# Evolution of Phenotypic and Molecular Drug Susceptibility Testing

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**Abstract**

Drug Resistant Tuberculosis (DRTB) is an emerging problem world-wide. In order to control the disease and decrease the number of cases overtime a prompt diagnosis followed by an appropriate treatment should be provided to patients. Phenotypic DST based on liquid automated culture has greatly reduced the time needed to generate reliable data but has the drawback to be expensive and prone to contamination in the absence of appropriate infrastructures. In the past 10 years molecular biology tools have been developed. Those tools target the main mutations responsible for DRTB and are now globally accessible in term of cost and infrastructures needed for the implementation. The dissemination of the Xpert MTB/rif has radically increased the capacity to perform the detection of rifampicin resistant TB cases. One of the main challenges for the large scale implementation of molecular based tests is the emergence of conflicting results between phenotypic and genotypic tests. This mines the confidence of clinicians in the molecular tests and delays the initiation of an appropriate treatment. A new technique is revolutionizing the genotypic approach to DST: the WGS by Next-Generation Sequencing technologies. This methodology promises to become the solution for a rapid access to universal DST, able indeed to overcome the limitations of the current phenotypic and genotypic assays. Today the use of the generated information is still challenging in decentralized facilities due to the lack of automation for sample processing and standardization in the analysis.

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S. Gagneux (ed.), *Strain Variation in the* Mycobacterium tuberculosis *Complex: Its Role in Biology, Epidemiology and Control*, Advances in Experimental Medicine and Biology 1019,
DOI 10.1007/978-3-319-64371-7_12

The growing knowledge of the molecular mechanisms at the basis of drug resistance and the introduction of high-performing user-friendly tools at peripheral level should allow the very much needed accurate diagnosis of DRTB in the near future.

**Keywords**
Molecular drug susceptibility test • Phenotypic drug susceptibility test

## 12.1 Introduction

Proper managing of tuberculosis requires starting an effective antitubercular therapy as soon as possible to prevent spreading of the disease and to increase the cure rate of the affected individuals (Uys et al. 2009; Dowdy et al. 2008). Appropriate therapy can only be provided if the drug susceptibility pattern of the infecting strain is known. Drug susceptibility testing (DST) for *M. tuberculosis* can be performed by conventional phenotypic methods or by molecular detection of genetic determinants associated with drug resistance. The two approaches have advantages and disadvantages and in the most difficult cases a combination of the two may be required. Conventional DST based on mycobacterial growth on both solid and liquid media is time-consuming, and challenged by technical difficulties and biosafety issues (Kim 2005; WHO 2012a, b; Jiang et al. 2013; Somoskovi and Salfinger 2015). The development of molecular technologies has led to the emergence of rapid diagnostic assays suitable for the detection of drug-resistant tuberculosis. Despite these advancements in technology and the large amount of data that are going to be collected by Whole Genome Sequencing (WGS) of drug resistant and drug sensitive *M. tuberculosis* strains, we cannot abandon completely phenotypic DST at this time. Achieving a more comprehensive understanding of the genotype-phenotype-clinical outcome associations could lead to a future when molecular DST will become the routine and phenotypic will be restricted as a referral test for few cases.

We can predict that deeper knowledge will be available in the near future allowing designing a full molecular DST for routine testing. Phenotypic tests and Minimal Inhibitory Concentration (MIC) will be reserved for the most challenging cases.

## 12.2 Phenotypic DST

Streptomycin, the first antituberculosis drug was first experimented in 1944 (Jones et al. 1944; Emmart 1945; Smith and Waskman 1947). Shortly after its introduction in clinical practice, the first cases of resistance were reported (Youmans et al. 1946). Not substantially different was the history of all other antimycobacterial agents; resistance was rapidly emerging in particular when drugs were used inappropriately or in monotherapy (Guernsey and Alexander 1978; Smith et al. 2013). It became clear that a combination of several effective drugs was essential to achieve cured in patients with tuberculosis. The need to develop a laboratory test able to predict antibacterial sensitivity to a specific drug soon emerged and in the 1960s the pioneering experiments of Canetti, at the Pasteur Institute of Paris, lead to the development of the "Proportion method" (Canetti et al. 1963). It is based on the empiric observation that when the proportion of resistant mutants within the *M. tuberculosis* population infecting the patient is approximately $\geq 1\%$, the probability of treatment failure is very high. The proportion method uses a set of media, each containing the "critical" concentration of a single drug, to test the growth of the strain in comparison with that obtained on a drug-free medium (the growth control). The susceptibility to single antimicrobials is inferred by determining the percentage between the counts of colonies grown on

the medium with the drug and on the control; whenever this proportion is ≥1% the strain is classified as resistant. Several years earlier the same researcher had developed the "Absolute Concentration" method (Canetti et al. 1969), a kind of MIC determination with multiple drug concentrations; in this case each laboratory was requested to define its critical concentration. The Resistance Ratio method relies, for each determination, on a parallel testing of the susceptible reference strain *M. tuberculosis* H37Rv (Kent and Kubica 1985). The results are interpreted on the basis of the ratio between the MICs of reference and test strains. The proportion method, thanks to its easy implementation and interpretation, rapidly prevailed and its principle is still at the basis of modern phenotypic susceptibility testing for *M. tuberculosis.* A feature shared by all methods on solid media is the long incubation time which, added to the time for culture, make results available for clinical use in not less than 2–3 months.

In the last 40 years the liquid media, suitable to shorten the incubation time, have progressively replaced the classical solid media for the culture of *M. tuberculosis*.

At present the commercial Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson) has monopolized the market worldwide. It is an automated system that infers the bacterial growth rate from the oxygen consumption. When used for the determination of antimicrobial susceptibility a set of tubes of liquid medium, added with critical concentrations of drugs, is inoculated with a standardized suspension of the strain to be tested; while the control tube is inoculated with the standardized suspension above, diluted 1/100. The tubes, with and without drug, are monitored in parallel and the software, upon quality validation, reports susceptibility or resistance on the basis of the comparison of respective growth curves (Rüsch-Gerdes et al. 1999; Tortoli et al. 2002).

Although the MGIT is widespread, it is not the only method available. The classical proportion methods on egg-based solid media, along with its variant on agar media (Middlebrook 7H10 or Middlebrook 7H11), are still considered the reference for several antibiotics.

Micro-dilution methods in liquid medium have also been developed which, combining inexpensiveness and ease reading, are especially suited for low-income countries. In the Microscopic Observation Drugs Susceptibility (MODS) assay the drug resistance is detected by the low magnification observation of the growth in liquid medium dispensed in microwells and added with drugs. The reading is made easy by the characteristic corded morphology of *M. tuberculosis* colonies (Moore et al. 2004). In another microtiter assay, the addition of a redox indicator is used to detect the bacterial growth (TeMA, MABA, ReMA, according the indicator used: tetrazolium, alamar blue, resazurin). The color change due to indicator reduction is consistent with bacterial growth and indicates resistance to the antimicrobial present in the micro-well (Collins and Franzblau 1997).

Differently from MODS and other microtitre assays, which are home-made, a microdilution method based on microtitre plates containing twofold concentrations of freeze-dried drugs has been recently commercialized. This method, still under validation, combines a number of potential benefits: the inclusion in a single test of both first- and second-line drugs, the possibility of MIC determination, it is user-friendly and relatively inexpensive (Hall et al. 2012).

