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USEFULNESS OF MOLECULAR METHODS FOR DETECTING DRUG RESISTANCE AND GENOTYPING IN MYCOBACTERIUM TUBERCULOSIS

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INDEX

SUMMARY

Tuberculosis (TB) is one of the major infectious diseases worldwide, and the emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis*, the causative agent, is a public health threat. In order to improve TB control, rapid diagnosis of the disease and implementation of an adequate treatment are required. In order to determine the strain susceptibility pattern, the reference method is phenotypic drug susceptibility testing. However, due to the slow growth rate of *M. tuberculosis*, results may be delayed for several weeks. Since the molecular basis and mutations associated with phenotypic resistance have been characterized for certain drugs, several molecular methods that detect these mutations have been developed for rapid genotypic drug susceptibility testing. Still, diagnostic accuracy studies assessing the usefulness of these methods are needed. In addition, it is essential to take into consideration the different impacts that each mutation may have on the phenotypic resistance in the *M. tuberculosis* strain and on the clinical outcome, in order to guide the clinician for an optimal choice of the anti-TB drugs for treatment. Furthermore, the study of the *M. tuberculosis* transmission by genotyping is needed in order to detect epidemiological links among TB cases and outbreaks, and thus control the spread of the disease. The most widely used genotyping methods are IS*6110* restriction fragment length polymorphism (RFLP), spoligotyping [spacer oligonucleotide typing, based on the polymorphism of the clustered regularly interspaced short palindromic repeats (CRISPR)], and mycobacterial interspersed repetitive units - variable number tandem repeats (MIRU-VNTR). However, these methods are either slow, laborious, time-consuming, low-throughput, or may present low-discriminatory power. Therefore, the objectives of this thesis are (1) to assess the usefulness of new molecular methods for detecting genotypic drug resistance, (2) to review the molecular methods used for TB management and the relationship between genotypic and phenotypic drug resistance and clinical outcomes, and (3) to evaluate and develop new methods for genotyping *M. tuberculosis* strains.

For the first objective, the diagnostic accuracy of different molecular methods for detecting drug resistance was assessed with clinical strains and clinical specimens (Articles I, II, III, and IV). Strains and specimens were analysed with the phenotypic drug susceptibility methods

BACTEC 460TB and MGIT 960, and with molecular methods based on reverse hybridization array (GenoFlow DR-MTB Array Test, DiagCor Bioscience, Hong Kong) and line probe assay (AID TB resistance, AID Diagnostika, Germany), multiplex PCR (Anyplex II MTB/MDR/XDR, Seegene, Korea), and pyrosequencing (Qiagen, The Netherlands). Each of these methods targets a set of defined mutations among the following genes or genomic regions: *rpoB* for rifampicin resistance; *katG* and *inhA* promoter for isoniazid resistance; *gyrA* for fluoroquinolones resistance; *rrs* and *eis* promoter for injectable drugs (kanamycin, amikacin, and capreomycin) resistance; *embB* for ethambutol resistance, and *rrs* and *rpsL* for streptomycin resistance. Results obtained with molecular methods were compared with those obtained with phenotypic methods. In case of discordance, the strains/specimens were analysed with another molecular method. As for the second objective, the molecular methods used for TB management and the relationship between gene/promoter mutations, phenotypic drug resistance, and clinical outcomes were reviewed (Articles V and VI). This latter review was carried out in the context of a collaboration between the TBNET and RESIST-TB groups to reach a consensus about the clinical use of molecular drug susceptibility testing. Finally, for the third objective, a microbead-based method (TB-SPRINT) aimed to perform simultaneously spoligotyping and detection of molecular resistance to rifampicin and isoniazid was evaluated (Article VII). In addition, a genotyping method based on the polymorphism of the IS*6110* insertion site combined with pyrosequencing, and termed PyroTyping, was developed (Article VIII). For both methods, results obtained with the new method were compared with conventional spoligotyping and IS*6110*-RFLP. Regarding TB-SPRINT, drug susceptibility results were compared with phenotypic and molecular methods.

Regarding the diagnostic accuracy of the different molecular methods for detecting genotypic drug resistance, the sensitivity and specificity values varied depending on the drug considered. The sensitivity was high (≥90%) for detecting rifampicin, amikacin, capreomycin, and streptomycin resistance; it was high or moderate (60-89%) for isoniazid and kanamycin; it was moderate or low (<60%) for fluoroquinolones; and it was moderate for ethambutol. In addition, the specificity of the molecular methods was high for isoniazid, rifampicin, and streptomycin; and it was high or moderate for fluoroquinolones, kanamycin, amikacin, capreomycin, and ethambutol. Despite these differences, the results of the different molecular methods were highly concordant. Discordant results between the molecular

method evaluated and the phenotypic method were obtained for some drugs. In most of the cases, results obtained with an alternative molecular method were concordant with the molecular method evaluated. Finally, the molecular methods studied present differences in terms of throughput capacity, equipment and training required, and interpretation of the results.

As for the relationship between genotypic and phenotypic resistance and clinical outcomes, the extent of knowledge varies depending on the drug considered. It is of note that depending on the mutation detected, the considered drug can still be used for treatment or should be excluded. Regarding isoniazid, the mutation *katG*315 is associated with high-level phenotypic resistance and unfavourable clinical outcomes (treatment failure, death, or relapse), therefore isoniazid should not be considered as an option for treatment. Conversely, mutations in *inhA* promoter are associated with low-level resistance to isoniazid, and cross-resistance to ethionamide and prothionamide, but have no effect on clinical outcomes. Hence, high-dose isoniazid is usually recommended for treatment. Regarding the rifamycins, the different mutations detected in *rpoB* have variable effects on the phenotypic cross-resistance between rifampicin, rifabutin, and rifapentine: some mutations confer high-level resistance to the three drugs, other mutations affect rifampicin but not the other rifamycins, and other mutations have a slight effect on susceptibility to the three drugs. In addition, some of the mutations are associated with unfavourable clinical outcomes. Therefore, depending on the mutation detected, rifampicin can still be an option for treatment or it should be excluded. As for fluoroquinolones, some mutations in *gyrA* confer phenotypic and clinical resistance, whereas other mutations are discussed controversially and the clinical implications are unclear. Finally, regarding the injectable drugs, different mutations in *rrs* and *eis* promoter are associated with partial cross-resistance between kanamycin, amikacin, and capreomycin. Nevertheless, studies assessing the effect of these mutations on clinical outcomes have not been performed yet.

