains, which of those mutations could potentially make the strain resistant to multiple drugs needs comprehensive sequence-structure analysis of the proteins that confer resistance. For instance, although some of the above sequences are fast evolving, some of those point mutations may still not confer resistance. Thus, the fast-evolving strains identified using the phylogenetic analysis may not necessarily be the most virulent or the multi-drug resistant strains. A comprehensive sequence-structure analysis of proteins can inform us how some of the key mutations could potentially confer resistance. Also, performing a thorough whole genome-wide mutational landscape survey can precisely inform the trends in fast-evolving strains.

***RpoB* and *KatG* mutational hotspots**

The genes rpoB and katG of Mycobacterium tuberculosis are responsible for encoding the ß subunit of RNA polymerase and catalase-peroxidase, respectively. Mutations occurring in the rpoB gene lead to resistance against rifampicin and are limited to a specific region within the gene called the rifampicin resistance determining region (RRDR), spanning 81 base pairs. Similarly, a mutation in the katG gene results in resistance to isoniazid. If both of these mutations are present in a single Mtb isolate, it indicates (MDR-TB), whereas the presence of either mutation alone indicates monoresistant TB.

The most widespread and deadliest mutations identified in India are Ser315Thr in katG and Ser450Leu in rpoB. This conclusion was drawn based on an analysis of various publications that focused on the patterns of mutations associated with MDR-TB (MDR-TB) in different regions (North and South) and highly affected states. The analysis primarily relied on data collected from these regions, considering factors such as sample size, resistance percentages, patient recovery rates, and more recent publications. Additionally, strains used in the laboratory experiments conducted in these studies revealed the most commonly utilized strain as *Mycobacterium tuberculosis* H37Rv.

4.3 Logistics of The Project:

There are multiple diagnostic kits that can be utilized for the detection of tuberculosis (TB), such as the Cartridge-Based Nucleic Acid Amplification Test (CB NAAT), the culture method, and chest X-ray imaging.

The CB NAAT method is widely recognised as a highly sensitive and reliable technique that is extensively employed in medical centers for the identification of drug-resistant strains of TB. The cost of CB NAAT kits exhibits considerable variation, ranging from tens to hundreds of dollars Regarding Xpert/MTB, a particular form of CB NAAT that can detect rifampicin resistance, the cost breakdown consists of equipment comprising 3.49% of the overall expenditure, human resources accounting for 8.45%, reagents and other inputs constituting 87.58%, and infrastructure representing 0.53% (Sachdeva and Shrivastava, 2018; Silva *et al.*, 2021)

The establishment of CB NAAT laboratories necessitates the presence of biosafety cabinets, refrigeration units, and dependable access to electricity and potable water coupled with costs associated with laboratory maintenance. Effective biohazardous waste management in CB NAAT testing, including collection, transportation, and disposal, adds to overall costs. Managing biohazardous waste from CB NAAT testing affects overall costs. Although a precise cost analysis for CB NAAT is unavailable, we can estimate based on July 2020 COVID-19 rRT-PCR tests, which cost approximately ₹566 ($7.5) per unit. The bulk of expenses (87%) were for laboratory consumables, with 10% allocated to human resources, and only 0.02% for stationary and allied items. A sensitivity analysis revealed that fluctuations in laboratory consumable prices had the most significant impact (21.7%), followed by the number of samples tested (3.9%), human resources salaries (2.6%), and equipment and rental costs (0.23% and 0.14%, respectively) (Minhas *et al.*, 2023).

Taking into account the various factors at play, the CODE-M diagnostic kit can be both cost-effective and specifically designed for the detection of MDR-TB. The overall expenditure for this assay is estimated to be approximately ₹522.58 ($6.37), with reagent costs amounting to approximately ₹57.43 ($0.7), representing approximately 10.7% of the total project expenditure. The reduction in reagent cost is attained by employing effective sourcing methods and implementing optimized formulation strategies. The assembly and filling process constitutes the most significant factor in determining the total price, accounting for 53.7% of the overall expenditure. Efficient manufacturing techniques, including labor optimization and economies of scale, reduced assembly and filling costs. Cartilage packing is the most economical component at 2.3% of total expenditure. Cost-saving strategies like reduced reagent expenses, streamlined processes, and economical packaging make our diagnostic kit affordable for MDR-TB testing. It's 55% cheaper than CB NAAT, with comparable sensitivity, ensuring cost-effective healthcare services for MDR-TB testing.