First-line drugs (isoniazid, rifampicin, pyrazinamide and rifampicin) are normally tested with the phenotypic approach; in case of simultaneous isoniazid- and rifampicin-resistance the test must be widened to second line molecules, at least to fluoroquinolones and injectables (amikacin, kanamycin and capreomycin).

In general, the phenotypic susceptibility testing produces reliable results, in particular for the two major antitubercular drugs, rifampicin and isoniazid. For ethambutol, a bacteriostatic drug, the results are less reliable (Madison et al. 2002) and DST for this drug is not considered a priority by the World Health Organization (WHO). Pyrazinamide testing has been reported as highly challenging by several laboratories, and results may not be fully reliable (Piersimoni et al. 2013).

Pyrazinamide is a prodrug and needs a low pH for activation, a condition that is difficult to control in an "in vitro" test (Table 12.1).

Critical Concentrations (CC) for the major antitubercular drugs were proposed by WHO in 2008, revised in 2012 and under revision in 2017. Table 12.2 shows the critical concentrations endorsed by WHO in 2012. Drugs used for treatment of rifampicin resistant tuberculosis and MDR tuberculosis are listed according to the new classification published in 2016 in the last WHO manual for drug resistant tuberculosis (http://www.who.int/tb/areas-of-work/drug-resistant tb/MDRTBguidelines2016.pdf). It must be noted that some of the CCs will be revised very soon as reported in the table legend.

CCs should be established at the epidemiological cut-off value (ECOFF) or one dilution higher. For the majority of the drugs, the ECOFF separates wild-type strains expected to be sensitive from those expected to be resistant to a selected drug. If drugs can be used at higher doses without high risk of toxicity, concentration higher than the CC can be tested to predict sensitivity to treatment when high doses of the drug can be used to achieve higher plasmatic concentration. In this case a "clinical breakpoint" can be established; for example, a clinical breakpoint has been recently established for moxifloxacin. For some drugs, CCs cannot be established due to the lack of data. For drugs such cycloserin, imipenem, amoxi/clavulanate *in vitro* testing is still not recommended due to the absence of reliable protocols.

As a general rule, it is advisable to test the drug in use for treatment and to perform susceptibility tests under quality assurance conditions and strictly adhering to the recommended protocols.

Recently, two new drugs, delamanid and bedaquiline, received conditional approval for treatment of MDR-TB cases. Interim CCs were recently discussed and will be reported officially by WHO at the end of 2017. Protocols for susceptibility testing on liquid and solid media have been published (Schena et al. 2016; Torrea et al. 2015).

Interpretation of discrepant results obtained in different high level laboratories, from phenotypic tests performed on different media or between genotype and phenotype has underlined the need of MIC determination for a correct management of difficult cases.

Plates for MIC determination are commercially available but have been only evaluated on a small scale so far (Hall et al. 2012). These microtitre plates allow the MIC determination for several first and second line antimycobacterial agents. Yu et al. (2016) have recently reported an agreement between plates and LJ of 99.2% for rifampicin, ofloxacin, amikacin, kanamycin and cycloserin, 98.4% for isoniazid and PAS and lower than 90% for ethambutol. The use of microtitre plates highly reduces the cost of DST compared to other liquid media but poses several questions on the feasibility in terms of implementation in laboratories located in high burden countries for the level of biosafety requested to handle the plates and the risk of cross-contamination. Automation in plate-reading and a "sealed" layout could improve the use of MIC plates in the future.

Although all the methods in liquid medium have drastically reduced the turnaround time, the cost of maintaining an adequate level of bio containment and the high rates of contaminated culture remain a major limit to implement phenotypic DST in TB high burden settings.

## 12.3 Molecular DST

### 12.3.1 Molecular Basis of Drug Resistance in *M. tuberculosis*

In *M. tuberculosis*, drug resistance is mainly caused by chromosomal mutations and evidences exclude horizontal transfer of genetic material as a source of resistance (Gillespie 2002; Marttila and Soini 2003). In a limited number of cases, mobile genetic elements (such as insertion sequences, *e.g.* IS*6110* see Chap. 3) can contribute to the insurgence of phenotypic drug resistance (Lemaitre et al. 1999).

**Table 12.1** Grouping of antitubercular drug and proposed CC according to WHO 2012

| Drug Groups[a] | Drug | DST method available | DST critical concentration (μg/ml) | | | |
|---|---|---|---|---|---|---|
| | | | Löwenstein-Jensen[b] | Middlebrook 7H10[b] | Middlebrook 7H11[b] | MGIT960 |
| First-line oral anti-TB agents | Ethambutol[c] | Solid, liquid | 2.0 | 5 | 7.5 | 5.0 |
| | Isoniazid | Solid, liquid | 0.2 | 0.2 | 0.2 | 0.1 |
| | Pyrazinamide | Liquid | – | – | – | 100.0 |
| | Rifampicin[d] | Solid, liquid | 40.0 | 1 | 1 | 1.0 |
| Injectable anti-TB agents (B) | Amikacin[e] | Solid, liquid | 30.0 | **4.0** | – | 1.0 |
| | Capreomycin | Solid, liquid | 40.0 | 4.0 | – | 2.5 |
| | Kanamycin | Solid, liquid | 30.0 | 4.0 | – | 2.5 |
| | Streptomycin | Solid, liquid | 4.0 | 2.0 | 2.0 | 1.0 |
| Fluoroquinolones (A) | Gatifloxacin[f] | Solid | **–** | **1.0** | **–** | **–** |
| | Levofloxacin | Solid, liquid | – | 1.0 | **–** | **1.5** |
| | Moxifloxacin[g] | Solid, liquid | **–** | **0.5/2.0** | **–** | **0.5/2.0** |
| | Ofloxacin[h] | Solid, liquid | **4.0** | **2.0** | **2.0** | **1.0** |
| Additional Core second line agents (C) | Clofazimine[i] | Liquid | **–** | **–** | **–** | **–** |
| | Cycloserine/Terizidone[j] | Solid | **30.0** | – | – | – |
| | Ethionamide | Solid, liquid | 40.0 | 5.0 | 10.0 | 5.0 |
| | Linezolid[k] | Liquid | – | **–** | **–** | 1.0 |
| | Prothionamide | Solid, liquid | 40.0 | – | – | 2.5 |

(continued)

**Table 12.1** (continued)

| Drug Groups[a] | Drug | | DST method available | DST critical concentration (μg/ml) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Löwenstein-Jensen[b] | Middlebrook 7H10[b] | Middlebrook 7H11[b] | MGIT960 |
| Add-on agents (D) (not part of the core MDR-TB regimen) | D1 | First-line oral anti-TB agents (Pyrazinamide, Ethambutol, Isoniazid HD) | | | | | |
| | D2 | Bedaquiline[l] | Solid, liquid | – | – | **–** | **–** |
| | | Delamanid[m] | Solid, liquid | – | – | **–** | **–** |
| | D3 | P-aminosalicylic acid | Solid, liquid | 1.0 | 2.0 | 8.0 | 4.0 |
| | | Imepenen-cilastatin | None | – | – | – | – |
| | | Meropenem | None | – | – | – | – |
| | | Amoxicillin/clavulanate | None | – | – | – | – |
| | | (Thioacetazone) | None | – | – | – | – |

