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Evaluation of rapid low-cost colorimetric methods for diagnosis of multidrug-resistant tuberculosis in limited-resource settings

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Evaluation of Rapid Low-Cost Colorimetric Methods for Diagnosis of Multidrug-resistant Tuberculosis in Limited-Resource Settings

A Thesis Submitted to

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in partial fulfillment of the requirements for

the degree of Master of Science in Biotechnology

By Mai M. H. Mansour

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Under the supervision of
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ABSTRACT

The American University in Cairo

Evaluation of Rapid Low-Cost Colorimetric Methods for Diagnosis of Multidrug-resistant Tuberculosis in Limited-Resource Settings

BY: Mai M. H. Mansour

Under the supervision of Dr. Hassan M. E. Azzazy and Dr. Moustafa Abdel Fadeel

One third of the world's population is currently infected with tuberculosis (TB), a consuming airborne disease whose main causative agent is *Mycobacterium tuberculosis*. The majority of these patients are found in the world's poorest areas. Treatment of TB is a lengthy and demanding process utilizing a cocktail of powerful drugs; however, multidrug resistant TB (MDR-TB) strains, defined by resistance to both isoniazid (INH) and rifampicin (RIF), are now emerging worldwide and threatening disease control efforts. The major problem facing efforts to combat MDR-TB spread is its early detection. Conventional fairly affordable methods for drug resistance detection are based on solid culture and are highly time consuming (3-6 weeks in addition to initial pathogen culturing). On the other hand, the more rapid liquid culture-based automated systems are costly to set up and maintain while the very rapid molecular assays (hours to few days) are simply too complex and unaffordable and non-sustainable in limited resource settings. The objective of this work was to evaluate the performance of two liquid culture-based colorimetric assays for detection of drug resistance; nitrate reducate assay (NRA) and colorimetric redox indicator (CRI) methods for detection of MDR-TB. The assays were tested on mycobacterial isolates from Egyptian patients and their performance was compared with microscopic observation drug susceptibility assay (MODS) and the commercial automated culture system MGIT 960. Concordance was 96.7% for CRI and 93.3%, at almost one-tenth of the MGIT cost, and close to that of MODS without the need for an inverted microscope. The NRA format used in this study is more convenient and higher in throughput than the initially developed format. Additionally, DNA was extracted from the mycobacterial isolates and16S rDNA was amplified and sequenced to gain insight on the molecular diversity of Egyptian strains. Moreover, the molecular basis of strain resistance was investigated by DNA sequencing of the genes most commonly containing resistance conferring mutations. Analysis of the 16S rDNA sequencing results confirmed the identity of the samples as *mycobacterium tuberculosis* and suggested possible presence of two different strains. On the other hand, the analysis of the resistance related genes found common resistance conferring mutations in the MDR samples.

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LIST OF ABBREVIATIONS

AFB: acid-fast bacilli

BCG: Bacillus Calmette-Guérin

CDC: Centers for Disease Control

CLSI: Clinical and Laboratory Standards Institute

CRI: Colorimetric Redox Indicator

DST: Drug Sensitivity Testing

ELISA: Enzyme-Linked Immunosorbent Assay

ELONA: Enzyme-Linked Oligonucleotide Assay

ETH: Ethambutol

GC: Guanine Cytosine

HBCs: High Burden Countries

HIV: Human Immunodeficiency Virus

IGRA: Interferon Gamma Release Assay

INH: Isoniazid

LAMP: Loop Mediated Isothermal Amplification

MDR: Multi-Drug Resistant

MODS: Microscopic Observation Drug Susceptibility assay

MTB: *Mycobacterium tuberculosis*

MTBC: *Mycobacterium tuberculosis* Complex

NOA: Nystatin, Oxacillin and Aztreonam.

NRA: Nitrate Reductase Assay

NTM: Nontuberculous Mycobacteria

PCR: Polymerase Chain Reaction

PZA: Pyrazinamide

RIF: Rifampicin

rDNA: Ribosomal DNA gene

SDA: Strand Displacement Amplification

SM: Streptomycin

SPR: Surface Plasmon Resonance

TAT: Turnaround Time

TB: Tuberculosis

TLA: Thin Layer Agar

WHO: World Health Organization

XDR: Extended Drug Resistant

ZN: Ziehl Neelsen

1. Background and Review of Literature

1.1. Tuberculosis

Tuberculosis (TB), once known as the "white plague" and "consumption", is one of the oldest known diseases. In addition to plaguing medieval and renaissance Europe, TB has been traced back and documented in ancient civilizations including Greek, Egyptian, and Pervian. To date TB remains a global health problem and pandemic, presenting significant challenges in both diagnosis and treatment [1]. The air-transmitted disease is caused primarily by the bacterium, *Mycobacterium tuberculosis* (MTB). TB was declared a global emergency in 1993 by the World Health Organization (WHO) and currently affects about one third of the world's population, with 8-9 million new TB cases annually. The estimated death toll in 2010 alone was 1.1 million among human immunodeficiency virus (HIV)-negative people, in addition to another 0.35 million among HIV-infected individuals [2-5]. TB remains a particularly alarming problem in sub-Saharan Africa where there are high co-infection rates with HIV (50% of TB patients in southern Africa are HIV-positive [6]). Overall globally, 13% of TB cases occur among HIV-positive individuals. There is only one available vaccine; the Bacillus Calmette–Guérin (BCG), a live attenuated vaccine, which has been in use since the 1920s, and whose actual benefits are questionable. It has a highly variable efficacy (reportedly 0-80%) and does not prevent occurrence of TB infection. It rather protects against severe forms of TB such as miliary TB, particularly in infants, thereby preventing only about 5% of deaths that could be averted via vaccination [7-11]. Despite the fact that incidence rates of TB have been declining over the past decade, TB remains an active global threat. It must also be noted that the actual TB burden extends beyond the direct death and has an extensive social impact, which is visible in the fact that about 10 million children were orphaned because of TB in 2009 [3]. The poorest of countries are typically hit the hardest by TB as the low standards of living, overcrowdness and poor healthcare facilities are direct causes of high infection rates, particularly in areas with high HIV prevalence [12]. Additionally, over the next decade it is estimated that the economic consequences of TB will claim $1 to $3 trillion, catastrophic figure for nations whose people get by with under $2 daily [13]. Asia and Africa bear the worst of the TB impact where they have 55% and 30 % of all TB cases worldwide, respectively (**Figure 1)**. Egypt-specific TB statistics are presented in **table 1.** Notably, in 2007

government expenditure on health in Africa was $34 per capita and in Southeast Asia, home to 30% of the world's poor, it was only $15 per capita. Moreover, almost 80% of all TB cases globally are found in 22 countries known as the high burden countries (HBCs) [3, 11, 14-15]. These countries include the 7 countries with the highest hunger rates globally, according to the United Nations Food and Agriculture Organization (FAO) [16]. Low socioeconomic conditions, undernourishment, and poor hygiene conditions aggravate the problems of TB, and great concordance between TB incidence rates regions and FAO hunger regions are a demonstration of this fact. Furthermore, children constitute about 15-20% of TB cases in endemic regions [3].

1.2. Mycobacterium tuberculosis (MTB)

Mycobacterium tuberculosis (MTB) is a member of the *Mycobacterium* genus of acid-fast bacteria, and was first discovered in 1882 by Robert Koch. MTB organisms are intracellular pathogens which are aerobic slow-growing (18-24 hr generation time) non-motile rod shaped bacilli, measuring 0.5 x 3 µm. It is the primary TB-causing member in the mycobacteria, although a few other members are capable of causing tuberculosis. These are known collectively as *M. tuberculosis* complex (MTBC) and include *M. bovis* (causes TB in cattle), *M. bovis* BCG (the attenuated strain used in vaccines), *M. africanum* (less pathogenic than MTB, causes TB-like symptoms, found mainly in West Africa), and *M. canettii* (found mainly in Horn of Africa). There is also *M. microti*, which is seldom the culprit in human TB cases but causes the disease in voles, in addition to *M. caprae* and *M. pinnipedi*, which are causative agents of TB in goats and deer, and seals, respectively; however, human infection has been demonstrated possible [8, 17-18].

Mycobacteria are characterized by having peptidoglycan cell walls with exceptionally high lipid content, mainly mycolic acid **(Figure 2)**, and thus cannot retain any conventional stains such as the basic aniline dye Gram stain. This means they cannot be classified as either Gram-positive or Gram-negative. However, they can be stained with basic lipid-soluble fuschin dyes as they cannot be decolorized with acidified alcohol containing 3% HCl, a property known acid-fastness [1, 7-8, 17-19]. The exceptional cell wall structure of MTB is a key virulence factor, as this hydrophobic cell wall is impermeable to many antibiotics, and contains channels and

porins that pump out drugs. Also, this lipid-rich rigid cell wall containing the carbohydrate lipoarabinomannan allows MTB to survive within the patients' macrophages that engulf the bacilli [18-21].

1.3. Transmission and pathogenesis of tuberculosis

TB is spread by droplet inhalation and merely 1-3 bacilli suffice for infection if inhaled [22]. The outcome of infection can be elimination of the bacilli and infection clearance, failure to control the infection and development of active symptomatic TB, or control of infection by the immune system without clearance of tubercle bacilli. In the latter case the patient is noninfective and exhibits no symptoms, however, he/she is bearer of dormant bacilli which may be reactivated if the person's immunity is severely compromised e.g. due to HIV infection or uncontrolled sepsis or diabetes mellitus [7, 19]. Typically less than 10% of individuals who become infected with TB bacilli will develop an active infection. Depending on degree of immunocompetency, this value can reach 30%, and coinfection with HIV makes progression to active TB disease 21-34 times more likely, and if active TB is left untreated, fatality is estimated to be 50% [8, 14, 22-23].

When infective droplets containing MTB are inhaled, a portion may be eradicated by the mucociliary systems in the airways. The evading bacilli that reach the lungs are engulfed by macrophages as an infection containment defense. However, MTB possesses an extraordinary ability to persist within the macrophages and counteracts their destruction mechanisms. The first line of defense for MTB against the destructive environment inside macrophages is the sturdy cell wall. On another front, MTB counteracts the acidification process initiated by the macrophages, thereby changing the pH within the macrophage from the deadly 4.5-5, to a near neutral 6.4. Moreover, the entire metabolic profile of MTB alters so as favor survival within the hostile macrophage environment with limited nutrients [7, 18-19].

The accumulation of macrophages and T-lymphocytes around the tubercle bacilli creates a granuloma in this region. Necrosis then sets in and gradually the bacilli are trapped within the characteristic lesions in a microenvironment with low oxygen, pH, and nutrients. The bacilli thus minimize all their metabolic functions and go into a non-replicative dormant state, and latent TB is established. If the patient immune system is not sufficiently strong, or if latent TB is reactivated, the bacilli

cannot be contained within the granulomas, which burst open and the bacilli spread. They can spread to the alveoli and cause pulmonary TB, or the infected macrophages can take the bacilli to other organs causing extrapulmonary TB (20% of all TB cases) e.g. lymphatic TB. The clinical symptoms of active TB are rather non specific, they include fatigue, night chills and sweats, malaise, progressive weight loss, low-grade fever, and coughing in pulmonary TB where sputum is usually tainted with blood [7, 12, 18-19].

1.4. Drug-resistant tuberculosis

MTB has an intrinsic resistance to a variety of antibiotics by virtue of its exceptional cell wall structure which also include multiple efflux mechanisms to counteract drug and antibiotics, in addition to β-lactamase activity. Additionally, drug resistance of TB can either be primary or acquired, **table 2** provides definitions for key terms related to drug resistance [21, 24], and multiple factors contribute to the development of drug resistance in a TB isolate. Resistance to a single drug can be the outcome of a spontaneous mutation in the respective resistance-conferring gene, an event which occurs in wild MTB strains every 10^6 to 10^8 replications. For the two main anti-TB drugs isoniazid (INH) and rifampicin (RIF), the rate of spontaneous resistance-conferring mutations are 2.56×10^{-8} and 2.25×10^{-10}, respectively [25]. The use of drug combinations in TB therapy reduces the likelihood of emergence of resistance, as the risk of a stain harboring two resistance mutations is less than 10^{-18} [21]. The primary cause of acquired TB drug resistance is prior unsuccessful or incomplete therapy, and thus drug resistance is significantly more likely in retreatment patients, who represent about 13% of notified TB cases worldwide [21, 26].

An issue with global ramifications is the rise of the incidence of drug-resistance in TB, particularly multidrug-resistant TB (MDR-TB) strains worldwide. MDR-TB strains demonstrate resistance to both rifampicin (RIF) and isoniazid (INH), the two main drugs of first line anti-TB antibiotics [27-30]. MDR-TB strains currently infect about 50 million patients worldwide [7, 31], with almost 500,000 new cases annually [32]. MDR-TB cases account for about 2.9% of all new TB cases worldwide [25] with the prevalence of MDR-TB in retreatment cases differing significantly between countries (30% to 80% [33]), with the countries of the former Soviet Union having a particularly significant increase in incidence of MDR-TB [4]. **Figure 3**

illustrates the global distribution of proportion of MDR-TB cases among notified new cases of TB. 85% of the global cases of MDR-TB can be found in 27 HBCs, with China and India alone comprising half of the global MDR-TB burden [34]. This makes up a combined total of 36 HBCs for TB and MDR-TB [3]. Another alarming fact is that an estimated 5-7% of the annual new MDR cases become extended drug-resistant TB (XDR-TB). XDR-TB strains are additionally resistant to a fluorquinolone and at least one of the injectable antibiotics: amikacin, kanamycin, and capreomycin [21].

Despite presenting a significant challenge, MDR-TB is treatable with second-line drugs (e.g. fluoroquinolones). However, early diagnosis is crucial for proper management of the patient [33, 35]. In 2008, 150,000 lives were lost to MDR-TB, mainly because the patients did not receive the required second-line drugs [25]. The fact that only about 7% of global MDR-TB cases are detected is extremely alarming [36]. In Egypt, less than 3% of newly diagnosed MTB cases are classified as MDR-TB, however, this figures rises to about 38% among retreatment cases [34]. Despite the increase in number of MDR-TB cases receiving treatment in 2010, they still account for a meager 16% of the notified MDR-TB cases that year. This is not surprising when considering that in the majority of countries less than 5% of both new and retreatment TB patients were tested for presence of MDR-TB strains [3].

1.5. Genome of *Mycobacterium tuberculosis*

MTB is noted for genetic homogeneity, and the genome of the reference virulent strain H37Rv was sequenced in 1998 and is about 4.4 Mb in size, with a high GC content (65.6%), and 4,000 genes [5, 8]. This is currently the only universal control available for drug sensitivity testing (DST), as a characterized pathogenic pan-susceptible strain [37]. The phenotypic and virulence variations observed among different MTB strains may be molecularly attributed to polymorphic genomic 'regions of difference' (RD), which measure about 0.5 kb [5, 8]. Correlation between expression levels of RD1 antigens with strain virulence has been demonstrated with the BCG vaccination strain, and the deletion of RD1 has been shown to be a key factor in the loss of strain pathogenicity [8, 38-39]. Certain RD loci, can also be of utility in strain lineage identification, such as MTB deleted region 1 (TbD1) whose presence or absence helps define MTB lineages [8].

The currently circulating strains of MTBC are believed to have emerged from a common ancestor, *M. prototuberculosis*, 40,000 years ago in East Africa, then spread in concordance with human population motion. Then about 20,000 years ago two lineages emerged, one which became exclusive to human infection and the other caused animal infections [8, 40]. Currently circulating MTB strains comprise six major clades with geographical association. Also, there is strain variability in disease development and interaction with host immune system [1, 41-42]. The Beijing strain, the most thriving current strain which showed about 500-fold population expansion almost 180 year ago, along with the Indo-Oceanic lineage are significantly more likely to cause disseminated tuberculosis and meningitis compared to European lineage strains. Also, multiple cases of drug resistance were found to be associated with strains belonging to *W* or *W*-like families, and animal model studies highlight the association of *W*-Beijing strain with high virulence and increased dissemination, and early mortality [12, 40-42]. **Figure 4** illustrates geographical strain migration.

Regarding Egypt, there is no clear data on the predominant circulating strains of MTB and the most recent study found, only indicates that there is unexpectedly high proportion of Manu lineage strains, but does not provide details of the circulating strains [43]. Such information is important not only for epidemiological analysis and infection tracing to aid disease control efforts , but also due to the presence of evidence that there is some correlation between genotypes and disease progression [44]. This is particularly noting that Egypt is expected to have mixed lineages in the circulating strains (**Figure 4).**

1.6. Treatment of tuberculosis

The availability of effective drugs make TB a manageable disease, however, the cost, both monetary and humanitarian, is by no means small. Treatment of TB is a lengthy and demanding process. A typical treatment course would last 24-36 weeks using a combination of drugs, which is one contribution to noncompliance of patients and premature interruption of therapy, leading to relapse and possible emergence of drug resistance [24-25]. The emergence of MDR-TB and XDR-TB along with HIV coinfection has compounded the treatment challenges. Treatment of MDR-TB requires a minimum of 18-24 months of therapy and has 60-70% success rates and mortality rates range from 10-15%, and can cost up to 200 times more than drug-

sensitive TB [25, 45]. On the scale of a nation's economy, about $4 billion are needed each year to sustain the infrastructure required for treatment of TB patients, which is a substantial financial burden on the struggling economies of developing low-income countries [13]. However, the use of the WHO directly observed treatment short course (DOTS) strategy between 1995 and 2010 resulted in 7 million saved lives and successful treatment of 46 million patients [3].

The first anti-TB to be discovered was streptomycin (SM) in 1944. However, due to extensive SM monotherapy, resistance developed rapidly to it [1, 21, 46]. The first line anti-TB drugs comprise isoniazid (INH), rifampcin (RIF), pyrazinamide (PZA), ethambutol (ETH), and streptomycin (SM), where INH and RIF are the two most potent drugs. **Table 3** describes these drugs and their actions, while their chemical structures are shown in **figure 5**. INH, the first oral anti-TB drug, was first discovered in 1952, and an INH monotherapy is favored for the latent TB treatment [1, 45-46]. The discovery of rifamycin compounds in 1957, strengthened the anti-TB arsenal with another oral drug; RIF [1, 24]. RIF must always be given in conjunction with another potent antimycobacterial as resistance readily emerges to it [26], and resistance to rifampicin is sometimes considered a marker for MDR [47]. **Table 4** presents the molecular basis of resistance to first-line drugs.

The standard treatment regimen for new TB patients is 2 months of INH, ETH, RIF, and PZA, followed by 4 months of INH, RIF, and ETH. Drugs are administered 5 to 7 times weekly in most regimens. As for retreatment patients, who made up 13% of TB notifications in 2007, DST is recommended prior to therapy initiation and should be the guide for therapy design [26, 45]. However, if DST is not promptly available, the general approach involves treatment duration of about 18-21 months. This involves use of at least 4 drugs to which are likely to be effective as resistance to them is not expected, including an injectable for 7 months followed by a minimum of 3 likely effective drugs. Drugs with expected cross-resistance must be excluded [25-26]. The need for using the drugs cocktail for TB treatment is the need to eliminate tubercle bacilli at different metabolic and growth phases, thereby each drug has a different role in treatment [25, 46, 48], as shown in **table 3. Figure 6** illustrates the target of different TB drugs of the different phases of TB in infection.

In case of intolerance to INH or resistance, 4 month of RIF is an alternative in such cases [45]. INH resistance accounts for almost half of all cases of TB drug resistance, amounting to almost 7.4% of all resistance cases. Additionally, it was

found that monoresistance to INH further aggravates the problem of MDR-TB, as it is a primary risk factor for acquired rifampicin (RIF) resistance [25]. The frequency of INH resistance *in vitro* is 1 in 10^5 -10^6 bacilli [46]. Isolate susceptibility to both RIF and INH makes detection of ETH or SM resistance inconsequential, as treatment regimen will not require modification, and DST for these drugs can only be performed if specifically relevant to a particular patient treatment [49]. In regions where prevalence of INH resistance is more than 10%, for new TB patients, a 2 month intensive treatment phase using INH, RIF, PZA, and ETH followed by a continuation phase including ETH, plus INH and RIF. Also, the standard recommendation for isoniazid monoresistance, despite uncertainty of effectiveness, is 2 months RIF, ETH, and PZA, followed by 8 months RIF and ETH [25].

1.7. Diagnosis of tuberculosis infection

The main strategies for TB diagnosis have not changed much for decades, and in most regions with high TB burden.The primary detection methods of active infection rely on finding the TB bacilli in patient sputum smears. This is a relatively simple, fast, low cost approach that dates back to more than 100 years, however, and yet it misses about 30-35% of positive cases [3, 31]. Culture identification is recommended for smear negative samples and DST, and chest x-ray may be performed sometimes in presence of suggestive clinical symptoms to aid diagnosis [3]. The inadequate performance of the current diagnostic strategies is reflected by the global detection rate of 62%, falling short of the 2005 goal of 70% set by the World Health Assembly, the decision-making entity of the WHO [50]. The magnitude of resource limitation for TB diagnosis is demonstrated by the fact that still 8 of the 22 HBCs lack the WHO recommended 1 microscopy center per 100,000 population [3]. **Table 5** summarizes the main diagnostic strategies available for TB and their limitations.

