History

Tuberculosis is a disease of antiquity. Skeletal remains of prehistoric humans dating back to 8000 BC, found in Germany, have shown evidence of the disease. Ancient Hindu and Chinese scripts also have documented the existence of this disease. It has been a disease of human beings for thousands of years. It was considered the ‘White Plague’ of eighteenth and nineteenth century. Chemotherapy was expected to reduce the disease burden by the late twentieth century. But the past two decades has seen the resurgence of tuberculosis because of arrival of Human Immunodeficiency Virus (HIV), along with the problem of emerging drug resistance. Often a lifelong infection, tuberculosis affects virtually all organ systems. With the advent of anti tubercular therapy, great strides were achieved in the control of tuberculosis. However, in recent times, this disease has assumed more importance because of its increased incidence in developing and developed countries and its deadly association with the Human Immunodeficiency Virus (HIV).

Epidemiology and Demography

In 1993, World Health Organization (WHO) declared TB a global emergency. Further, the World Health Report (1999) released by WHO further emphasized the global emergence with almost one third of the world’s population being affected resulting in nearly three million deaths per annum. In the year 2000, 8.2 million new TB cases had already occurred worldwide, 60% of all these cases were in Asia. According to the seventh WHO Annual Report (2003) on global tuberculosis control, the global incidence rate of TB is growing at approximately 0.4% per year. This rate is much faster in Sub-Saharan Africa and in countries of the former Soviet Union. It is not possible to extrapolate the western data available to suit Indian research as more than 90% of our population is infected with Mycobacterium tuberculosis given the high endemicity of this disease in the Indian subcontinent.
DOTS Progress report (India) from 1995-2002 reveals that the tuberculosis remains a serious public health problem with an annual incidence of 2 million out of which nearly 1 million are infectious smear positive pulmonary cases\textsuperscript{35}. One smear positive patient infects up to 20 contacts annually in absence of chemotherapy\textsuperscript{36}. One person dies from TB in India every minute\textsuperscript{35}. Further, an alarming increase in infection due to the human immunodeficiency virus (HIV) has accelerated this situation and it is believed that, as of now, about 3.5 million people in India are infected with HIV\textsuperscript{2}. There is a grave concern in India regarding the increase in HIV-associated TB and the emergence of MDR-TB in both magnitude and severity of TB\textsuperscript{37}. Several studies show that age distribution of 30-45 years was common among the tuberculosis patients\textsuperscript{38,39}. In the various surveys carried out (during the 1955 to 2010) to estimate the prevalence of tuberculosis found that the male to female ratio vary between 2:1 to 5:1\textsuperscript{40}. A nation-wide survey conducted from 1955–1958 revealed that tuberculosis (TB) was highly prevalent throughout the country\textsuperscript{41}. Surveys carried out thereafter in geographically defined areas revealed that the prevalence of TB continued to be high, though varied, in different parts of the country\textsuperscript{40}. Further, a study carried out by Subramani et al (2008) shows that the TB is much more affected among inhabitants of rural areas than the urban population\textsuperscript{42}.

**Diagnosis of TB**

Diagnosis of tuberculosis relies on clinical features, histopathology, demonstration of acid fast bacilli, and isolation of M. tuberculosis from clinical specimens, serology, tuberculin testing and radiological examination are supplemental.

**Clinical features**

Early pulmonary tuberculosis is usually asymptomatic. Non-specific constitutional symptoms such as anorexia, fatigue, weight loss and evening rise of temperature and night sweats may ensue. Late manifestations are gradual in onset.
These include chest pain, haemoptysis, cough more than 2 weeks or longer, persistent low grade fever (>37ºC for 2 weeks). Chest X ray may show patchy or nodular infiltrate in the apical or sub apical posterior areas of the upper lobes and the superior segment of a lower lobe. A bilateral involvement is usually seen and may be seen with cavity formation. In 2009, Daley et al carried out as study on sodium hypochlorite pretreatment methods, which showed that cough more than two weeks (98.3%), experiencing chest pain (55.1%), fever (86.5%). Another study carried out by Bonnet et al (2011) explains the frequency of anorexia (78.6%), weight loss (80.3%), night sweats (78.4%), chest pain (78.9%), haemoptysis (9.8%), cough more than 2 weeks or longer (100%), persistent low grade fever [(>37º C for 2 weeks)77.6%], respectively.39,43

**Laboratory diagnosis**

The most commonly used methods are acid fast staining and culture

**Acid fast staining**

For over 100 years now, the only rapid test for presumptive diagnosis of tuberculosis was the smear examination of the patient’s specimen for acid-fast bacilli. Smear from a non-processed sputum specimen may be positive if specimen contains more than 10,000 AFB/ml. The sensitivity of this method can be increased to detect even 1,000 AFB/ml, if the specimen is concentrated through appropriate processing and the smear examined by means of fluorescent microscopy 44. In spite of this, the sensitivity is not beneficial thus leading to missed diagnosis. Also, though smear negative cases are considered less infectious than smear positive cases, they have nevertheless been shown to transmit disease45