In bold the CC that will be updated in 2017

[a]Modified according to WHO Guidelines for the programmatic management of drug-resistant tuberculosis

[b]Indirect proportion method recommended. Other solid media methods (resistance ratio) have not been adequately validated for second-line drugs. Concentrations for the absolute concentration method were not evaluated

[c]Ethambutol 5 ug/ml in MGIT is not equivalent to other methods. Ethambutol testing in 7H11 not equivalent to 7H10

[d]Rifampicin borderline resistance can missed by MGIT and can cause discrepant phenotypic/genotypic results

[e]Amikacin. Critical concentration on 7H10 it is expected to be decreased

[f]Gatifloxacin the drug is not widely available. Few data are available to establish the CC

[g]Moxifloxacin. Two concentrations were proposed in 2012. Testing for moxifloxacin at 2 mg/L underestimate resistance and truncates the distribution of mutant strains. It is anticipated that new recommendations will decrease the CC for moxifloxacin and levofloxacin in order to reflect the distribution of wild type and mutated strains. A clinical breakpoint for moxifloxacin is under discussion

[h]Ofloxacin is not used for therapy . it is suggested to use the fluoroquinolone included in regimens

[i]Clofazimine provisional CC for MGIT will be proposed in 2017

[j]Cycloserine. Critical concentration in LJ will be withdrawn due to concerns of unreliable testing and reproducibility

[k]Linezolid temptative CC, CC on solid media will be proposed in 2017

[l]Bedaquiline CC will be proposed in 2017

[m]Delamanid CC will be proposed in 2017

**Table 12.2** Main genomic regions associated with drug resistance in M. tuberculosis

| Drug | Gene(s) containing drug resistance-conferring mutations |
|---|---|
| Amikacin | *rrs* |
| Bedaquiline | *mmp*L5, *mmp*S5, *Rv0678*, *atp*E, *pep*Q |
| Capreomycin | *rrs*, *tly*A |
| Clofazimine | *Rv0678*, *pep*Q |
| Delamanid | *ddn*, *fgd*1, *fbi*A, *fbi*B and *fbi*C |
| Ethambutol | *emb*A, *emb*B, *emb*C, *ubi*A |
| Ethionamide | *inh*A, *ndh*, *eth*A, *eth*R |
| Fluoroquinolones | *gyr*A, *gyr*B |
| Isoniazid | *kat*G, *inh*A, *ndh*, *fur*A, *kas*A |
| Kanamycin | *rrs*, *gid*B, *eis*, *tap*, *whi*B7 |
| Linezolid | *rpl*C, *rrl* |
| Pyrazinamide | *pnc*A, *rps*A, *pan*D |
| Rifampicin | *rpo*B |
| Streptomycin | *rps*L, *rrs*, *gid*B, *tap*, *whi*B7 |

Drug resistance can be defined according to different criteria. Clinical resistance is based on breakpoints determining the likelihood of therapeutic failure during treatment. Even though this definition is useful for clinical practice, it does not consider low-level resistance mechanisms that increase the MIC without reaching the breakpoint, thus representing an hallmark of a possible evolutionary trend towards high-level (clinically relevant) resistance (Baquero 2001; Martínez et al. 2015). Alternatively, resistance could be referred according to an epidemiological definition: the European Committee on Antimicrobial Susceptibility Testing (EUCAST) established common ECOFFs defined as the MIC value corresponding to the upper limit of the wild-type population of a specific bacterial species (Kronvall 2010). In this case, low-level resistance can be also determined. Table 12.2 summarizes main genomic regions involved in the emergence of drug resistance to preeminent anti-tubercular drugs (see Chap. 14).

Rifampicin is an excellent example for molecular evaluation of phenotypic resistance: more than 95% of the mutations causing drug resistance are mapping in a short (81 nucleotides in length) "hot-spot" region in the *rpo*B gene (Ramaswamy and Musser 1998). Despite the variability of mutations found, four mutations (D516V, H526Y, H526D, and S531L – refer to Andre 2016 for corresponding *M. tuberculosis* codon numbering system (Andre et al. 2016)) account for more than 70% of resistant clinical isolates. Different mutations can also be associated to different fitness (Comas et al. 2011; Brandis et al. 2012; de Vos et al. 2013). Due to the lower rate of spontaneous mutations, rifampicin resistance alone is rarely observed, and the majority of rifampicin-resistant isolates have already accumulated mutations conferring resistance to isoniazid (Coker 2004). This observation initially leads policy-makers to consider rifampicin-resistance as a marker for MDR tuberculosis. However, further data showed that rifampicin resistance is not a good surrogate marker at the global level for MDR-TB and its reliability is strictly dependent on the rate of primary resistance to rifampicin in the setting considered (WHO 2004).

Isoniazid resistance is caused by mutations affecting different genomic regions: approximately 60% and 20% of resistant cases are caused respectively by mutations affecting the codon 315 in the *kat*G gene and the position −15 in the *inh*A gene promoter region (Seifert et al. 2015). Different mutations in these regions slightly increase the percentage of isolates with at least one reported mutation. The mutation frequency was

also found to be different according to the geographical distribution (Seifert et al. 2015). Other genomic regions involved in isoniazid resistance are in the *ndh*, *fur*A and *kas*A genes, however their contribution is usually below 1% (Banerjee et al. 1994; Kelley et al. 1997; Sreevatsan et al. 1997; Banerjee et al. 1998; Slayden and Barry 2000; Lee et al. 2001; van Doorn et al. 2003; Vilchèze et al. 2005). Recently, another potential target of the drug has been proposed: the MymA protein, a flavin-containing monooxygenase was found to be inhibited by isoniazid in modeling and biochemical analyses (Saraav et al. 2017). Further studies will help in understanding any role of this putative target in the development of isoniazid resistance. Other studies focusing on bacterial persistence underlined the complexity of the interaction between antibiotics and bacteria. In particular, studies on isoniazid showed that single-cell dynamics of the isoniazid-activating enzyme catalase-peroxidase (KatG) are driving the success of the drug; thus, persistence to isoniazid was likely due to reversible phenotypic tolerance rather than stable genetic mutations (Wakamoto et al. 2013). Several mechanisms of bacterial persistence have been described, and many were also found in *M. tuberculosis* (Nathan 2012; Dartois et al. 2016; Harms et al. 2016).

Ethambutol resistance is caused by mutations affecting the *emb*CAB operon, and the *emb*R gene encoding for its regulator (Telenti et al. 1997). Most frequent mutations are observed at codon 306 of the *emb*B gene (approximately 70% of resistant cases) (Zhang and Yew 2015). Recently, mutations affecting the *ubi*A gene have also been associated with ethambutol resistance (Safi et al. 2013; He et al. 2015). Researchers started to characterize the frequency of mutations at this locus in clinical isolates. However, they seems associated to particular geographical regions (Xu et al. 2015; Lingaraju et al. 2016).