1.7.1. Phenotypic and biochemical identification

1.7.1.1.Microscopic examination

Smear microscopy relies on the examination of sputum smears for acid-fast bacilli (AFB) stained using acid-fast Ziehl Neelsen (ZN) stain (primary dye is carbolfuschin) (**figure 7**). For detection of positive samples, a minimum of 300 microscopic fields

must be observed, and AFB smear microscopy requires the presence of at least 5,000 to 10,000 bacilli per mL of sputum, a lower bacillary load would be missed. AFB smear microscopy is useful for detection of active TB infection as non-viable bacilli with damaged cell walls may lack the acid-fastness upon which the staining is based [4, 17, 48, 51-53]. The recommended procedure for use of smear microscopy to detect pulmonary TB involves the examination of two sputum smears, and morning collection specimens are preferred [3, 11].

The detection rates are highly variable ranging from 20-80%. Detection rates below 20% are observed in HIV patients, and extrapulmonary TB, and MDR strains cannot be detected, and almost 60% of all TB notifications are either smear-negative or extrapulmonary cases. Also, AFB smears cannot differentiate MTB from nontuberculous mycobacteria (NTMs) with similar looking bacilli, which can pose a problem in area where TB is not endemic [3-4, 27, 53]. An estimated minimum 17% of new infections are acquired from others who tested smear-negative [24]. One approach which is suggested to improve AFB smear sensitivity is sputum concentration prior to examination either by passive sedimentation or treatment with bleach or ammonium sulphate, followed by centrifugation [11]. 11-26% improvement in sensitivity has been reported when the latter method was used [54].

A number of recent improvements have been developed to enhance the performance of smear microscopy. One of which is the use auramine/rhodamine fluorescent dyes for staining smears and the use of fluorescence microscopes for examination. Despite providing a notable enhancement in sensitivity, throughput, and speed of detection, this modification also comes with a significant increase in cost, as the stain, the microscopes and their bulbs are expensive. A possible compromise between cost and performance advantages of fluorescence microscopy may be provided by the newly developed light-emitting diode (LED) microscopes, which are cheaper than regular fluorescence microscopes and can emit regular white light as well as fluorescence wavelengths. The use of LED smear microscopy has been endorsed by the WHO, and several commercial products for TB diagnosis are now available [3, 11, 52, 55].

1.7.1.2. Immunological assays

The main utility of immunological assays in TB diagnosis is screening for latent TB infection. One of the oldest and most widely used assays is the tuberculin skin test

(TST), also known as the Mantoux test. This test involves intradermal injection of TB purified protein derivative (PPD) and then examining the area of injection after 2-3 days for induration. TST detects the cell-mediated immune reaction to the infection, and the cut off diameter of the induration for a positive reaction depends on the risk group to which the patient belongs. False positive results can be observed in previously vaccinated or exposed individuals, and cross reaction with other mycobacteria, as some antigenic components of PPD are conserved among mycobacteria. Also, false negatives are observed in case of immunocompromised patients and those with recent active infection [2, 12, 14, 19].

Another immunological assay; the interferon gamma release assay (IGRA), measures interferon gamma release by T-cells in response to exposure to certain MTB antigens. Initially PPD was used and then other proteins with higher specificity to MTB came into use instead, which are encoded by genes in the RD1 region of the genome e.g. early secreted antigenic target 6 (ESAT-6) [2, 12]. This region is very specific to MTB as it is not found in the BCG vaccine strain and most NTMs, and IGRAs have demonstrated superior specificity to TST and similar sensitivity [2, 8, 56]. However, clinical performance of IGRAs is inadequate for detection of extrapulmonary TB. Three commercial FDA-approved IGRAs are available; T-SPOT TB test (Oxford Immunotech, Abingdon, UK), QuantiFERON-TB Gold (Cellestis Ltd, Carnegie, Australia), and the QuantiFERON-TB Gold in tube (Cellestis Ltd, Carnegie, Australia). After demonstration of significantly superior performance to TST, IGRAs are currently the only available methodology for accurate detection of latent TB infection, a critical determination for the success of disease control efforts [2, 12, 19, 52, 56-58].

On a different front, immunological assays are being developed in attempt to provide fast, direct, non-invasive diagnosis of TB by detection of relative antigens in urine. This could also mean the possibility to develop point-of-care tests which would greatly benefit low-income regions with endemic TB. Another important advantage of detection of TB antigens is avoiding false results due to modulations of the host immune response as is the case with HIV patients when testing for antibodies. Additionally such antigens are generally associated with actively replicating pathogens and thus allow distinguishing between active and latent TB infections [6, 50, 59-60].

Lipoarabinomannan (LAM) is a mycobacterial cell wall glycolipid which was originally found in patients' serum, but was later found to be excreted in urine. LAM is currently detected using enzyme-linked immunosorbent assay (ELISA) and is particularly sensitive in HIV-positive patients and in smear-positive patients. Nevertheless, the overall sensitivity and specificity of LAM detection assays are not yet adequate for conclusive diagnosis of active TB infection [50, 59-60]. However, LAM urine testing combined with smear microscopy can be of value for TB detection in areas of high HIV prevalence [6]. In addition to LAM, a number of other urine antigens are being investigated. These include MTB ornithine carboamyltransferase, phosphoadenosine phosphosulphate reductase, homoserine O-acetyltransferase, and MoaA-related protein as markers of active pulmonary TB infection [31, 61].

1.7.1.3. Culture methods

The current gold standard for diagnosis of MTB infection is bacterial culture, which is very sensitive, however, it is extremely time consuming (typically 3-4 weeks), and has substantial infrastructure and biosafety requirements. Solid media include the conventional and popular egg-based Lowenstein Jenssen (LJ), and the agar-based Middlebrook 7H10, and the newly developed TK medium [4, 18, 48, 52]. LJ cultures are highly sensitive and can detect as few as 10-100 bacilli, and **figure 8** shows the typical colony morphology of MTB grown on them. The TK medium, which has comparable sensitivity to LJ, has the advantage of having a colorimetric indicator incorporated within it, which indicates the mycobacterial growth even prior to colony visualization. However, larger studies are needed for performance validation [17-18, 58, 62]. Sputum specimens to be cultured are first decontaminated and liquefied using N-acetyl-L-cysteine (NALC)-NaOH (2%) treatment, and then planted on the culture medium. NALC is a mucolytic agent while the NaOH kills normal flora in the sputum. The treatment includes a centrifugation for concentration of bacilli.However, overtreatment of the sputum using this approach e.g. using excessive reagent volume with respect to the sample volume, can kill almost one third of the mycobacteria in the clinical specimen [11, 18, 58].

Conventional culture methods also incorporate subsequent multiple biochemical properties allow differentiation of MTB from other mycobacteria. MTB has the capacity to reduce niacin and nitrate but yields a negative result with catalase,

tellurite, and thiophene-2-carboxylic acid hydrazide (TCH) tests. Additionally, identified and differentiated from NTM using media that contains para-nitrobenzoic acid (PNB), which inhibits MTB growth [18, 63].

The development of liquid media such as the Middlebrook 7H9 has resulted in notable improvement in rate of mycobacterial recovery from primary specimens. Greater sensitivity is provided by use liquid media and case detection yield can be up to 10% higher than solid media [64]. A decrease of detection time has also been provided along with aiding the development of more efficient semi-automated and automated commercial detection systems since the 1980s. These include radiometric Bactec 460 TB System (BD Diagnostic Systems, MD, USA), and the non-radiometric Mycobacterial Growth Indicator Tube (MGIT) 960 system (BD Diagnostic Systems, MD, USA). Both systems use Middlebrook 7H9 media supplemented with a growth supplement; oleic acid, albumin, dextrose, and catalase (OADC), and a mixture of antimicrobial agents; polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA). Notably, the MGIT 960 and other similar systems from different suppliers, all show a higher rate of contamination than the older Bactec 460, whose production is being discontinued. However, the latter has the major disadvantage of radioactive waste generation [11, 58, 65]. Despite high sensitivity, throughput, and speed, these automated systems require costly infrastructure setup, maintenance, reliable power supply, and have a relatively large footprint, all of which are factors hindering their routine use in low-resource settings. Liquid media offer speed and higher mycobacterial recovery rates in cultures. However, they are more costly and more liable to contamination, in addition to the need for stricter biosafety and staff training measures due to risk aerosol formation [14, 17-18, 52, 58]. It is to be noted that a contamination rate under 10% in liquid culture is regarded as acceptable [11]. Nevertheless, the overall performance advantage of culture using liquid media has prompted WHO recommendation of their use in low and middle-income regions [3, 11]. **Table 6** describes the operation principles and performance of notable commercial culture methods

1.7.2. Molecular identification methods

One of the most common targets for bacterial identification, including mycobacteria is the highly conserved 16S rRNA gene, and can be utilized for phylogenetic analysis.

Sequencing of the 16S rDNA remains the reference method for mycobacterial identification, although other options include restriction fragment length polymorphism (RFLP) analysis of amplified 16S rDNA regions or probe hybridization. The genomic targets for such analysis are primarily in the 5' section of the 16S rRNA gene and amount to about 500 bp in size [66-68]. The 16S rDNA contains 9 hypervariable regions (V1-V9) which have the sequence diversity used for bacterial speciation [66]. V2 which spans nucleotides 137-242 was found to be useful in detecting mycobacterial species and differentiating them [66]. Two hypervariable regions were also recognized as particularly valuable for mycobacterial species identification and differentiation; region A which spans positions 123 to 273, and region B which spans positions 430 to 500. Region A is usually suitable for identification of most mycobacteria as unique sequences are exhibited by them in that region [67, 69-72]. A minimum of 94.3% similarity between mycobacterial species is found in the 16S rDNA [22]. Other target genes for mycobacterial identification include the 16S-23S internal transcribed spacer (ITS), and the genes *rpoB, gyrB,* and *hsp65* [11, 67-68, 72]. MTB-specific genomic regions make up about 600 kb, about half of which have resulted from lateral gene transfer [22].

A large repertoire of molecular techniques, both commercial and in-house, is available for MTB identification. The majority of these techniques are amplification-based e.g. conventional and real-time PCR, strand displacement amplification (SDA), followed by electrophoresis detection or probe hybridization. Isothermal amplification methods like loop-mediated isothermal amplification (LAMP) are also available. To date, no commercial molecular assay has gained FDA approval for detection of MTB from extrapulmonary specimens. However, several have been cleared for direct detection of MTB in processed sputum samples. Gen-Probe Amplified MTD (Gen-probe Inc., USA) is FDA-approved for use with both smear-positive and smear-negative pulmonary specimens. [11, 51, 58]. **Table 7** presents the main commercial molecular detection assay for MTB detection and their target regions and performance parameters.

A common target for in-house molecular amplification assays for MTB identification, is the IS6110 region, however, there is notable variation in sensitivity and specificity between different studies [51]. Notably, IS6110 RFLP has the highest power of discrimination for strain typing and evaluation of genetic diversity, inspite of its high technical demands [42] Although, molecular assays can shorten detection

time to a few hours, their routine use is not always deemed practical, especially in low-income regions. This is on account of thehigh cost of equipment set up and maintenance, and requirement for highly trained personnel [51, 73].

1.7.3. *Other detection approaches*

The quest for efficient strategies detection of TB continues on all fronts and targets various disease markers. Rotherham *et al.* [74] used aptamers to successfully detect a heterodimer of the promising antigenic markers ESAT-6 and 10-kDa culture filtrate protein (CFP-10) in TB-positive sputum samples. The detection was done using and enzyme-linked oligonucleotide assay (ELONA). The choice of using aptamers, which composed of single stranded DNA, is attributed to their higher specificity and lower cost of production compared to antibodies (10-50 time cheaper) conventionally used for antigen detection.

Baptista *et al.*[75] resorted to using the increasingly promising gold nanoparticles (AuNPs), with their exquisite optophysical properties. The exquisite optical properties of AuNPs are attributed to a phenomenon known as surface plasmon resonance (SPR) and a closely related phenomenon called plasmon-plasmon interaction. A colloidal solution of spherical AuNPs is red, however, when nanoparticles aggregate, interaction of locally adjacent AuNPs (plasmon-plasmon interaction) shifts this color to blue [75-76]. The group used nanoprobes composed of AuNPs linked to thiol-modified oligonucleotides for the detection of the DNA of MTBC members, after first-round PCR amplification. The simple colorimetric assay is based on resistance of AuNPs to salt-induced aggregation upon hybridization of the nanoprobes with the complementary target due to formation of dsDNA providing charge stabilization and steric hindrance preventing the aggregation of AuNPs. Therefore, following PCR and nanoprobe addition, MTBC-positive samples remain red as due to hybridization of the nanoprobes with their target, while in the negative samples the AuNPs are allowed to aggregate and the color of the solution turns blue [75]. The same group developed the assay further to a miniaturized platform that could be used at the "point-of-need". The assay was performed in a 384-well paper microplate (5 µl reaction volume per well) thereby enhancing color visibility due the contrast with the white paper background.

The result was documented using a smartphone camera and analyzed using a simple RGB program anlaysis program on the same device [77].

1.8. Guidelines for detection of active and latent TB and infections

The National Institute for Health and Clinical Excellence (NICE) in the United Kingdom has put forth recommendations for guidance of detection efforts for TB in 2011[78]. These recommendations were based on analysis of a multitude of systematic reviews addressing the issue of diagnosis of TB infection. For diagnosis of active TB infection a chest x-ray is recommended as the starting point. If the x-ray results are suggestive of TB infection, sputum smear microscopy and culture are performed (using at least 3 sputum samples). Rapid molecular tests are recommended for patients with positive sputum smears in case the test result would cause a modification of patient treatment. As for latent TB detection, use of IGRAs is recommended for individuals whose Mantoux test is positive [78].

1.9. Detection of drug resistance

The current most commonly used detection method of active TB, sputum microscopy, cannot detect drug resistant strains– more advanced methods are needed for DST [3, 27]. Conventional DST methods have been slow and labor-intensive due to the lengthy time required for culturing MTB. Performance of DST is not a routine step while testing for TB infection in most countries with high TB burden, but is typically done in reaction to the patient's failure to respond to standard therapy. This means that drug resistance and DST would be performed anywhere from 2 to 8 months after start of treatment, with no positive effect while enduring all the adverse drug effects [24]. Conventional methods include the resistance ratio method, the absolute concentration method, and the current gold standard proportion method. All of these methods have rather long turn-around time (TAT), where solid media culture methods using egg or agar-based media require 3-6weeks. This is in addition to the initial pathogen isolation procedures which take 2-6 weeks. More recently, liquid culture-based methods have been developed which have shorter TAT (albeit still relatively long for optimal clinical interventions), but may be significantly more expensive. This is a crucial factor in limited-resource countries which typically have the highest TB burden [32, 47, 79-82]. The more advanced and expensive liquid media-based

methods shorten TAT for DST to 1-2 weeks [83]. Notably, the use of line probe assays and automated liquid culture based DST is recommended by WHO [14]. **Tables 7** and **8** summarize the main features of the phenotypic and genotypic DST methods which will be presented in the coming sections.

1.9.1. Conventional DST methods

1.9.1.1. Phenotypic DST methods

DST testing can be performed directly on clinical specimen, or indirectly by testing pure isolates cultured from clinical specimens. Direct testing of primary clinical specimens saves the time needed to first culture MTB from sputum (at least 2 weeks) and then perform the DST on the culture. Several commercial assays can be used to perform direct DST, however, despite the time disadvantage DST is generally performed indirectly so asto avoid the ~15% of failed tests attributed to problems and interferences from contaminating bacteria or NTMs in the clinical samples [24, 58]. The popular proportion method remains gold standard for DST, almost 50 years after its initial development, which can be performed using different solid media including the most popular LJ, and Middlebrook 7H10 agar. Medium tubes with and without the drugs to be tested are inoculated with 100 µL of the bacterial suspension are inoculated per LJ tube with and without the drug to be tested and incubated at 37 ºC. After 28 days of incubation the number of colonies in each tube is counted. The ratio of number of colonies counted in the drug containing tubes compared to those in the drug-free tubes is calculated. If the proportion exceeds 1% (for INH and RIF) or 10% for other first line drugs, and second line drugs except *para*-amino-salicylic acid, the isolate is designated resistant. In order to designate an isolate as susceptible, incubation must be extended and on the 42nd day if the proportion does not exceed 1%, the isolate is considered susceptible [47, 51, 84].

The absolute concentration is based on growing the strains to be tested in presence of the drugs at critical concentrations, and the drug-susceptible reference strain H37Rv is also grown in parallel for quality control. After 4 weeks of incubation, if the drug-free growth control has 100 colonies while the drug-containing culture has less than 20 colonies, the isolate is considered susceptible [24].

The resistance ratio (RR) method, involves culturing the samples and the H37Rv strains on solid media with different drug concentrations and without the drug as growth control. The ratio of the MIC of the patient isolated to that of H37Rv is

calculated after 4 weeks of incubation at 37 ºC, where the MIC is defined here as the drug concentration at which the isolate growth has less than 20 colonies. An isolate is defined as resistant to the tested drug if its RR is 8 or more, and susceptible if it is 2 or less. Despite being the most accurate, RR method is not the most favored on account of its labor-intensiveness and cost. However, one advantage of the RR methods is that it allows MIC determination. [24].

On the commercial front, several systems are already available and used for MTB detection, which provide the facility for high throughput DST of MTB. The MGIT 960 system is the only one recommended by the WHO for surveillance of drug resistance (first-line drugs) [84], and none of the commercial systems is FDA-approved for second-line drugs [58]. The automated step in the MGIT 960 is the reading of the tubes, while all other preparatory steps are manual. Consequently, the main advantage is the reduction in turnaround time to a few days, the overall workload is not significantly less than the regular manual method. However, the reading throughput is substantially higher [47, 84]. Characteristics of notable commercial phenotypic DST systems are presented in **table 5.**

1.9.1.2. Genotypic DST methods

Many of the commercial molecular MTB detection systems are also suited, or versions of them for resistance detection (**table 7**), and for in-house assays, similar principles are utilized for resistance detection. Commonly, utilized strategies include probe hybridization, real-time PCR with hybridization probes, while DNA sequencing remains the reference standard. Genotypic methods offer the tremendous advantage of bringing down the time for DST to 1-2 days instead of weeks. However, this comes at a high monetary cost and with practical reservations [51, 55]. A major issue is the fact that the data on resistance–inducing mutations are incomprehensive, and not all mutations are translated into phenotypic resistance. Moreover, molecular methods do not reflect the proportion of the resistant bacteria in the sample [55]. Additionally, the likelihood of false negative results is not to be ignored. This is because absence of a mutation does not necessarily mean the isolate is phenotypically susceptible to the drug. It is not an uncommon finding that isolates demonstrate phenotypic drug-resistance while molecular testing shows they lack known resistance-conferring mutations [49].

Initially developed over 15 years ago, line probe assays (LPAs) utilize PCR amplification with labeled primers, followed by solid phase reverse hybridization. The labeled PCR amplicon is incubated in solution on nitrocellulose or nylon membranes strips on which target-specific probes are immobilized in parallel lines on solid support. Unbound DNA is then washed off, the hybridized probes are visualized by color development through enzymatic reaction utilizing the labeled amplicon, revealing a pattern of probe hybridization which reflects the resistance status [84-86]. There are currently three LPAs on the market; GenoType® MTBDR/MTBDR*plus* and Geno-Type® MTBDR*sl* (Hain Lifescience, Nehren, Germany) and INNO-LiPA Rif.TB (Innogenetics, Zwijndrecht, Belgium) [11, 55].

One of the main issues with use of molecular assays is the high risk of large scale cross contamination, which is why fully automated closed systems are preferred despite cost. Additionally, since PCR amplification is an initial step in many genotypic assays, presence of polymerase inhibitors is a potential problem [55, 84]. However, this was addressed in another successful test which is a hemi-nested real-time PCR based assay for RIF DST Xpert MTB/RIF (Cepheid Inc., CA, USA) which has the advantage of automation and being a closed system, thereby minimizing risk of cross contamination. The combination of speed and specificity of LPAs and Xpert MTB/RIF assays have led to the WHO recommendation for the expansion for their use in screening for drug resistance and initiation of measures to lower cost for accessibility by impoverished areas with high burden TB [3, 55, 58, 87]. Both INNO-LiPA RifTB and Xpert MTB/RIF can detect extrapulmonary TB with high specificity (pooled specificity 94% and 96%, respectively), but with limited sensitivity (pooled sensitivity 68% and 63%, respectively) [55].