**Culture**

A major challenge to clinical microbiology is the detection of *Mycobacterium tuberculosis* (MTB) quickly, easily and accurately. Isolation of *Mycobacterium tuberculosis* remains the gold standard for the laboratory
diagnosis of tuberculosis. The conventional solid media require a minimum of 6-8 weeks, thus leading to late diagnosis. Substantial improvements in the mean time to detection and the total number of isolates recovered can be realized by using a broth based system such as BACTEC (BBL) or the broth/agar system SEPTI-CHECK AFB (BBL). This expedites the growth of Mycobacterium tuberculosis from the smear positive and most of smear negative specimens within 2-3 weeks. The advent of molecular tools for detecting specific nucleotides of Mycobacterium tuberculosis complex by direct amplification methods/polymerase chain reaction, provide a precise rapid final diagnosis. These techniques are not cost effective in a developing country like India. Also these tests are yet to be standardized for all specimens and are not recommended for smear negative sputum samples [13, 14]. They have low specificity and can lead to false positive diagnosis.

Serology

Different serological methods ELISA are used to detect the presence of antimycobacterial antibodies against antigens like 38kDa antigen which is specific to Mycobacterium tuberculosis complex in serum and body fluids like cerebrospinal fluid. Intratracheal synthesis of MTB specific immunoglobulin G occurs in tuberculous meningitis, which may be detected. Though serology is not very important in diagnosis of pulmonary tuberculosis, but it has a role in diagnosis of extra pulmonary tuberculosis such as tubercular meningitis.

ELISPOT assay (Enzyme linked immunospot assay)

This test is used to study the tuberculosis specific cellular immune response using mycobacterial specific antigens like ESAT-6 (early secretory antigen 6) and CFP (culture filtrate antigen).
Antitubercular drug susceptibility testing

Drug susceptibility testing is mandatory due to the development of drug resistant strains of Mycobacterium tuberculosis.

Antituberculosis drug resistance

Drug resistance in mycobacteria is defined as neither decreased susceptibility nor resistance to a particular anti tuberculosis drug resulting in the failure of the treatment regimen. Tuberculosis drug resistance may be either primary (transmission of resistant organisms) or secondary (resistance acquired in the host related to inadequate treatment). The level of primary resistance in the community is considered to reflect the efficacy of control measures in the past, while the level of acquired resistance is a measure of ongoing TB control programs. World health organization and international union against tuberculosis have replaced the term primary resistance by the term “drug resistance among new cases” and acquired resistance by the term “drug resistance among previously treated cases”.

MDR and XDR TB

Origin of drug resistance

Multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least Isoniazid and Rifampicin (the two most important first-line drugs), appeared after the introduction of Rifampicin in 1966. However, most MDR cases occurred in patients receiving prolonged, inappropriate therapy; while sporadic outbreaks of primary transmission occurred, the magnitude and impact was relatively limited.

The fight against TB in the last two decades has been further challenged with the emergence of two key obstacles: The first man-made phenomenon is the emergence of the resistant forms of TB, against which the treatment is much more difficult and at times impossible. The second obstacles have been the emergence and pandemic spread of the Human Immunodeficiency Virus (HIV).

More recently, the emergence of extensively drug resistant tuberculosis (XDR-TB), defined as TB resistant to Isoniazid, Rifampicin, quinolones and at least one
of three injectable second line drugs (Kanamycin, Capreomycin, or Amikacin), in every region of the world has raised further alarms about the future of TB control. In 2006, a review of global DST data conducted by researchers at the CDC found 347 isolates of XDRTB worldwide, accounting for 2% of all TB isolates surveyed and 15% of MDR-TB isolates; data from African and Asian countries, other than South Korea, were notably lacking.\(^{52}\)