Lastly, as for the evaluation of TB-SPRINT spoligotyping and development of PyroTyping, the concordance between these novel methods and the reference methods with respect to the clustering or discrimination of the strains studied was 96.9% and 100%, respectively. According to spoligotyping results from both evaluations, the strains included were classified

as Haarlem, Latin-America and Mediterranean, T, Africanum, East African Indian, X, Beijing, Bovis, Central Asian, or the lineage could not be assigned. Moreover, TB-SPRINT simultaneously detected mutations associated with rifampicin and isoniazid resistance, and it was highly sensitive and specific compared with phenotypic drug susceptibility testing and DNA sequencing/pyrosequencing, although the number of drug-resistant strains analysed was low to draw a robust conclusion. TB-SPRINT and PyroTyping are rapid methods, relatively easy to perform, and high-throughput, and interpretation of the results does not require a complex bioinformatics analysis, although both methods require specific equipment. It is of note that the choice of the genotyping method, based either on the polymorphism of the CRISPR region or the IS*6110* insertion site, depends on the objective of investigation, with regard to the potential discriminatory power needed. Finally, both microbead-based hybridization and pyrosequencing can be used for detecting genotypic drug resistance, increasing the clinical value of these methods.

In conclusion, the molecular methods evaluated for detecting first- and second-line drug resistance present variable sensitivity and specificity when compared to phenotypic methods depending on the drug considered. However, the sensitivity and specificity values of these molecular methods are comparable between them. Current knowledge on the relationship between molecular and phenotypic drug resistance and clinical outcomes has been investigated for some drugs but it is unclear for other drugs. Large studies are needed to establish these relationships and to identify the subset of mutations predictive of treatment failure, in order to tailor an adequate treatment and make rational use of the limited drugs available. Finally, the performance and discriminatory power of the genotyping methods based on hybridization on microbeads and pyrosequencing are variable, hence, the selection of the method depends on the objective of the investigation. Furthermore, the potential simultaneous detection of drug resistance with these methods increases their clinical value for patient management.

ABBREVIATIONS

AFB	Acid-fast bacilli
AMK	Amikacin
CAP	Capreomycin
CFZ	Clofazimine
CRISPR	Clustered regularly interspaced short palindromic repeats
DST	Drug susceptibility testing
EMB	Ethambutol
ETO	Ethionamide
FLQ	Fluoroquinolone
GFX	Gatifloxacin
HIV	Human immunodeficiency virus
HR	Isoniazid and rifampicin
HREZ	Isoniazid, rifampicin, ethambutol, and pyrazinamide
INH	Isoniazid
IS	Insertion sequence
KAN	Kanamycin
LFX	Levofloxacin
LPA	Line probe assay
MDR	Multidrug resistance
MFX	Moxifloxacin
MGIT	Mycobacteria growth in tube
MIRU	Mycobacterial Interspersed Repetitive Unit
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
PAS	*p*-aminosalicylic acid
PTO	Prothionamide
PZA	Pyrazinamide
RBT	Rifabutin
RIF	Rifampicin

RFLP	Restriction fragment length polymorphism
STR	Streptomycin
TB	Tuberculosis
TB-SPRINT	Tuberculosis spoligo rifampicin isoniazid typing
VNTR	Variable Number of Tandem Repeat
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively drug resistance

LIST OF ARTICLES ORIGINATED FROM THIS THESIS

Article I. Evaluation of GenoFlow DR-MTB Array Test for detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis*. Molina-Moya B, Kazdaglis G, Lacoma A, Prat C, Gómez A, Villar-Hernández R, García-García E, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Domínguez J. Journal of Clinical Microbiology. 2016; 54:1160-1163.

Article II. Diagnostic accuracy study of multiplex PCR for detecting tuberculosis drug resistance. Molina-Moya B, Lacoma A, Prat C, Pimkina E, Diaz J, García-Sierra N, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Ausina V, Dominguez J. Journal of Infection. 2015; 71: 220-230.

Article III. AID TB resistance line probe assay for rapid detection of resistant *Mycobacterium tuberculosis* in clinical samples. Molina-Moya B, Lacoma A, Prat C, Diaz J, Dudnyk A, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Ausina V, Dominguez J. Journal of Infection. 2015; 70: 400-408.

Article IV. Pyrosequencing for rapid detection of *Mycobacterium tuberculosis* second-line drugs and ethambutol resistance. Lacoma A*, Molina-Moya B*, Prat C, Pimkina E, Diaz J, Dudnyk A, García-Sierra N, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Ausina V, Dominguez J. Diagnostic Microbiology and Infectious Diseases. 2015; 83: 263-269.
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Article V. Recent advances in tuberculosis diagnosis: IGRAs and molecular biology. Molina-Moya B, Latorre I, Lacoma A, Prat C, Dominguez J. Current Treatment Options in Infectious Diseases. 2014; 6: 377-391.

Article VI. Clinical implications of molecular drug resistance testing for *Mycobacterium tuberculosis*: a TBNET/RESIST-TB consensus statement. Domínguez J, Boettger EC, Cirillo D, Cobelens F, Eisenach KD, Gagneux S, Hilleman D, Horsburgh R, Molina-Moya B, Niemann S,

Tortoli E, Whitelaw A, Lange C; TBNET; RESIST-TB networks. International Journal of Tuberculosis and Lung Disease. 2016; 20: 24-42.

Article VII. Molecular characterization of *Mycobacterium tuberculosis* strains with TB-SPRINT. Molina-Moya B, Gomgnimbou MK, Lafoz C, Lacoma A, Prat C, Refrégier G, Samper S, Domínguez J, Sola C. American Journal of Tropical Medicine and Hygiene. 2016. Submitted.