1.9.2. Emerging DST methods

About 60% of the global number of TB patients present in remote and peripheral healthcare centers lacking the resources and infrastructure for advanced and molecular diagnostic systems [88] . Consequently, a number of new techniques have emerged to circumvent the disadvantages of high cost and long TAT of conventional culture DST, some of which have already gained WHO endorsement. Other promising approaches are yet to accumulate a sufficient body of supporting evidence e.g. mycobacteriophage-based assays, others. The latter include the microscopic

observation drug susceptibility (MODS) assay, and the colorimetric assays; the nitrate reductase assay (NRA) and the colorimetric redox indicator (CRI) method [32, 84, 89].

1.9.2.1. Microcolony growth examination methods

1.9.2.1.1. Microscopic observation drug susceptibility assay (MODS)

The proof-of-concept of MODS as a rapid liquid culture-based method for simultaneous detection and DST directly from decontaminated sputum was first provided by Caviedes *et al.* [90], and illustrated the validity of direct detection of resistance to RIF and INH with sensitivity of 92% and specificity of 93%.

Processed sputum samples (or isolate bacterial suspensions) are inoculated into supplemented Middlebrook 7H9 broth with and without drugs (INH and RIF) at critical concentrations (0.4 and 1 µg/mL, respectively), in 24-well microtiter plates (**figure 9)**. The plates are sealed in zip-lock bags and incubated for 7 days at 37 ºC. The plates are then microscopically examined for the characteristic cording pattern of MTB organisms (**figure 10**)**.** If no characteristic MTB cords are detected in the drug-free wells, the reading is repeated every other day up to 21 days, after which the sample is declared negative [91-92]. The most recent meta-analysis found MODS to have pooled sensitivities of 88.6% and 96.8% for detection of INH and RIF resistance, respectively, in direct specimens (n=7), and pooled specificities of 98.5% and 99%, with an average TAT of 11.6 days. As for indirect testing the pooled sensitivities were 93% and 100% for INH and RIFresistance detection, respectively, and the pooled specificities were 100% for both drugs, with an average TAT of 6.5 days (n=2) [89].

The main advantage of MODS is that it utilizes common facilitiesavailable in TB laboratories, including those with limited resources, with which the personnel are typically familiar. Also, direct DST testing can simply be performed in biosafety level 2 laboratories and the costly sophisticated level 3 set up is uncalled for. MODS combines the relatively rapid growth of culture in liquid media, with a much more cost-effective methodology. However, this test method is still fairly labor intensive and technically demanding, particularly in inoculation, where contamination risk is greatest [32, 47, 49, 82, 84]. Average contamination rate in MODS studies was found to be 7.4% [89]. Caution must be exercised when using MODS for direct testing in regions with high NTM prevalence as the evidence is still lacking with respect to the

assays ability to differentiate them from MTB, particularly *M. chelonae*. One proposed solution for this issue is the inclusion of PNB-containing well which would specifically inhibit MTB growth [84, 89].

1.9.2.1.2. Thin Layer Agar (TLA) Method

The thin layer agar (TLA) method is also known the microcolony method, and can detect mycobacterial growth on agar media within about 10 days. This method uses quadrant agar plates with drug-free and drug-containing quadrants (at critical concentrations). The quadrants are inoculated with the processed sputum and incubated at 37 ºC with 5% CO_2. The plate is then examined with a light microscope with 100x magnification for colony growth [84, 93-94]. One version of TLA includes PNB in one of the quadrants to ensure growth is MTB complex and not an NTM [94]. The current body of evidence available for TLA remains insufficient for endorsement of its worldwide use [55], where a recent meta-analysis identified only 3 assessment studies of TLA, all showing 100% concordance with the reference proportion method. Also, a thorough cost analysis is not yet available [89].

1.9.2.2. Colorimetric methods

1.9.2.2.1. Colorimetric Redox Indicator (CRI) Assay

The CRI assay detects the metabolic activity of viable mycobacteria that reduce a redox indicator added to the medium after the isolates are incubated with each test drug. The reduction causes a change in the color of the indicator, which varies based on the used indicator (e.g. Alamar blue or resazurin changes from blue to pink; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) changes from yellow to violet). The metabolic reactions of the viable mycobacteria reduce resazurin (blue) to resorufin (pink), while in case of MTT (yellow), it is reduced to insoluble formazan (violet), which is then solubilized prior to result reading [79, 95].

The assay is performed in a microtiter plate, where the isolates to be tested are incubated for 7 days at 37ºC with a serial dilution of the drugs in broth, and growth and sterility controls are also included. Then the redox indicator is added, the plate reincubated, and the plate read the following day (**figure 11**) [73, 96].

The CRI assay was initially developed for screening and evaluation of anti-tuberculosis drugs and utilized the propriety redox indicator Alamar blue [97] and the microplate format was introduced by Franzblau *et al.* in 1998 [98]. It was later

established that Alamar blue and resazurin are basically the same active compound (**figure 12**) and undergo the same reaction in the CRI assay. However, unlike Alamar blue, resazurin, usually supplied as a sodium salt, is a non-proprietary reagent [97].

Different studies investigated the performance of the CRI assay using MTT, Alamar blue, or resazurin, and found them to yield comparable results [79], however, there are some factors to be considered in the indicator choice, including reagent propriety which affect both cost and access to reagent. As for MTT, the main issue associated with its use is the need for an additional solubilization step for theformazan, which requires the plate to be opened one more time, thus posing an additional risk of assay contamination and biosafety hazard due to possible aerosol formation [97]. Moreover, there is a risk of false-resistance results with INH due to its potential interference with formazan formation [99].

The Alamar blue and resazurin CRI has demonstrated reliable and consistent results with regards to INH and RIF susceptibility testing, and subsequently for MDR-TB detection. However, some discrepancies with regards to MICs were found when testing ETH and SM, but had little impact on final designation of sample as resistant or susceptible. The assay can also be carried out using an inoculum from both solid and liquid cultures, with no adverse effect on performance [100]. The resazurin-based CRI for MDR-TB detection was first introduced by Palomino *et al.* [96], which is now WHO-endorsed and a standardized protocol is now available [64, 101]. An evaluation study of the assay in Madagascar yielded sensitivity of 95% for both INH and RIF resistance detection, while the specificity was 97.3% for INH and 100% for RIF. The total TAT for CRI, including initial isolation was 4-5 weeks versus 7-10 weeks for LJ-based proportion method [102]. Overall, the reported TATs for these assays are much shorter than the 3-6 weeks needed for conventional methods, the TATs range between 7-14 days for CRI, exclusive of initial isolation [79].

Moreover, a study by Martin *et al.* [103] incorporated nicotinamide in a modified CRI to perform DST for the typically problematic pyrzinamide, on account of its requirement for low pH in the media. Additionally, CRI is being evaluated for detection of XDR-TB by testing second-line drugs and fluoroquinolones [97, 104]. Despite low expectations of success due to high contamination chances, use of direct CRI testing on sputum has been attempted using MTT, with promising results, but evidence remains too scarce for constructive evaluation [73, 97, 105].

1.8.2.2.2 Nitrate reductase assay (NRA)

Similar to CRI, the NRA utilizes the biochemical activity of viable Mycobacteria in the sample. The basis of NRA is the fact that MTB can reduce nitrate to nitrite, which is one of the distinctive biochemical properties of MTB among mycobacteria. This ability is lacked by *M. bovis*, another MTBC member. In this assay the sample is incubated with and without the test drug in liquid media containing potassium or sodium nitrate at 1000 µg/mL concentration. Resistant strains will remain viable in the presence of the drugs, convert the nitrate in the medium to nitrite and result in the change of the indicator Griess reagent color to pink. Griess reagent contains an azo dye that changes color upon reaction with nitrite in the medium (**figure 13).**It is composed of 50% (v/v) hydrochloric acid, sulfanilamide 0.2% w/v, and N-(1-naphtyl)ethylene-diamine dihydrochloride 0.1% (w/v) mixed in ratio of 1:2:2. [81, 106-107].

NRA can be performed using either solid or liquid media [32, 47, 81, 108], and the solid media-based format has been endorsed by the WHO for both direct and indirect DST [64]. Direct NRA was found to yield results in median time of 10 days (range 10-23 days), and detected MDR-TB with a sensitivity of 97% and specificity 98% [107]. The indirect LJ-based NRA does not provide significant TAT advantage over conventional methods based on use of solid media [47].

A few reports illustrate the use of NRA based on liquid media with good performance [99, 108-110]. In one approach, the assay was performed using 7H9 broth with and without INH and RIF at critical concentrations, in screw-cap tubes to minimize risk of aerosol formation while benefiting from the high mycobacterial recovery rate of liquid media. Despite providing more safety, the use of the liquid-based NRA in tubes is rather cumbersome and impractical in terms of throughput, particularly if MIC determination is to be carried out. Kumar *et al.* reported the use of liquid-based NRA in 96-well microtiter plates [110-111]. Despite safety concerns, this format allows handling of smaller culture volumes while allowing for MIC determination and increasing throughput as multiple samples can be tested in the same plate with multiple drugs. The microplate assay showed 96% concordance with the proportion method for detection of INH resistance and 100% for RIF, with results available within 8 days [110].

By detecting the nitrate reduction activity of MTB prior to colony formation, NRA offers sensitivity and speed. However, a potential limitation albeit minor, is the

assay's inability to detect the rare nitrate reductase-negative strains of MTB. Although, these represent less than 1% of strains, this caveat must always be taken into consideration [112]. Another point which must also be considered in use of NRA is that Griess reagent cannot detect the reduction of nitrate, beyond nitrite, to nitric oxide. The issue is mitigated by addition of a small amount of zinc dust to negative tubes, if the nitrate in the medium has already been reduced beyond nitrite, the color will not change and the result will be considered a positive. However, if the color changes to pink due to rapid reduction of nitrate by zinc, this means it was a true negative in the beginning and no nitrate reduction was undertaken by mycobacteria [112-113]. The analysis by Martin *et al.* [81] reported overall TATs of 5 to 12 days for indirect NRA, and 14 to 18 days for direct NRA. Direct testing of sputum using NRA on liquid media is promising but current data is insufficient for proper evaluation [81].

1.9.2.3. Mycobacteriophage-based assays

Another promising strategy that is yet to accumulate enough evidence to warrant recommendation of large scale or routine use is the mycobacteriophage assay. This assay utilizes the natural specificity of certain phage types for infection and replication within mycobacteria [24, 55, 65, 84].

There are two approaches utilizing mycobacteriophage which were used for development of drug resistance detection assays, and a single commercial provider; Biotec Labs Ltd, Ipswich, UK. In the first approach, the basis of detection is phage amplification, and is the basis of the only commercial phage-based MTB resistance detection assay; the CE-marked *FASTPlaque* assay (Biotec Labs Ltd, Ipswich, UK). D29 lytic phage is incubated with the decontaminated sputum samples, with and without RIF, and then a virucidal agent is added to eliminate excess phage outside of the mycobacterial cells. Progeny phage generation in the viable MTB cells result in plaque formation on a lawn of *M. smegmatis* (non-pathogenic fast-growing mycobacterium) on agar plates, and RIF resistance can thus be detected within 2 days [65, 84, 114]. According to the most recent meta-analysis, an average of 21% of direct sputum tests using this strategy yielded uninterpretable results. The manufacturer introduced the use of an antibiotic supplement containing nystatin, oxacillin and aztreonam (NOA). This reduced bacterial and fungal contamination by 68% but increased the assay cost. the pooled sensitivity of the direct commercial phage

amplification assay was 93.6%, and 95.7% for the indirect assay, and the pooled specificities were 96.3% and 94.1%, respectively [114].. The direct assays were shown to suffer from unexplained false-resistance results as well as contamination problems [84, 114], where.

The other approach, the luciferase reporter phage assay, is based on using bioengineered phages incorporating a luciferase gene, and is used on isolates. These phages are incubated with the sample, with and without the drug, and the medium contains the substrate luciferin. In viable mycobacteria, the engineered phage is able to produce a light signal in presence of luciferin and cellular adenosine triphosphate, which can be detected using a luminometer, and a result is obtained within 2 days [65, 84]. Commercialization of this assay was attempted by Sequella Inc., USA into a complete system termed Bronx Box. However, these attempts were recently abandoned. The mycobacteriophage assays have demonstrated the advantages of rapidity and high sensitivity; however, their drawbacks hinder their practical application. At the heart of thesassays are substantial cost elements such as that of the engineered phage, the NOA supplement, and the need for a luminometer for signal detection (in case of the luciferase assay). Moreover, the time-saving capability of direct sputum testing is hindered by siginifcant rates uninterpretable results, and when this is combined with the cost factor, the actual practicality of such assays remains questionable [30, 114].

The above mentioned assays are appropriate examples of approaches that are principally sound, but are tackled by the practical and logistical challenges of TB detection and resistance testing, including the nature of the primary specimens, and slow growing nature of MTB. This is the elusive nature of the balance between efficient analytical performance and turnaround time, and cost effectiveness and practicality, bearing in mind the financial and logistical settings of the areas and markets where TB DST assays are most needed.

2. Project Aims

1. Evaluation of the performance of the colorimetric redox indicator (CRI) and the nitrate reducate assay (NRA) methods for detection of multidrug resistant tuberculosis (MDR-TB), defined by resistance to both isoniazid (INH) and rifampicin (RIF), in limited resource settings with adequate performance. The performance is compared with microscopic observation drug susceptibility assay (MODS) and the commercial MGIT 960.

2. PCR amplification and DNA sequencing of part of the 16S rDNA from ten random clinical samples being tested using CRI & NRA. This is to confirm that the samples' identities as *Mycobacterium tuberculosis* (MTB), and to determine if there are any strain variations among these Egyptian clinical samples.

3. PCR amplification and DNA sequencing of gene segments containing common resistance-conferring mutations, for selected samples. This is to gain insight regarding the molecular basis of the detected phenotypic resistance of the samples. The target genes are *katG* and *inhA* for INH resistance, and *rpoB* for RIF resistance.

3. Materials and Methods

3.1. Samples

Thirty four mycobacterial isolates on LJ medium, from the sample archive of the Naval Medical Research Unit 3 (NAMRU-3) were tested in this study. The samples were isolated from sputum samples, collected between 2009 and 2011, from Egyptian patients diagnosed with TB, processed using NALC-NaOH method.The samples were collected through research project NAMRU3.2010.004: Integration of Microscopic Observation Drug Susceptibility (MODS) assay technology into tuberculosis surveillance in Egypt between 2009 and 2011, from patients diagnosed with TB. The reference strain H37Rv (pathogenic, but sensitive to INH and RIF) was also included. The available samples contain 9 drug-resistant isolates, resistant to INH and/or RIF. Five isolates were resistant to both RIF and INH and are thus considered as MDR, 3 isolates were INH-monoresistant, and 1 was RIF-monoresistant.

3.2. Media Preparation

3.2.1. MODS

7H9 broth was prepared by weighing 5.9 g Middlebrook broth base powder (BD, NJ, USA cat. # 271310), 1.25 g casitone (BD, NJ, USA cat.# 225930) and dissolved in 900 mL of distilled water, along with 3.1 mL glycerol. The broth was then autoclaved and incubated for 24 h at 37°C and checked for sterility (indicated by lack of turbidity after incubation).

3.2.2. CRI and NRA

7H9-supplemented (7H9-S) broth consisting of Middlebrook 7H9 broth, 10% OADC supplement (oleic acid, albumin, dextrose, catalase) (BD, USA, cat. #211886), 0.5 % glycerol, and 0.1 % casitone was prepared as follows. 0.94 g of 7H9 powder were weighed and dissolved in 180 mL of distilled water, then 0.2 g of casitone were added and dissolved. This was followed by addition of 1 mL of glycerol, and the broth was autoclaved. After autoclaving and cooling, 20 mL of OADC were added and mixed well. For use in NRA, 1 mL of filter-sterilized $NaNO_3$ (Sigma- Aldrich, cat. # S5022-1KG) (20% w/v) was added to make 7H9-SN broth. Both media were then incubated for 24 h at 37 °C and checked for sterility.

3.3. Preparation of Drug Solutions

3.3.1. MODS

An INH stock of 8 mg/mL was prepared by dissolving 20 mg INH powder (Sigma-Aldrich, cat. # 377-50G) in 2.5 mL distilled water. A RIF stock was of 8mg/mL was prepared by dissolving 20 mg RIF powder (Sigma-Aldrich, cat. # R3501-5G) in 1.25 mL dimethyl sulfoxide (DMSO) and then adding 1.25 mL distilled water. Both solutions were filtered using 0.2 µm syringe filters (CHM, Spain, cat.# BM316) and aliquoted and stored at -20ºC.

3.3.2. CRI and NRA

An INH stock of 1 mg/mL was prepared by dissolving 2 mg INH powder in 2 mL distilled water. A RIF stock of 10 mg/mL was prepared by dissolving 20 mg RIF powder in 2 mL absolute methanol. Both solutions were filtered using syringe filters and aliquoted and stored at -20ºC.

3.4. Testing of Samples Using MGIT 960 System

All samples were tested using BACTEC MGIT 960 (BD, NJ, USA), according to manufacturer protocol.

3.5. MODS Testing

The assay was performed as previously described in [91]. 4.5 mL of the prepared 7H9 broth were placed in sterile screw-cap tubes, and 0.5 mL OADC were added to each tube. Several mycobacterial colonies were harvested from LJ slants into sterile screw-capped tube containing 100 µL of freshly prepared sterile 0.04% Tween 80 (BD, NJ, USA, cat # 231181) solution and 4-5 sterile glass beads. The tubes were vortexed for 2-3 minutes, then allowed to stand for 5 minutes. 3 mL of the Tween 80 solution were then added and vortexed again for 20 seconds and the tubes were allowed to stand for 30 minutes. The supernatant was transferred to another sterile tube and the suspension's turbidity was adjusted to 1.0 McFarland, and 5 µL of each bacterial suspension was then added to each supplemented media tube to make up the final inoculum. Stock INH and RIF solutions were diluted in broth to working concentrations of 4 µg/mL and 10 µg/mL, respectively. 100 µL of the supplemented broth were placed in the first two wells of each column of a sterile 24-well microplate (BD ,NJ, USA, cat.# 353047), to act as drug-free controls, and 100 µL of each of the

working drug solutions were placed in the other two wells 900 µL of the inoculum were placed in each of the respective wells of the microplate for each sample. One column included only 1 mL broth in all well, with no inoculum to serve as a negative control. The plates were then covered with their lids, sealed with autoclave tape, placed in ziplock bags, and incubated at 37℃. After incubation for 5 days, the plates were then examined under inverted light microscope with 40x magnification (after visual examination of wells for turbidity to rule out contamination) to observe the characteristic cording pattern of MTB (**figure 10)**. If no cords the plate was reincubated and examined again at 7 days, or every two days up to 21 days.

3.6. CRI Testing

The assay was performed according to the protocol by Martin and Palomino [101]. 100 µL of 7H9-S broth were placed in each well of a 96-well microplate (Greiner Bio One, cat. # 655061), excluding outer perimeter wells. The drug stock solutions were diluted in 7H9-S broth to prepare a working INH solution with a 4 µg/mL concentration and a RIF solution with an 8 µg/mL concentration. 100 µL of each drug working solution were placed in the first row of columns 2-9, then 2-fold serial dilutions of the drugs were made down the columns. Inoculums from 21-28 day old isolates from LJ cultures were prepared in 7H9-S broth and adjusted to 1.0 McFarland standard. The inoculum was diluted 1:20 using 7H9-S broth, and 100 µL of the diluted inoculum were used to inoculate the plate (**figure 14)**. Growth control wells without drugs for each sample were included as well as sterility control wells with only 7H9-S broth, and outer perimeter wells were filled with sterile distilled water. The plate was placed in ziplock bag and incubated for 7 days at 37ºC then 30 µL of 0.01% resazurin solution in distilled water were added to each well and the plate sealed and reincubated overnight then read.

3.7. NRA Testing

The assay was performed based on the method of Kumar *et al.* [110] with modifications. 50 µL of 7H9-SN broth were placed in all wells of a sterile, flat-bottom 96-well microtiter plate, except column 1 and 12. The drug stock solutions were diluted in 7H9-S broth to prepare a working solution of INH (16 µg/mL) and a working solution of RIF (32 µg/mL). 50 µL of each working drug solution were placed in the first row of columns 2-9, then 2-fold serial dilutions of the drugs were

made down the columns. Inoculums from 10-21 day old isolates from LJ cultures were prepared in 0.05% Tween 80 in 7H9-S broth and adjusted to 1.0 McFarland standard. The inoculum was diluted1:10 in 7H9-SN and the plate was inoculated with 50 µL of the diluted inoculum in each well (**figure 15).** Growth control wells without drugs for each sample were included as well as sterility control wells with only 7H9-SN broth and no inoculum. The plate was placed in ziplock bag and incubated for 7 days at 37ºC then 50 µL of Griess reagent (Sigma-Aldrich, cat. # 03553-100ML) were added to each well and the plate sealed and read.

