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## Chapter 19: Drug Resistance and Drug Resistance Detection

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### 19.1. Introduction

Drug resistance in tuberculosis (TB) is a matter of great concern for TB control programs since there is no cure for some multidrug-resistant TB (MDR-TB) strains of *M. tuberculosis*. There is concern that these strains could spread around the world, stressing the need for additional control measures, such as new diagnostic methods, better drugs for treatment, and a more effective vaccine. MDR-TB, defined as resistance to at least rifampicin (RIF) and isoniazid (INH), is a compounding factor for the control of the disease, since patients harboring MDR strains of *M. tuberculosis* need to be entered into alternative treatment regimens involving second-line drugs that are more costly, more toxic, and less effective.

Moreover, the problem of extensively drug resistant (XDR) strains has recently been introduced. These strains, in addition to being MDR, were initially defined as having resistance to at least three of the six main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and para-aminosalicylic acid) (CDC 2006). More recently, at a consultation meeting of the World Health Organization (WHO) Global Task Force on XDR-TB, held in Geneva, a revised laboratory case definition was agreed: “XDR-TB is TB showing resistance to at least rifampicin and isoniazid, which is the definition of MDR-TB, in addition to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin.” ([http://www.who.int/tb/xdr/taskforcereport\\_oct06.pdf](http://www.who.int/tb/xdr/taskforcereport_oct06.pdf)). XDR-TB now constitutes an emerging threat for the control of the disease and the further spread of drug resistance, especially in HIV-infected patients, as was recently reported (Gandhi 2006). For this reason, rapid detection of drug resistance to both first- and second-line anti-tuberculosis drugs has become a key component of TB control programs.

### 19.2. Drug resistance surveillance

#### 19.2.1. Benefits and recommendations

The surveillance of drug resistance in TB is a critical component of the monitoring system of the disease. The benefits of drug resistance surveillance are numerous

and include the strengthening of laboratory networks, the evaluation of TB control program performance, and the collection of important data for appropriate treatment strategies. Furthermore, global drug resistance surveillance identifies areas of high resistance, warning the health authorities to initiate the appropriate correction measures. To adequately establish drug resistance surveillance at a national level, three recommendations have been provided: the sampled specimens should be representative of the patients from the area under study and the sample size should be statistically determined to allow standard epidemiological analysis; the patient's history should be obtained and medical records carefully reviewed to determine whether the patient has received previous treatment in order to distinguish primary from acquired resistance; and the laboratory techniques used for determining the drug susceptibility to anti-tuberculosis drugs should be selected from those that are internationally recommended (WHO/IUATLD 1998). In 1996, the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) launched the Global Project on Drug Resistance Surveillance based on data collected and reported by an international network of laboratories acting as Supranational Reference Laboratories. The network includes twenty-six Supranational Reference Laboratories distributed in the five WHO regions and is coordinated by the Prince Leopold Institute of Tropical Medicine in Antwerp, Belgium.

### **19.2.2. Global trends in drug resistant tuberculosis**

Since the establishment of the WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance, three global reports have been produced (WHO 1997, 2001, 2004). The first two reports covered data from 35 and 58 settings respectively. The main conclusions of those two reports were that drug-resistant TB was present in all settings surveyed, MDR-TB was identified in most settings, and good TB control practices were associated with lower or decreasing levels of resistance.

The third and last report available, published in 2004, covers data from 77 settings and had the main goal of expanding knowledge of the prevalent global patterns of resistance and exploring trends in resistance over time. The data were collected between 1999 and 2002 and represented 20 % of the total global number of new smear-positive TB cases. This third report also contributes to address two issues not thoroughly dealt with in previous reports: the importance of conducting surveillance on re-treatment cases, and stressing the issue of the role of the laboratory in TB control (WHO 2004, Aziz 2006).

The prevalence of drug resistance among new patients is a very important indicator for a TB control program. The prevalence of resistance among previously untreated patients also reflects program performance over a long period of time and indicates the level of transmission within the community. The prevalence of drug resistance among patients with a history of previous treatment, on the other hand, has received less attention, since surveillance of this population is more complex. Re-treatment patients are a heterogeneous group composed of chronic patients, those with treatment failure, those who have relapsed, and those who have returned after defaulting. Sometimes this population represents more than 40 % of smear-positive cases. The prevalence of drug resistance varies greatly among subgroups of this population. Chronic cases and treatment failures are at a greater risk of having resistant and MDR-TB. Relapses and default patients are more likely to have drug resistance than new cases, but are almost always at a lower risk for MDR-TB than failures and chronic cases. One of the recommendations of the last report is that all subgroups of re-treatment cases be notified separately and their outcomes reported; furthermore, surveillance of resistance should be conducted on a representative sample of this population.

The second issue stressed in the third resistance report is that of the role of the laboratory. While laboratory services are fundamental for TB control, they are often the weakest components of the system. The importance of the laboratory in the control of TB should be recognized and they should be able to perform sputum smear microscopy, culture, and drug susceptibility testing of a high quality as standard components of TB control. Culture and drug susceptibility testing should be performed by national reference laboratories. Recognizing the pressing need to improve laboratory performance, a Subgroup on Laboratory Capacity Strengthening was established within the DOTS Expansion Working group in 2002 (Portaels 2006). The major objective of the subgroup is to assist high-TB burden and other countries in strengthening TB laboratory capacity and to provide high quality diagnostic services.