Article VIII. PyroTyping, a novel pyrosequencing-based assay for *Mycobacterium tuberculosis* genotyping. Molina-Moya B, Lacoma A, Garcia-Sierra N, Blanco S, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Prat C, Arnold C, Domínguez J. Nature Methods. 2016. Submitted.

INTRODUCTION

1. INTRODUCTION

1.1. Epidemiology and resistance situation

Tuberculosis (TB) is one of the major infectious diseases worldwide. Despite TB mortality has fallen 47% since 1990, and 43 million lives were estimated to be saved between 2000 and 2014, this disease still remains a global health problem [1]. According to the World Health Organization (WHO) Global Tuberculosis Report, in 2014, 9.6 million people were estimated to have developed the disease worldwide and 1.5 million people died (1.1 million human immune deficiency virus (HIV)-negative and 0.4 million HIV-positive) (Figure 1). However, 6 million (63%) new TB cases, and 260,000 previously treated cases were reported to WHO. Among the new TB cases, 5.1 million (85%) were pulmonary and 890,000 (15%) were extrapulmonary [1].

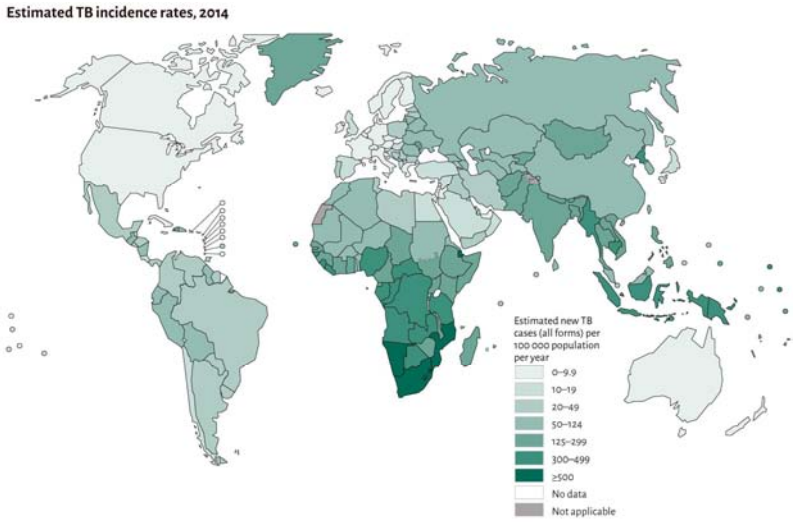


Figure 1. Estimated TB incidence rates in 2014 (WHO Global Tuberculosis Report 2015) [1].

Although almost all the TB cases can be treated and cured, the emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis*, the etiologic agent of TB, pose challenges to global TB control [1]. The estimated number of multidrug-resistant (MDR) TB cases was 480,000 in 2014, accounting for 3.3% of new TB cases and 20% of previously treated cases, and an estimated 190,000 people died of MDR-TB, although only 123,000 (26%) cases were detected and reported [1]. Nevertheless, there was an improvement on the proportion of TB

patients tested for drug resistance, partly due to the implementation of molecular tests: 58% of previously treated patients and 12% of new cases were tested in 2014, in contrast with 17% and 8.5%, respectively, in 2013 [1]. Furthermore, extensively drug-resistant (XDR) *M. tuberculosis* strains had been reported in 105 countries by 2015, and an estimated 9.7% of people with MDR-TB had XDR-TB [1].

In the European countries there were 340,000 estimated TB cases in 2014, with an incidence of 37 per 100,000 people [1]. In contrast with the global data, MDR-TB accounted for 15% of new and 48% of retreatment cases, and the coverage of drug susceptibility testing for both fluoroquinolones and second-line injectable drugs was the lowest [1].

In Catalonia there were 1,074 notified cases in 2015, with an incidence of 14.3 cases per 100,000 people [2]. The case notification rate was higher for foreign-born patients than for native patients (39.5 and 9.1 cases per 100,000 inhabitants, respectively), although 47.1% of the cases corresponded to foreign-born patients. MDR-TB accounted for 1.3% of TB cases in 2015.

During the last years, new tests for diagnosing drug-resistant TB and new drugs for treatment have been developed [3]. However, these tests should be evaluated, and more diagnostics, drugs, and vaccines are needed in order to achieve the targets set in the End TB Strategy [1].

1.2. Evolution of drug resistance in *Mycobacterium tuberculosis* complex

There are two types of drug resistance in *M. tuberculosis*: phenotypic resistance (or drug tolerance) and genetic resistance. Phenotypic resistance is due to epigenetic changes in gene expression and protein modification that reduce susceptibility to several drugs in non-replicating bacilli, whereas genetic resistance is due to mutations in chromosomal genes in replicating bacilli [4]. Phenotypic resistance may be caused by efflux pump expression induced by stress or subinhibitory concentration of drugs [4]. Phenotypic resistance may facilitate the development of mutations causing genetic resistance [5], and vice versa [4].

M. tuberculosis is intrinsically resistant to several antibiotics, due to the mycolic acids-rich impermeable cell wall [6]. In addition, its *whiB7* regulon includes genes involved in drug efflux (*tap*), a putative macrolide exporter (Rv1473), the ribosomal methyltransferase *erm*, and the aminoglycoside acetyltransferase *eis*, which may reduce susceptibility to several drugs, including macrolides and aminoglycosides [6].

Emergence of drug resistance is explained by the "fall and rise" effect [7]. When a patient is treated with a single anti-TB drug, the subpopulation of susceptible bacilli will decline, but spontaneously resistant bacilli will be selected and the resistant subpopulation will increase. The number of bacilli required for the emergence of a spontaneous resistant bacilli is $1x10^2$-10^4 for pyrazinamide (PZA), $1x10^5$-10^6 for isoniazid (INH), streptomycin (STR), ethambutol (EMB), and fluoroquinolones (FLQ), $1x10^7$-10^8 for rifampicin (RIF), and $1x10^3$-10^6 bacilli for other drugs [7]. Amplification of the drug resistance profile is due to multiple cycles of "fall and rise" due to treatment with a single drug [7]. Therefore, resistance to multiple drugs takes time to develop and is the result of ineffective anti-TB treatment [7,8].