3.8. DNA Extraction

Extraction of mycobacterial DNA was performed using a modified enzymatic method [115]. The sealed LJ slant tubes containing the bacterial growth were first heated in an 80ºC water bath for 1 hr. The bacterial cells were then harvested in 2 mL Tris-EDTA (TE) buffer, pH=8, then frozen for 4 hrs, to weaken the cell wall. The pellet was then thawed and resuspended in the TE buffer. Delipidation to aid cell lysis, was then performed by adding an equal volume of chloroform/methanol mixture (2:1) and rocking the tubes for 2 minutes. The suspension was then centrifuged at 5000 g for 20 minutes, and the bacteria formed a tight band at the interface of the aqueous and organic layers, which were discarded and the bacterial band retained. The tube containing the bacterial band was heated with the cap opened at 55ºC for 10 minutes to remove any traces of chloroform, and then the bacterial band was resuspeneded in 2 mL TE buffer. 200 µL of Tris-HCl, pH = 9, were then added, followed by 10 µL of lysozyme, stock concentration was 10 mg/mL. The tubes were then incubated overnight at 37ºC, and then 10 µL of DNAse-free RNAse (Promega, cat # M6101), stock concentration 10 mg/mL, were added, and the tubes reincubated at 37ºC for 30 minutes. Then a DNA extraction kit (Qiagen, USA, cat.#51304) was used in accordance with the protocol for gram-positive bacteria after the lysozyme digestion step.

3.9. Quantification of extracted MTB DNA

Concentration of extracted DNA was determined by spectrophotometric measurement at 260 and 280 nm using NanoDrop spectrophotometer (Thermo Scientific, USA).

3.10. Sequencing of 16S rDNA

3.10.1. Primer design

A pair of primers targeting a conserved part of the 16S rDNA of *Mycobacterium* genus was designed Vector NTI Advance® v.11.5 (Invitrogen). The conserved region was chosen based on multiple sequence alignment analysis against *Mycobacterium* genomes sequences published in NCBI Gene Bank using the same software package.

3.10.2. PCR amplification of 16S rDNA region

A 514 bp region of the 16S rDNA gene was amplified using standard PCR using our designed primers, for 10 randomly chosen isolates from the initial 34 isoaltes . First, the extracted MTB DNA was digested using *Bam*HI restriction enzyme (Promega), according to manufacturer protocol. *Bam*HI was selected due to absence of a restriction site within the target region. Dimethylsulfoxide (Sigma-Aldrich, cat.#276855) was added in the master mix (Promega, USA, cat.#M7142), at 5% final concentration, and the mix was used for PCR. A 50 µL PCR reaction was prepared by mixing 25 µL PCR Master Mix 2X, 2.5 µLof 10 µM of each of the forward and reverse primers, 5 µL of DNA template, and 15 µL nuclease-free water. PCR was done in 25 cycles: initial denaturation at 95℃ for 2 min, denaturation at 95℃ for 30 s, annealing at 61°C for 1 min, extension at 72°C for 45 s, and final extension at 72°C for 2 min in a thermal cycler (Veriti, Applied Biosystems CA, USA). Amplification was verified by electrophoresis of the amplicon on 2% agarose gel and visualization of the ~500 bp band using ethidium bromide staining. A 100 bp ladder (Promega, USA, cat.#G2101) was used for band size comparison.

3.10.3. DNA sequencing of 16S rDNA

The same primers that were used for PCR amplification of the 16S rDNA region were used for sequencing (forward and reverse) the amplicon using ABI 3730xl DNA sequencer (Applied Biosystems, CA, USA) according to manufacturer's protocols.

3.10.4. 16S DNA sequence data analysis

Vector NTI 11.5 software was used to assemble, trim, and then align all sequencing results along with the full 16S sequence of the reference strains H37Rv (GenBank no. X52917.1), and they were also analyzed using NCBI Blast for alignment against available DNA sequences in the NCBI database.

3.11. Sequencing of INH and RIF resistance-related genes

3.11.1. Primer design

Two pairs of primers were obtained from the literature to target *katG* [116] , and *rpoB* [117] genes, while another pair targeting *inhA* regulatory region was designed using the Vector NTI 11.5 software (**table 9)**.

3.11.2. PCR amplification of resistance-related regions of katG, inhA, and rpoB

The target regions of *katG, rpoB,* and the regulatory region of *inhA* of 3 phenotypically drug resistant samples, 3 phenotypically susceptible samples, and the reference drug-susceptible strains H37Ra, were amplified by conventional PCR. First, the extracted MTB DNA was digested using *Eco*RI restriction enzyme (Promega, USA, cat# R4014), according to manufacturer protocol. *Eco*RI was selected due to absence of a restriction site within the target region. Dimethylsulfoxide (DMSO),(Sigma-Aldrich, cat. #276855) was added in the master mix (Promega, USA, cat.# M7142), to yield final concentration of 5%, and the mix was then used for PCR. A 50 µL PCR reaction was prepared by mixing 25 µL PCR Master Mix 2X, 2.5 µL of 10 µM of each of the forward and reverse primers, 5 µL of DNA template, and 15 µL nuclease-free water. PCR was done in 25 cycles: initial denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing for 1 min (temperature used for each primer is listed in **table 9)**, extension at 72°C for 45 s, and final extension at 72°C for 2 min in a thermal cycler (Veriti, Applied Biosystems CA, USA). Amplification was verified by electrophoresis of the amplicon on 2% agarose gel and visualization of the bands using ethidium bromide with comparison to a 100 bp ladder (Promega, USA, cat.#G2101).

3.11.3. DNA sequencing of katG, inhA, and rpoB

The same primers that were used for PCR amplification of the *inhA, katG* and *rpoB* regions were used for sequencing (forward and reverse) the amplicon using ABI 3730xl DNA sequencer (Applied Biosystems, CA, USA) according to manufacturer protocols.

3.11.4. DNA sequence data analysis for katG, inhA, and rpoB

Vector NTI 11.5 software package was used to assemble, trim, and then align all sequencing results along with the full sequence of the reference strain H37Ra, and they were also analyzed using NCBI Blast.

3.12. Biosafety measures

All steps for preparation of MODS, CRI, and NRA were performed in a level 2 biosafety cabinet (BSC-2), with the operator wearing N-95 mask. For plate reading and documentation, the plates were always maintained in the sealed ziplock bags, and the N-95 mask was always on when the plates were removed from the BSC to be examined under the microscope (in case of MODS) or examined using the plate mirror and photographed. As for the DNA extraction process, the heat decontamination, and harvesting the cells into the TE lysis buffer steps are performed inside the BSC while wearing the N-95 mask. The remaining steps were performed outside the BSC with common level 2 laboratory safety precautions.

4. Results

4.1. Samples

Thirty four mycobacterial isolates, comprising the sample pool were characterized using both MODS and MGIT, with complete concordance between both methods (**table 10**). Five samples were found to be MDR, 3 monoresistant to INH, 1 monoresistant to RIF, and 25 were sensitive to both drugs. The MGIT testing was performed on the standard panel of first-line drugs SM, INH, RIF, ETH (SIRE panel). 6 isolates also demonstrated resistance to SM and 2 to ETH. One sample was only included in the molecular studies and not in the culture-based resistance detection using CRI and NRA, for safety reasons, as MGIT results indicated its resistance to all 4 drugs in the SIRE panel.

4.2. Phenotypic detection of drug resistance

4.2.1. Colorimetric redox indicator assay (CRI)

CRI assay was performed on 31 isolates; 4 isolates were MDR, 3 monoresistant to INH, 1 monoresistant to RIF, 23 sensitive to both drugs. The MICs of the resistant isolates ranged from 0.125 to > 1 µg/mL for INH, and from 0.5 to >2 µg/mL for RIF, and thus one isolate which was INH monoresistant by MGIT and MODS was found sensitive by CRI. Concordance was 96.7% with MGIT and MODS reference methods, and assay reproducibility was established by random repeat testing of isolates. Performance of CRI is presented in **table 11**. An example of a CRI plate result is shown in **figure 16.**

4.2.2. Nitrate reductase assay (NRA)

NRA was performed on 30 isolates, 4 MDR, 3 monoresistant to INH, 1 monoresistant to RIF, and 22 sensitive to both drugs. The MICs of the resistant isolates were determined, The MICs of the resistant isolates ranged from 5 to > 8 µg/mL for INH, and from 10 to >16 µg/mL for RIF. Concordance was 93.3% with MGIT and MODS, and false resistance to RIF was obtained for one INH monoresistant isolate, and another INH resistant isolate yielded a false sensitive result for INH. Assay reproducibility was demonstrated by random repeat testing of isolates. Performance of NRA is presented in **table 12**. An example of an NRA plate result is shown in **figure 17.**

4.2.3. Turnaround times (TAT)

CRI median TAT was 8 days (range 7 to 10 days), and that of NRA was 7 days (range 6 to 8 days). MGIT TAT ranged from 5 to 13 days, and MODS from 7 to 21 days.

4.2.4. Age of tested isolates in CRI and NRA

Since both CRI and NRA are indirect testing methods, that require initial bacterial isolation, the age of isolates affected the TAT. Optimum age of isolates testing was determined to be 18-29 days for CRI, and 13-21 days for NRA, which yielded clearest and most consistent results. Although, valid CRI result were obtained for isolates ranging in age from 12-32 days, and 12-28 days for NRA (one MDR isolate 32 days old yielded valid results).

4.2.5. Cost analysis

The cost of both assays was estimated with respect to used media, drugs, indicator, and microplates and other consumables. The cost of testing an isolate for resistance to both INH and RIF using CRI and NRA was found to be $4.5 and $5.2, respectively.

4.3. PCR amplification and sequencing of 16S rDNA

4.3.1. Selection of PCR primers

The target region for the designed primers was chosen to be a highly conserved part of the 16S rDNA of *Mycobacterium* genus, as multiple alignment analysis against *Mycobacterium* genomes sequences, including the reference strain H37Rv published in NCBI Gene Bank showed high degrees of homology (**figure 18)**.

4.3.2. PCR amplification of 16S rDNA region

A clear band of ~500 bp size was visualized on agarose gel electrophoresis thus supporting amplification of the target region (**figure 19)**.

4.3.3. DNA sequencing

The PCR amplicons for the 16S rDNA were sequenced and the sequencing results showed clear sharp bands and low background noise (**figure 20**).

4.3.4. Blast analysis

Blast analysis of sequencing results for 16S rDNA (forward and reverse sequences assembled) using NCBI nucleotide Blast software yielded results supporting sample identities as *Mycobacterium tuberculosis* (**figure 21)**. E-value was 0.0 indicating the high significance of the obtained results. Alignment of the sequences and construction of the relationship dendrogram showed that all the samples clustered closely except for two samples (#321 and #361), which clustered together differently (**figure**s **22** and **23**).

4.3.5. Mutation analysis

The amplified part of 16S rDNA encompasses the *rrs* gene, which is known to harbor point mutations correlated with resistance to SM. The sample sequences and that of the reference H37Rv (GenBank no. X52917.1) were aligned using AlignX Vector NTI 11.5. Analysis of the sequences' alignment for polymorphisms showed two point mutations at nucleotide 513 (A→T) and 526 (C→T) based on *E. coli* numbering system, in two samples known to be SM-resistance by MGIT analysis (**figure 24**).

4.4. PCR amplification and sequencing of inhA, katG and rpoB regions

4.4.1. Selection of PCR primers

All primers for *katG*, *inhA*, and *rpoB* were verified for specificity by multiple alignment analysis against *Mycobacterium* genomes sequences published in NCBI Gene Bank using the Vector NTI 11.5 software package.

4.4.2. PCR amplification of inhA, katG and rpoB regions

A clear band of ~400 bp size for *katG* amplicon, and at ~600 bp size for *inhA* and *rpoB* amplicons were visualized using agarose gel electrophoresis thus supporting amplification of the target regions.

4.4.3. DNA sequencing

The PCR amplicons for the *inhA, katG* and *rpoB* genes were sequenced and the sequencing results showed clear sharp peaks and low background noise.

4.4.4. Mutation analysis

The *katG* sequences obtained from the isolates, and the sequence of the reference strain and that of the full gene for the reference H37Ra (GenBank accession # NC_ 009525) were aligned using AlignX Vector NTI 11.5. The process was repeated with the *rpoB* sequences. Analysis of the sequences' alignment for polymorphisms showed a point mutations in the *katG* sequences of samples #314 and #344, both MDR, in codon 315. As for *rpoB*, a point mutation in codon 526 in sample #344 (MDR), and another mutation in codon 531 in sample #314, which is also MDR. No mutations were found in the *inhA* regulatory region. The observed mutations and corresponding amino acid changes are listed in **table 13. Figures 25** and **26** present the sequence alignments showing the described mutations.

5. Discussion

5.1. Evaluation of colorimetric DST assays CRI and NRA

The regular challenge facing DST for MTB infection is striking the balance between cost and speed while maintaining accuracy. The most rapid direct molecular methods are very costly and require high expertise, and even the slower automated culture systems are also expensive with high infrastructure and maintenance requirements. The more affordable conventional culture methods have a very long TAT, this is on top of the specific technical challenges of each individual assay. The aim of this study was to evaluate the performance of two colorimetric liquid culture-based assays that could fit the requirements for TB DST in the regions where resources are limited, and especially where MDR-TB is a particular issue.

A selection of 34 mycobacterial isolates grown on LJ media from processed sputum obtained from Egyptian TB patients, was characterized using for growth and DST using the in-house method MODS and the commercial liquid culture system MGIT 960. Both MODS and CRI are in-house methods endorsed by the WHO for DST, while the microplate format used in this study has not been sufficiently investigated.

The primary point of strength in MODS is its suitability for use for direct sputum testing, which is a significant advantage for speeding up reporting of results. However, this comes at the cost of increased contamination risk, as is the case with most direct DST approaches. Additionally, the need for inverted microscopes (cost about $1000[49], and are not part of standard laboratory setting), highly trained personnel for result determination, which is also highly subjective, weigh up to the cons of this rapid method.

CRI and NRA results were 96.7% and 93.3% concordant with those of MGIT and MODS and, two of the validly tested samples were smear-negative. The performance of CRI is more established by virtue of the existing body of literature although more large scale studies in the target populations are needed. As for the presented NRA format, there is limited data, and further testing is warranted by the potential advantages. These include high specificity to MTB, shorter overall turnaround time, and immediate result generation after indicator addition. The use of a liquid-media based approach would positively affect TAT and ease of performance, as

solid media for use in NRA have to be prepared in-house. Although this is a process typically familiar to laboratory staff in limited-resource areas, which also may have a cost advantage over buying ready commercial reagent, this is a lengthy labor-intensive procedure, and reproducibility of batches would be a concern. Additionally, there is an uncertainty regarding actual drug potency when incorporated in LJ media, particularly that loss of potency may occur due to the high temperature used during inssipation [11, 110]. Actually, this one reason why the egg-based LJ medium is not regarded as suitable for DST, along with possible interference of its components with drug activity. Even for the reference proportion method, OADC-supplemented Middlebrook 7H10 agar is recommended medium by the CDC and CLSI [11]. A collateral advantage is the fact that the indicator Griess reagent kills the mycobacteria [11], thus reducing biohazard risk during documentation and disposal, albeit this does not indicate any change in the standard biosafety measures. Despite the availability of a tube version of NRA utilizing liquid culture, we opted for the optimization of a microplate format. This allows handling of smaller culture volumes while allowing for MIC determination and increasing throughput as multiple samples can be tested in the same plate, which is more manageable and easier to handle in the restricted space of a biosafety cabinet. Both assays utilize non-propriety reagents, and do not require any specialized or costly equipment. This is an important advantage for areas with low infrastructure and unstable power supply where sophisticated equipment like the MGIT 960 system cannot be reliably maintained. **Table 14** compares the cost and infrastructure requirements for the 4 methods used in this study.

The evaluated colorimetric assays fit in between the performance of conventional solid culture-based DST and the sophisticated cutting edge molecular assays, with regards to cost, simplicity, and turnaround time. At the same time, they offer reliable DST results. Capacity building for TB-afflicted regions, especially HBCs would require substantial time and resources to reach up to the recommended and desired DST, and is also affected by multiple factors beyond the medical scope e.g. political and economic environment. Therefore, CRI and NRA are suitable tools for the interim phase, as supported by WHO endorsement.

Establishment of breakpoint concentrations that reflect clinically significant drug resistance is an important process, yet it is no simple feat and requires MICs distribution analysis of the target population [37, 118]. Thus, a point that must be

noted with regards to results interpretation is the fact that the used protocol for CRI breakpoint concentration to designation of an isolate as drug resistant were ≥0.25 µg/mL for INH, and ≥0.5 µg/mL for INH, which was also used to NRA. However, this was established based on testing of the study population. One isolate (#361) showed a clear degree of resistance by CRI with MIC of 0.125 µg/mL, which is below the standard break point. However, given that all drug-sensitive isolates did not demonstrate any mycobacterial growth under the same assay conditions. Although this isolate had to be considered to have a discordant result by CRI and NRA under the used cutoffs, it may actually be considered to be intermediately resistant. Such a determination and it clinical relevance is to be assessed by a larger scale study on the Egyptian population.

5.2. Molecular characterization of clinical isolates

Multiple alignment using Align X of the Vector NTI 11.5 software package of the sequenced 16S rDNA revealed polymorphisms at positions 513, and 526 of *rrs* were found in three samples, two of which have been found to be SM resistant by MGIT. Mutations at these locations, in general the region around nucleotide 530 (**table 4**), (clinical frequency 2% for position 526) have been shown to be correlated with resistance to SM, and degree of resistance may also be relevant [119-122]. Samples #314 and #321 are streptomycin resistant according to MGIT results from NAMRU-3, however, no data on MIC are available. Therefore exact correlation with MIC cannot be determined, although current information in the literature correlates *rrs* with low to moderate resistance [21, 123]. Mutations related to SM resistance in the *rrs* gene (16S rDNA) are actively being studied in the current literature.

On the resistance front, the two MDR sample for which *katG* and *rpoB* were sequenced demonstrated common high-confidence resistance-conferring mutations, none of which were observed in the drug-susceptible strains. The observed mutations are consistent with those commonly described in the literature (**table 4).** Also, they are consistent with those which were found to be of highest frequency in studies performed on Egyptian samples [124-125]. As for the INH monoresistant sample #361, the lack of mutations in either *katG* or *inhA*, combined with the observed discrepancy between its MGIT and MODS results and those of CRI and NRA, may

suggest either the presence of mutations in other uncommon genes or that there is a different origin of the phenotypic resistance such as an efflux mechanism.

Finally, on a general note, one of the important, yet underestimated, factors to consider in method evaluation is the prior familiarity of the person performing the evaluation of the method. Higher performance indicators are typically reported when the method is evaluated by its industry or original developer, and about 40% of studies on diagnostics of TB, HIV, and malaria encompass industry association of some sort [114]. This creates a hidden bias, and in this study, the fact that the operator had no prior familiarity with TB DST, or the tested methods, offers a fairly true assessment of their degree of applicability and trainability in new settings.

6. Conclusions and Future Perspectives

This study has evaluated the performance of low-cost rapid TB DST colorimetric methods; CRI and NRA in Egypt, a moderate-resource setting. Both assays demonstrated highly concordant results with reference methods and rapid TAT. Also, their cost estimates (exclusive of labor and overheads) are around $5 (for testing an isolate for 2 drugs with MIC determination) (**table 14**) versus MGIT cost per test $32-56 (test only at critical drug concentrations) [49]. It is also to be noted that the cost of NRA can be further reduced by preparing the Griess reagent in-house as opposed to using a readymade commercial preparation as was done in this study. This performance indicates suitability for use in reference laboratories with limited resources, without the need for extensive staff training or equipment. Additionally, result documentation is much simpler when compared to MODS.

The evaluated assays, CRI and NRA, provide low cost, relatively low complexity, rapid indirect phenotypic DST. These assays are best suited for use in central laboratories in resource poor areas with high TB prevalence. The use of colorimetric detection adds to saving in both number and training of laboratory personnel. Reading simplicity would increase number of sample reads per technician, who also require only basic training, as opposed to MODS which requires specially trained personnel for cording pattern detection and plate reading. Also, subjectivity in result reading is minimized in color-based detection, which would only pose a problem in case of the reading being performed by color-blind individual [83].

Another advantage of these colorimetric assays is the possibility of quantification of mycobacterial load, as the indicator color is reflective of the amount of viable mycobacteria [97]. This can be done either spectrophotometrically, or more simply by computer analysis of the image taken of the plate. Considering that both indicators; resazurin and Griess reagent, yield a degree of pink color, the image can be analyzed for RGB content using any of the basic image editing software, freely available on computer. A simple basic computer code can also be specially designed. This is an upgrade of the assays that is to be pursued.