Pyrazinamide resistance is caused by mutations affecting the *pnc*A gene encoding the pyrazinamidase enzyme required to convert pyrazinamide to its active form pyrazinoic acid (Zhang and Mitchison 2003). Resistance-conferring mutations are spread along the entire gene, including the promoter region, and a clear hot-spot region cannot be identified. In addition, more than 600 different mutations (including indels) have been described in the literature, making the detection of individual mutations or a restricted subset of them useless for diagnostic purposes (Miotto et al. 2014; Ramirez-Busby and Valafar 2015; Whitfield et al. 2015). In some cases, insertion of the IS*6110* mobile genetic element in the gene has been also reported (Gillespie 2002). According to the most recent systematic review available, mutations in the *pnc*A locus are responsible of 83% of pyrazinamide resistant cases (Ramirez-Busby and Valafar 2015). Mutations affecting two novel genetic loci have been associated with the emergence of pyrazinamide resistance: evidences suggest that *rps*A and *pan*D genes are involved in the mechanism of action of the drug (Yang et al. 2015; Pandey et al. 2016), however only a relatively small number of clinical isolates have been characterized for these genomic regions, and the clinical relevance of these targets remains questionable. Pyrazinamide monoresistance is an extremely rare phenotype (with the exception of *M. bovis* and *M. canettii*) and any phenotypic resistance in the absence of *pncA* mutations should be interpreted with extreme caution.

Streptomycin resistance is caused by mutations in the *rps*L gene, with codons 43 and 88 as the most affected ones. Mutations in this genetic locus account for approximately 50% of streptomycin-resistant clinical isolates. An additional 10% of resistant strains harbor mutations in regions 530 and 912 of the *rrs* gene (Sreevatsan et al. 1996). Other genes described as involved in streptomycin resistance are *gid*B, *tap* and *whi*B7 genes (Okamoto et al. 2007; Wong et al. 2011; Reeves et al. 2013). Mutations in the *rrs* gene but at different region (region 1400) are also associated with resistance to amikacin, kanamycin and capreomycin (Alangaden et al. 1998; Maus et al. 2005). In addition, mutations in the promoter region of *eis* gene and in the *tly*A gene are responsible of kanamycin and capreomycin resistance, respectively (Johansen et al. 2006; Zaunbrecher et al. 2009). According to Georghiou et al., the *rrs* 1401 mutation alone is found in 70–80% of amikacin and capreomycin

resistant isolates, and in 60% of strains resistant to kanamycin (Georghiou et al. 2012). The contribution of mutations in the promoter region of the *eis* gene to kanamycin resistance is variable across different geographical regions, however in some settings it can rise up to more than 60% of resistant cases (Hoshide et al. 2014). The contribution of mutations in *tly*A gene in determining capreomycin resistance remains low to moderate (5–10%) (Campbell et al. 2011; Georghiou et al. 2012).

Similarly to the other anti-tubercular drugs, resistance to fluoroquinolones is promoted by sub-optimal bacterial-drug exposure (Miotto et al. 2015). Main drug resistance-associated mutations are found in the quinolone resistance determining regions (QRDRs) of the *gyr*A and *gyr*B genes. In particular, 80% of resistant cases harbor mutations in the QRDR of the *gyr*A gene, whereas mutations in the *gyr*B gene contribute for less than 1% of resistant cases (Avalos et al. 2015). Several studies showed that the levels of resistance to the different fluoroquinolones (namely levofloxacin, ciprofloxacin, ofloxacin, moxifloxacin, gatifloxacin) are associated with the type of mutation present in the *gyr*A and *gyr*B genes, however MIC values for isolates with the same mutation vary widely (Kam et al. 2006; Nosova et al. 2013; Kambli et al. 2015; Chien et al. 2016; Farhat et al. 2016a, b). Concordance in resistance testing among fluoroquinolones was found to be low (Farhat et al. 2015; Coeck et al. 2016). These observations lead to consider the possibility to use later-generation fluoroquinolones (moxifloxacin, gatifloxacin) to treat cases resistant to earlier generation (levofloxacin, ciprofloxacin, ofloxacin) fluoroquinolones (resistance detected by phenotypic tests using CC published in 2012). According to Farhat et al. (2015), nearly 30% of strains had moxifloxacin MICs in the intermediate range, likely below the peak serum concentrations of the drug, and thus clinically treatable with standard doses of moxifloxacin (despite distribution of drugs in the granulomas could not reflect the serum level of the antibiotic, as suggested by Dartois and Barry 2013). Similarly, a previous study described ofloxacin-resistant cases showing improved treatment outcomes when treated with moxifloxacin (Jo et al. 2014). Van Deun et al. suggested that specific *gyr*A gene mutations can be used to predict poor treatment outcome in MDR tuberculosis, and in particular mutations other than Ala substitution at codon 94 are associated with high-level resistance to gatifloxacin and moxifloxacin (Rigouts et al. 2016).

Data for drugs recently introduced in the treatment of MDR and XDR tuberculosis such as linezolid, delamanid, bedaquiline and clofazimine are scarce, both in terms of genetic loci involved and prevalence (Bloemberg 2015; Xu 2017). Further work is needed to fill present knowledge gaps. Resistance to linezolid is mainly caused by mutations in *rpl*C and *rrl* genes (Hillemann et al. 2008; Beckert et al. 2012; Makafe et al. 2016), whereas resistance to delamanid is mediated by mutations affecting *ddn*, *fgd*1, *fbi*A, *fbi*B and *fbi*C genes (Stover et al. 2000; Choi et al. 2001; Matsumoto et al. 2006; Manjunatha et al. 2006; Feuerriegel et al. 2011). Resistance to delamanid has been observed in strains never exposed to the drug and presenting mutations in *ddn* (Schena et al. 2016). Mutations affecting *mmp*L5, *mmp*S5, *pep*Q, *Rv0678*, *atp*E genes cause resistant to bedaquiline (Andries et al. 2005; Huitric et al. 2010; Andries et al. 2014; Hartkoorn et al. 2014; Almeida et al. 2016; Segala 2012). Mutations in *Rv0678* and *pep*Q were found to confer cross-resistance between bedaquiline and clofazimine, an antileprosy drug recently gaining attention for treating MDR tuberculosis (Hartkoorn et al. 2014; Almeida et al. 2016). In particular, mutations in Tv0678 were linked to the upregulation of *mmp*S5 and *mmp*L5 genes. Resistance mediated by *pep*Q mutation seems to be associated with increased drug efflux, but this is not due to upregulation of *mmp*L5 and *mmp*S5 expression as in the case of resistance mediated by mutations in the *Rv0678* gene.

The frequency of mutations in these genomic regions remain to be determined in clinical isolates and only limited data on small number of samples are available.

The mycobacterial cell wall permeability barrier and active multidrug efflux pumps represent

a relevant role in the development of phenotypic drug resistance in *M. tuberculosis* (De Rossi et al. 2006; Escribano et al. 2007; Louw et al. 2009; da Silva et al. 2011). Studying these specific aspects of the biology of *M. tuberculosis* requires more sophisticated experiments and the identification of mutations related to these mechanisms is more complex; therefore, there are very limited data available on clinical isolates covering these mechanisms of resistance (see Chap. 14).

For drugs sharing the same molecular target cross-resistance is commonly observed. This phenomenon has been reported for fluoroquinolones, second-line injectable drugs, but also for isoniazid and ethionamide. However, it should be noted that certain mutations confer different level and pattern of resistance among the different drugs. Table 12.3 summarizes some of the main information available on cross-resistance and its molecular bases, together with main references.