Genetic resistance has been mainly attributed to random genetic mutations in the chromosome, since there is no evidence of horizontal gene transfer in *M. tuberculosis* or resistance mutations acquired through transposition [6,9,10]. Genetic mutations are mainly due to errors introduced during DNA replication [10,11], and, subsequently, mutant bacilli are selected [4]. The most common type of mutation are single nucleotide changes, but small insertions and deletions (indels) or larger deletions have also been described [12]. Most of

these mutations silence drug-activating enzymes, modify the drug target, or increase the gene product targeted by the drug [12]. However, other mutations result in up-regulation of bacterial efflux pumps, causing phenotypic resistance [13], and facilitating the development of genetic resistance [4,5]. Resistance to multiple drugs is caused by sequential mutations in different genes, and single chromosomal mutations causing genetic resistance to more than one drug have not been described [7].

Some reports concluded that *M. tuberculosis* strains resistant to one or more drugs would not be widely disseminated due to the reduced fitness associated with genetic drug resistance [4]. However, some resistance-conferring mutations have low or no fitness cost, and other mutations with high fitness cost can be accompanied by compensatory mechanisms; consequently, some drug-resistant strains have been actively transmitted [4].

1.3. Drugs for treatment of tuberculosis and mechanisms of resistance

Until recently, the anti-TB drugs have been categorised as Groups 1, 2, 3, 4 and 5 [14]. The updated regrouping of anti-TB drugs is presented in Table 1 [15]. For some anti-TB drugs, the mechanisms of phenotypic resistance and the responsible genes and mutations are characterized, whereas for other drugs there is still incomplete knowledge.

Table 1. Classification of the anti-TB drugs. Adapted from WHO 2016 [15].

First-line drugs		
		Isoniazid
		Rifampicin
		Ethambutol
		Pyrazinamide
Second-line drugs		
A. Fluoroquinolones		Levofloxacin
		Moxifloxacin
		Gatifloxacin
B. Second-line injectable agents		Amikacin
		Capreomycin
		Kanamycin
		Streptomycin
C. Other core second-line agents		Ethionamide / Prothionamide
		Cycloserine / Terizidone
		Linezolid
		Clofazimine
D. Add-on agents (not part of the core MDR-TB regimen)	D1	Pyrazinamide
		Ethambutol
		High-dose isoniazid
	D2	Bedaquiline
		Delamanid
	D3	p-aminosalicylic acid
		Imipenem-cilastatin
		Meropenem
		Amoxicillin-clavulanate
		Thioacetazone

1.3.1. First-line drugs

1.3.1.1. Isoniazid

Isoniazid (INH) was discovered in 1952 [16] and it is a drug active only against mycobacteria, and mainly against *M. tuberculosis* complex [4,7]. It is a prodrug that is activated by the catalase-peroxidase encoded by the *katG* gene [4,17]. The active species (isonicotinic acyl

radical or anion) forms an adduct with NAD (nicotinamide adenine dinucleotide). The INH-NAD adduct inhibits the enoyl acyl carrier protein reductase InhA [18], and other targets, inhibiting the synthesis of mycolic acids of the cell wall [4,7]. INH is the anti-TB drug with the most potent early bactericidal activity, and it is active only against rapidly replicating bacilli [7].

The major mechanism of INH resistance involves *katG*, and the most common mutation is the S315T (AGC to ACC or ACA) [4]. Mutations in the *katG* promoter region (*furA*-*katG* intergenic region), affecting *katG* expression, have also been detected in INH-resistant strains [4]. Another mechanism of INH resistance consists on mutations in the promoter region of *mabA*(*fabG1*)/*inhA* operon causing overexpression of InhA, or mutations in the genomic region coding for the InhA active site [4]. The most common mutations are located in *inhA* promoter positions -16 (A to G), -15 (C to T), and -8 (T to C or A). Mutations in *inhA* confer cross-resistance between INH and ETO/PTO, since these drugs are structurally related and share the same genetic target [4,7]. In addition, a percentage of low-level INH-resistant strains do not harbour mutations in *katG* or *inhA* [4], and resistance may be due to new mechanisms [4]. In this line, mutations associated with INH resistance have been identified with WGS in more than 40 genes [19].

1.3.1.2. Rifampicin (rifamycins)

Rifampicin (RIF) was discovered in 1963 [16] and it is the most effective drug against *M. tuberculosis*, and also against a wide range of microorganisms [7]. RIF specifically binds to and inhibits the β subunit of the DNA-dependent RNA polymerase, encoded by *rpoB*, and interferes with the synthesis of mRNA [4,7]. Moreover, once RIF has bound to the RpoB target, it induces hydroxyl radical formation [4,20]. RIF has good bactericidal and sterilising activity, which enables the shortening of treatment [4,7,21]. In addition to RIF, other rifamycins available are rifabutin (RBT) and rifapentine. RBT is used to substitute RIF in TB-HIV patients [16], and RBT or rifapentine may be used in the treatment of RIF-resistant TB [7].

More than 95% of mutations associated with phenotypic RIF resistance are located in the 81-bp hotspot region of *rpoB*, known as the RIF resistance-determining region [4,7]. Mutations in *rpoB* generally result in cross-resistance between rifamycins [7]: most of the RIF-resistant

strains are also rifapentine-resistant [7], but some strains are RBT-susceptible [4]. The most frequent mutations among RIF-resistant strains are S531L (TCG to TTG), H526Y/D (CAC to TAC/GAC), and D516V (CAG to GTC), although other mutations at these and other codons in the RIF resistance-determining region may also be associated with RIF resistance [4]. Interestingly, some mutations in *rpoB* are also found in phenotypically RIF-susceptible strains [4].

1.3.1.4. Ethambutol

Ethambutol (EMB) was discovered in 1961 [16] and it is active only against mycobacteria [7]. EMB targets arabinosyl transferase, encoded by *embB*, and inhibits the polymerisation of arabinogalactan and lipoarabinomannan, two components of the mycobacterial cell wall [4,7]. Thus, EMB inhibits mycolic acid synthesis, and also triggers a cascade of changes in lipid metabolism of mycobacteria, leading to the disaggregation of bacteria clumps into smaller clusters [7]. Despite this mode of action, EMB has poor bactericidal activity on both extracellular and intracellular bacilli. However, it prevents the selection of resistance to INH and RIF [7,10].