In this third report, data were collected through routine or continuous surveillance of all TB cases (in 38 settings) or from specific surveys of sampled patients (in 39 settings). These were reported on a standard reporting form, either annually or on completion of the survey (WHO 2004).

The results show that in new TB cases with data available from 75 settings (55,779 patients) the prevalence of resistance to at least one drug (any resistance) ranged from 0 % in some Western European countries to 57.1 % in Kazakhstan (median = 10.2 %). Median prevalence of resistance to individual drugs was: streptomycin (SM), 6.3 %; INH, 5.9 %; RIF, 1.4 %; and ethambutol (EMB), 0.8 %. Prevalence

of MDR-TB ranged from 0 % in eight countries to 14.2 % in Kazakhstan and Israel (median = 1.1 %). The highest prevalences of MDR-TB were observed in Tomsk Oblast (Russian Federation) (13.7 %), Karakalpakstan (Uzbekistan) (13.2 %), Estonia (12.2 %), Liaoning Province (China) (10.4 %), Lithuania (9.4 %), Latvia (9.3 %), Henan Province (China) (7.8 %), and Ecuador (6.6 %). Trends in drug resistance were determined in 46 settings (20 with two data points and 26 with at least three). Significant increases in prevalence of any resistance were found in Botswana, New Zealand, Poland, and Tomsk Oblast (Russian Federation). Cuba, Hong Kong SAR, and Thailand reported significant decreases over time. Tomsk Oblast (Russian Federation) and Poland reported significantly increased prevalences of MDR-TB. Decreasing trends in MDR-TB were observed in Hong Kong SAR, Thailand, and the USA.

Among previously treated cases with data available from 66 settings (8,405 patients) the median prevalence of resistance to at least one drug (any resistance) was 18.4 %, with the highest prevalence being 82.1 % in Kazakhstan. Median prevalence of resistance to individual drugs was: INH, 14.4 %; SM, 11.4 %; RIF, 8.7 %; and EMB, 3.5 %. The median prevalence of MDR-TB was 7.0 %. The highest prevalence of MDR-TB was reported in Oman (58.3 %) and Kazakhstan (56.4 %). Countries of the former Soviet Union had a median prevalence of resistance to the four drugs of 30 %, compared with 1.3% in all other settings. However, these data should be interpreted with caution given the small number of subjects tested in some settings. Trends in drug resistance in this group were determined in 43 settings (19 with two data points and 24 with at least three data points). A significant increase in the prevalence of any resistance was observed in Botswana. Cuba, Switzerland, and the USA showed significant decreases. The prevalence of MDR-TB significantly increased in Estonia, Lithuania, and Tomsk Oblast (Russian Federation). Decreasing trends were significant in Slovakia and the USA.

The annual incidence of MDR-TB cases was estimated in 69 settings. In most Western and Central European countries, the estimated incidence was fewer than 10 cases each. Estonia, Latvia, Lithuania and two Oblasts in the Russian Federation were estimated to have between 99 and 248 MDR-TB cases. For Henan and Huber Provinces of China, more than 1,000 cases each were estimated, and for Kazakhstan and South Africa, more than 3,000.

The report also evaluated RIF resistance as a predictor of MDR-TB, in order to explore the significance of rapid testing for RIF resistance to identify cases likely to have MDR-TB. The positive predictive value, a function of the sensitivity and specificity of RIF resistance testing and the prevalence of MDR-TB and non-MDR-TB RIF resistance, was highest among previously treated cases in settings with

high MDR-TB prevalence and low non-MDR-TB RIF resistance. The report also confirmed that, globally, more isolates were resistant to INH than to any other drug (range 0–42 %). INH and SM resistance were more prevalent than RIF or EMB resistance. Resistance to INH, SM, RIF and EMB was the most prevalent pattern among previously treated cases and the proportions of isolates resistant to three or four drugs were significantly greater than among new cases, suggesting an amplification of resistance. It appears that monoresistance to either INH or SM is the main gateway to the acquisition of additional resistance.

Tables 19-1 and 19-2 below show a summary of the prevalence of drug resistance and MDR-TB in new TB cases and previously treated patients, respectively, according to the five WHO regions in the world.

Table 19-1: Median prevalence of drug resistance, polyresistance and MDR-TB among new TB cases by region (%)

<b>Region</b>	<b>Any resistance</b>	<b>Polyresistance</b>	<b>MDR-TB</b>
Africa	7.1	1.3	1.4
Americas	9.7	2.1	1.1
Eastern Mediterranean	9.9	2.5	0.4
Europe	8.4	1.1	0.9
South-East Asia	19.8	4.0	1.3
Western Pacific	11.4	2.5	0.9
<b>Overall median</b>	<b>10.2</b>	<b>1.9</b>	<b>1.1</b>

Adapted from Reference WHO, 2006

Table 19-2: Median prevalence of drug resistance, polyresistance and MDR-TB among previously-treated TB cases by region (%)