The primary limitation of this study is the number of samples and expansion of this study to include a larger number of Egyptian patients is warranted. Also, a larger proportion of the samples is to be MDR so as to further explore he prevailing resistance-conferring mutations. The mutations are to be correlated with the

subsequent degree of phenotypic resistance, which would aid disease detection and control efforts. This is taking into consideration the possibility of MTB strain heterogeneity in Egypt, indicated by the two clustering patterns obtained from the analysis of the 16S rDNA of 10 out of the 34 samples (**figure 22).** The lack of data on Egyptian strains, except an unexpected high proportion of Manu lineage [43], warrant such an investigation. Additionally, for proper large scale implementation of CRI and NRA and accurate clinically relevant interpretation of their results, MICs for resistant Egyptian isolates are to be analyzed. They are then to be used to verify whether the currently used breakpoint concentrations are suitable for the target population or if their modification is warranted.

7. Tables

Table 1. Egypt TB statistics. [3, 126]

Parameter	*Number/Percentage*	*Year*
Incidence	18 per 100,000, estimated total of 15,000 cases	2010
Prevalence	28 per 100,000, estimated total of 23,000 cases	2010
Mortality	less than 1 per 100,000, estimated 660 cases	2010
Case detection rate	64%	2010
% of MDR-TB in new cases	2.2%	2010
% of MDR-TB in retreatment cases	38%	2010
Estimated cure rate (among new smear-positive pulmonary cases)	72%	2009
% of notified new TB cases tested for MDR:	1%	2009
Total number of confirmed MDR-TB among new and retreatment patients	204	2009
Number of smear laboratories	0.4 per100,000 population	2010
Number of DST laboratories	less than 0.1 per 5 million population	2010

Table 2. Definitions of key terms related to drug resistance of tuberculosis.

Term	*Definition*	*Reference(s)*
Primary drug resistance	Resistance to a drug which is detected in an isolate from a patient who has not previously received, or has received less than one month of drug therapy	[24, 127]
Acquired drug resistance	Resistance to a drug which is detected in an isolate obtained from a patient actively undertaking antimyobacterial therapy or has previously been treated.	[21, 24]
Multi-drug resistance (MDR)	Resistance of an isolate to both RIF and INH	[24, 30]
Drug-resistant isolate	An isolate is generally considered resistant to a given drug if more than 1% of the bacteria exhibit resistance to that drug (applies to the majority of anti-TB drugs).	[24]
Minimum inhibitory concentration (MIC)	The first concentration of the drug that prevents isolate's growth and change of indicator color.	[101]
Critical concentration	A single concentration calibrated as midway between the highest and lowest MICs of different wild type strains, which is defined as the concentration which kills 95% or more of the wild type bacteria, not previously exposed to a drug. It is used as a cut off in qualitative drug susceptibility assays.	[24, 123]
Direct DST	DST performed on primary clinical specimens without prior bacterial culturing.	[58]
Indirect DST	DST performed on pure isolates cultured from primary clinical specimens	[58]

DST: drug susceptibility testing; INH: isoniazid; MIC: minimum inhibitory concentration; RIF: rifampicin; TB: tuberculosis

Table 3. Main first-line TB drugs, mode of action, biochemical description, and resistance conferring mutations [21, 25-26, 46].

Drug	*Description*	*Mode of Action*	*Administration Method*	*Utility in Treatment*	*Main Possible Adverse Effects*	*Molecular Basis of Resistance*
Isoniazid (INH)	Isonicotinylhydrazine, a prodrug which is transformed to the active form by the catalase peroxidase enzyme of the bacteria	Interferes with the enzymatic synthesis of mycolic acid.	Mainly oral, but in the critically ill, it may be injected intramuscularly or intravenously.	Bactericidal affecting actively replicating bacilli only.	- Systemic or cutanous hypersensitvity early in treatment. - Lethargy - Neuropathy	Mutations in *inhA, katG*
Rifampicin (RIF)	A macrocyclic lipid-soluble antibiotic which is a semisynthetic raifamycin derivative.	Disruption of DNA synthesis, by binding to the β subunit of the RNA polymerase.	Oral, and possibly intravenous for the critically ill.	Bactericidal, affects both replicating bacteria and those with low metabolic rates. Both intracellular and extracellular bacilli are potently sterilized by RIF.	- Gastrointesitnal reactions. - Pruritus without rash. - Exfoliative dermatitis is more often encountered with HIV patients.	Mutations in *rpoB*
Ethambutol (ETH)	[(2*S*,2'*S*)-2,2'-(ethylenediimino)-1,2-diyldiimino)di-1-butanol],	Interferes with biosynthesis of the MTB cell wall components arabinogalactan, and lipoarabinomannan.	Oral.	Bacteriostatic agent effective only on actively growing bacilli. It is used with other first-line TB drugs to delay emergence of resistance.	- Dose-dependent optic neuritis, normall reversible. - Occasional peripheral neuritis in legs.	Mutations in *embB*
Streptomycin	Aminoglycoside	Interferes with mRNA	Deep intramuscular	Killing actively	- Injections are	Mutations in

(SM)	antibiotic	translation by binding to the 30S ribosomal subunit and the thus inhibits bacterial protein synthesis.	injection. Also, available for intravenous injection.	replicating bacteria, but has no effect on intracellular and non-replicating bacteria	painful, and rash or sterile abscesses may appear at the injection site. - Numbness around the mouth right after the injection. - Possible cutaneous hypersensitivity. - Nephrotoxicity	*rpsL* and *rrs*
Pyrazinamide (PZA)	Synthetic nicotinamide analogue, which is a prodrug converted by MTB to the active compound pyarazinoic acid.	Disrupts membrane potential of MTB and causes cytoplasmic acidification by drawing in protons.	Oral.	Potent sterilization activity aimed particularly at intracellular bacilli in the low pH environment with macrophages. It is also weakly bactericidal. Its sterilizing effects allow shortening of treatment duration.	- Gastrointestinal reactions. - A degree of hyper uricemia. - Hepatotoxicity.	Mutations in *pncA*

Table 4. Molecular basis of resistance to first-line anti-TB drugs [21, 119-120, 123, 128].

Drug	*Most Commonly Mutated Gene(s) in Resistant Strains (mutation target region)*	*Normal Gene Product/role*	*Relative Frequency in Resistant Strains*	*Typical Association with Phenotypic Resistance*	*Typical MICs (Test Media)*	*Common High Confidence Mutations*	*Mutation Frequency*
Isoniazid (INH)	*inhA* (regulatory region)	Enoyl-acyl carrier protein reductase	6-30%	Moderate to high level resistance	>1 mg/L	Nucleotide (-)15	6-30%
	katG (entire gene)	Catalase/peroxidase	50-95%	Low level resistance	0.02–0.2 mg/L(7H9/7H10)	Codon 315	50-95%
Rifampicin (RIF)	*rpoB* (81 bp hotspot region)	β-subunit of RNA polymerase	90-95%	Dependant on specific mutation, but mostly high level resistance	0.05–0.1 mg/L (7H9/7H10)	Codon 516,Codon 531 (Ser531Leu, His 526Tyr, His526Asp)	30-75% (54%, 11%, 7%)
Ethambutol (ETH)	*embB* (many codons)	Arabinosyltransferase	47-89%	Low to moderate level resistance	1–5 mg/L (7H9/7H10)	Codon 306	20-70%
Streptomycin (SM)	*rpsL* (codons 43 and 48)	Ribosomal protein S12	40-95%	High level resistance	2–8 mg/L (7H9/7H10)	rpsL 42 Arg, rpsL 42 Thr, rpsL 42 Asn	88%, <1%, <1%
	Rrs	16S rRNA	N/A	Moderate level resistance	N/A	rrs 523 C, rrs 522 T, rrs 526 T	6%, 3%, 2%
Pyrazinamide (PZA)	*pncA* (many codons)	Pyrazinamidase responsible for conversion of the prodrug to active form	62-97%	Mostly high level resistance	16–50 mg/L (LJ)	N/A	N/A

Table 5. Main diagnostic strategies for TB infection.

Test	*Detected marker*	*Advantages*	*Limitations*	*Reference(s)*
Smear examination	TB bacilli	- Simple. - Cheap. - Good specificity, particularly in TB endemic areas.	- Variable sensitivity (20-80%). - Not very useful in patients with low sputum bacillary load e.g. children and HIV patients. - Highly dependent on skill of smear examiner.	[3, 11, 30, 129]
Tuberculin skin test	Antibody response to TB proteins	- Simple. - Cheap. - Suitable for detection of latent infection.	- Variable specificity. - Can give false positive results with vaccinated individuals or previous exposure.	[2-3]
Interferon-γ-release assays	Cellular immune response to TB infection	- Limited infrastructure and equipment required. - Moderate personnel training needed. - Good specificity to MTB. - Useful for use with BCG-vaccinated individuals.	- Ineffective in case of severely immunocompromised individuals or the very young.	[2-3, 58]
Liquid and solid culture	TB bacterial growth	- High specificity. - Can be adapted for determination of drug sensitivity.	- Complex. - High personnel training required. - Costly infrastructure and consumables required.	[3]
Nucleic acid tests (e.g. PCR, real-time PCR, SDA, LAMP, LPAs)	TB bacterial DNA	- High specificity and sensitivity. - Fast. - Decreased contamination risk in case of real-time PCR.	- High equipment and consumables cost. - Extensive staff training and expertise needed	[3, 30, 130]

BCG: Bacillus Calmette–Guérin; LAMP: loop-mediated isothermal amplification; LPAs: line probe assays; PCR: polymerase chain reaction, SDA: strand displacement amplification

Table 6. Performance parameters for notable commercial phenotypic DST systems

System	*Media*	*Basis of growth detection*	*DST (Drugs)*	*DST TAT*	*References*
Bactec 460 (BD Diagnostic Systems, MD, USA)	Middlebrook 7H12 broth	Detection of mycobacteria growth by detecting $^{14}CO_2$ liberated by actively growing bacteria from ^{14}C-labeled palmitic acid in the modified MiddleBrook 7H12 broth	First-line	9.3 days	[24]
√Bactec MGIT 960 (BD Diagnostic Systems, MD, USA)	Middlebrook 7H9 broth	An oxygen-quenched fluorophore (tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate,) is embedded in the bottom of the culture tube. As the bacteria grows and the oxygen is consumed the fluorescence signal increases. The automated system reads out the tubes automatically, while in the manual version the tubes are scanned using a UV light and positive growth is indicated by orange fluorescence. In DST fluorescence in the drug containing tubes is compared with that of the drug-free growth tube to determine positive growth and thus resistance in the drug tube.	First-line. Some studies also indicate possible use with ofloxacin and kanamycin	- DST TAT: 5-14 days after primary isolation, where TAT for TB detection is 4-13 days. - A sample is declared negative after 42 days of no growth.	[47, 49, 131]
BacT/Alert MB (bioMeriuex, NC, USA)	Modified Middlebrook 7H9 broth	A colorimetric sensor is incorporated in the medium which measures the production of carbon dioxide by the growing mycobacteria, and growth is indicated by change in color of indicator from green to yellow.	First-line	6-8 days	[24, 65]
√VersaTrek (Trek Diagnostic Systems, OH, USA) [formerly known as the ESP Culture System II]	Enriched Middlebrook 7H9 broth	The system monitors pressure changes in the headspace of the culture vial, generated by the mycobacterial metabolic activity.	First-line	4-8 days	[65, 132]

√ FDA approved. DST: drug sensitivity testing; TAT: turnaround time.

Table 7. Commercial molecular assays for MTB detection and drug susceptibility testing.

Assay	*Samples*	*Assay principle*	*Target*	*Analytical Performance*	*References*
√ COBAS® Amplicor® MTB Test (Roche Diagnostics, Switzerland)	Pulmonary specimens	PCR amplification of 584 bp fragment of 16S rRNA gene. the biotin labeled amplicon is then hybridized to MTBC specific probes coated to microtiter plate, followed by colorimetric detection.	16S rDNA	• *Smear-positive samples:* Sensitivity: 97% Specificity:>95% • *Smear-negative samples:* Sensitivity: 40-73% Specificity:>95% • TAT: 6.5 hrs	[51, 68, 133]
COBAS® TaqMan® MTB Test (Roche Diagnostics, Switzerland)	Pulmonary specimens	Real-time PCR reaction for the amplification of genus specific region of 16S rRNA gene. The amplicon is detected by genus specific Taqman® probes	16S rDNA	• *Smear-positive samples:* Sensitivity: 96.9 % Specificity:100% • *Smear-negative samples:* Sensitivity: 79.5 % Specificity: 98.7 % • TAT: 2.5 hrs after sample preparation	[133]
√ AMPLIFIED MTD (Mycobacterium Tuberculosis Direct) Test *(Gen-Probe,, CA, USA)*	Pulmonary specimens	The test qualitatively detect MTBC rRNA by utilizing Transcription-Mediated Amplification (TMA) for amplification of genus specific 16S rRNA target and Hybridization Protection Assay (HPA) for chemiluminescent detection of amplified targets.	16S rDNA	• *Smear-positive samples:* Sensitivity: 92-100% Specificity:>95% • *Smear-negative samples:* Sensitivity: 40-93% Specificity:>95% • TAT: 2.5 to 3.5 hrs	[51, 134-135]
√ AccuProbe MYCOBACTERIUM Culture Identification Test *(Gen-Probe, CA, USA)*	Cultured isolates	The test utilize nucleic acid hybridization technique and HPA for the identification of Mycobacterium isolated from cultures	16S rDNA	• Sensitivity: 99.2% • Specificity: 99.9% • TAT: 2-3 hrs after growth of isolates	[136-137]
INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium)	Solid and early Liquid culture	A reverse hybridization assay. First biotinylated primers are used to amplify 16-23S spacer region. The biotinylated amplicon is then hybridized with specific oligonucleotide probes immobilized on membrane based strips in parallel lines.	16S-23S rDNA spacer region	• Sensitivity: 100% • Specificity: 94.4%	[51, 138]

		Following hybridization color is developed using alkaline phosphatase labeled streptavidin and the substrate to develop purple/brown color at positive bands.			
INNO-LiPA Rif.TB (Innogenetics, Gent, Belgium)	Solid and early liquid culture	The test principle is the Same as INNO-LiPA MYCOBACTERIA v2.	*rpoB*	• *Smear-positive samples:* Pooled sensitivity: 93% Pooled specificity: 83% (reports range from 80 to 100%) • *Smear-negative samples:* Pooled sensitivity: 65% Pooled specificity: 96% • TAT: <2 hrs • Estimated cost per test $15-45	[49, 51, 55, 139]
GenoType® MTBC (Hain Lifescience, Nehren, Germany)	Culture	Amplification of RNA isolated from sample by) technique (NASBA). Finally the amplified product is hybridized membrane based strip in a reverse hybridization approach	23S rRNA	• Sensitivity: 92% • Specificity: 100% • TAT: 5 hrs	[49, 68]
GenoType MTBDRplus (Hain Lifescience, Nehren, Germany)	Sputum or culture	The test include three steps, DNA extraction from clinical specimen or cultured material (solid or Liquid) followed by multiplexed amplification with biotinylated primers. The biotin labeled amplicons are then hybridized to membrane bound probes.	*rpoB* *katG* *inhA*	• *MTBC detection* Sensitivity: 94.4% • *RIF resistance* Sensitivity: 96.2% Specificity: 90.2% • *INH resistance* Sensitivity: 97.3% Specificity: 83.3% • TAT: 1-2 days • Estimated cost per test $15-45	[49, 52, 68, 139-140]
Xpert MTB/RIF (Cepheid Inc., CA, USA)	Sputum	Automated real-time PCR targeting specific resistance related regions.	*rpoB*	• *Smear-positive samples:* Pooled sensitivity: 98% Pooled specificity: 99% • *Smear-negative samples:* Pooled sensitivity: 75% Pooled Specificity: 99% • TAT: 6-48 hours	[49, 55]

√ FDA approved. HPA: Hybridization protection assay; INH: isoniazid; NASBA: Nucleic Acid Sequence Based Amplification; RIF: rifampicin.

Table 8. Comparison of different non-commercial phenotypic drug susceptibility assays for *M. tuberculosis*.

Assay	*Medium*	*Sample*	*TAT/Cost*	*Advantages*	*Limitations*	*References*
Slide DST	Liquid	Sputum	10 days	- Simplicity. - Safety	- Valid only for sputum positive cases. - No available controls.	[65, 84]
Thin layer agar	Middlebrook 7H10 or 7H11 agar	Pulmonary and extrapulmonary specimens	13 days	- Simple requirements of agar and conventional light microscope.	- A CO_2 incubator is required.	[55, 65, 84, 94]
E-Test	Agar	Isolate	5-10 days	- Rapid resistance detection within 5-10 days. - MIC determination.	- Requires very high inoculum concentration	[65]
MODS*	Middlebrook 7H9 broth	Sputum or isolates	- TAT: 7-9 days - Cost per test $1.3 to 3.5	- Speed and possible direct detection. - Low cost	- Special personnel training needed. - Highly subjective to readers' skills. - False-resistance chances are high due to misidentification of artifacts as TB cords.	[49, 65, 141]
CRI (using resazurin indicator)*	Middlebrook 7H9 broth	Isolates	- TAT: 8 days. - Pooled sensitivity 97% for INH, and 98% for RIF. - Pooled specificity 98% for INH, 99% for RIF. - Demonstrated suitability for	- MIC determination. - Sample throughput is high. - Simple reading and interpretation.	- Biosafety concern over repeated opening of the culture plate.	[47, 64, 79, 97, 142]

			both first and second-line drugs. - Cost $3.75 per sample per drug			
NRA*	- LJ	Sputum or isolates	Combined for direct and indirect testing: - Sensitivity 97% for both INH and RIF resistance - Specificity 99% for INH and 100% for RIF resistance - Cost $3-4	- Direct rapid testing. - Ease of result reading	- Possible false-susceptibility results with rare nitrate reductase-negative strains.	[49, 64]
	- Middlebrook 7H9 broth	Sputum or isolates	Direct Testing: - Sensitivity 100% for both INH and RIF - Specificity 99.2% INH and 99.3% for RIF - Cost about $3.7 per isolate for indirect test at critical drug concentration.	- MIC determination with increased throughput. - Ease of result reading. - MTB specificity.	- Possible false-susceptibility results with rare nitrate reductase-negative strains.	[81, 108-109]

*Assay sensitivity and specificity for MODS, CRI, and LJ-based NRA are based on WHO multicenter studies [64].
CRI: colorimetric redox indicator assay; INH: isoniazid; MODS: microscopic observation drug susceptbility; NRA: nitrate reductase assay; RIF: rifampicin; TAT: turnaround time

Table 9. PCR primers used for amplification of resistance-related genes.

Gene	***Forward Primer***	***Reverse Primer***	***Annealing Temp. (°C)***	***Amplicon Length (bp)***	***Reference***
inhA	TCCACACCCTGCGGCACGTA	TGGCGTTGATGACCTTCTCG	51	590	---
katG	TTGACCTCCCACCCGACTTG	AGCTCGTATGGCACCGGAAC	51	620	[116]
rpoB	CAGACCACGATGACCGTTCC	GAGCCGATCAGACCGATGTT	50	443	[117]

Table 10. Samples results by MODS and MGIT.

Resistance pattern	***No. of Samples***
INH monoresistant	3
RIF monoresistant	1
MDR (INH + RIF resistant)	5
Sensitive	25

Table 11. CRI clinical performance (N=31)

Drug	***Sensitive***	***Resistant***	***Sensitivity***	***Specificity***	***PPV***	***NPV***
Isoniazid	24/24	6/7	100%	96%	85.7%	100%
Rifampicin	26/26	5/5	100%	100%	100%	100%
Both drugs (MDR)	23/23	4/4	100%	100%	100%	100%

Table 12. NRA clinical performance (N=30).

Resistance type	*Sensitive*	*Resistant*	*Sensitivity*	*Specificity*	*PPV*	*NPV*
Isoniazid	23/23	6/7	85.7%	100%	100%	95.8%
Rifampicin	24/25	5/5	100%	96%	83.3%	100%
Both drugs (MDR)	22/22	4/4	100%	100%	100%	100%

Table 13. Mutations observed in the sequenced resistance-related gene region of isolates demonstrating phenotypic resistance.

Sample #	*Phenotypic Resistance*	*Gene*	*Observed Mutation (Amino Acid Change)*	*Position*
314	INH RIF SM ETH	*inhA*	---	---
		katG	AGC→ACC (Ser → Thr)	Codon 315
		rpoB	TCG→TTG (Ser →Leu)	Codon 531
344	INH RIF	*inhA*	---	---
		katG	AGC→ACC (Ser → Thr)	Codon 315
		rpoB	CAC→TAC (His→Tyr)	Codon 526
361	INH	*inhA*	---	----
		rpoB	----	----
		katG	----	----

ETH: ethambutol; INH: isoniazid; RIF: rifampicin; SM: streptomycin; wt: wild type

Table 14. Comparison between the cost and infrastructure requirements of MGIT, MODS, CRI, and NRA [65, 87].