**Mechanism of drug resistance**

Tuberculosis drug resistance can be either primary (transmission of resistant organisms) or secondary (resistance acquired in the host related to inadequate treatment). There are four broad categories of mechanisms of acquired resistance to drugs by *M. tuberculosis*: 1) the creation of a lipid-rich cell wall that can reduce the permeability of drugs (and arrest phagosome maturation); 2) the production of enzymes that degrade or modify compounds, rendering them useless; 3) the efflux of drugs through protein pumps, described for Isoniazid and Ethambutol; and 4) the spontaneous chromosomal mutations that affect key drug targets.\(^{53}\) Among these, the fourth mechanism is considered to be the most important. Mobile or horizontal transmission of resistance, such as plasmid mediated resistance, does not occur in *M. tuberculosis*. Random genetic mutations occur with low but predictable frequencies in the range of one mutation per $10^6$ to $10^9$ organisms. The frequency of mutations conferring resistance to particular agents varies from the range of $10^3$ for many second line drugs (Thiacetazone, Ethionamide, Capreomycin, Cycloserine, and Viomycin) to an intermediate level (around $10^6$) for some first and second line drugs (Isoniazid, Streptomycin, Ethambutol, Kanamycin, and Pamino salicylic acid) to the lowest levels for Rifampicin, on the order of $10^8$ to $10^{10}$. When large populations of *M. tuberculosis* are formed in a host and selective pressure is placed by a chemotherapeutic agent, the small population of *M. tuberculosis* that has evolved resistance to the agent will continue to multiply while the susceptible *M. tuberculosis* is suppressed. This
enables the drug resistant organism to become the dominant organism in the host. In order to prevent this scenario from occurring, the central strategies in therapy are to: 1) administer several chemotherapeutic agents, such that if there are organisms resistant to one or two agents, they will be killed by the other agents; 2) provide therapy for an adequate duration in order to ensure eradication of populations of M. tuberculosis\textsuperscript{54}. Compared with therapy for drug susceptible tuberculosis, treatment of MDR-TB requires a longer duration, is considerably more complicated, expensive, and toxic, and treatment success rates are typically lower. Therefore, detection of MDR TB is necessary

**Risk factors of MDR**

**Genetic factors**

Evidence suggest that the host genetic predisposition as the basis for the development of MDRTB occurring through gene acquisition and loss is the major underlying event in the emergence of successful drug resistant M. tuberculosis complex\textsuperscript{55 -58}. Spontaneous chromosomally borne mutations occurring in M. tuberculosis at a predictable rate are also thought to confer resistance to anti-TB drugs\textsuperscript{59-60}

**Incomplete and inadequate treatment**

In 2004, a review article published by Sharma and Mohan strongly suggests that the most powerful predictor of the presence of MDR-TB is a history of treatment of TB, though some individuals who did not have previous TB treatment can be infected by MDRTB. Many new cases of MDR-TB are created each year by physician’s errors (drugs, dosing intervals, duration)\textsuperscript{60}. In 1993, Professor Michael Iseman, the US “guru” of MDR-TB, has shown that two to four errors are needed to turn a fully susceptible organism in to a case of MDR-TB. MDR-TB develops due to error in TB management such as the use of single drug to treat TB, the addition of a single drug to a failing regimen, the failure to identify pre-existing resistance, the initiation of an inadequate regimen using first
line anti tubercular drugs and variations in bioavailability of anti-TB drugs predispose the patient to the development of MDR-TB \textsuperscript{60-61}. Shortage of drugs has been one of the most common reasons for the inadequacy of the initial anti-TB regimen, especially in resource poor settings\textsuperscript{62}. Other major issues significantly contributing to the higher complexity of the treatment of MDR-TB is the increased cost of treatment.

**Inadequate treatment adherence**

Non-adherence to prescribed treatment is often underestimated by the physician and is difficult to predict. Certain factors such as psychiatric illness, alcoholism, drug addiction, and homelessness do predict non-adherence to treatment\textsuperscript{60}. Poor compliance with treatment is also an important factor in the development of acquired drug resistance. In 1993, a study conducted in South India observed that only 43\% of the patients receiving short course treatment \((n=2306)\) and 35\% of those receiving standard chemotherapy \((n=1051)\) completed 80\% or more of their treatment\textsuperscript{63}. The various reasons for default included travel to different places, symptom relief, adverse drug reactions and inability to afford treatment\textsuperscript{64}. MDR-TB requires a two- to four-fold longer period of treatment compared with the drug susceptible TB. Shortest treatment course so far validated for drug susceptible TB is six months long. Most of the problems from which drug resistance originates are related to length of treatment (especially considering tolerability). The longer time that is required to treat MDR-TB clearly implies an additional risk of poor treatment adherence and consequently of treatment failure\textsuperscript{65}. Some other factors also play important role in the development of MDR-TB such as poor administrative control on purchase and distribution of the drugs with no proper mechanism on quality control and bioavailability tests\textsuperscript{66}. Tuberculosis control program implemented in past has also partially contributed to the development of drug resistance due to poor follow up and infrastructure.
Other factors
Over all multivariate analysis showed that being male, having a history of TB and previous or current treatment for more than 4 weeks, advanced disease with cavitations, and a history of imprisonment remained as highly significant risk factors for single drug resistance and MDR-TB. This study also examined the role of social factors in drug resistance. Smoking was found to be associated with Isoniazid resistance but more evidence is needed to explain this association. In North India, of the risk factors studied for MDR-TB, bacillary load and previous treatment of TB were found significant (p<0.05). HIV status, tobacco smoking, excessive alcohol intake, age, sex, education and socio-economic status had no relation to infection with MDR TB.