Region	Resistance	Polyresistance	MDR-TB
Africa	16.7	1.8	5.9
Americas	24.6	3.7	7.0
Eastern Mediterranean	63.3	5.8	48.3
Europe	15.9	2.6	4.7
South-East Asia	39.9	7.3	20.4
Western Pacific	32.8	6.1	15.5
<b>Overall median</b>	<b>18.4</b>	<b>3.2</b>	<b>7.0</b>

Adapted from Reference WHO, 2006

### 19.3. Methods for detection of drug resistance

Early detection of drug resistance constitutes one of the priorities of TB control programs. It allows initiation of the appropriate treatment in patients and also surveillance of drug resistance. Detection of drug resistance has been performed in the past by so-called 'conventional methods' based on detection of growth of *M. tuberculosis* in the presence of the antibiotics. However, due to the laboriousness of some of these methods, and most of all, the long period of time necessary to obtain results, in recent years new technologies and approaches have been proposed. These include both phenotypic and genotypic methods. In many cases, the genotypic methods in particular have been directed towards detection of RIF resistance, since it is considered a good surrogate marker for MDR-TB, especially in settings with a high prevalence of MDR-TB. Genotypic methods have the advantage of a shorter turnaround time, no need for growth of the organism, the possibility of direct application in clinical samples, lower biohazard risks, and the feasibility of automation; however, not all molecular mechanisms of drug resistance are known. Phenotypic methods, on the other hand, are in general simpler to perform and might be closer to implementation on a routine basis in clinical mycobacteriology laboratories. The following section describes the phenotypic and genotypic methods as well as the new methodologies recently proposed for drug resistance detection in TB.

### 19.3.1. Conventional phenotypic methods

In general, phenotypic methods assess inhibition of *M. tuberculosis* growth in the presence of antibiotics to distinguish between susceptible and resistant strains. This is possible since *M. tuberculosis* isolates from patients never treated before are very uniform in their level of susceptibility, as shown by the narrow ranges of minimal inhibitory concentrations (MIC) of the main anti-tuberculosis drugs (Heifets 1996). The classical definition for a drug resistant *M. tuberculosis* strain is that it displays a degree of susceptibility significantly lower than that of a wild strain that has never been in contact with the drug (Canetti 1963, Canetti 1969).

Phenotypic methods based on cultivation of *M. tuberculosis* in the presence of antibiotics have been most commonly performed on egg-based or agar-based solid media, and can also be performed as a direct or indirect method. For the direct method, antibiotic-containing and control media are inoculated with a decontaminated and concentrated clinical specimen, while for the indirect method the antibiotic-containing and control media are inoculated with a bacterial suspension of the isolated strain. There are three conventional phenotypic methods for drug susceptibility testing based on solid media: the proportion method, the resistance ratio method and the absolute concentration method (Canetti 1963, Canetti 1969, Kent 1985). More recent methods are based on liquid media including the BACTEC radiometric and the Mycobacterial Growth Indicator Tube methods.

#### The proportion method

The proportion method is the most commonly used method worldwide amongst the three methods mentioned above. It allows the precise determination of the proportion of resistant mutants to a certain drug. Briefly, several 100-fold serial bacilli dilutions are inoculated into drug-containing and drug-free (control) media. One of those dilutions should produce a number of colonies that is easy to be counted. The number of colonies obtained in the drug-containing and control media are enumerated and the proportion of resistant mutants is then calculated. When performed in Löwenstein-Jensen medium tubes, the test is first read after 28 days of incubation at 37°C. If the proportion of resistant bacteria is higher than 1 % for isoniazid, rifampicin and para-aminosalicylic acid, or 10 % for the other drugs, the strain is considered resistant and the results are final; otherwise, the test is read again at 42 days of incubation to assess if the strain is susceptible to a certain drug (Heifets 2000). If the test is performed on agar, a Middlebrook 7H10/11 is used and the medium is incubated in a 10 % CO<sub>2</sub> atmosphere. Results are interpreted after 21 days of incubation or even earlier if they show the strain to be resistant (Kent

1985). The critical concentrations of the main drugs used in the proportion method are shown in Table 19-3.

Table 19-3: Critical concentration of main antibiotics in the proportion method ( $\mu\text{g/mL}$ )

Antibiotic	Löwenstein-Jensen	7H10 agar	7H11 agar
Isoniazid	0.2	0.2, 1.0	0.2, 1.0
Rifampicin	40.0	1.0	1.0
Ethambutol	2.0	5.0	7.5
Streptomycin	4.0	2.0	2.0, 10.0
Pyrazinamide	100	-	-
PAS	0.5	2.0	8.0
Kanamycin	20.0	5.0	6.0
Ethionamide	20.0	5.0	10.0
Ofloxacin	2.0	2.0	2.0
Capreomycin	20.0	10.0	10.0
Cycloserine	40.0	-	-

Adapted from: Kent 1985; WHO/CDS/TB/2001.288; and NCCLS 2000

### The resistance ratio method

This method is based on the resistance ratio, which corresponds to the MIC of a test strain divided by the MIC of the drug-susceptible reference strain H37Rv tested at the same time. Thus, it compares the resistance of an unknown strain with that of a standard laboratory strain. For the performance of the test, parallel sets of tubes containing two-fold dilutions of the tested drug are then inoculated with a standardized inoculum of both test and reference strain. Reading of the test is performed after 4 weeks of incubation at 37°C. Tubes containing 20 or more colonies are considered as positive for growth and the MIC is defined as the lowest concentration of drug in the presence of which the number of colonies is lower than 20.