The main mechanism of EMB resistance involves the *embCAB* operon. The most common mutations are located in *embB* codon 306, which are detected in less than 70% of EMB-resistant strains [4]. Different amino acid substitutions have been identified in *embB*306: M306V (GTG) and M306I (ATA, ATC, and ATT) [22]. Other mutations have been detected in *embB* codons 319, 406, and 497, and also in the *embC* and *embA* genes [23,24]. However, *embB*306 mutations have also been detected in EMB-susceptible MDR-TB strains [25]. Finally, mutations in *ubiA*, encoding the DPPR (decaprenyl-phosphate 5-phosphoribosyltransferase) synthase involved in cell-wall synthesis, have been identified [4,26].

1.3.1.3. Pyrazinamide

Pyrazinamide (PZA) was discovered in 1954 [16] and is active only against mycobacteria, especially *M. tuberculosis*. PZA is a prodrug that is converted to the active form pyrazinoic acid by the pyrazinamidase/nicotinamidase encoded by the *pncA* gene [4,27]. Pyrazinoic acid accumulates in acidic pH [7] and interferes with membrane energy production and inhibits RpsA (ribosomal protein S1) involved in trans-translation and aspartate decarboxylase PanD

involved in pantothenate and co-enzyme A synthesis, among other effects [4]. PZA has a very poor bactericidal ability but a powerful sterilising activity on non-growing intracellular bacilli in acidic environments [4,7], which enables the shortening of treatment [21].

The main mechanism of PZA resistance involves mutations in *pncA*, which are highly diverse and scattered along the gene, and are detected in 72–99% of phenotypically resistant strains [4]. Nevertheless, non-synonymous mutations in *pncA* have also been detected in some PZA-susceptible strains [4]. Strains without *pncA* mutations may harbour mutations in *rpsA* or in *panD* [4].

1.3.2. Second-line drugs

1.3.2.1. Group A: Fluoroquinolones

Fluoroquinolones (FLQ) are broad-spectrum drugs that target DNA topoisomerases, thus, they are repurposed drugs for treatment of drug-resistant TB [14,16]. FLQs inhibit the α subunit of DNA gyrase, encoded by *gyrA* gene, and have acceptable bactericidal and sterilising action [7]. The FLQs recommended for treatment of TB are levofloxacin (LFX), moxifloxacin (MFX), and gatifloxacin (GFX) [15], although ofloxacin has been widely used as well.

The main mechanism of FLQ resistance involves *gyrA*, and to a lesser extent, *gyrB*, coding for the β subunit of DNA gyrase [4,28]. Variable cross-resistance between the different FLQs has been described. Although all FLQs share the same target, strains show different minimum inhibitory concentrations for each FLQ depending on the mutation considered [7]. The most common mutations are located in the quinolone-resistance-determining region of *gyrA*, particularly A90V (GCG to GTG), S91P (TCG to CCG), and D94A/N/Y/H/G (GAC to GCC/AAC/TAC/CAC/GGC) [28]. In, addition, mutations in codons 74, 80, and 88 have also been detected [4].

Efflux pumps may also play a role in FLQ resistance in *M. tuberculosis*, since suboptimal drug exposure may facilitate acquisition of genetic resistance through mutations in *gyrA* and *gyrB* [4]. Another important factor is heteroresistance, which refers to the coexistence of distinct proportion of subpopulations that differ in the nucleotides, i.e. wild-type or mutations, at a drug resistance locus [4,29].

1.3.2.2. Group B: Second-line injectable drugs

The group of injectable drugs includes streptomycin (STR) (1944), kanamycin (KAN) (1957), capreomycin (CAP) (1963), and amikacin (AMK) (1972) [15]. These drugs are active against *M. tuberculosis* and other bacteria. Among these drugs, STR, KAN, and AMK are aminoglycosides, and CAP is a polypeptide. The aminoglycosides inhibit protein synthesis through irreversible binding to the 30S ribosomal subunit, causing misreadings of the mRNA [4,7]. The polypeptides inhibit translocation of peptidyl-tRNA and block the initiation of protein synthesis [4,7]. Although CAP has a different chemical structure and mechanism of action, the mechanism of antibacterial and metabolic activity is similar [7]. The injectable drugs are bactericidal and have strong extracellular activity. Only one of these drugs has to be chosen for a drug-resistant TB regimen, and the choice between KAN, AMK, and CAP depends on effectiveness and implementation considerations [15].

The main mechanisms of STR resistance involve *rpsL*, coding for the S12 protein, and *rrs*, coding for 16S rRNA [4]. The most common mutations are K43R and K88Q/R in *rpsL*, and mutations in the 530 loop of *rrs* [30]. Strains without mutations in *rpsL* or *rrs* may harbour mutations in *gidB*, coding for a guanosine methyltransferase specific for 16S rRNA [4]. In addition, mutations in the promoter region of *whiB7*, leading to an increased expression of the tap efflux gene and *eis*, have been detected in strains presenting cross-resistance between STR and KAN [4,7].

Cross-resistance between KAN, AMK, and CAP has been reported. Different mutations in *rrs* (A1401G, C1402T, and G1484C/T) are associated with partial cross-resistance between KAN, AMK, and CAP [4]. In addition, mutations in the promoter region of *eis* (G-37T, C-14T, C-12T, and G-10A), encoding aminoglycoside acetyltransferase, have been detected in strains resistant to KAN and AMK [31,32].

1.3.2.3. Group C: Other core second-line agents

1.3.2.3.1. Thioamides

Thioamides are derivatives of isonicotinic acid, active only against *M. tuberculosis* and, to a lesser extent, against other mycobacteria [4,7]. The main thioamides used are ethionamide (ETO) and prothionamide (PTO). ETO is a prodrug that is activated by the mono-oxygenase EtaA/EthA and inhibits the enoyl acyl carrier protein reductase InhA, hence, the synthesis of mycolic acids of the cell wall [4]. The structure and activity of PTO are almost identical to those of ETO [4]. Due to the shared target between INH, ETO, and PTO, there is cross-resistance between these drugs [7]. There is also cross-resistance with other thioamides, such as thioacetazone, since EthA activates drugs from this class [4]. There are uncertainties about the reproducibility and reliability of drug susceptibility testing (DST) for ETO [7,14]. Mutations in *inhA* or *ethA* confer ETO resistance [33].