### 12.3.2 Molecular Identification of the Drug Susceptibility Profile of *M. tuberculosis*

The bottleneck in the molecular detection of drug resistance in *M. tuberculosis* is the limited knowledge of the relevant mutations responsible of the resistant phenotype (Zhang and Yew 2015). There are several genotypic approaches to detect known mutations causing drug resistance; the use of the polymerase chain reaction (PCR) in the diagnosis of tuberculosis was introduced in 1990 (Patel et al. 1990) and few years later, starting on 1993 (Telenti et al. 1993), PCR-based assays have been developed for the detection of drug resistance in *M. tuberculosis* (for reviews on early developed methods please refer to Caws 2001; García de Viedma 2003). Together with undeniable advantages in terms of both ease of use and reduced time-to-results, the introduction of molecular assays for direct drug susceptibility profiling of *M. tuberculosis* in specimens also reduced biosafety risks associated with the manipulation of live pathogens, especially in resource-limited settings (WHO 2008; Parsons et al. 2011; WHO 2013; Somoskovi and Salfinger 2015).

Although molecular techniques can detect a single bacillus in a specimen (at least in theory), sensitivity can be hampered by the presence of inhibitors in clinical specimens and loss of nucleic acids during specimen processing. In addition, the need to detect mutations affecting multiple genes (*e.g.* those required to identify delamanid or bedaquiline resistances) and/or the multiplicity of mutations on a single target (*e.g.* mutations in *pnc*A gene associated with pyrazinamide resis-

**Table 12.3** Genes involved in cross resistance to anti-TB drugs

| Cross-resistances | Genomic region | Example mutations | References |
|---|---|---|---|
| Amikacin, kanamycin, capreomycin | *rrs* | a1401g, a1484t | Blumberg et al. (2003), Maus et al. (2005), and Georghiou et al. (2012) |
| Bedaquiline, clofazimine | *Rv0678* | S63R, R134Stop | Hartkoorn et al. 2014 |
| | *pep*Q | A14ins c, R271del c, L44P | Almeida et al. (2016) |
| Ciprofloxacin, levofloxacin, ofloxacin, moxifloxacin | *gyr*A | G88C, A90V, S91P, D94N, D94G, D94Y N538D, E540V, | Malik et al. (2012), Nosova et al. (2013), Imperiale et al. (2014), and Willby et al. (2015) |
| | *gyr*B | R485C + T539N | |
| Levofloxacin, ofloxacin | *gyr*B | D500H, D500N | Malik et al. (2012) |
| Isoniazid, ethionamide | *inh*A | c-15t | Imperiale et al. (2014) and Rueda et al. (2015) |
| Rifampicin, rifabutin | *rpo*B | Q513E, Q513K, Q513L, Q513P, S531L, S531F, S531W, H526D, H526Y, H526R, D516A + R529Q | Bodmer et al. (1995), Sintchenko et al. (1999), Goldstein (2014), Jamieson et al. (2014), Imperiale et al. (2014), and Berrada et al. (2016) |

tance) considerably limit the choice to the techniques with a sufficient capacity for multiplexing.

Starting in 2008, molecular tools detecting rifampicin resistance-associated mutations have been formally endorsed by the World Health Organization, as they represent cost-effective rapid diagnostics for fast detection of resistant cases. In particular, based on evidence and expert opinion, the WHO endorsed the use of molecular line probe assays (LiPAs), and the Xpert MTB/RIF assay (Cepheid) for the rapid detection of MDR tuberculosis cases (WHO 2008, 2013; Gilpin et al. 2016).

The commercially available LiPAs are based on targeted amplification of specific regions of the *M. tuberculosis* genome followed by hybridization of the amplicons to oligo probes immobilized on nitrocellulose strips. The INNO-LiPA Rif.TB (Innogenetics) has been designed to detect rifampicin resistance alone by targeting the hot-spot region of the *rpo*B gene with five wild-type probes and four probes specific for most frequent rifampicin resistance-associated mutations (D516V, H526Y, H526D, and S531L). Reported pooled sensitivity and specificity are 97% (95% CI 95.0–98.0) and 99% (95% CI 98.0–100.0), respectively (Morgan et al. 2005). The Genotype MTBDR*plus* assay by Hain Lifescience detects both rifampicin and isoniazid resistance. The test targets the hot-spot region of the *rpo*B gene with eight wild-type probes and four probes specific for most common rifampicin resistance-associated mutations (D516V, H526Y, H526D, and S531L). The detection of isoniazid resistance is enabled by one wild-type probe plus two mutated probes for the *kat*G gene (S315T, nucleotidic substitutions agc/acc and agc/aca) and two wild-type probes plus four mutated probes for the promoter region of the *inh*A gene (c-15t, a-16g, t-8c, and t-8a). The pooled sensitivity for the detection of rifampicin resistance was reported 98.1% (95% CI 95.9–99.1), with a specificity of 98.7% (95% CI 97.3–99.4). Results for isoniazid showed lower sensitivity (84.3%, 95% CI 76.6–89.8) but high specificity (99.5%, 95% CI 97.5–99.9) (Ling et al. 2008). More recently a non-inferiority study of the new version of the GenoType MTBDR*plus* assay (version 2) and a newly developed LiPA assay named Nipro NTM + MTBDRTB assay (Nipro Corporation) was published. Both tests have been designed to detect rifampicin and isoniazid resistance. The GenoType MTBDR*plus* assay ver. 2 targets the regions already described for the MTBDR*plus* ver.1 assay. Similarly, the Nipro assay targets the hot-spot region of the *rpo*B gene for rifampicin resistance (5 wild-type probes plus four mutated probes targeting D516V, H526Y, H526D, and S531L substitutions), whereas for isoniazid resistance the assay targets four wild-type probes plus two mutated probes for the *kat*G gene (S315T, and S315N) and one wild-type probe plus four mutated probes for the promoter region of the *inh*A gene (c-15t, a-16g, t-8c, and t-8a). Non-inferiority of MTBDR*plus* ver.2 and Nipro assays to MTBDR*plus* ver.1 was demonstrated for rifampicin and isoniazid resistance detection (Nathavitharana et al. 2016).

The Genotype MTBDR*sl* (Hain Lifescience) is the only molecular test designed for the detection of resistance to second-line drugs. The assay targets the QRDR of the *gyr*A gene (three wild-type probes plus six mutated probes targeting mutations A90V, S91P, D94A, D94N/Y, D94G, and D94H) and the region 1400 of the *rrs* gene (two wild-type probes plus two mutated probes targeting a1401g, and g1484t). In addition, the assay targets the codon 306 of the *emb*B gene for ethambutol resistance. The pooled sensitivity for detecting fluoroquinolone resistance on isolates was 83.1% (95% C.I. 78.7–86.7) and the pooled specificity was 97.7% (95% C.I. 94.3–99.1), respectively. Similar performances were found for direct testing in clinical specimens. Performance on second-line injectable drugs resistance (amikacin, kanamycin, and capreomycin) showed a pooled sensitivity of 76.9% (95% C.I. 61.1–87.6) and a pooled specificity of 99.5% (95% C.I. 97.1–99.9), respectively (Theron et al. 2014). The new version of the MTBDR*sl* assay (version 2) in addition to *gyr*A and *rrs* genes targets also the QRDR of the *gyr*B gene for fluoroquinolone resistance (one wild-type probe plus two mutated probes targeting N538D, and E540V), and the promoter region of the *eis* gene for kanamycin resistance (three wild-type probes

plus one mutated probe targeting c-14t); the target region for ethambutol has been removed. The new version of the assay showed improved performances (Tagliani et al. 2015; Brossier et al. 2016), and very recently, the WHO also provided recommendations for the use of the MTBDR*sl* for the detection of resistance to second-line antituberculosis drugs (WHO 2016).