An isolate with a resistance ratio value of 2 or less is considered susceptible, while a resistance ratio of 8 or more defines the isolate as resistant (Kent 1985, Heifets 2000).

#### **The absolute concentration method**

This method uses a standard inoculum of the test strain grown in a two-fold dilution drug-containing media and drug-free control. The resistance of a strain is expressed in terms of the lowest concentration of a certain drug that inhibits all or almost all the growth of the strain. The critical concentrations included in the medium are similar to the ones used in the proportion method (see Table 19-3) but the drug concentration considered as 'critical' should be determined in each laboratory (Heifets 2000). For the interpretation of the test, the reading is performed after 4 weeks of incubation at 37°C, or at 5-6 weeks if there is not enough growth. A strain is considered to be susceptible if the number of colonies on the drug-containing medium is less than 20 with a 3+ or 4+ (confluent) growth on the drug-free control.

#### **The BACTEC radiometric method**

The radiometric method is based on the commercial system BACTEC TB-460 (Becton Dickinson, Sparks, MD), which uses an enriched Middelbrook 7H9 liquid medium containing <sup>14</sup>C-labeled palmitic acid as the sole carbon source (12B vial). Growth of the mycobacteria and consumption of the labeled fatty acid will produce <sup>14</sup>CO<sub>2</sub> that is detected inside the 12B vial by the BACTEC apparatus and expressed as a growth index. In the presence of a certain drug, susceptibility can be measured by inhibition of the daily increases in the growth index. For the performance of the test, a test vial containing the drug under study and a drug-free control are inoculated with a standard inoculum and incubated at 37°C. The vials are then read in the BACTEC 460-TB apparatus on a daily basis. Since two control vials are inoculated with a 100-fold serial dilution of the inoculum, results can be interpreted as in the proportion method with the 1 % proportion of growth. The BACTEC radiometric method has been approved by the Food and Drug Administration (FDA) of the United States (US) and is also considered to be the 'gold standard' for drug susceptibility testing to first-line anti-tuberculosis drugs (Roberts 1983, Heifets 1999). More recently, critical concentrations for second-line drugs have also been proposed and tested successfully for most drugs in a multicenter evaluation (Pfyffer 1999). The major advantage of the BACTEC radiometric method is the capacity to detect drug resistance faster than with the solid media-based methods; the major disadvantage is the cost of the system and the need for disposal of the radioactive waste from used vials.

### The Mycobacterial Growth Indicator Tube

The Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson, Sparks, MD) is part of the 'new generation' of TB diagnostic tools both in its manual version as well as in its more recently introduced automated format (Pfyffer 1997, Idigoras 2000). It is based on fluorescence detection of mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. Consumption of oxygen in the medium produces fluorescence when illuminated by a UV lamp. In the manual system, for the performance of the test a drug-containing tube and a control tube are inoculated with the standardized mycobacterial suspension and incubated at 37°C (day 0). Starting on the third day (day 2), the tubes are controlled daily with an UV lamp. The presence of an orange fluorescence in the drug-containing tube at the same time as in the control tube or within two days of positivity in the control is interpreted as resistance to the drug; otherwise, the strain is considered to be susceptible. The test is valid if the growth control gives a positive signal until the 14<sup>th</sup> day of incubation (day 12) (Palomino 1999). The MGIT system in its manual version has also been successfully used as a direct method using decontaminated clinical specimens (Goloubeva 2001).



Figure 19-1: MGIT tubes showing a positive and a negative reaction

The MGIT has also been recently introduced as an automated system. The BACTEC MGIT960 (Becton Dickinson, Sparks, MD) is based on the same principle of oxygen consumption and a fluorescence signal, but the tubes are incubated and controlled inside the MGIT960 apparatus. For the performance of the test, drug-containing and drug-free control vials are inoculated with a standardized inoculum of the *M. tuberculosis* isolate and entered into the machine in a special rack-carrier with a printed barcode; this is read by the machine when entering the tubes to identify the test and apply the adequate algorithm for susceptibility or resistance interpretation. All readings are performed inside the machine and the results are printed as susceptible or resistant (Ardito 2001).

Many studies have now been published on the application of the MGIT system for the rapid detection of resistance to first- and second-line antituberculosis drugs (Johansen 2004, Rusch-Gerdes 2006). In all these studies, the MGIT system has shown very good results with a high correlation with the conventional methods on solid media and the BACTEC TB-460 system. The BACTEC MGIT960 system has recently been approved by the US FDA for the detection of drug resistance to first-line drugs.

Other automated systems, such as those already described in Chapter 14, have been used for the rapid detection of drug resistance in *M. tuberculosis*, but they have not been used on a routine basis in the clinical mycobacteriology laboratory (Ängeby 2003, Ruiz 2000). Recent developments of phenotypic formats for rapid drug resistance detection will be presented in section 19.3.3 below.

### 19.3.2. Genotypic methods

Genotypic methods for drug resistance in TB look for the genetic determinants of resistance rather than the resistance phenotype, and involve two basic steps: nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains; and a second step of assessing the amplified products for specific mutations correlating with drug resistance (García de Viedma 2003, Palomino 2005).