1.3.2.3.2. Cycloserine / Terizidone

Cycloserine was discovered in 1955 [16] and it is active against several species of gram-positive bacteria and *M. tuberculosis* [7]. Cycloserine blocks the action of D-alanine racemase (Alr), involved in the conversion of L-alanine to D-alanine, which is a substrate for the D-alanine:D-alanine ligase (Ddl) [4]. These enzymes are involved in the synthesis of peptidoglycan of the cell wall [4,7]. Cycloserine is a bacteriostatic agent used in the treatment of drug-resistant TB [4,7]. Terizidone is a combination of two molecules of cycloserine [4,7]. The mechanism of cycloserine resistance in *M. tuberculosis* remains unclear [4].

1.3.2.3.3. Linezolid

Linezolid was discovered in 1996 and is active against a broad spectrum of gram-positive bacteria, and also *M. tuberculosis* [7]. It binds to the 23S rRNA peptidyl transferase of the 50S ribosomal subunit and forms a secondary interaction with the 30S subunit, inhibiting the formation of the initiation complex for protein synthesis [4]. Linezolid has a significant bacteriostatic activity [4].

Mutations G2061T or G2576T in *rrn*, coding for the 23S rRNA, have been detected in high-level linezolid resistant strains [4]. Low-level resistant strains had no mutations in *rrn*, but the mutation T460C in *rplC*, encoding ribosomal protein L3, was putatively involved in linezolid

resistance [4]. Only 30% of linezolid-resistant MDR strains had mutations in *rrn* or *rplC*, suggesting other unknown resistance mechanisms [4].

1.3.2.3.4. Clofazimine

Clofazimine was discovered in 1956 and has good activity against mycobacteria, including *M. tuberculosis* [4]. The mechanisms of action may include production of reactive oxygen species [4], inhibition of energy production through inhibition of NADH dehydrogenase, and disruption of the membrane that could lead to a reduction in ATP production [4]. Clofazimine may have both intracellular and extracellular activity, and might act as a facilitator for other drugs [7].

The molecular basis of clofazimine resistance is not completely understood. Mutations in *rv0678*, coding for a transcriptional repressor of MmpS5-MmpL5 efflux pump, are the main mechanism of resistance. Mutations in *rv1979c* and *rv2535c* were also detected in clofazimine resistant strains [34]. Mutations in *rv0678* caused resistance to both clofazimine and bedaquiline [4].

1.4.2.4. Group D: Add-on agents

This group includes drugs that are not considered core second-line agents for treatment of drug-resistant TB (Table 1) [15]. Phenotypic and genotypic DST for some of these drugs is not routinely performed.

1.4. Treatment of tuberculosis

The first objective of the treatment is to cure the patient and to prevent death from active TB. However, the implementation of and adherence to an adequate treatment serves also to prevent relapse of TB, reduce transmission in the community, and prevent the selection and spread of drug-resistant *M. tuberculosis* strains [35]. The treatment of TB is based in combination therapy with bactericidal and sterilizing drugs for which the *M. tuberculosis* strain is susceptible during several months [35]. Drugs are associated to reduce the emergence of drug resistance and to eliminate the subpopulations of *M. tuberculosis* bacilli that are at different replication states [7,16]. The actively replicating subpopulation is eliminated by bactericidal drugs in the first days/weeks of treatment, reducing the patient infectiousness and increasing the chances for survival [7], whereas the slowly or non-replicating subpopulation is eliminated by sterilizing drugs in the following weeks/months, reducing the chance of relapse [10,16]. The main challenges of this long treatment are drug intolerance, toxicities, and patient compliance [10]. A strategy to ensure patient compliance is the directly observed therapy [36].

1.4.1. Treatment of drug-susceptible tuberculosis

The standard first-line regimens are assigned for defined patient groups according to the WHO guidelines [35]. For new patients presumed or known to have drug-susceptible TB, the regimen consists on a two-month intensive phase with INH, RIF, PZA, and EMB, and a four-month continuation phase with INH and RIF (2HRZE/4HR) (Table 2) [35]. Nevertheless, regimens should be adjusted appropriately upon availability of DST results [35].

Globally, the treatment outcomes in 2013 were success in 86%, failure in 1%, death in 4%, lost to follow-up in 4%, and not evaluated in 4% of new and relapse TB cases [1].

1.4.2. Treatment of drug-resistant tuberculosis

Resistance to at least INH and RIF is defined as multidrug resistance (MDR). Detection of MDR-TB is essential to implement an adequate treatment, since treatment with first-line drugs is much less effective and resistance can be further amplified [35]. It is of note that RIF-resistant TB is considered as MDR-TB for treatment purposes [15].

Treatment of MDR-TB should be individually adjusted according to the DST results. Nevertheless, a recommended empirical regimen for MDR-TB can be prescribed [15]. This regimen is composed of at least five effective drugs, from the groups recently updated by the WHO (Table 1), during the intensive phase of treatment: a FLQ from group A (LFX, MFX, GFX), a second-line injectable drug from group B (AMK, CAP, KAN, STR), at least two drugs from group C (ETO/PTO, cycloserine, linezolid, clofazimine), and PZA (group D1) (Table2) [15]. It is also recommended to strengthen the regimen with high-dose INH and/or EMB (group D1). Agents from groups D2 and D3 may be used to complete a regimen with five effective drugs [15]. The treatment should be prolonged during at least 20 months: an intensive phase of 8 months minimum and a continuation phase of 12-18 months [15]. Nevertheless, a short-course MDR-TB regimen of 9-12 months, derived from the Bangladesh regimen, has been recently recommended by the WHO, in cases where FLQ and second-line injectable drugs are likely to be effective, among other criteria [15]. This regimen consists of an intensive phase of treatment of 4 to 6 months with GFX (or MFX), KAN, PTO, CFZ, high-dose INH, PZA, and EMB, and a continuation phase of 5 months with GFX (or MFX), CFZ, PZA, and EMB (Table2) [15]. Nevertheless, it has been recently assessed that in the European region of the WHO only 4% of the MDR-TB cases could benefit from this regimen [37].