Despite the good performances of the LiPAs on clinical isolates and smear-positive clinical specimens, it should be noted that the WHO does not recommend the use of these assays for smear-negative samples. MTBDR*sl* assay (version 2) is recommended to triage patients for the short MDR regimen. Although not perfect, this assay can identify patients that are resistant to all second line injectable drugs (presenting the a1401g mutation in *rrs*) from those that may still respond to amikacin (position 1402, *rrs* gene). Patients presenting mutations in the promoter region of *eis* could also still be treatable with capreomycin.

At present guidelines for interpreting the genotype to predict drug responses are under preparation and more data are collected to support the interpretation of mutation pattern for clinical management of DR tuberculosis.

The Cepheid Gene Xpert MTB/RIF system is a fully automated real time PCR-based assay for the detection of *M. tuberculosis* DNA and mutations associated with rifampicin resistance, directly in clinical specimens (Boehme et al. 2010). The assay uses semi-nested PCR to amplify the hot-spot region of the *rpo*B gene (RRDR) resistance is detected by five molecular beacons targeting both wild-type sequences and the most common mutated codons. For the detection of rifampicin resistance, the pooled sensitivity and specificity were 95% and 98%, respectively (Steingart et al. 2014). Due to the high sensitivity of the assay, the Xpert MTB/RIF is also recommended for smear-negative specimens, and extra-pulmonary samples (WHO 2013; Denkinger et al. 2014). Despite these recommendations the sensitivity on paucibacillary samples remains suboptimal compared to liquid culture. Additional limitations for this test are the suboptimal negative predictive value (Theron et al. 2014), the capacity to detect heteroresistance to rifampicin (Zetola 2014), low capacity to detect the C533G mutation (Rufai et al. 2014) and occasional rifampicin resistant false positive cases due to delays in the signal generated by the probes D and E (Williamson 2012) or detection of silent mutations such as F514F. A new generation of Xpert assay, named Ultra, was recently developed and evaluated in a non inferiority study including the previous version (https://www.finddx.org/wp-content/uploads/2017/03/Ultra-WHO-report_24MAR2017_FINAL.pdf). The Ultra is an improved version of the previous test (G4) working on the same platform after upgrade of software. The main differences between G4 and Ultra are: the increased volume of the PCR chamber, the target genes for detection of MTB (two multicopy genes IS6110 and IS 1081) and faster reaction kinetics. Rifampicin resistance is detected using the melting temperature curves of RRDR-specific probes. Samples are defined positive according to five semiquantitative categories. The first four (high, medium, low and very low) correspond to the G4 categories while the last one, "trace", is new.

The Ultra cartridges have been endorsed by WHO in April 2017. In the first prospective multicenter study comparing G4 and Ultra, the Ultra showed an increased sensitivity (+17%) in smear negative respiratory samples and HIV coinfected subjects (+ 14%). Specificities of Ultra and G4 for case detection were 95.6% and 98.3%, overall, and 93.5% and 98.4% among patients with a history of tuberculosis. The decreased specificity in patients with history of tuberculosis is mainly in the category of results labeled as "trace". Samples resulting positive as "trace" will not be further analyzed for rifampicin resistance. Large amount of data will be needed to define the diagnostic value of the "trace" category. "Trace" positive subjects could be managed differently based on local TB epidemiology, patient's history and immunological status (Chakravorty et al. 2017).

As already mentioned, the clinical value of molecular assays relies upon our knowledge of the mutations involved in the emergence of drug resistance in *M. tuberculosis*, evidences

suggest that future drug resistance diagnostics will need to be able to detect high numbers of mutations to impact on the management of patients with drug-resistance tuberculosis (Farhat et al. 2016b). However, additional considerations should be taken into account in the evaluation of such tools. First, whereas in settings with a high prevalence of rifampicin resistance and MDR-TB, these tests may be a valuable component of an MDR-TB management strategy, molecular tests for rifampicin resistance alone cannot accurately predict resistance in areas with a low prevalence of rifampicin resistance (Arentz et al. 2013; WHO 2013; Drobniewski et al. 2015). Thus, careful evaluation of the setting should performed prior introduction of molecular assays. Second, although some molecular tools are often easier to be performed compared to phenotypic DST, interpretation challenges may arise. Whereas rare or novel mutations usually do not account for the majority of resistance determination based on the absence of wild-type probe hybridization, continuous evaluation of geographical mutation frequencies might be needed for maximizing the impact of molecular diagnostics (Seifert et al. 2016; Sanchez-Padilla et al. 2015). Similarly, false-positive rifampicin resistance detection or detection of *M. tuberculosis* DNA by Xpert MTB/RIF assay in culture-negative patients can be confusing and detrimental for patient management (Huh et al. 2014; Lippincott et al. 2015). Third, clear data on the relationship between genotype, phenotype and response to treatment are limited. Phylogenetic polymorphisms, mutations associated with hyper-susceptibility and/or different level of resistance, and differences related to the phenotypic testing method used as reference have been described. Distinctive geographical distributions of drug resistance-associated mutations further complicate the clinical interpretation of genetic polymorphisms (Aubry et al. 2006; Rigouts et al. 2013; Feuerriegel et al. 2014; Hoshide et al. 2014; Van Deun et al. 2015; Kambli et al. 2015; Singh et al. 2015; Coeck et al. 2016; Berrada et al. 2016; Kambli et al. 2016).

Target product profiles in terms of minimal requirements, performances and controls for developing new molecular diagnostic assays for drug-resistant tuberculosis and guidelines for their successful evaluation have been developed to guide the development of new assays (Wells et al. 2013; Kik et al. 2014; WHO 2015; Denkinger et al. 2015). Currently, large efforts are devoted to fill the gaps in our understanding of the genotype-phenotype relationships. We can now take advantage from the onset of next generation sequencing (NGS). NGS is making whole genome sequencing (WGS) affordable in the broader field of microbiology (Punina et al. 2015; Gilchrist et al. 2015). Several automated or semi-automated tools for interpreting *M. tuberculosis* drug resistance in WGS data are already available (Steiner et al. 2014; Flandrois et al. 2014; Bradley et al. 2015; Feuerriegel et al. 2015; Coll et al. 2015). Recent studies highlight the need for standardized databases for interpreting genotype-phenotype correlation in clinical contexts (Witney et al. 2015). At this end, a large collaborative project involving academic institutions, public health agencies, and nongovernmental organizations has been established to develop a tuberculosis relational sequencing data platform (ReSeqTB) for improving understanding of the relationships between genotype, phenotype and clinical outcomes (Starks et al. 2015; Schito and Dolinger 2015; https://platform.resqtb.org/). Another consortium named "CRyPTIC" (Comprehensive Resistance Prediction for Tuberculosis, www.crypticproject.org/) aims at developing a sufficient number of sequences to unveil all possible variants leading to drug resistance.