#### Desoxyribonucleic acid (DNA) sequencing

Sequencing DNA of PCR-amplified products has become the most widely used genotypic method for detecting drug resistance in *M. tuberculosis*; it is accurate and reliable and it has become the reference standard for mutation detection. It was performed several years ago by manual procedures, but in our days, it is performed with automatic sequencers (Victor 2001). DNA sequencing has been widely used

for characterizing mutations in the *rpoB* gene in RIF-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs (Telenti 1993, García de Viedma 2003, Jalava 2004). Drug resistance detection in *M. tuberculosis* has also been described by pyrosequencing technology (Arnold 2005, Jureen 2006). This technology is a short-read (30–50 bp) sequencing technique, which is based on the quantitative detection of pyrophosphate released following nucleotide incorporation into a growing DNA chain (Ronaghi 1999). However, not all molecular mechanisms of drug resistance for *M. tuberculosis* are known and it would be rather difficult and expensive to implement it routinely for the detection of drug resistance mutations for several drugs (Hazbón 2004).

### **Solid-phase hybridization techniques**

There are currently two commercially available solid-phase hybridization techniques for the rapid detection of drug resistance in TB: the Line Probe Assay (INNO-LiPA Rif TB Assay, Innogenetics, Ghent, Belgium) for the detection of resistance to RIF and the GenoType MTBDR assay (Hain Lifesciences, Nehren, Germany) for the simultaneous detection of resistance to INH and RIF.

The LiPA assay was introduced several years ago and is based on reverse hybridization of amplified DNA from cultured strains or clinical samples to ten probes covering the core region of the *rpoB* gene of *M. tuberculosis*, immobilized on a nitrocellulose strip (De Beenhouwer 1995). From the pattern of hybridization obtained, the presence or absence of mutated or wild regions is visualized by a colorimetric reaction and the strain can be considered as resistant or susceptible to RIF (Rossau 1997). Many studies have been conducted on the application of the LiPA assay for detection of RIF resistance; most of them have been performed on *M. tuberculosis* isolates and just a few have applied the test directly in sputum samples (Jureen 2004, Traore 2006). It has been proposed as a good initial indicator of multidrug resistance with a sensitivity of 98.5 % for detecting RIF resistance (Traore 2000). In a recent systematic review and meta-analysis of studies that applied the LiPA test, 12 of 14 studies performed in isolates had sensitivity greater than 95 % and specificity of 100 %. Four studies that applied LiPA directly to clinical specimens had 100 % specificity, and the sensitivity ranged from 80 % to 100 % (Morgan 2005). In a very recent and large study, not included in the meta-analysis mentioned above, the utility of the LiPA test for detecting RIF resistance was assessed in 420 sputum samples originating from different countries (Traore 2006). There was a 99.6 % concordance between the RIF resistance obtained by culture and by the LiPA test, confirming that with an adequate DNA extraction method, the LiPA test allows rapid detection of resistance to RIF directly from sputum samples.

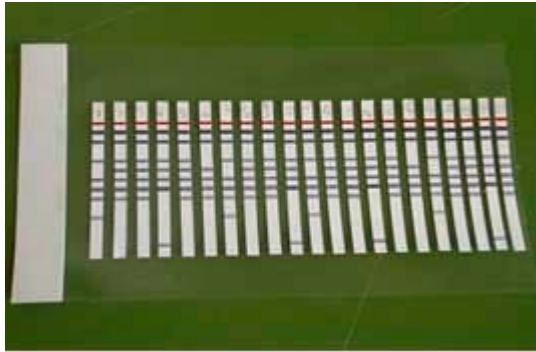


Figure 19-2: LiPA strips showing different mutations

The GenoType MTBDR, on the other hand, detects resistance to INH and RIF in culture samples based on the detection of the most common mutations in the *katG* and *rpoB* genes respectively (Makinen 2006). It also utilizes PCR and reverse hybridization to probes immobilized on a DNA strip. In a recent study that evaluated the GenoType MTBDR assay in 143 *M. tuberculosis* isolates, 99 % of the MDR strains were found to have mutations in the *rpoB* gene and 88.4 % of strains with mutations in the codon 315 of the *katG* gene were also correctly identified (Hillemann 2005). The correlation with DNA sequencing was 100 %, and good sensitivity and specificity was obtained when compared to the conventional tests. As with other genotypic tests, there is interest in the application of these techniques directly to sputum samples. There are only two studies that address this issue. In the study by Hillemann et al., the GenoType MTBDR was tested directly in 42 smear-positive sputum samples obtaining a concordance of 100 % when compared to conventional drug susceptibility testing (Hillemann 2006). In another more recent study, the GenoType MTBDR was evaluated in 143 smear-positive sputum samples and it was able to correctly identify INH resistance in 48 (84.2 %) of the 57 specimens containing strains with resistance to high level of INH (0.4 µg/mL), and RIF resistance in 25 (96.2 %) of the 26 specimens containing RIF-resistant strains (Somoskovi 2006). There is currently interest in expanding these studies to TB-endemic countries to assess the usefulness of this type of assay for the rapid detection of multidrug resistance in TB ([http://www.finddiagnostics.org/news/press/hain\\_oct06.shtml](http://www.finddiagnostics.org/news/press/hain_oct06.shtml)).