	MGIT	***MODS***	***CRI***	***NRA***
Special equipment needed	Yes	Yes	No	No
Propriety reagents	Yes	No	No	No
Operator training requirements	Moderate	High	Low	Low
DST Cost per isolate for both RIF and INH	$32-56 [49]	$1.4-3.5[49]	$4.5 (this study)	$5.2 (this study)
Laboratory infrastructure	Advanced	Regular	Regular	Regular
Throughput	Very high (up to 960 samples per run)	Moderate	High	High
MIC determination	No	No	Yes	Yes

CRI: colorimetric redox indicator assay; DST: drug susceptibility testing; INH: isoniazid; MGIT: Mycobacterial Growth Indicator Tube; MIC: minimum inhibitory concentration; MODS: microscopic observation drug susceptbility; NRA: nitrate reductase assay; RIF: rifampicin.

8. Figures

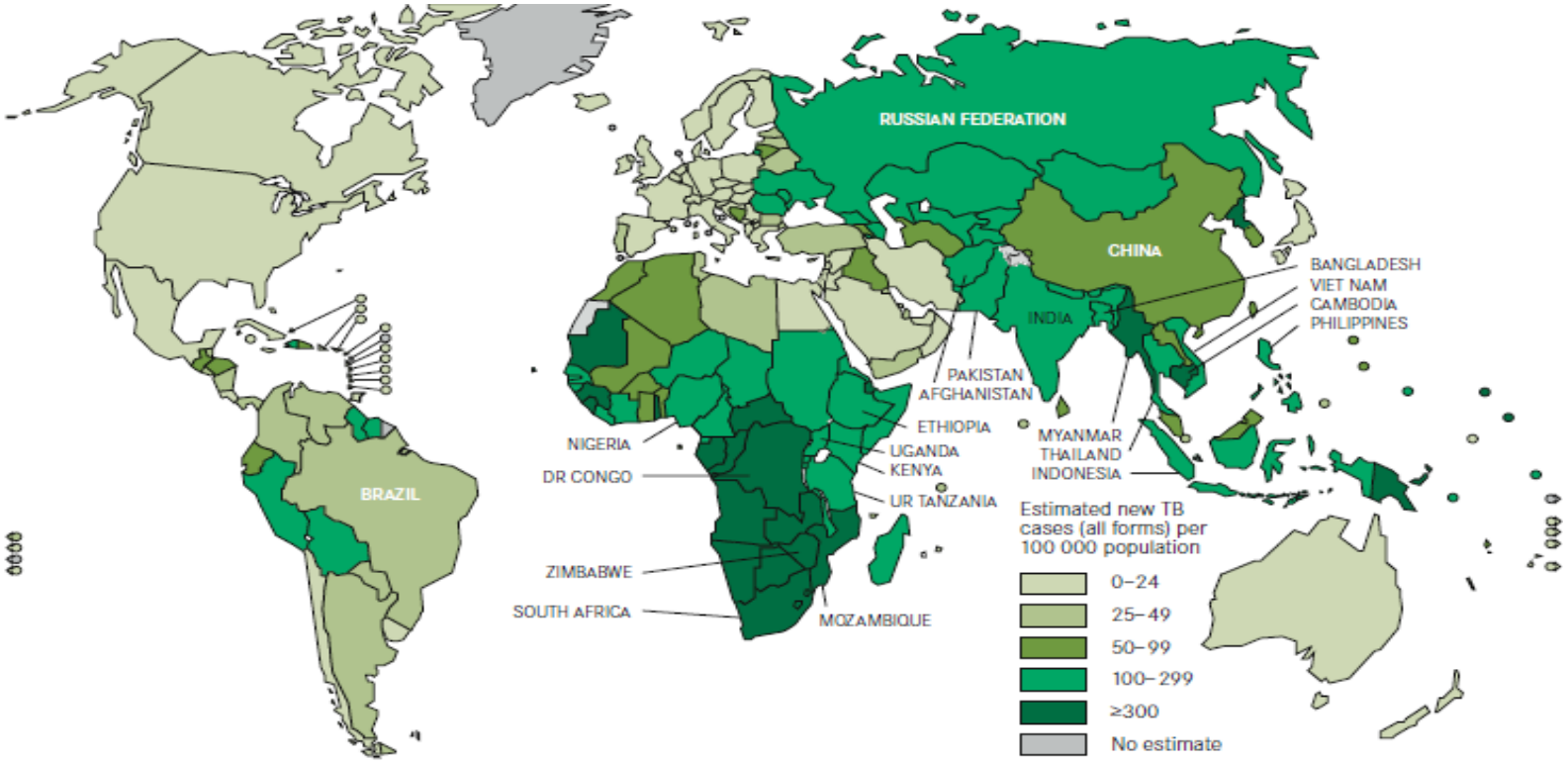


Figure 1. Estimated worldwide TB incidence rates in 2010. Reprinted from [3].

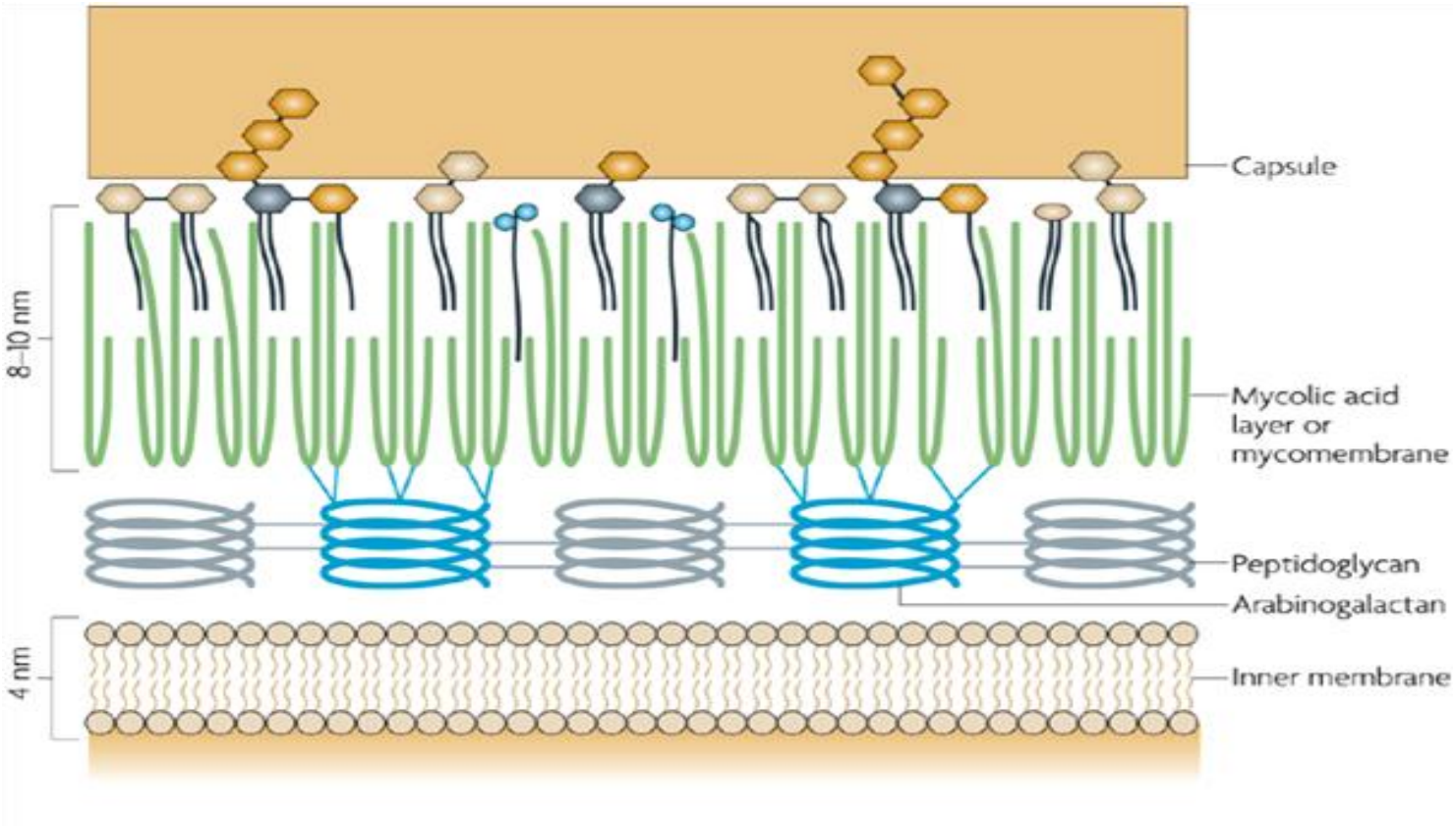


Figure 2. **Structure of mycobacterial cell wall.** Reprinted from [143].

The exceptional high content of mycolic acid and peptidoglycan in the cell wall is the origin of the acid-fastness property of MTB.

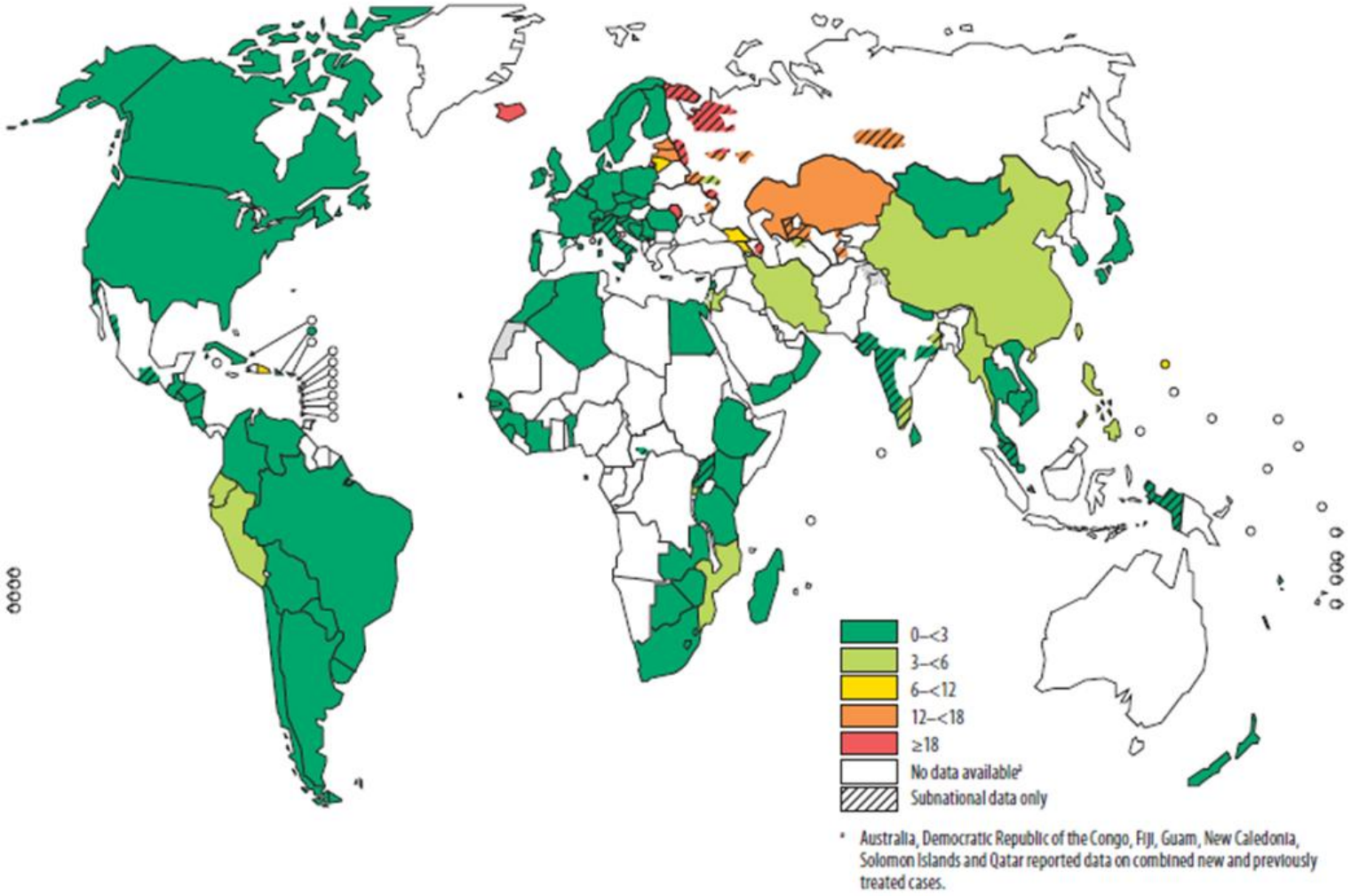


Figure 3. Global distribution of percentage of MDR-TB cases among newly notified TB cases. Reprinted from [34].

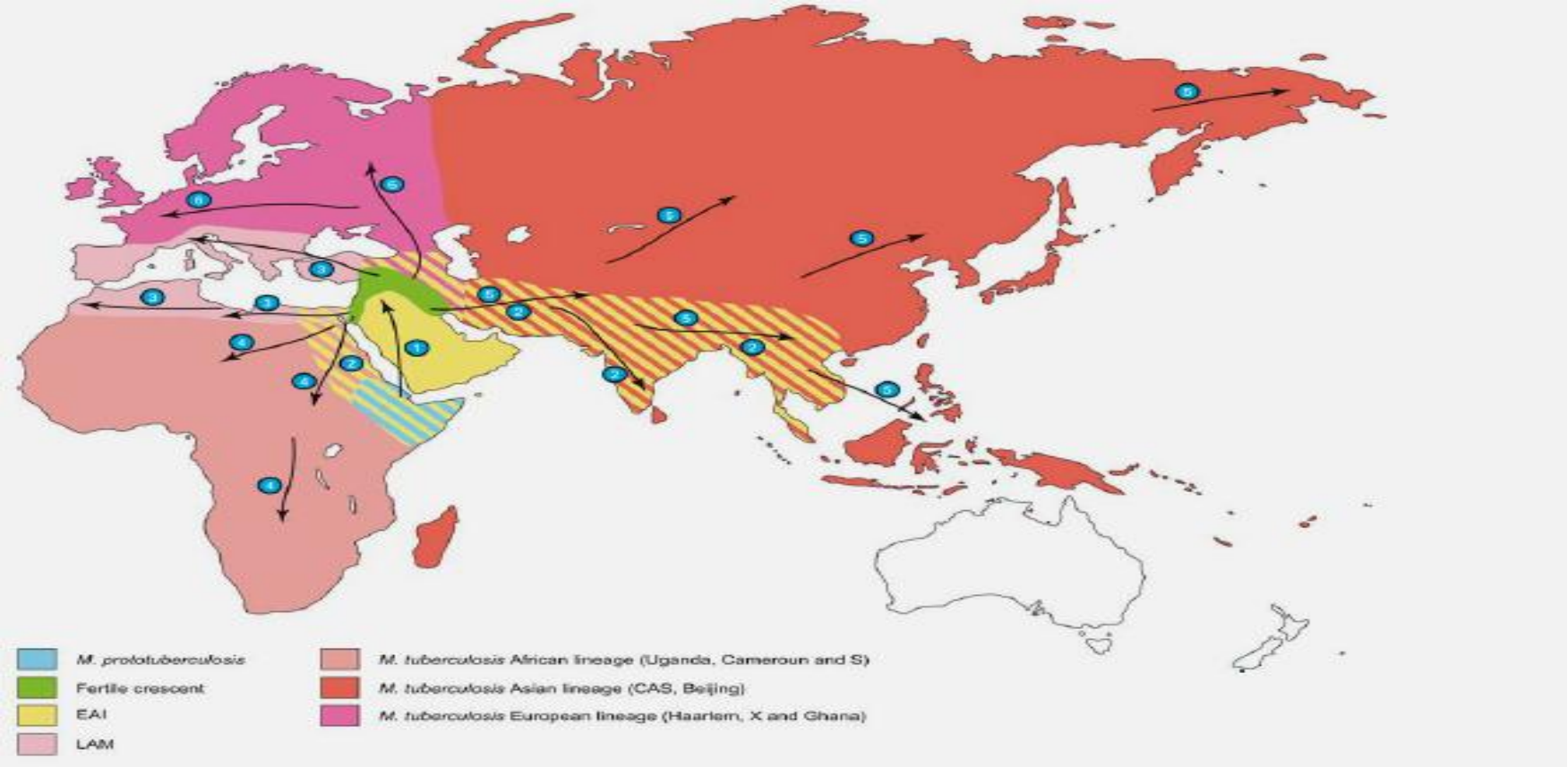


Figure 4. *M. tuberculosis* evolutionary scenario and geographical spread."The main migrations events are numbered and correspond to: 1, *M. prototuberculosis*, the ancestor of the MTBC, this bacterium reached the Fertile Crescent some 40,000 years ago by sea or land; 2 and 3, two distinct basal lineages arose, EAI and LAM and spread out of Mesopotamia some 10, 000 years ago; 4, 5 and 6, later on (8–5000 years ago) derived populations from clade 1 followed main human migration patterns to Africa, Asia and Europe, giving rise to locally adapted tubercle strains and further diversifications" Reprinted from [40].

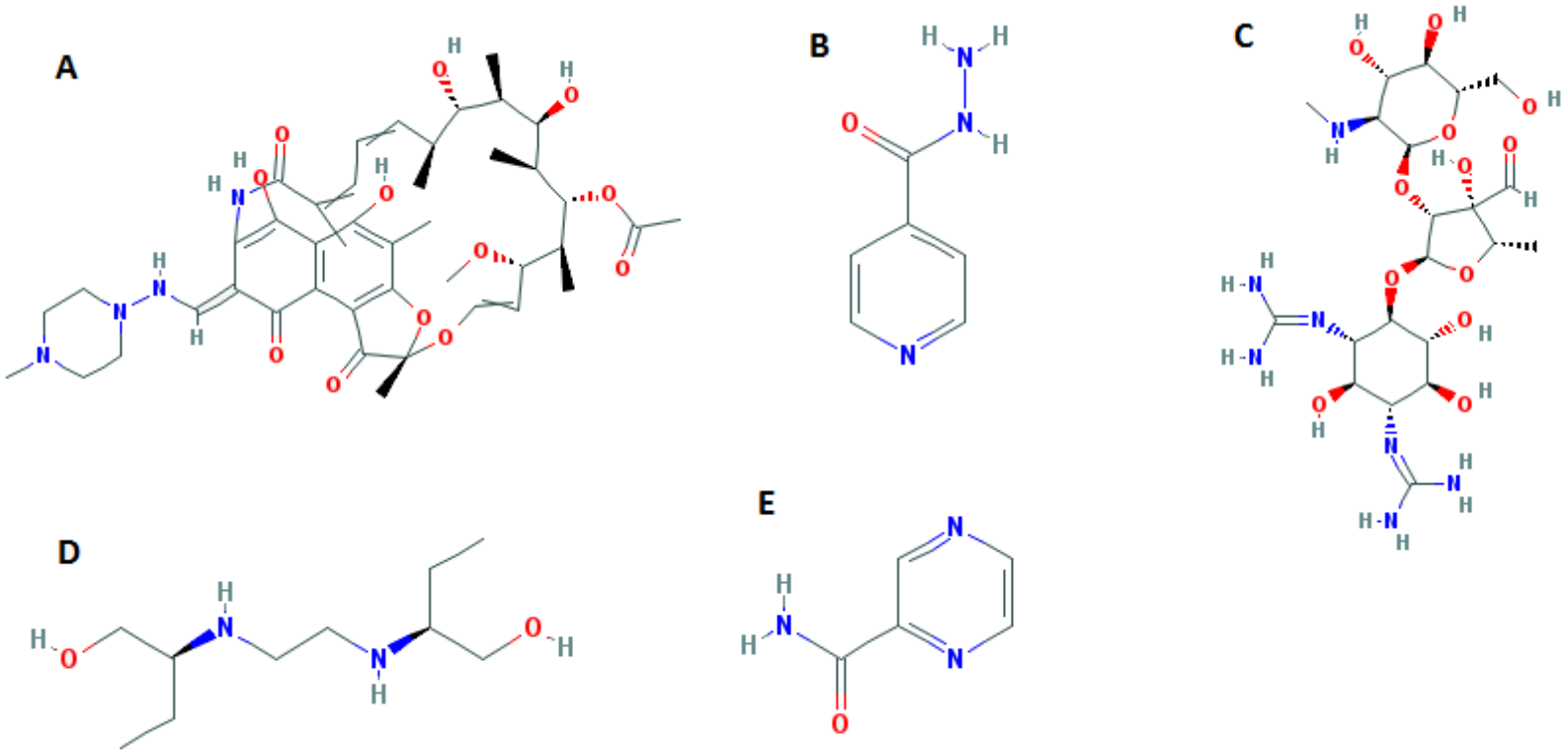


Figure 5. Chemical structures of first-line anti-TB drugs. A) Rifampicin, B) Isoniazid, C) Streptomycin, D) Ethambutol, E) Pyrazinamide. Reprinted from [144].