## 12.4 Discrepancies Between Phenotypic and Genotypic Tests

Since the introduction of molecular tests for the diagnosis of drug resistant tuberculosis, several reports showing conflicting results were published. Discrepancies between molecular methods to detect drug resistance (including

**Table 12.4** Main reasons for genotype/phenotype discrepancies

| |
|---|
| Mutation is out side the region targeted by the molecular assay |
| Mutation confers low level resistance and CC is set to high |
| Presence of unknown mechanisms conferring DR |
| Trivial errors in the performance of DST |
| Presence of heteroresistance not detected by molecular methods |
| Molecular assay detect silent mutations |
| Errors due to probe interaction/binding in LPA or other molecular assays |

rifampicin resistance) and traditional phenotypic methods have caused confusion and in many settings have decreased the confidence in molecular tests. This has resulted in delay in starting treatment or inappropriate treatment when priority was given to phenotypic data. These discrepancies could be due to real "false positive" or "false negative" results of the tests used for the determination (both genotypic or phenotypic) or can be linked to more complex reasons. Table 12.4 lists the most frequent reasons for the discrepancies. As already mentioned, phenotypic tests performed on different media may yield conflicting results when the MIC of the strain for the drug tested is close to the critical concentration (Coeck et al. 2016). Technical issues related to the methodology used for DST, quality of media and drugs, experience of the staff can strongly affect the reliability of the test. Testing *M. tuberculosis* sensitivity by phenotypic methods should only be performed in laboratories maintaining a high standard in performance and with a consistent workload.

Additional causes for discrepancies are linked to the use of CCs established at value that are too high and don't represent the true distribution of the wild type and mutant bacterial population. When drugs can be used at higher doses without causing serious side effects, a clinical breakpoint can be established, and in some case it is recommended to test the drug at two concentrations. Phenotypic sensitivity at the higher concentration should not be interpreted as a "true discrepancy" but as useful information for the clinical management of the patient. Moxifloxacin is a candidate drug for testing at two concentrations.

Our knowledge on the mechanisms conferring drug resistance is still limited, and in some case determinants that are causing resistance are not properly investigated. The implementation of whole genome sequencing has highly improved our knowledge of genomic variants causing drug resistance. For some minority variants the association to drug resistance will need to be confirmed by reverse genetic experiments. Some molecular assays are not able to discriminate "silent" from "non-silent" mutations: F514F is the most common silent mutation in rpoB, if detected by molecular assay the sample may be misinterpretated as rifampicin-resistant.

Table 12.5 summarizes some of the most common mutations causing DR and expected phenotype.

## 12.5 Whole Genome Sequencing as Novel Approach to Susceptibility Testing

The whole genome sequencing (WGS) approach offers a powerful alternative for diagnosis of drug-resistant tuberculosis, promising a rapid and accurate determination of all the clinically-relevant mutations (Drobniewski et al. 2015; Witney et al. 2015; Walker et al. 2015). Indeed, using this methodology, clinicians could promptly obtain relevant information on the best therapy to adopt, receiving information on sensitivity to the first-line drugs, as well as to second-line and new agents. In addition, it is now accepted that the emergence of drug resistance is not always caused by point mutations affecting only single genes, but the presence of other mechanisms, such as compensatory mutations,

**Table 12.5** Most common mutations causing DR and the expected phenotype

| Antimicrobial agent | Gene | Common mutations | Less common mutations | Expected phenotype |
|---|---|---|---|---|
| Isoniazid | *katG* | S315T | S315T, S315N, S315I, S315L, additional mutations in *katG* coding region | S315T is the most common mutation conferring resistance to INH, is associated to medium to high level of resistance, is the most represented mutation in MDR strains |
| | *inhA* promoter | −15C/T | −8T/C, −8T/A, −8T/G, −9G/T, −16A/G, −17G/T | Often associated with low-level INH resistance, confers ethionamide resistance. |
| | | | | Some strains may tests sensitive by phenotypic DST |
| | *inhA coding region fabG1* | Different codons may be affected | | Mutations in the coding regions are non detected by LPA commercial assays and/or assays targeting the promoter. Presence of mutations in the coding region in association with mutations in the promoter increases the level of resistance. |
| | *ahpC promoter* | −48C/T | −52G/A, −54G/A, −51C/T, −52G/T, −49A/G, −57G/A | Associated with INH resistance. Few data available |
| Rifampin | *rpoB* | S531L | L511P, L533P, D516Y, H526N, | S531 L is the most frequent mutation identified in MDR TB, associated with resistance to all rifamicins |
| | | H526Y | S522L, H526L, H526A, H526C, D516F, D516V | F514F is the most common silent mutation. It can cause misinterpretation of resistance to rifampicin if not recognised. |
| | | H526D | Q513A, Q513E, H526Y, H526D, H526R, S531W, S531F, S531L, V176F. | L511P, L533P, D516Y, H526N "disputed" mutations. Are associated to rifampicin resistance if tested on solid media, may test sensitive when tested in MGIT. Associated to poor clinical outcome should be interpreted as conferring resistance and rifampicin should not be counted as a fully active drug in the therapeutic regimen. |
| | | | | S522 L, H526L, H526A, H526C, D516F, D516V: those mutations have been associated to rifampicin resistance and rifabutin sensitivity. This is still a disputed issue and more data on mic distribution for rifabutin are needed |
| Ethambutol | *embB* | M306V | M306L, M306I D354A, G406D, etc | M306V is the most frequent mutation associated with R to EMB. |
| | | | | Not all mutations in *embB* are associated with EMB-R. Discrepant phenotypic/genotypic results are expected due to the CC used for in vitro testing |

(continued)

**Table 12.5** (continued)

| Antimicrobial agent | Gene | Common mutations | Less common mutations | Expected phenotype |
|---|---|---|---|---|
| Pyrazinamide | *pncA* | No predominant mutations | | Pyrazinamide monoresistance is associated to *M.bovis* and *M.bovis*/BCG due to the presence of a characteristic mutation in position 57. For all other cases phenotypic resistance to pyrazinamide in the absence of mutations should be interpreted as false resistance. *pncA* mutations are widely distributed throughout the gene and its promoter. |
| | | | | Some mutations such as E37V, D110G, V163A, A170V and V180I, are not associated with PZA-R |
| | *panD* | | I49V, I115T | Few data to support the role of *panD* as main determinant for PZA resistance. It is causing resistance in *M. canetti* |
| Quinolones | *gyrA* | D94G | D94Y, D94H, D94A, D94N, S91P; G88A, mutations outside hot spot | D94G causes resistance to all fluoroquinolones (including moxifloxacin and gatifloxacin) in the presence of this mutation fluoroquinolone treatment is not recommended or if performed should be condered potentially not effective despite results of "in vitro" phenotypic testing |
| | | A90V | | A90V is associated resistance to levofloxacin and to lower level resistance to moxifloxacin and gatifloxacin |
| Amikacin | *rrs* | 1401A/G | 1484G/T | 1401A/G is the most frequent mutation; confers resistance to AMK and all second line injectables |
| | | | | 1484G/T may be associated with R to AMK. |
| | *eis* | | −14C/T | may confer very low level resistance |
| Capreomycin | *rrs* | 1401A/G | 1402C/T, 1484G/T | 1401A/G is the most common mutation; Usually associated with R to CAP. |
| | | | | 1402C/T and 1484G/T mutations may also be associated with R to CAP. |
| | *tlyA* | No predominant mutations | | Mutations are widely distributed throughout the gene. |
| | | | | Not all mutations are associated with R to CAP. |
| | | | | role of the different mutations is still disputed |
| Kanamycin | *rrs* | 1401A/G, | 1402C/T, 1484G/T | 1401A/G is the most common mutation and associated with high level R to KAN. |
| | | | | 1402C/T may be associated with low-level R to KAN. |
| | *eis promoter* | −10G/A | | Confer KAN-resistance, short MDR regimen with kanamycin cannot be used for treatment. May induces increased mic to Amikacin. Capreomycin could still be effective |
| | | −12C/T | | |
| | | −14C/T | | |
| | | −37G/T | | |
| | | | | −12C/T may confer low level R to KAN. |