The global drug resistance scenario
During the period 1994-2002, a total of 109 surveillance projects on anti-TB drug resistance in 90 countries were completed. This included 43 per cent of all the countries in the world covering approximately 42 % of the world’s population and 34 % of the reported TB cases. However, the Global Project had the highest coverage in the Americas (95%) and the Western Pacific Region (49%), while the lowest coverage was observed in South-East Asia (11%). The median prevalence of MDR-TB in new cases of tuberculosis was 1.1 per cent (range 0-14.2%). Among previously treated cases median prevalence of resistance to any drug was 33.4 per cent (range 0-93.8%). The median prevalence of MDR-TB among treated cases was 7.0 per cent, ranging from 0 per cent in eight geographical settings to a maximum of 58.3 per cent in Oman. Table I. shows the global ant tuberculosis drug resistance situation.

<table>
<thead>
<tr>
<th>Table I. Global ant tuberculosis drug resistance situation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Initial</td>
</tr>
</tbody>
</table>
Drug resistance scenario in India

Although drug resistance in tuberculosis has been reported frequently during the last four decades, the available information from India is localized, inaccurate or incomplete. In order to formulate a national treatment policy, reliable and periodic updates on the prevalence of drug resistance for the entire country is needed, which would serve as an indication of the transmission of drug resistant organisms as well as the efficacy of the NTP. In view of the large size of the country and several other administrative as well as financial constraints, surveys of drug resistance at a national level are logistically difficult to undertake. Most of the published reports on drug resistance in India, with the exception of studies reported from the Tuberculosis Research Centre (TRC) in view of the detailed and repeated questioning methods used for eliciting history of previous treatment from the patients. Table 2 and 3 shows the distribution of drug resistance among newly diagnosed and previously treated TB cases in India.

<table>
<thead>
<tr>
<th>Location</th>
<th>period</th>
<th>no. of isolates</th>
<th>Any resistance to (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9Centres-</td>
<td>1964-</td>
<td>1838</td>
<td>14.7 12.5 ND 6.5 ND</td>
</tr>
</tbody>
</table>

**Table II. Summary of studies on initial drug resistance among M.tuberculosis isolates in India (newly diagnosed TB cases)**
<table>
<thead>
<tr>
<th>Location</th>
<th>period</th>
<th>no. of isolates</th>
<th>Any resistance to (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>R</td>
</tr>
<tr>
<td>Gujarat**</td>
<td>1985-89</td>
<td>2779</td>
<td>11.6</td>
</tr>
<tr>
<td>Pondicherry</td>
<td>1985-91</td>
<td>1841</td>
<td>8.1</td>
</tr>
<tr>
<td>Kolar</td>
<td>1987-89</td>
<td>292</td>
<td>5.1</td>
</tr>
<tr>
<td>Raichur</td>
<td>1988-89</td>
<td>244</td>
<td>11.4</td>
</tr>
<tr>
<td>Jaipur</td>
<td>1989-91</td>
<td>1009</td>
<td>7.6</td>
</tr>
<tr>
<td>New Delhi</td>
<td>1990-91</td>
<td>324</td>
<td>ND</td>
</tr>
<tr>
<td>Military Hosp, Pune</td>
<td>1992-93</td>
<td>473</td>
<td>8.2</td>
</tr>
<tr>
<td>Tamil Nadu</td>
<td>1997</td>
<td>384</td>
<td>6.8</td>
</tr>
<tr>
<td>North Arcot</td>
<td>1999</td>
<td>282</td>
<td>12.4</td>
</tr>
<tr>
<td>Raichur</td>
<td>1999</td>
<td>278</td>
<td>7.2</td>
</tr>
<tr>
<td>Wardha**</td>
<td>2000</td>
<td>197</td>
<td>7.6</td>
</tr>
<tr>
<td>Jabalpur**</td>
<td>2000</td>
<td>273</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*Tuberculosis Research Centre, unpublished data

**Tuberculosis Research Centre, interim findings, unpublished data

S, streptomycin; H, isoniazid; R, rifamicin; ND, not done

Table III. Summary of studies on initial drug resistance among M.tuberculosis isolates in India (previously treated TB cases)
<table>
<thead>
<tr>
<th>Location</th>
<th>Year</th>
<th>Isolates</th>
<th>H (%)</th>
<th>R (%)</th>
<th>X (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gujarat22</td>
<td>1983-86</td>
<td>1259</td>
<td>81.1</td>
<td>33.0</td>
<td>30.2</td>
</tr>
<tr>
<td>Wardha21</td>
<td>1982-89</td>
<td>302</td>
<td>47.0</td>
<td>12.6</td>
<td>9.6</td>
</tr>
<tr>
<td>North Arcot1</td>
<td>1988-89</td>
<td>560</td>
<td>67.0</td>
<td>12.0</td>
<td>10.9</td>
</tr>
<tr>
<td>Raichur17</td>
<td>1988-89</td>
<td>111</td>
<td>52.3</td>
<td>17.1</td>
<td>17.1</td>
</tr>
<tr>
<td>New Delhi25</td>
<td>1990-91</td>
<td>81</td>
<td>60.5</td>
<td>33.3</td>
<td>33.3</td>
</tr>
<tr>
<td>TamilNadu(4 districts)</td>
<td>1996</td>
<td>162</td>
<td>-</td>
<td>-</td>
<td>20.3</td>
</tr>
<tr>
<td>Tamil Nadu State11</td>
<td>1997</td>
<td>16</td>
<td>(50.0)</td>
<td>(25.0)</td>
<td>(25.0)</td>
</tr>
<tr>
<td>North Arcot12</td>
<td>1999</td>
<td>16</td>
<td>(81.0)</td>
<td>(69.0)</td>
<td>(69.0)</td>
</tr>
<tr>
<td>Raichur12</td>
<td>1999</td>
<td>11</td>
<td>(100.0)</td>
<td>(100.0)</td>
<td>(100.0)</td>
</tr>
<tr>
<td>Wardha*</td>
<td>2000</td>
<td>9</td>
<td>(78.0)</td>
<td>(78.0)</td>
<td>(78.0)</td>
</tr>
<tr>
<td>Jabalpur*</td>
<td>2002</td>
<td>31</td>
<td>87.1</td>
<td>80.6</td>
<td>80.6</td>
</tr>
</tbody>
</table>