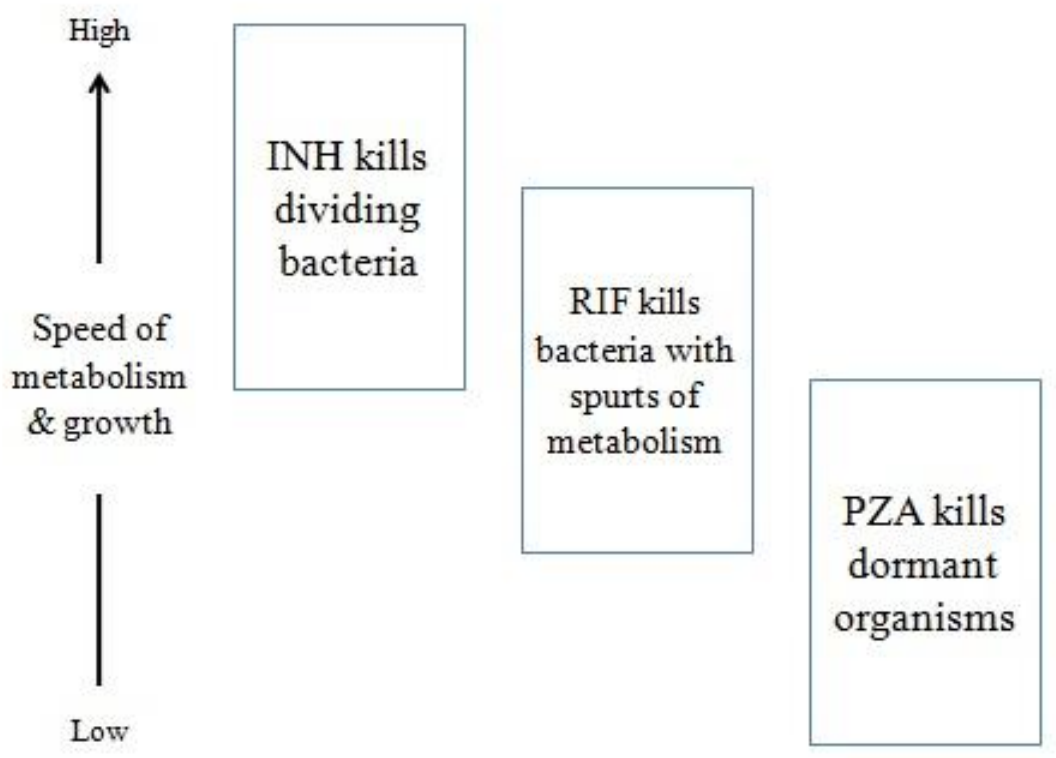


Figure 6. Action of different drugs in treatment of TB infection. Each drug targets the bacteria at a different metabolic states, to ensure killing of all bacteria in the body. Reprinted from [48].

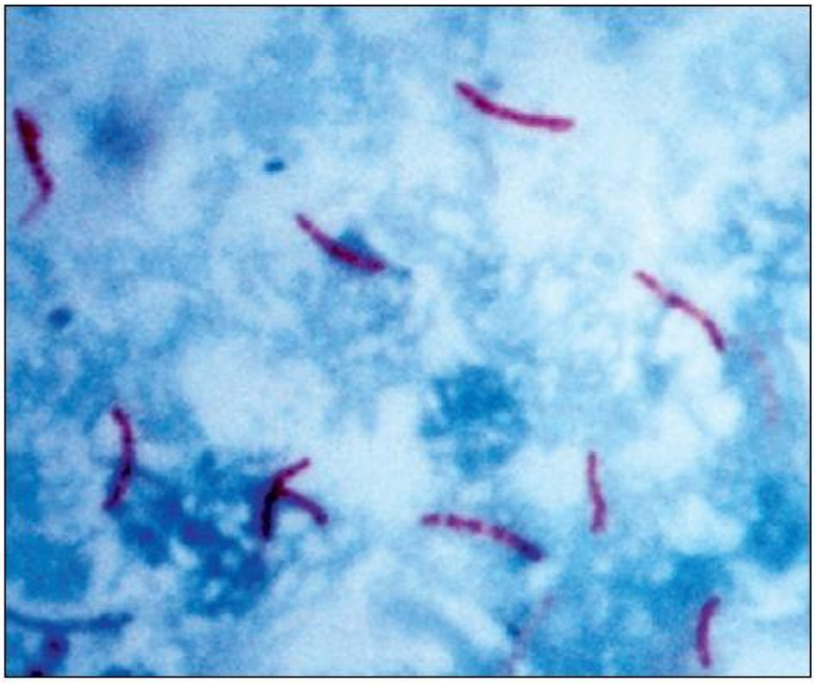


Figure 7. Acid-fast staining of MTB bacilli in sputum (x1000 magnification) [12].

Figure 8. Colonies of MTB on LJ medium [145].

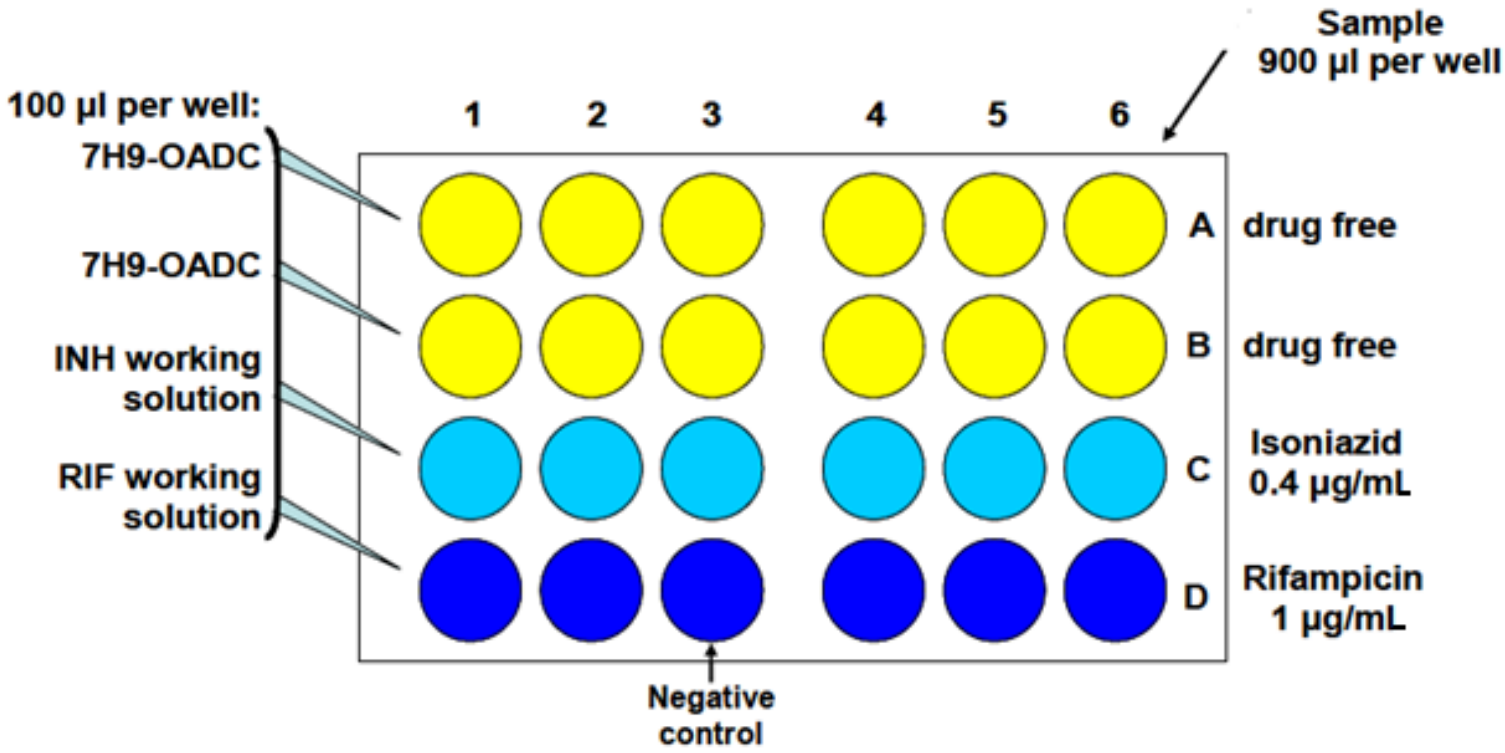


Figure 9. **MODS plate layout.** The samples are incubated with 7H9-supplemented broth in 24-well microplates. For each sample two well contain each of the drugs at critical concentration and the other two do not and act as growth control. The plate is then sealed and incubated at 37º C for a week and then examined under an inverted microscope. Reprinted from [92].

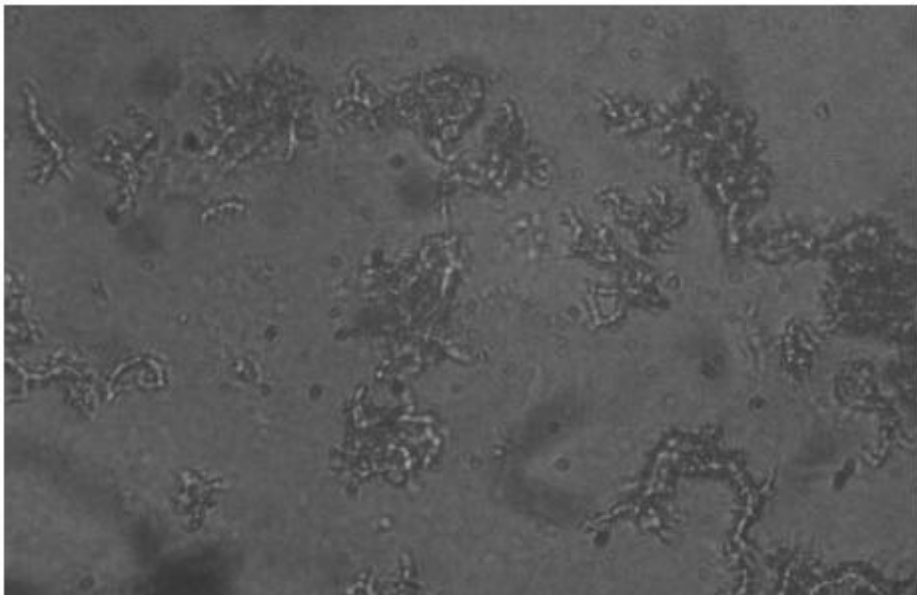


Figure 10. Characteristic cord-like appearance of MTB under the microscope in MODS (20x). Reprinted from [146].

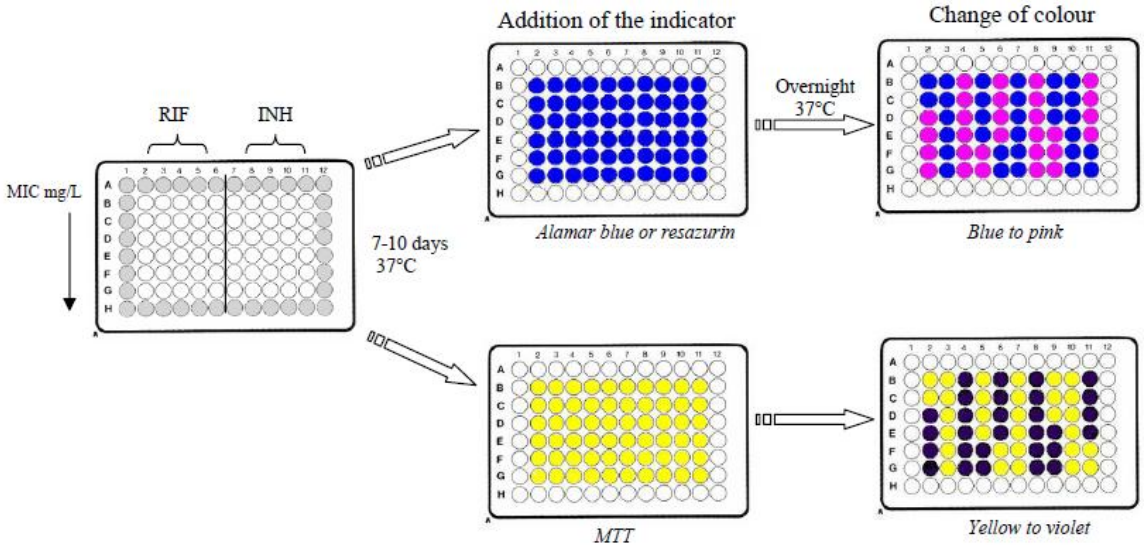


Figure 11. **CRI overview.** Samples are incubated in broth with a serial dilution o f the drugs in 96-well microtiter plate. The plate is then incubated for 7-10 days at 37 ºC, and then a redox indicator is added e.g. resazurin or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The plate is then reincubated overnight and then the plate is observed for color change of the indicator reflecting bacterial growth. Successful bacterial growth in drug-containing wells indicates bacterial resistance to that drug. Reprinted from [79].

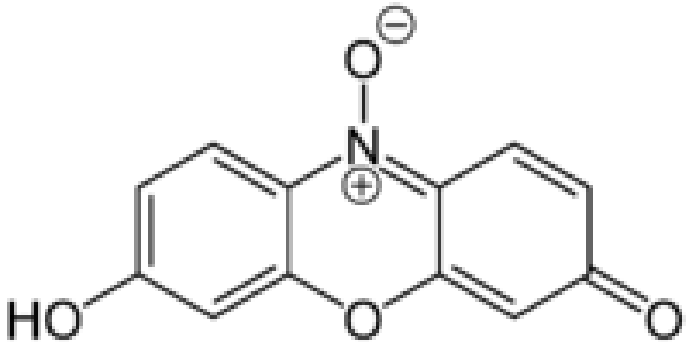


Figure 12. Chemical structure of resazurin.

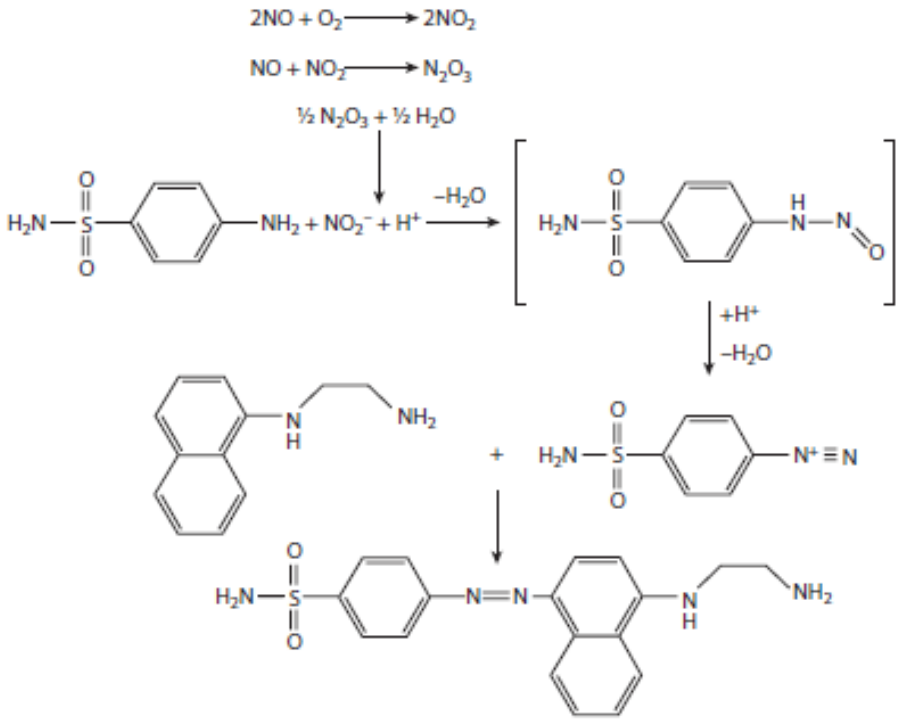


Figure 13. Reaction of Griess indicator used in nitrate reductase assay. A diazotization reaction is employed in the assay where sulphanilic acid reacts with nitrite to form a diazonium salt intermediate, which then reacts with *N*-(1-naphthyl)ethylenediamine to form the pink azo dye. The color formation indicates the presence of viable bacteria converting the nitrate in the media to nitrite, where the latter reacts with the Griess reagent to form the colored product [147].

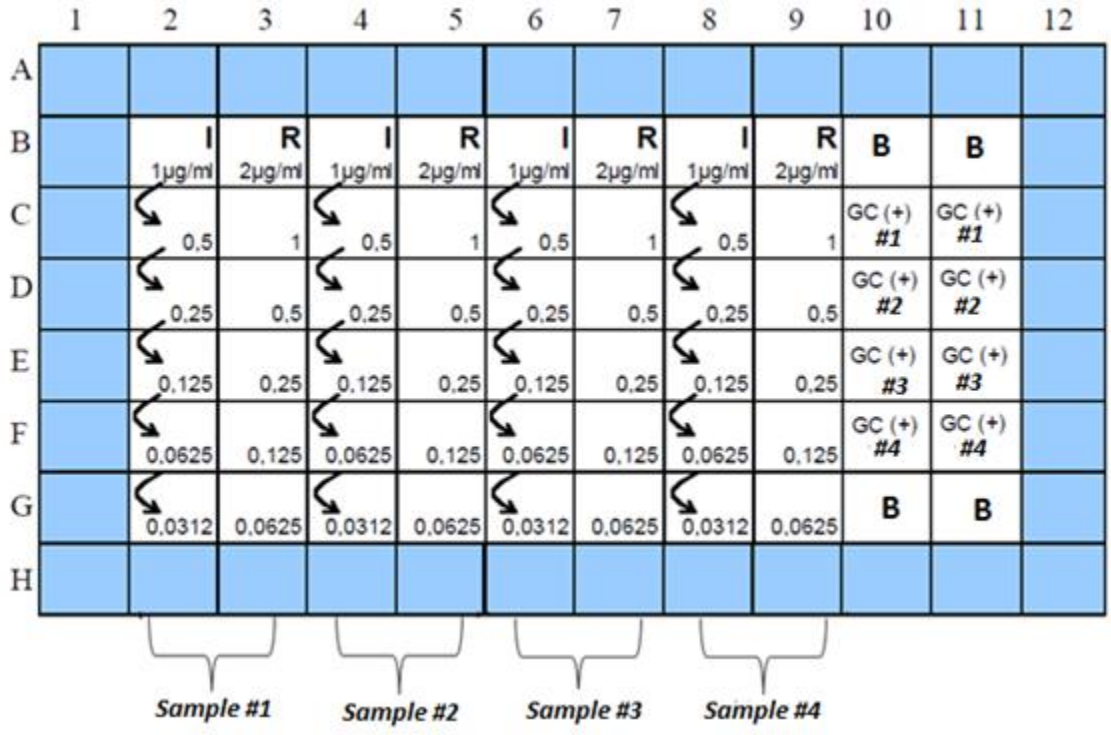


Figure 14. CRI plate layout. Two columns are dedicated to each sample, and each column contains a serial dilution of each drug (I → isoniazid, R→ rifampicin). Blank (B) controls containing only media are included for sterility check, and duplicate growth controls (GC) are also included for each sample and contain only the sample and media without drugs.

	1	2	3	4	5	6	7	8	9	10	11	12
A		**INH** 20 µg/mL	**RIF** 20 µg/mL	**INH** 20 µg/mL	**RIF** 20 µg/mL	**INH** 20 µg/mL	**RIF** 20 µg/mL	**INH** 20 µg/mL	**RIF** 20 µg/mL	**B**	**B**	
B		10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	**GC (+) #1**	**GC (+) #1**	
C		5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	**GC (+)#2**	**GC (+)#2**	
D		2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	**GC (+) #3**	**GC (+) #3**	
E		1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	1.25 µg/mL	**GC (+) #4**	**GC (+) #4**	
F		0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	0.625 µg/mL	**GC (+) #5**	**GC (+) #5**	
G		0.3125 µg/mL	0.3125 µg/mL	0.3125 µg/mL	0.3125 µg/mL	0.3125 µg/mL	0.3125 µg/mL	0.3125 µg/mL	0.3125 µg/mL	**B**	**B**	
H		0.156 µg/mL	0.156 µg/mL	0.156 µg/mL	0.156 µg/mL	0.156 µg/mL	0.156 µg/mL	0.156 µg/mL	0.156 µg/mL			

Figure 15. NRA plate layout. Two columns are dedicated to each sample, and each column contains a serial dilution of each drug (I → isoniazid, R→ rifampicin). Blank (B) controls containing only media are included for sterility check, and duplicate growth controls (GC) are also included for each sample and contain only the sample and media without drugs.