(continued)

**Table 12.5** (continued)

| Antimicrobial agent | Gene | Common mutations | Less common mutations | Expected phenotype |
|---|---|---|---|---|
| Bedaquiline | *atpE* | D28N, A63V | | Mutations in C ring of the ATP synthase may be associated with BDQ resistance. |
| | *mmpR* | No predominant mutations | | A new publication indicated mutations in mmpR may be associated with BDQ-R. |
| Delamanid | *fbiA (Rv3261), fbiB (Rv3262), fbiC (Rv1173), fgd1 (Rv0407)* | | | Mutations in genes involved in coenzyme F420 biosynthesis and metabolism has been proposed as possible mechanisms of resistance to DLM (Choi KP *et al*., *J. Bacteriol*. 2002) *several mutations have been observed but data correlating to DR are not yet available* |
| | *ddn* | | | *Stop codons in the ddn have been associated to high level resistance* |

could explain the discrepancies observed between phenotypic and genotypic results. With the increasing number of genomic loci identified by WGS as linked to resistance, the value of this approach will increase in particular for use in laboratory routine (Drobniewski et al. 2015; Pankhurst et al. 2016). Recent studies underlined that over 100 genetic regions are involved in the drug resistance pathways and that mutations found within these regions could play relevant roles. WGS therefore appears the most suitable approach for a comprehensive analysis, given an appropriate validation of all the mutations by MIC and allelic exchange experiments, and considering the correlation with clinical outcomes (Zhang and Yew 2015). At the moment, WGS can be used to rapidly identify the known conferring-resistance mutations and, consequently, to guide individualized treatment decisions, even supporting for some drugs the phenotypic DST results, due to the reliability issues of the latter (Koser 2013). Among the advantages of WGS over the molecular tools currently recommended by the WHO, there is the possibility to provide information on the specific nucleotide substitutions that confer different levels of phenotypic resistance (e.g. mutations affecting codons 90 and 94 in gyrA) and the analysis of large genomic regions not limited to hotspot fragments (e.g. *pncA* complete coding and promoter sequencing; mutations outside the *rpoB* RRDR and *gyrA*-*gyrB* QRDR). WGS can also provide information to support conventional contact tracing for epidemiological studies, given its high discriminatory power in determining phylogenetic lineages (see Chap. 4), and in tracking the circulating strains and their relatedness (Drobniewski et al. 2015; WHO/UNITAID 2015; Witney et al. 2015). Thus, it may be possible to diagnose drug resistance and monitor transmission events at the same moment, with considerable impact on public health strategies (Arinaminpathy 2015). WGS platforms have been already adopted in many TB supranational and national reference laboratories, as well as in research laboratories: several groups are working to reduce the complexity of such technologies, from the hardware to the analysis part, with the final aim to make this technology accessible to all (Chap. 3). Already, several Countries are moving towards a centralized genomic approach for detection of sensitivity at least to first line antitubercular drugs. In addition WGS provides detailed information on the prevalence of strains and drug resistance patterns in the different settings, thus helping the strategies adopted by TB control programs at local and national levels (WHO/UNITAID 2015; Zignol et al. 2016). The cost of WGS varies depending on the technologies and numbers of sample analysed, and it has now probably reached the price range of the other tests performed in the hospital laboratories. The cost benefit depends also on the time needed to provide results, with a reduction of around 4 weeks compared to phenotypic

DST, avoiding also the use of ineffective and expensive drugs and hospital isolation sectors for long period of time (Drobniewski et al. 2015; Witney et al. 2015). Despite the great opportunity to provide a comprehensive analysis of MTB primary cultures including species identification, simultaneous determination of resistance to all the anti-TB drugs through the interrogation of the known molecular targets, and genotyping and phylogenetic investigation to track the transmission events, the use of the generated information is challenging in decentralized facilities due to the computational capacity and bioinformatics skills required, and to the lack of standardized reference, analysis pipelines, and interpretation tools (Schito and Dolinger 2015).

Moving from culture-based WGS to direct analysis from clinical samples with fully automated platforms could be the next step to make this approach suitable for high burden settings. Commercial tests based on NGS of specific targets are under development and will be available in a near future.

## 12.6 Clinical Considerations

Moving into the era of "personalized medicine" requires an appropriate and accurate classification of the bacterial strains causing TB for both the sensitivity patterns and the genotype. Treatment of TB and of drug-resistant TB in particular is still very long and associated with toxicity and irreversible side effects. Treatment initiation in the absence of data on the susceptibility of the strain to the drugs selected should be avoided whenever possible. Each patient deserves a reliable drug sensitivity test done under the best conditions in a quality assured laboratory.

The introduction of additional therapeutic options, ranging from the adoption of the short MDR regimen to the introduction of new or repurposed drugs, requires a "triaging" of the patient with MDR-TB in the shortest possible time, it is clear that only molecular tools can respond to this need.

In the past few years DST for *M. tuberculosis* has evolved from a mostly "home made" test performed in few laboratories with doubtful results with turnaround time of months to a much needed high-tech test. The promise of WGS is now the "all in one" approach, with a prediction of the resistance pattern associated to epidemiological and genotypic information from clinical samples.

Although we recognize that rapid molecular tests are still unable to predict sensitivity or resistance in 100% of cases, they are still able to guide therapy in the high majority of cases allowing not starting or early discontinuation of potentially toxic therapy in cases in which resistance can be predicted.

It is becoming clear that the concept of "one gold standard method" for testing *M.tuberculosis* susceptibility to antibiotics is challenged by the fact that the different tests are providing results that at first may appear conflicting. This is causing confusion among clinicians and reluctance in modifying therapy. We need to accept that each drug may have a different testing standard and that for some drug the genotypic results will overrule the phenotype.

In some cases the use of MIC will provide substantial information to decide on the discontinuation of a therapy.

In the future, the same investment should be made in training clinician in the interpretation of molecular tests and MIC-based test, that we have devoted to train microbiologists in the use of molecular tests in order to translate into clinical action the information that the technology will allow to collect.

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