Second-line drugs are more toxic and expensive than first-line drugs. Minor adverse effects, which can be managed with symptomatic treatment, are common, but some severe adverse effects have also been reported [7,38]. The treatment outcomes among MDR-TB patients in 2012 were success in 50% of the cases, failure in 10%, death in 18%, and lost to follow-up or not evaluated in 22% [1]. It is of note that the treatment success rates of MDR-TB may be underestimated using the current WHO definitions [39], as outlined in a recent report that compared the treatment outcomes using the WHO definitions and the new simplified definitions proposed [40].

Resistance to at least INH and RIF (i.e. MDR), plus additional resistance to any FLQ, and at least one of the second-line injectable drugs (AMK, CAP, and KAN) is defined as extensively drug resistance (XDR). Treatment of XDR-TB should include six active drugs (or more) during the intensive phase, and four drugs during the continuation phase [21,41]. Most regimens are individually tailored based on DST results, but in general, the principles and duration for these

regimens are the same as for MDR-TB [41,42]. The treatment outcomes among XDR-TB patients in 2012 were success in 26% of the cases, failure in 19%, death in 30%, and lost to follow-up or not evaluated in 25% [1].

Table 2. Summary of the recommended empirical regimens for treatment of TB. Adapted from World Health Organization, 2016 [15].

Treatment	Drugs	Treatment duration
Drug susceptible TB	- INH - RIF - PZA - EMB	6-month regimen: - 2 months intensive phase (HRZE) - 4 months continuation phase (HR)
MDR-TB	- A FLQ (group A):LFX, MFX, GFX - An injectable (group B): AMK, CAP, KAN, STR - At least two drugs (group C):ETO/PTO, cycloserine, linezolid, clofazimine - PZA (group D1)	20-month regimen: - 8 months intensive phase - 12-18 months continuation phase
MDR-TB short regimen	- A FLQ (group A): GFX (or MFX) - An injectable (group B): KAN - Two drugs (group C): PTO and CFZ - High-dose INH, PZA, and EMB (group D1)	9-12-month regimen: - 4 months intensive phase - 5 months continuation phase

HRZE: isoniazid, rifampicin, pyrazinamide, ethambutol; HR: isoniazid and rifampicin.

1.4.3. New treatment options

Several new or repurposed anti-TB drugs are in advanced phases of clinical development [1,42]. Bedaquiline and delamanid are the first new anti-TB drugs approved by the Food and Drug Administration in 40 years, and WHO issued policy guidance for safe and rational use of these drugs in 2013 and 2014, respectively [43,44]. Given the uncertainty on potential advantages and risks, bedaquiline or delamanid may be added to a WHO-recommended regimen in adult patients with pulmonary MDR-TB. However, data on the simultaneous use of these drugs is not still available [42,44].

Several trials are currently in progress to assess new treatment regimens for drug-resistant and drug-susceptible TB using different combinations and doses of anti-TB drugs [21,42]. Other strategies are also being explored, such as routes of administration, adjunct host-directed therapies for potentially enhancing immune responses, or co-administration of efflux pump inhibitors to partly restore susceptibility to anti-TB drugs [42]. Finally, in order to guarantee efficacy of treatment, adherence, adequate follow-up, and support of patients should be assured [3].

1.5. Principles of phenotypic drug susceptibility testing

Phenotypic DST of *M. tuberculosis* assesses the ability of strains to grow in the presence of a given drug. Phenotypic DST methods are classified as direct -if performed from the specimen- or indirect -if performed from the culture. Due to the slow growth rate of *M. tuberculosis*, culture and phenotypic DST takes several weeks [45].

Phenotypic resistance is considered to be clinically significant when at least 1% of the total bacterial population is able to grow in the presence of a drug at a given critical concentration. If at least 1% of the organisms are resistant, the whole population is considered resistant, whereas if less than 1% of the organisms are resistant, the whole population is considered susceptible [45].

The diagnostic accuracy and reproducibility of phenotypic DST are generally high for RIF, INH, FLQ, and injectable drugs, but they are lower for EMB, STR, and PZA [7,46-48]. In addition, phenotypic DST is not available in many geographical areas, and standardised methodologies for some drugs have not been established.

There are several phenotypic DST methods, which can be performed in solid or liquid media and in commercial and non-commercial systems.

1.5.1. Phenotypic drug susceptibility testing on solid media

There are three main phenotypic DST methods that can be performed on solid media (agar-based media, egg-based media): the absolute concentration method, the resistance ratio method, and the proportion method. These methods yield equivalent results, although the proportion method is the one most often used [7]. *M. tuberculosis* culture on solid media may take up to 8 weeks to grow, and phenotypic DST may take a further 2 to 8 weeks [45].

For the three methods, each strain tested is inoculated in several culture tubes containing different drug concentrations and in drug-free control tubes [45]. It is of note that for the proportion method the control tubes are inoculated with a dilution of the tested strain [45]. After a minimum four-week incubation at 37ºC, cultures are read. In the absolute

concentration method, the tested strain is considered resistant when there is growth (defined as more than 20 colonies) at the critical concentration, whereas the tested strain is considered susceptible if there is not enough growth [45]. In the resistance ratio method, the resistance ratio is the minimal concentration that inhibits growth of the tested strain divided by the minimal concentration that inhibits growth of H37Rv, the reference susceptible strain included in each set of tests. The tested strain is considered resistant when the resistance ratio is 8 or more, whereas the tested strain is considered susceptible when the resistance ratio is of 2 or less [45]. Finally, in the proportion method, the number of resistant bacilli units corresponds to the number of colonies in the drug-containing tube, and the number of viable bacilli units corresponds to the number of colonies in the control tube inoculated with the lowest dilution that is positive. The tested strain is considered resistant when at least 1% of the bacilli are resistant at the critical concentration [45]. The critical concentration is the lowest concentration of drug at which >95% of susceptible bacilli are unable to grow [49]. The critical concentrations of many anti-TB drugs are published [50,51].