Brackets indicate that the percentage is based on isolates less than 25
*TRC, unpublished interim findings
H, isoniazid; R, rifampicin
Superscript numerals indicate reference nos

Detection of drug resistance
Genotypic methods and phenotypic methods
Genotypic methods

Table IV. Genotypic methods for the detection of MDR TB\(^2\)
Novel molecular assays for detecting drug resistance offer several potential advantages, including lower turnaround times and minimal (or possibly no) initial culture. Many of the key gene mutations conferring resistance have been identified, permitting the development of in-house and commercial molecular tests. Considerable problems remain in the development of tests for clinical use. The majority of these tests are more costly than current methods, the exact ratio of resistant to susceptible organisms that produces clinical resistance is unclear, and the presence of common gene mutations is not always associated with drug resistance (i.e., silent mutations). Nevertheless, the mutations associated with resistance are now well-known for some drugs. Novel non-culture based drug resistance tests can be divided into genotypic systems, in which the drug target and nature of the gene mutation is known, and phenotypic systems, in which an outcome (i.e., death of the bacillus) is measured and previous knowledge of the precise underlying resistance mechanism is not required. Realistically, a combination of both types of test is required. Mutations within an 81-bp region (codons 507–533) of the rpoB gene, encoding the b chain of the
DNA-dependent RNA polymerase, confer rifampicin resistance in the confidence interval of 90–95% of all clinical isolates examined. The presence of a mutation may be detected by a genotypic test and, in nearly all cases, is predictive of clinical drug resistance. However, isoniazid drug resistance is more complex, involving mutations in at least four genes (katG, inhA, ahpC and oxyR) or gene complexes, with not all mutations affecting the phenotype.

Most genotypic assays involve three main steps: (i) sample preparation (ii) amplification of a specific region of a gene; and (iii) detection of the mutation. Once appropriate primers have been selected, the region of interest can be amplified and mutations detected as described below.

**DNA sequencing and other rapid molecular genotyping assays**

DNA sequencing is the reference standard, as all mutations will be detected and, unless they are silent, will be predictive of resistance. Automation has simplified the process, bringing sequencing within the capability of large academic and reference centers, although automated analyzers are relatively expensive to purchase and operate.

Automated analyzers using fluorescent chemistry methods can provide accurate sequence data within 48 h. As indicated in Table 2, automated sequencing has been used in both research and clinical settings to identify mutations for resistance to most first-line TB drugs. Microarrays and macroarrays using a solid phase hybridization approach offer an alternative approach to direct sequencing as part of initial screens for mutations. Other alternative techniques for mutation identification include PCR single-strand conformation polymorphism analysis, pyrosequencing (a short-read sequencing assay developed and applied as a screen to detect common mutations in katG and rpoB in early cultures), mutation-specific priming, and restriction enzyme analysis.

These methods are generally PCR-based, with amplification of the key regions...
involved in drug resistance being followed by a method that detects wild-type sequences or mutations in the amplified fragments.

**Phenotypic methods**

Symposia organized by the WHO and the International Union Against Lung and Tuberculosis produced agreed definitions for drug resistance, and three categories of acceptable methods for the detection of drug resistance were defined:

1. The absolute concentration method (effectively the MIC);
2. The resistance ratio method;
3. The proportion method.

1. **The absolute concentration method**

Drug is incorporated into solid agar or Lowenstein–Jensen medium as two-fold dilutions, or is used in a broth dilution method. Solid media methods are standardized more easily. Resistance is defined as the lowest concentration of the drug that inhibits growth (<20 colonies). Drug concentrations, and particularly inoculum size, must be carefully standardized with reference to wild-type cultures. Variation in inoculum size is the major source of error in this method.