Both solid-phase hybridization methods have proven relatively simple to perform; however, basic expertise in molecular biology and PCR techniques is required. As with other genotypic methods, the sensitivity of the test depends on the amount of DNA present in the sample, and the presence of inhibitors could also cause false-negative results (Palomino 2006).

Another solid-phase reverse hybridization test for rapid detection of RIF resistance is rifologotyping. This is an *in house* low-cost assay for the detection of RIF resistance-associated mutations in the *rpoB* gene of *M. tuberculosis*. The test was developed at the National Institute of Public Health and the Environment (<http://www.rivm.nl/en/>) in the Netherlands and initially evaluated at the Cetrán-golo Hospital in Argentina (Morcillo 2002). It also involves a combination of DNA amplification and reverse-line blot hybridization. DNA of the *rpoB* gene of *M. tuberculosis* is amplified by PCR with specific primers and the PCR products are hybridized to oligonucleotides on a DNA membrane, encoding the wild type *rpoB* sequence, and the most frequent mutations in RIF-resistant strains. Amplified products from RIF-resistant strains will fail to hybridize to one or more of the wild type oligonucleotides, and in most cases, will hybridize to one of the mutant oligonucleotides bound to the membrane. RIF-resistant strains can be detected within a few hours with an enhanced luminescent reaction. In this evaluation, a total of 135 *M. tuberculosis* isolates were tested with the rifologotyping assay and the results compared with the proportion method and the MGIT960 system. The rifologotyping assay correctly identified 90 of the 97 RIF-resistant isolates (sensitivity 92.8 %) while all the RIF-susceptible isolates were also correctly identified.

A minor modification of this assay has also been tested in a multicenter study to detect resistance to RIF, INH, SM and EMB in clinical isolates of *M. tuberculosis* (Mokrousov 2004). Oligonucleotides specific for wild type and mutant alleles of selected codons in the genes *rpoB*, *inhA*, *ahpC*, *rpsL*, *rrs*, *embB*, were immobilized on a nylon membrane. For validation of the test, the membranes were sent to seven laboratories in different geographical locations. The reproducibility for *rpoB* mutation detection was performed on a blinded set of reference DNA samples and overall concordant results were obtained. However, when further mutation analysis was performed on local strains, only 132 (85.2 %) of 155 RIF-resistant and 28 (51.0 %) of 55 EMB-resistant isolates were correctly identified. Resistance to INH was successfully identified in 16.9 % and 13.2 % of strains harboring mutations in the *inhA* and *ahpC* promoter region respectively. Likewise, mutations in *rrs* and *rpsL* conferring resistance to SM were identified in 15.1 % and 10.7 % of SM-resistant strains respectively. Nevertheless, the accuracy of this method for RIF resistance detection has recently been confirmed in another study that used a slightly modified

version of the rifoligotyping assay (Senna 2006). This study evaluated 157 isolates of *M. tuberculosis* and when compared to standard drug susceptibility testing had sensitivity and specificity of 93 % and 100 % respectively. Furthermore, high agreement was also obtained with DNA sequencing.

### **Real-time PCR techniques**

Real-time PCR techniques have also been introduced recently for the rapid detection of drug resistance in TB. Different probes have been used for detection, such as the TaqMan probe, Fluorescence Resonance Energy Transfer probes, molecular beacons and biprobes (Shamputa 2004). The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel. Real-time PCR techniques have been applied to *M. tuberculosis* strains and, more recently, directly to clinical samples (Sajduda 2004, Ruiz 2004, Espasa 2005). Results are generally obtained in an average of 1.5-2.0 hours after DNA extraction. Real-time PCR could eventually be implemented in reference laboratories with the required capacity to properly set up the technique and in settings where it can contribute to the management of TB patients.

### **Microarrays**

Microarrays, also known as biochips or DNA chips, have been proposed as genotypic methods for detecting drug resistance in *M. tuberculosis*. They are based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized on a solid support, such as miniaturized glass slides. They have been tested to detect resistance to INH and RIF (Gryadunov 2005). The recently described CombiChip Mycobacteria Drug-Resistance detection DNA chip is an oligonucleotide microchip coupled with PCR for the detection of resistance to INH and RIF. It was compared with sequencing and drug susceptibility testing in 69 INH- and/or RIF-resistant and 27 drug-susceptible *M. tuberculosis* isolates (Kim 2006). It allowed identification of 84.1 % of INH-resistant isolates, based on the *katG* codon 315 and *inhA15* mutations, and 100 % of RIF-resistant isolates based on seven codons: *rpoB511*, *rpoB513*, *rpoB516*, *rpoB522*, *rpoB526*, *rpoB531*, and *rpoB533*. The overall specificity was 100 % and 95.3 % for detecting INH and RIF resistance respectively. For the time being, and due to the high cost involved, the use of microarrays for detecting drug resistance in *M. tuberculosis* is still beyond the reach of most clinical mycobacteriology laboratories, especially in high-burden countries.

### 19.3.3. New phenotypic methods

The laboriousness and long time required by conventional methods to give results and, on the other hand, the requirement for expensive equipment and the need for skilled technical personnel for most molecular techniques, continue to stimulate the search for alternative and affordable methods for drug resistance detection in TB. The next section will describe several new developments for *M. tuberculosis* that have already been tested both in culture isolates and directly in clinical sputum samples.