5. Future Directions

5.1 Rise of XDR-TB

With the rise of antibiotic resistance in mycobacterium tuberculosis, some strains have surfaced that are resistant to even the second line of antibiotics for TB treatment. These TB infections from these strains are termed as Extreme Drug Resistant TB (XDR-TB). XDR TB is a rare type of multidrug-resistant tuberculosis (MDR TB) that is resistant to isoniazid, rifampin, a fluoroquinolone, and a second-line injectable (amikacin, capreomycin, and kanamycin) OR isoniazid, rifampin, a fluoroquinolone, and bedaquiline or linezolid. Currently the prevalence of XDR-TB cases in India is 1.9%, but it might soon be on rise if not handled properly (Goyal *et al.*, 2017). Current kits used to diagnose MDR-TB might need to update themselves to detect resistance for several other antibiotics in near future.

5.2 Hardware

Detecting MDR-TB in rural areas of India is challenging because of the high cost of diagnosis and limited transportation of equipment to these remote regions.

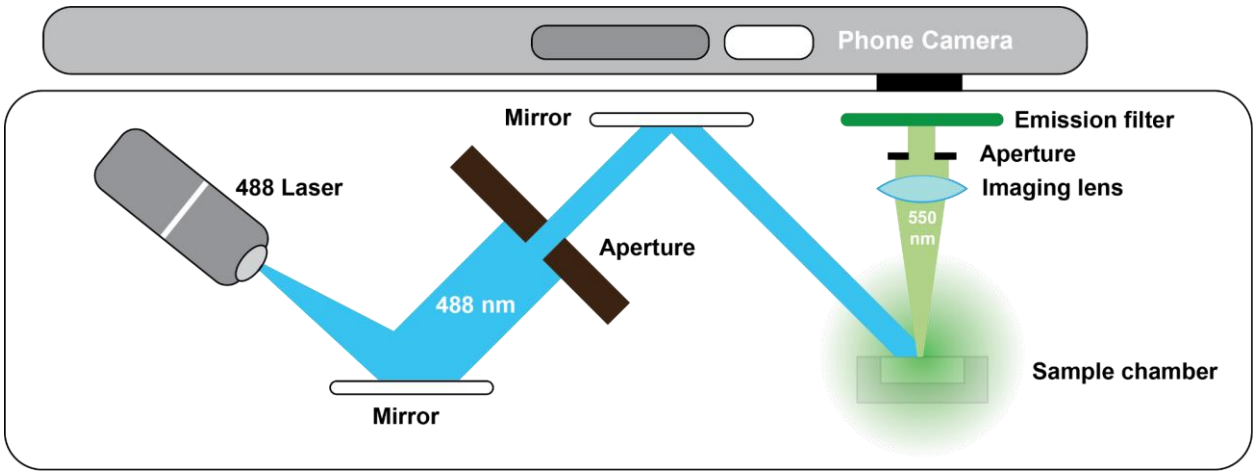


Figure 5: Hardware design showing the MDR TB detection using Mobile phone.

5.3 Expanding the CODE-M for the diagnosis of other diseases

Incorporating Cas14a in genetic mutation identification enhances diagnostic kit adaptability by eliminating the need for a PAM sequence in guide RNA, enabling easy customization for detecting TB strains and various illnesses through guide RNA sequence modification. Cas14's SNP detection capability aids in distinguishing pathogenic strains, crucial for addressing antimicrobial resistance. Access to cDNA libraries for different diseases further improves its effectiveness.

CRISPR-Cas14-based systems show promise in targeting viral and bacterial infections (e.g., HIV, hepatitis, influenza, TB, MRSA) and genetic disorders (e.g., sickle cell disease, cystic fibrosis). In oncology, it can identify genetic mutations linked to cancers (e.g., breast, lung, colorectal, leukemia). It can also be applied to neurodegenerative disorders (e.g., Alzheimer's, Parkinson's and Huntington's disease) and autoimmune disorders (e.g., rheumatoid arthritis, multiple sclerosis, lupus, celiac disease). Overall, Cas14-based methods advance molecular diagnostics, improving disease detection and characterization.