2. **The resistance ratio method**

This is a refinement of the absolute concentration method, in which variations in the MIC for a given isolate are controlled when the isolate is tested on different batches of drug-containing medium. The resistance ratio is defined as the MIC for the test isolate divided by the MIC for a standard susceptible strain, e.g., H37Rv, or for recently isolated susceptible wild-type strains. If the ratio is 2 or less, the isolate is considered as susceptible or if the ratio is 8 or more, then the isolate is considered as highly resistant. Intermediate or low-level resistance is difficult to measure accurately. Inoculum size needs to be standardized, but the critical
concentration does not need to be determined because of the direct comparison with susceptible isolates\textsuperscript{86}

3. **Proportion method**

In this method, the strain is classified as susceptible if its constituent cell population contains below a critical proportion of resistant cells, and as resistant if above this proportion. The proportion varies with different drugs, e.g., 1\% for isoniazid and rifampicin. This correlates with an effective clinical outcome. In practical terms, the proportion of drug-resistant mutants is obtained from the ratio of the number of colonies growing on drug-containing medium and on drug-free medium\textsuperscript{89-90}

The other phenotypic methods are broadly classified into mainly two categories;

a. **Evaluation of the performance of various colorimetric assays (Redox dyes) with conventional proportion method.**

b. **Evaluation of the performance of non colorimetric assay with conventional proportion method.**

a. **Evaluation of various colorimetric assays (Redox dyes) with conventional proportion method (using LJ media)**

Abate et al (1998) conducted a study at Statens Serum Institute, Copenhagen to standardize a colorimetric assay using a redox dye 3-(4,5-dimethylthiazol-2-yl)-2,5 di phenyl tetrazolium bromide(MTT) to rapidly detect rifampicin resistant Mycobacterium tuberculosis. This assay was performed using 3 ml of Dubos broth as the basal media and RIF concentration of 2 µg/ml. Further, the results obtained were compared with Bactec Reference method (RIF -2 µg/ml) and the strains were interpreted, either resistant or susceptible, based on the optical density values recorded at 570 nm on the third and sixth day. Indeed, the results obtained by the MTT assay perfectly matched the results obtained by the Bactec method. In addition, the findings demonstrated that MTT assay is simple, inexpensive assay and can be used as a rapid screening
method for the identification of rifampicin-resistant strains in low-income countries.

Franzblau et al (1998) evaluated the performance of microplate-based alamar blue assay (MABA) to determine the MICs of INH, RIF, streptomycin (SM), and ethambutol (EMB) for 34 Peruvian Mycobacterium tuberculosis isolates (including both pan sensitive and multidrug-resistant strains) and the H37Rv strain. For the assay, the bacterial suspensions were prepared directly from solid media and the assay performed in microtitre plates containing Middlebrook 7H9 broth. Interestingly, the results for all isolates were available within 8 days. However, discordant results were observed on initial tests for 3 of 16 INH-susceptible isolates, 5 of 31 EMB-susceptible isolates, and 2 of 4 SM-resistant isolates (by the BACTEC 460 system) as well. Although, the overall agreements between the MICs obtained by MABA and the results obtained with the BACTEC 460 system were 87.9% for initial results and 93.6% after retesting 12 of 17 samples with discrepant results. In nutshell, the authors concluded that the MABA is a simple, rapid, low-cost, appropriate technology, which does not require expensive instrumentation.

Caviedes et al (2002) evaluated the performance of Tetrazolium Microplate Assay (TEMA) and Microplate alamar blue assay (MABA). For the assay, the M. tuberculosis suspensions were prepared by emulsifying growth from LJ slants (20-30 days old culture) in 100 µl of Tween 80 and 0.2% bovine serum albumin and the turbidity was adjusted to McFarland standard no.1. The assay was carried out in microplates containing Middlebrook 7 H9 GC broth (culture media) and incubated at 37°C for 30 days. However, the results were read after 24 h and daily for 30 days. The workers reported that the MTT based methods is faster than Alamer Blue as it uses ethanol instead of a lysis Buffer. Further, the authors recommend that TEMA as a promising alternative method for
use in developing countries, where tuberculosis is an important and often fatal disease.\textsuperscript{6}

**Palomino et al (2002)** carried out a study at the Institute of Tropical Medicine, Belgium, to detect multidrug resistant Mycobacterium tuberculosis using resazurin microplate assay (REMA). In the current study, eighty clinical isolates were tested against INH and RIF and the results were available within 7 days. Further, these methods were compared with the indirect proportion method (the reference method) and it was observed that the REMA is inexpensive and rapid. In addition, REMA showed excellent specificity and sensitivity when compared to the proportion method. Therefore, the authors recommended the REMA to detect MDR TB along with other anti tubercular drugs.\textsuperscript{16}