#### Phage-based methods

There are currently two formats of phage-based assays that have been described for the rapid detection of drug resistance in *M. tuberculosis*. The first one, also known as phage-amplified biologically, was originally described by Wilson *et al.* in *M. tuberculosis* isolates (Wilson 1997); and the second format is based on reporter mycobacteriophages expressing luciferase (Jacobs 1993).

Phage-based methods that rely on the biological amplification of mycobacteriophages have gained wider application in the last years. They are based on the ability of *M. tuberculosis* to support the growth of an infecting mycobacteriophage. The number of endogenous phages, representing the original number of viable *M. tuberculosis* bacilli, is then determined in a rapidly-growing mycobacterium, such as *M. smegmatis* (McNerney 2001). The *in house* phage amplification test and the commercially available *FastPlaque TB* assay have been tested for the detection of RIF resistance both in *M. tuberculosis* isolates and directly on clinical specimens. In a study performed in 129 isolates from a hot-spot area of MDR-TB, the *in house* mycobacteriophage amplification assay showed 100 % sensitivity, 97.7 % specificity, and 95.2 % predictive value for detecting RIF-resistant *M. tuberculosis*; the test was smoothly integrated into the routine work flow of a low-resource reference laboratory (Simboli 2005). The *FastPlaque TB* has been evaluated in a comparative study with the proportion method on Middlebrook 7H11 agar for determining RIF-resistance directly in smear-positive sputum samples (Albert 2004). The study showed 100 % sensitivity and specificity with results available within two days.



Figure 19-3: *In house* phage amplification method

The luciferase reporter phage method is based on the efficient production of a light signal by viable mycobacteria infected with specific reporter phages expressing the firefly luciferase gene. Light production is dependent on phage infection, expression of the luciferase gene, and the level of cellular ATP (Jacobs 1993). Signals can be detected within minutes after the infection. *M. tuberculosis* isolates susceptible to INH or RIF, result in extinction of light production, while drug-resistant strains continue to produce light. Luciferase reporter tests have now been evaluated against the four first-line antibiotics with an overall agreement of 98.6 % compared with the BACTEC TB-460 system (Banaiee 2003). Furthermore, in a recent study two detection methods, photographic and luminometric, were compared and the sensitivity for detecting INH and RIF resistance was 100% concluding that both methods were appropriate as screening tests for MDR-TB surveillance (Hazbón 2004).

A recent systematic review and meta-analysis summarizes the accuracy of phage-based methods for detecting RIF-resistance in *M. tuberculosis* (Pai 2005). The study concluded that, based on published evidence, phage-based assays performed on *M. tuberculosis* isolates appear to have high sensitivity, but variable and slightly lower specificity. Not enough evidence is available on the accuracy of these assays when performed directly on sputum samples.

### Colorimetric methods

Several colorimetric methods have been proposed in the last few years for the rapid detection of drug resistance in *M. tuberculosis*. They use redox indicators or tetrazolium salts to detect mycobacterial growth. The tests are based on the reduction of

the colored redox indicator added to the culture medium after *M. tuberculosis* has been exposed *in vitro* to different antibiotics. Resistance is detected by a change in color of the indicator, which is directly proportional to the number of viable mycobacteria in the medium (Palomino 2004).

Alamar blue (Trek Diagnostics, Ohio, USA) is a proprietary reagent that was the first to be used to detect drug resistance in *M. tuberculosis*. The reagent is blue in the oxidized state but changes to pink when reduced. In a study that evaluated the activity of INH, RIF, EMB, and SM on clinical isolates of *M. tuberculosis*, MICs were obtained after 1-2 weeks of incubation with an overall accuracy of 97 %, compared to the agar proportion method (Yajko 1995). Alamar blue has been tested in several other studies to detect drug resistance in *M. tuberculosis* and to assess the activity of antimycobacterial drugs using a microplate format (Collins 1997, Franzblau 1998, Palomino 1999). In all these studies, Alamar blue has performed very well, especially for the detection of resistance to INH and RIF.

The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT is a yellow compound that, when reduced by metabolically active cells, it produces crystals of insoluble purple MTT formazan that can be measured with a spectrophotometer after solubilization (Mosmann 1983). MTT has also been proposed in a colorimetric assay for the rapid detection of resistance to RIF (Mshana 1998, Abate 1998). The test, performed on 92 clinical isolates of *M. tuberculosis*, matched the results obtained with the BACTEC radiometric method used as the gold standard. More recently, the MTT test has also been applied in the detection of resistance to other anti-tuberculosis drugs with good results (Foongladda 2002, Caviedes 2002, Morcillo 2004). With the purpose of speeding up the detection of drug resistance in clinical samples, MTT has also been applied as a direct assay in sputum samples for RIF-resistance detection. The sensitivity and specificity of this direct MTT assay matched those of the standard indirect drug susceptibility testing on 7H10 agar with 98.5 % of the samples giving interpretable results within two weeks (Abate 2004).