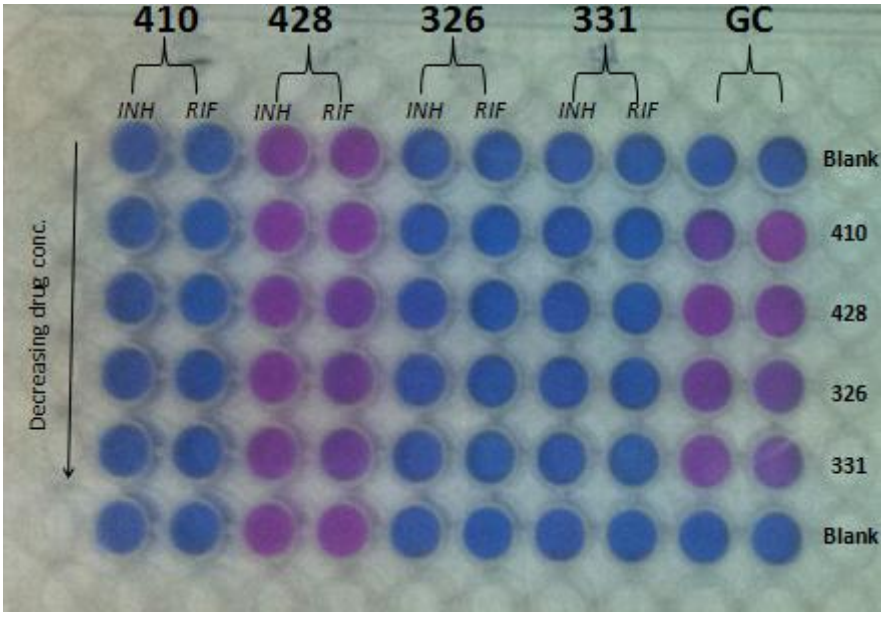


Figure 16.Typical CRI plate result. Mycobacterial growth is detected by change of indicator color from blue to pink. *GC* is the growth control. Results of this plate are: sample 410 is sensitive to both drugs, sample 428 is resistant to both drugs (MDR), sample 326 is sensitive to both drugs, and sample 331 is sensitive to both drugs.

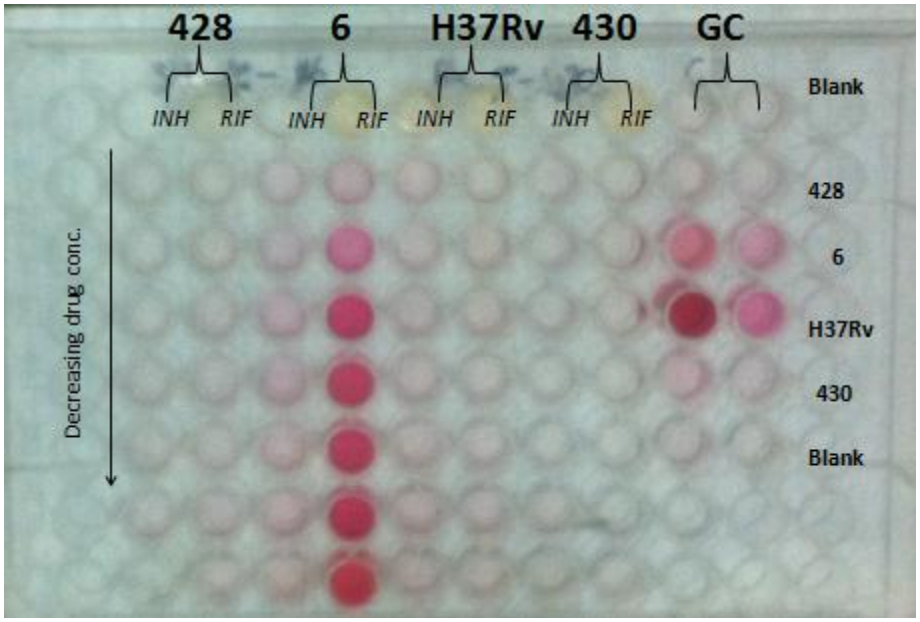
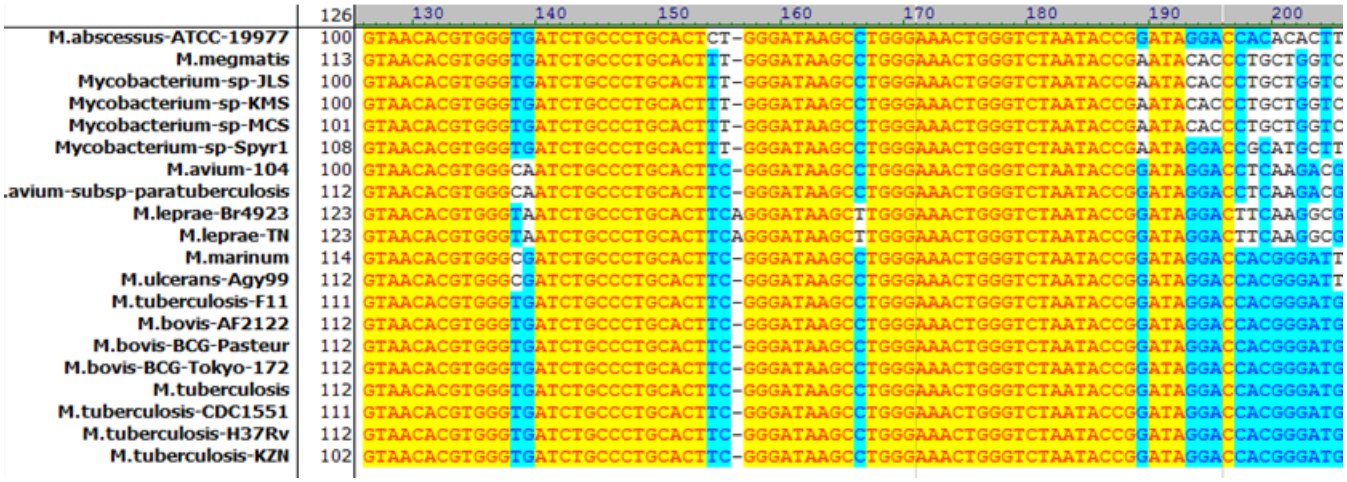


Figure 17.Example of NRA plate result. Mycobacterial growth is detected by change of indicator color from very faint pink to deep or reddish pink. *GC* is thegrowth control. Results of this plate are: *6* is RIF-resistant, *H37Rv* is sensitive to both drugs. Sample 428 and 430 failed to show growth in the control wells . These samples were retested.

A



B

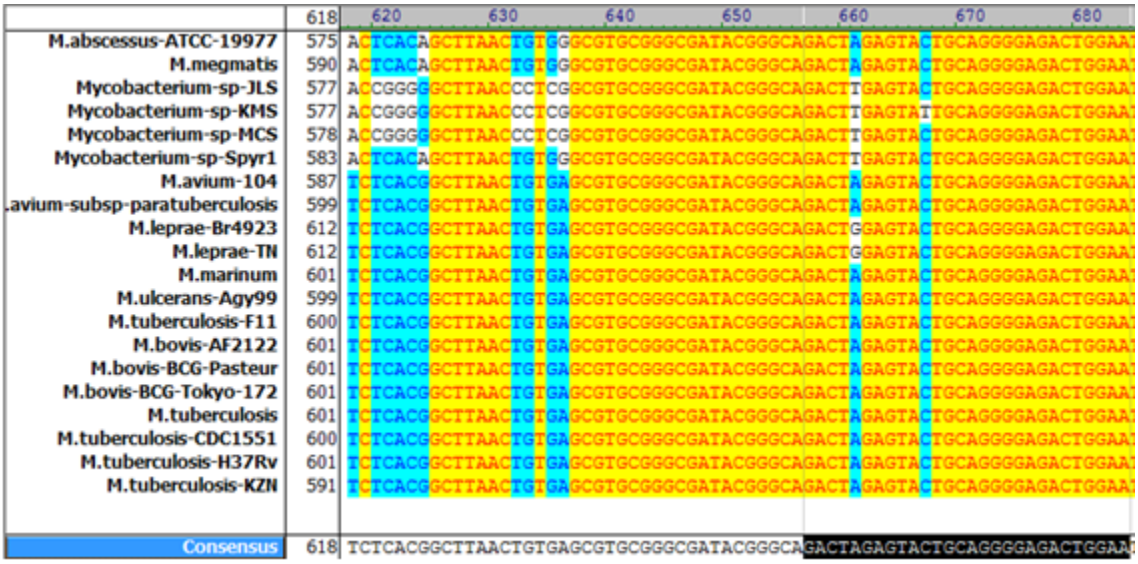


Figure 18. Alignment results for forward (A) and reverse (B) primers for amplification of 16S rDNA region using Vector NTI 11.5 software.

The alignment shows the high degree of conservation of the chosen region throughout *Mycobacterium* genomes.

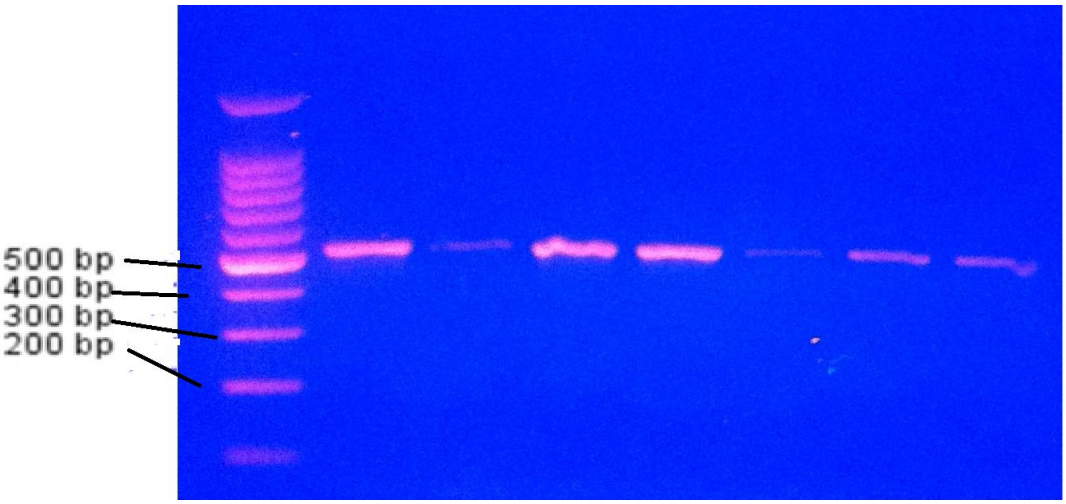


Figure 19. Agarose gel electrophoresis result for the 16S rDNA amplion. The band at~500 bp matches the expected amplicon size.

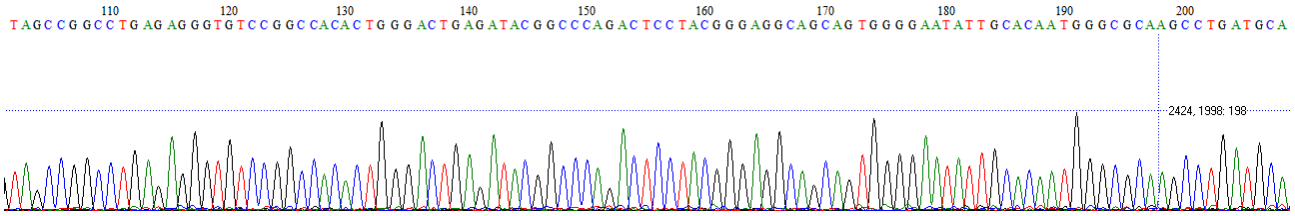


Figure 20. Part of the16S rDNA sequencing result for sample #314. The peaks are clear and sharp peaks and background noise is low.

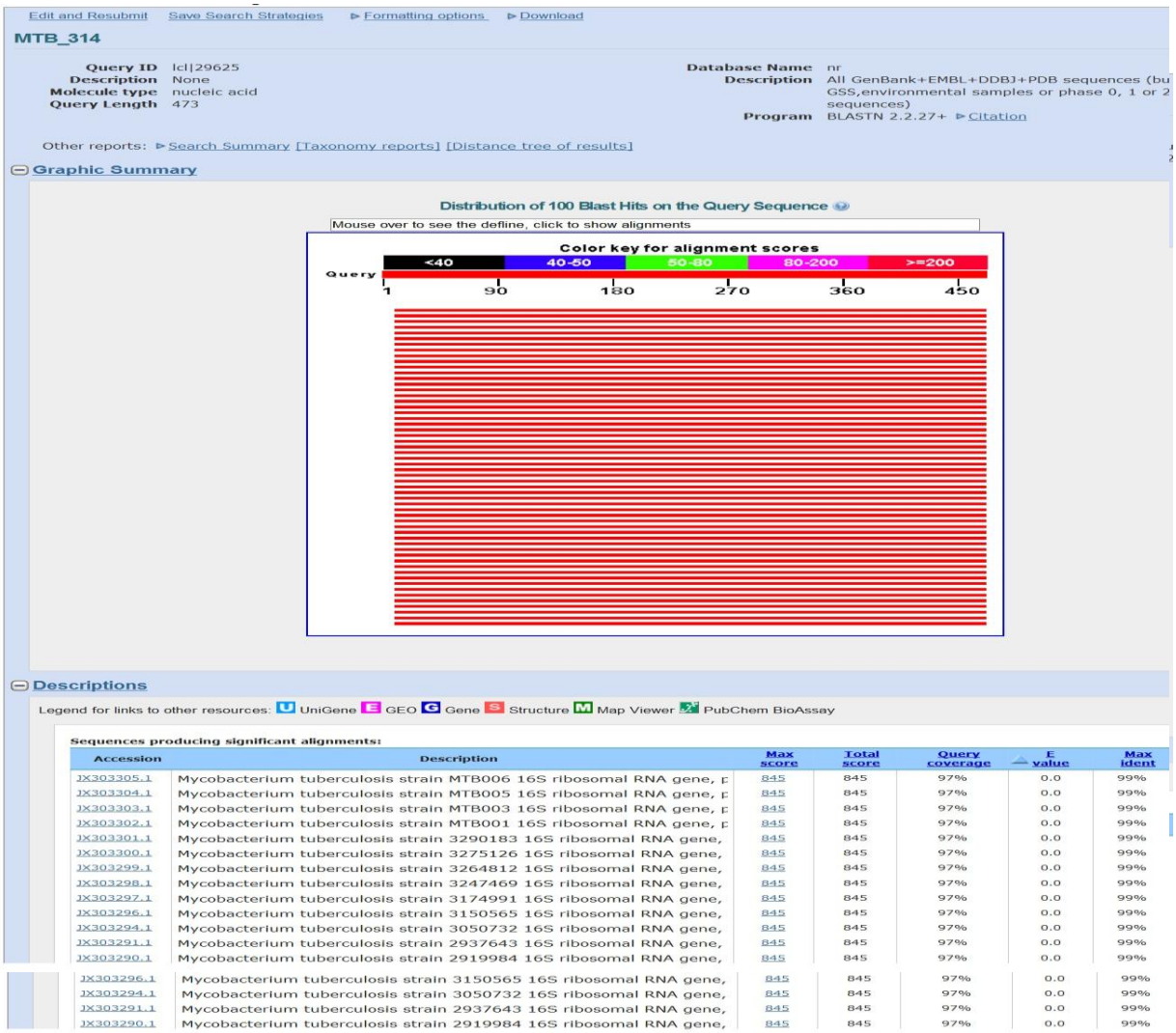


Figure 21. Blast analysis results for sample #314. This confirms sample identity as *Mycobacterium tuberculosis.*

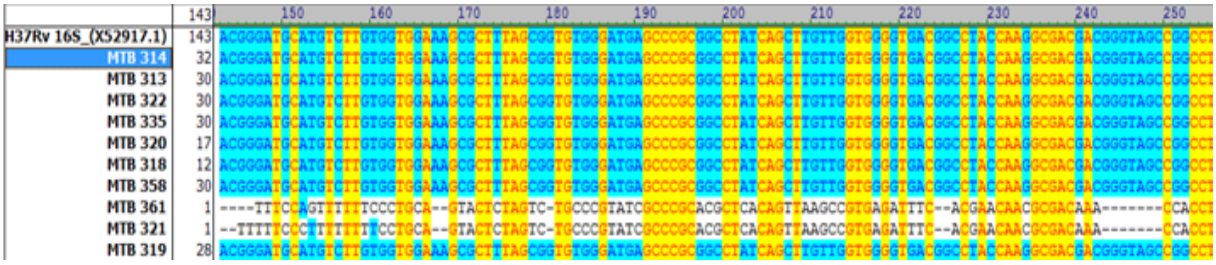


Figure 22. Alignment of 16S rDNA sequences. The alignment shows high homology between 8 out of the 10 sample while the remaining two are partially homologous.

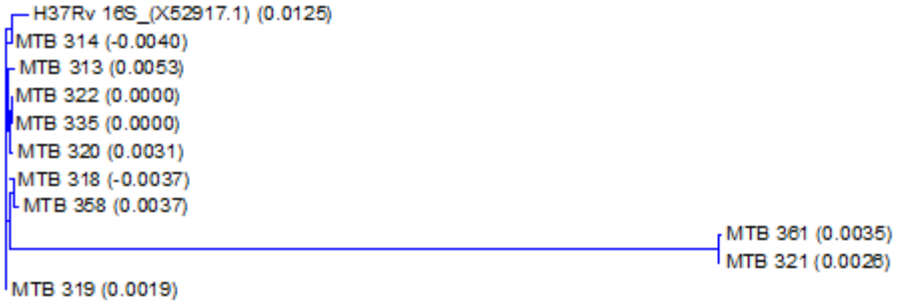


Figure 23. Dendrogram for relationship between samples based on 16S sequencing. Two samples clustered away from the remaining samples indicating possible strain difference.

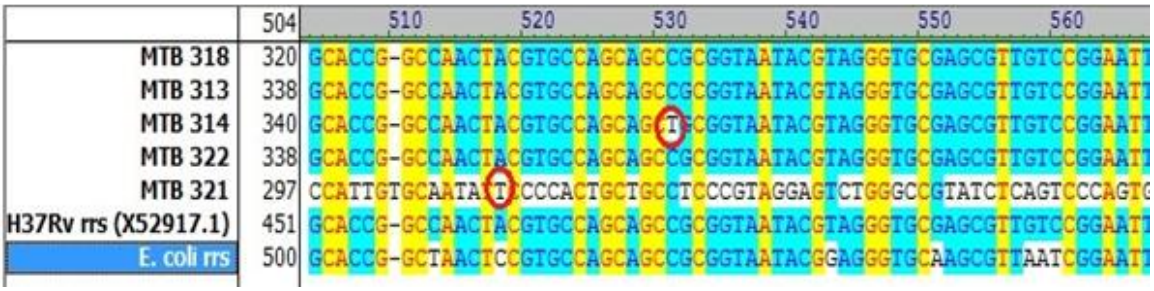


Figure 24. Alignment 16S rDNA for samples demonstrating phenotypic resistance to streptomycin. Two samples show mutations at positions 513 and 526 (*E. coli* numbering).

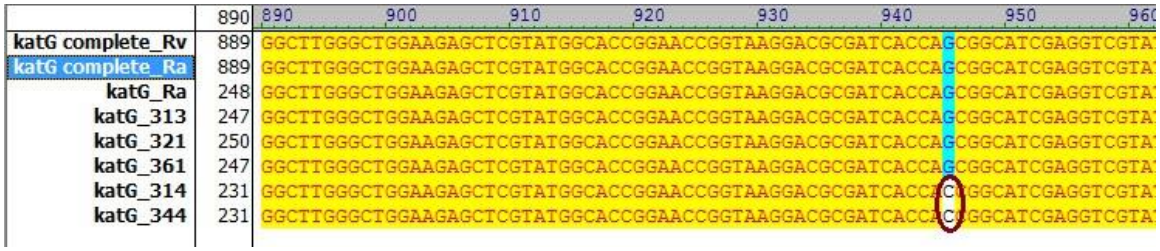


Figure 25. Alignemnt of *katG* sequences with marked mutations in codon 315. These marked mutations are known to confer resistance to INH and the two samples in which they were detected are phenotypically MDR, resistant to both INH and RIF.

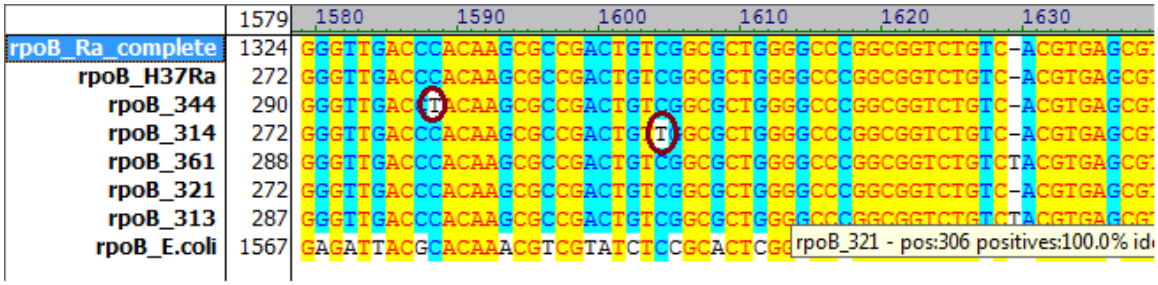


Figure 26. Alignment of *rpoB* sequences with marked mutations in codons 526 and 531(*E. coli* numbering). These marked mutations are known to confer resistance to RIF and the two samples in which they were detected are phenotypically MDR, resistant to both INH and RIF.

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