**Foongladda et al (2002)** developed a MTT assay for the susceptibility testing of rifampicin and isoniazid against clinical isolates of Mycobacterium tuberculosis. The MTT tube assay conducted using one ml of Middlebrook 7H9 broth and rifampicin and isoniazid in recommended concentration. Further the results were available within 4 days and 7 days for RIF and INH, respectively. When MTT assay results of 279 M. tuberculosis clinical isolates were compared with those of the conventional proportion method on LJ medium, high specificity and sensitivity values of 100% and 94.1%, respectively, for RIF susceptibility testing, and 99.5% and 89.2%, respectively, for INH susceptibility testing were obtained. In addition, the workers also noted that the MTT method is simple, inexpensive and rapid. Moreover, the high level of agreement with the conventional proportion method suggests a potential to rapidly detect drug-resistant M. tuberculosis particularly in developing countries, where only basic microbiological equipment are accessible.\textsuperscript{11}

**Angeby et al (2002)** developed a nitrate reductase assay (NRA) that depends on the ability of Mycobacterium tuberculosis to reduce nitrate to nitrite.
and the reduction could be detected using specific reagents, which produce a color change. The current study analyzed 57 M. tuberculosis strains with various resistance patterns. For the assay, the bacteria were inoculated on LJ medium, either without drugs or with RIF, INH, SM, EMB with potassium nitrate incorporated. After incubation for 7, 10, or 14 days, the Griess reagent was added and the nitrate reduction could be detected by a color change. Further, the sensitivities and specificities for drugs as determined by the NRA method compared to those determined by the BACTEC 460 method and it was found to be 100 and 100% for RIF, 97 and 96% for INH, 95 and 83% for SM, and 75 and 98% for EM, respectively. Interestingly, the results were available within 7 days in majority of the cases. Therefore, the workers highlighted that NRA is a rapid, inexpensive and could correctly identify most resistant and sensitive M. tuberculosis strains. Moreover, it has the potential to become an interesting alternative to existing methods like the proportion and BACTEC methods.

**Martin et al (2003)** performed a study at the Institute of Tropical Medicine, Belgium to detect the emergence of multidrug-resistant tuberculosis. The current study examined 150 Mycobacterium tuberculosis isolates against the second-line drugs ethionamide, kanamycin, capreomycin, ofloxacin, and para-amino salicylic acid using the resazurin microtiter assay (REMA) and the proportion method. By visual reading, MICs were obtained after 8 days in REMA. However, an excellent correlation was obtained between REMA and the proportion method results as well. Further the current study depicted that REMA is inexpensive, rapid, and simple to perform, and implementation of the assay is feasible for low-resource countries.

**Banfi et al (2003)** developed a new rapid micro dilution method to evaluate drug susceptibility of Mycobacterium tuberculosis employing resazurin as an indicator of mycobacterial growth. The reference strain H37Rv and 13 M. tuberculosis susceptible or multidrug-resistant clinical strains were used for
the current study. The MICs of isoniazid, rifampicin, streptomycin and ethambutol were determined by the Micro dilution Resazurin Assay (MRA) and further the results were compared with agar indirect proportion method (reference method) as well. Indeed, the workers observed a complete agreement between MRA and agar indirect proportion method in the current study. In addition, MRA results were found to be rapid, reliable, simple and inexpensive to detect the drug resistance of M. tuberculosis to first-line drugs.10

Morcillo et al (2004) developed a colorimetric microplate-based assay to detect multidrug-resistant Mycobacterium tuberculosis (MDR-TB). The efficacy of current assay was validated by analyzing one hundred and one clinical isolates using proportion method (with Middlebrook 7H11 agar) as gold standard. The assay was carried out in Microtitre plates using various concentrations of drugs; Kanamycin, capreomycin, ethionamide, para-aminosalicylic acid and clarithromycin and the general indicator MTT at 5.0 mg/ml was used for visualizing cellular growth and viability. Interestingly, the MICs by M-MTT were obtained at an average of 8 days and correlated with those obtained using the proportion method. In addition, the workers evaluated the cost of MIC determination for all the drugs tested and it was found to be 5.00 US Dollars. Hence the current study highlighted that M-MTT could be used as a simple, rapid, low-cost technology to test the susceptibility of MDR-TB strains to several second-line and alternative drugs.12

Nateche et al (2006) assessed the performance of the REMA for the detection of resistance to INH and RIF in 136 clinical isolates of Mycobacterium tuberculosis. The MICs were determined and the results were compared with the conventional proportion method on Löwenstein-Jensen medium. Indeed, excellent results were obtained for the REMA plate method with a sensitivity of 100 % for both INH and RIF and a specificity of 98.3 and 99.2 %,
respectively. Therefore, the authors suggested that the REMA plate method is a good alternative for use in resource-limited countries.

Mohammadzadeh, et al (2006), carried out a study to evaluate a colorimetric method using TTC for antibiotic susceptibility testing of M. tuberculosis isolates. For the assay, eleven (MDR M. tuberculosis strains) and twelve (M. tuberculosis susceptible isolates) were used and the test was performed with a critical concentration of 0.2 µg/ml INH and 2µg/ml RIF in 7H9GC broth with 0.625 mg TTC/ml. Each isolates were inoculated under these conditions and inspected daily for color changes; the results were obtained after a mean of 4.9 days. Interestingly, it was observed that the sensitivity and specificity of this method were 100% and 92%, respectively, for both antibiotics. In addition, the workers noted the speed, technical ease and cost-effectiveness of TTC assay and hence suggested TTC assay would be a good alternative method for drug susceptibility testing of M. tuberculosis isolates.