As a result of studies identifying resazurin as the main component of the Alamar blue reagent (O'Brien 2000), this redox indicator was also introduced in a rapid test to detect drug resistance in *M. tuberculosis* (Palomino 2002). The resazurin micro-titer assay (REMA) allowed rapid detection of multidrug resistance in *M. tuberculosis* isolates with an overall accuracy of 97 % as compared to the proportion method in Löwenstein-Jensen medium. The REMA also showed its usefulness for the detection of resistance to other anti-tuberculosis drugs, including common second-line drugs, quinolones and pyrazinamide (Martin 2003, Lemus 2004, Martin 2005a, Martin 2006).

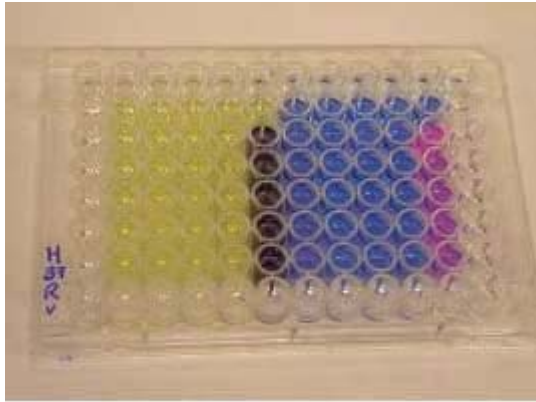


Fig.19-4: Microplate showing MTT and REMA test

In a multicenter study to assess the performance of the REMA and MTT assays in different settings, the resistance of *M. tuberculosis* coded strains to INH, RIF, EMB and SM was determined by REMA, MTT and the proportion method. Excellent results were reported for RIF, INH and EMB, with levels of specificity and sensitivity between 96 % and 99 % (Martin 2005b). Furthermore, a recent systematic review and meta-analysis of colorimetric redox indicator methods to detect multi-drug resistance in *M. tuberculosis* found evidence of a high sensitivity and specificity for the rapid detection of MDR-TB (Martin 2007). Colorimetric methods represent a good alternative for the rapid detection of drug resistance in laboratories with limited resources.

#### **The nitrate reductase assay**

The nitrate reductase assay (NRA) is a quite simple technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium. *M. tuberculosis* is cultivated on Löwenstein-Jensen medium in the presence of an antibiotic and its ability to reduce nitrate is measured after 10 days of incubation. Resistant strains will reduce the nitrate, which is revealed by a pink-red color in the medium, while susceptible strains will lose this capacity as they are inhibited by the antibiotic (Ängeby 2002). The assay has been evaluated in several studies for first-line drugs and ofloxacin with good results (Montoro 2005, Martin 2005a). It has the added advantage of using the same format and culture medium as the standard proportion method. In a recent

multicenter study that evaluated the performance of the NRA for detecting resistance to the first-line drugs, the test performed very well for INH, RIF and EMB with an accuracy of 96.6 % to 98 %. Lower values, were obtained for SM (Martin 2005a). However, the NRA was easily implemented in settings with limited laboratory facilities. Two recent studies applied the NRA directly on sputum samples and produced variable results for sensitivity and specificity; the best results were obtained for INH and RIF resistance detection (Musa 2005, Solís 2005). These two studies have shown the feasibility for implementation of the NRA as a direct test on sputum samples that warrant further evaluations in target populations.



Fig. 19-5: The nitrate reductase assay showing a susceptible and a resistant strain. GC= growth control

### The microscopic observation broth-drug susceptibility assay

As already introduced in Chapter 14, the microscopic observation broth-drug susceptibility assay (MODS) has been described for the early detection of growth and rapid drug susceptibility testing method for *M. tuberculosis*. It is based on the observation of the characteristic cord formation of *M. tuberculosis* that is visualized microscopically in liquid medium with the use of an inverted microscope (Caviedes 2000). In this study, TB-positive sputum samples were tested for susceptibility to INH and RIF by MODS. The results compared to those obtained with the colorimetric method using Alamar blue. They obtained 89 % concordance between the two methods with results available in an average of 9.5 days. The method has been proposed as a rapid, inexpensive, sensitive, and specific method for *M. tuberculosis* drug susceptibility testing, appropriate for use in developing countries.

In a recent operational study performed in Peru, the performance of the MODS assay was compared to an automated mycobacterial culture system, and the method of proportion on Löwenstein-Jensen for the direct detection of resistance to INH, RIF, EMB, and SM in sputum samples (Moore 2006). The median time for results was 7, 22, and 68 days for MODS, automated mycobacterial culture, and method of proportion respectively. The agreement between MODS and the reference standard for drug susceptibility testing was 97 % for INH, 100 % for RIF, and 99 % for INH and RIF combined (MDR). Lower values of agreement were obtained for EMB (95 %) and SM (92 %). They concluded that a single MODS culture of sputum provided a more rapid and sensitive detection of MDR-TB. One minor disadvantage of MODS is the requirement for an inverted microscope for observation of the mycobacterial growth.

### The thin-layer agar method

The Thin Layer 7H11 agar (TL7H11) method or microcolony method, already described in Chapter 14, has also been adapted for the rapid detection of multidrug resistance directly from sputum samples. The TL7H11/INH/RIF has been shown in preliminary studies to be accurate for the detection of MDR-TB as compared to the reference proportion method, with results available in one week (Robledo 2006). Further evaluation studies are expected in target populations to assess the performance of this method in different settings.

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