Discussion:

Emerging as a cornerstone in MDR-TB management, molecular diagnostics revolutionize the landscape with their ability to swiftly, precisely, and individually identify and profile drug-resistant *Mtb* strains. This not only expedites diagnosis but also enhances precision, a crucial factor in addressing this global health challenge. Additionally, the tailored nature of molecular diagnostics allows for customized treatment plans, optimizing therapeutic regimens for individual patients, even in the face of emerging resistance mutations. This holds great promise for MDR-TB patients, offering the potential for improved outcomes and strengthening the global effort against drug-resistant strains. The integration of these advanced techniques into standard clinical practice is poised to bring about transformative changes. Swift identification of MDR-TB is essential in halting its spread. Traditional methods, while accurate, are time-consuming, leading to delays in treatment initiation. Molecular diagnostics bypass this, expediting targeted therapies, crucial in improving patient outcomes. Looking ahead, the global adoption of molecular diagnostics promises to significantly reduce transmission rates and enhance patient prognosis in the battle against MDR-TB. Additionally, data generated through molecular profiling deepens our understanding of MDR-TB epidemiology, guiding public health interventions and surveillance efforts. The integration of these advanced techniques symbolizes hope in the pursuit of a TB-free world.

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Supplementary materials:

Table 1: Loop-Mediated Isothermal Amplification (LAMP) primer sequences for KatG and RpoB

Description	**LAMP primer sequence (5'-3')**
KatG target sequence	5'GCCGATCTGGTCGGCCCCGAACCCG AGGCTGCTCCGCTGGAGCAGATGGGCT TGGGCTGGAAGAGCTCGTATGGCACCG GAACCGGTAAGGACGCGATCACCACC GGCATCGAGGTCGTATGGACGAACAC CCCGACGAAATGGGACAACAGTTTCCT CGAGATCCTGTACGGCTACGAGTGGGA GCTGACGAAGAGCCCTGCTGGCGCT 3'

F3 (forward)	AGGCTGCTCCGCTGGA
B3 (backward)	AGCAGGGCTCTTCGTCAG
FIP (Forward Internal Primer)	GGTGATCGCGTCCTTACCGGTGCAG ATGGGCTTGGGC
BIP (Backward Internal Primer)	ACCGGCATCGAGGTCGTATGGCACT CGTAGCCGTACAGGAT
LF (Loop Forward)	GCCATACGAGCTCTTCCA
LB (Loop Backward)	CGACGAAATGGGACAACAG

<u>RpoB target sequene</u>	5’GCCGGTGGAAACCGACGACATCGAC CACTTCGGCAACCGCCGCCTGCGTACG GTCGGCGAGCTGATCCAAAACCAGATC CGGGTCGGCATGTCGCGGATGGAGCG GGTGGTCCGGGAGCGGATGACCACCC AGGACGTGGAGGCGATCACACCGCAG ACGTTGATCAACATCCGGCCGGTGGTC GCCGCGATCAAGGAGTTCTTCGGCACC AGCCAGCTGAGCCAATTCATGGACCAG AACAACCCGCTGTCGGGGTTGACCCAC AAGCGCCGACTGTCGGCGCTGGGGCCC GGCGGTCTGTCACGTGAGCGTGCCGGG CTGGAGGTCCGCGACGTGCACCCGTCG CACTACGGCCGGATGTGCCCGATCGAA ACCCCTGAGGGGCCCAACATCGGTCTG ATCGGCTCGCTGTCGGTGTACGCGCGG GTCAACCCGTTCGGGTTCATCGA 3’
F3 (forward)	CCGCAGACGTTGATCAACAT

B3 (backward)	CCCCTCAGGGGTTTCGA
FIP (Forward Internal Primer)	CAGCGGGTTGTTCTGGTCCATGGTCGC CGCGATCAAGG
BIP (Backward Internal Primer)	CCGGCGGTCTGTCACGTGAAGTGCGCA GGGTGCA
LF (Loop Forward)	AGCTGGCTGGTGCCGA

Table 2: sgRNA sequences for KatG and RpoB target mutations.

Gene	Spacer sequence	MDR-TB Mutation	Spacer sequence for the mutant	Phenotypic expression (drug resistant) conferred
KatG	5’ gcgatcaccagcggcatcga 3’	agc→acc	5’ gcgatcaccaccggcatcga 3’	Drug resistance to Isoniazid
RpoB	5’ cgccgactgtcggcgctggg 3’	tcg→ttg	5’ cgccgactgttggcgctggg 3’	Drug resistance to Rifampicin