Farnia, et al (2008) analyzed the performance of malachite green microtube (MGMT) drug susceptibility assay directly on sputum specimens (n = 80) and indirectly on Mycobacterium tuberculosis clinical isolates (n = 60). This technique is based on the malachite green dye, which changes color in response to M. tuberculosis growth. Further the authors recommend that the MGMT assay is simple and rapid and does not require expensive instruments. Therefore advised to use in low resource setting countries.

b. Evaluation of non colorimetric assay with conventional indirect proportion method (using LJ media)

Caviedes et al (2000) evaluated the performance of MODS and MABA to test the drug susceptibility pattern of M. tuberculosis. In the current study, the sputum samples from hospitalized patients in Peru were analyzed by using staining, culture, and PCR. Further the sputum samples positive for tuberculosis were tested for susceptibility to isoniazid and rifampin using
MABA and MODS assay. Interestingly, there was a concordance between MODS and MABA in 89% of cases. However, MODS assay yielded results rapidly (median, 9.0 and 9.5 days, respectively). In addition, the authors noticed that MODS is a rapid, inexpensive, sensitive, and specific method for MTB detection and susceptibility testing.\(^{21}\)

**Ha et al (2010)** performed a study to evaluate the performance of MODS for the early diagnosis of TB in HIV-positive patients. In the current study a total of 738 consecutive sputum samples collected from 307 HIV-positive individuals suspected of TB were tested by smear, MODS, and the mycobacteria growth indicator tube method (MGIT). Further the diagnostic sensitivity and specificity of MODS compared to the microbiological gold standard (either smear or MGIT) which was found to be 87 and 93%, respectively. However, the sensitivities of smear, MODS, and MGIT were 57, 71, and 75%, respectively, against clinical gold standard (MODS versus smear, \(P<0.001\); MODS versus MGIT, \(P=0.03\)). In this study the clinical gold standard was defined as patients who had a clinical examination and treatment consistent with TB, with or without microbiological confirmation. Interestingly, the current study observed the sensitivities of MODS and MGIT were 38 and 45%, respectively (\(P=0.08\)) for the diagnosis of smear-negative patients. However, the median times to detection using MODS and MGIT were 8 and 11 days, respectively, and they were 11 and 17 days, respectively, for smear-negative samples. Most interestingly, the original bacterial/fungal contamination rate of MODS was 1.1%, while it was 2.6% for MGIT. Further, the cross-contamination rate of MODS was found to be 4.7%. Hence the authors concluded that MODS is a sensitive, specific and rapid test for the detection of HIV-associated TB\(^{22}\)

**Bwanga et al (2011)** conducted a study to compare two direct techniques-NRA and MODS for rapid detection of MDR-TB. In the current study, smear positive sputum was collected from 245 consecutive re-treatment TB patients
attending a TB clinic in Kampala, Uganda. The collected samples were processed at the national reference laboratory and tested for susceptibility to rifampicin and isoniazid with direct NRA, direct MODS and the indirect LJ proportion method as reference. Of the 245 smear positive sputum, 229 specimens were confirmed as M. tuberculosis. However, interpretable results were obtained in 217 (95%) cases with either the NRA or MODS assay. Indeed, a high sensitivity, specificity and kappa agreement value, 97%, 98% and 0.93 with the NRA; and 87%, 95% and 0.78 with the MODS, respectively were obtained. Moreover, the median time to results was found to be 10, 7 and 64 days with NRA, MODS and the reference technique, respectively. Therefore, the authors concluded that the direct NRA and MODS assay can detect drug resistance almost eight weeks earlier than with the reference method.

Ghiraldi et al (2011) carried out a study to evaluate the performance of MODS with the broth microdilution method (BMM), absolute concentration method (ACM), and pyrazinamidase (PZase) activity to determine the susceptibility pattern of pyrazinamide to Mycobacterium tuberculosis isolates. In the current study, the workers analyzed 34 M. tuberculosis clinical isolates, 24 sensitive and eight resistant to pyrazinamide, and the control strain M. tuberculosis H37Rv (ATCC 27294). Moreover, the MODS, BMM, ACM and PZase determination provided results in average times of 6, 18, 28 and 7 days, respectively. Further all methods showed excellent sensitivity and specificity (p<0.05). Of the methods studied, the MODS proved to be faster, efficient, inexpensive, and easy to perform. However, the authors recommended that additional studies evaluating the MODS in differentiating pyrazinamide-resistant and pyrazinamide-susceptible M. tuberculosis must be conducted with a larger number of clinical isolates.