1.5.2. Phenotypic drug susceptibility testing in liquid media

There are different broth-based systems commercially available. The first most used system was the radiometric BACTEC 460TB (Becton Dickinson Diagnostic Systems, USA), based on the detection of labelled $^{14}CO_2$ released upon metabolization of (1-^{14}C)palmitic acid due to mycobacterial growth [52]. The radiometric BACTEC 460TB was later substituted by the non-radiometric MGIT 960 (Mycobacteria growth in tube, Becton Dickinson Diagnostic Systems, USA). This method is based on the detection of an oxygen-quenched indicator, which fluoresces upon consumption of the oxygen due to growth [53]. Another method is the VersaTREK Myco susceptibility kit (TREK Diagnostics, USA), based on detection of pressure changes due to the oxygen consumption as a result of microbial growth [54]. Finally, another method is BacT/ALERT 3D system (bioMérieux, USA), which detects a colour change in a sensor in the culture bottle, produced by the pH decrease due to the CO_2 released as a result of growth [55].

1.6. Principles of genotypic drug susceptibility testing

Genotypic DST is based on the detection of mutations associated with drug resistance with molecular methods. As mentioned for phenotypic DST, genotypic DST can also be classified as direct or indirect. The general procedure of molecular methods consists on DNA extraction from either the specimen or the cultured strain, PCR amplification of specific genomic regions, and detection of the mutations with different methods. Several methods have been developed to detect the main mutations involved in drug resistance.

1.6.1. DNA sequencing

DNA sequencing has been the reference standard molecular method for detecting the main mutations involved in drug resistance [56-58]. In dye-terminator sequencing, after PCR, another round of amplification is performed with four dideoxynucleotide (ddNTP) chain terminators labelled with different fluorescent dyes. Subsequently, fragments are separated by capillary electrophoresis and fluorescence is detected, producing a chromatogram where wild-type sequences or mutations can be identified (Figure 2).

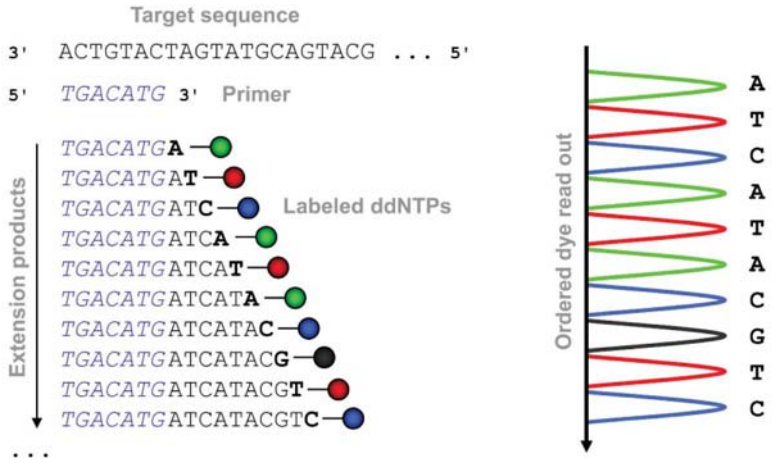


Figure 2. Principle of DNA sequencing by the chain-termination method. Adapted from Kircher et al [59].

1.6.2. Reverse hybridization line probe assay or arrays

Detection of mutations by solid-phase reverse hybridization can be performed with line probe assay (LPAs) on strips (Figure 3) or arrays. The method consists on denaturalization of the PCR product and hybridization to specific probes immobilized to a nitrocellulose membrane (strip or array). After the hybridization step, the streptavidin-alkaline phosphatase conjugate is added, and streptavidin binds to the biotinylated PCR product. Subsequently, the substrate chromogen NBT/BCIP (Nitro blue tetrazolium / 5-Bromo-4-chloro-3-indolyl phosphate) is added, resulting in the formation of a purple precipitate and colour development where the alkaline phosphatase is fixed. Mutations can be directly detected by hybridization on mutation probes and/or indirectly detected by absence of hybridization of the wild-type probes.

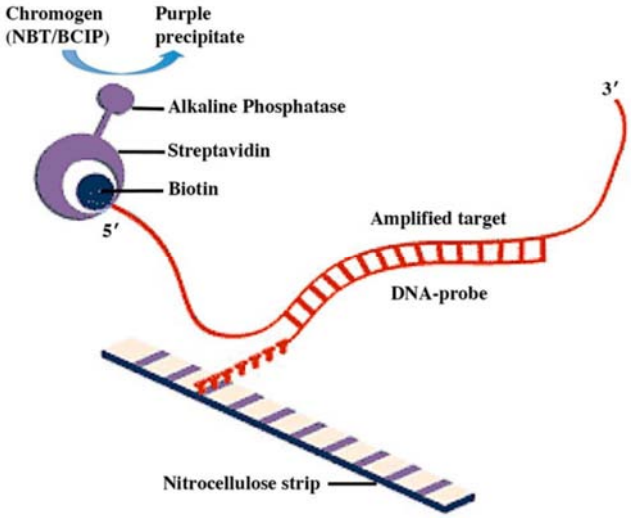


Figure 3. Principle of solid-phase reverse hybridization (Micalessi et al) [60].

Different LPAs that detect resistance to several anti-TB drugs are commercially available: INNO LiPA Rif.TB [61] (Fujirebio, Belgium), GenoType MTBDRplus [62,63] and GenoType MTBDRsl [64,65] (Hain Lifescience, Germany), AID TB Resistance assay [66] (AutoimmunDiagnostika, Germany), and Nipro LiPA [67] (Nipro Corporation, Japan). It is of note that a recent study demonstrated the noninferiority of GenoType MTBDRplus version 2 and NiproNTM+MDRTB 2 to GenoType MTBDRplus version 1 [68].

LPAs are endorsed by the WHO for rapid screening of RIF and INH resistance [69] and second-line drugs [15]. These tests are frequently used for the initial diagnosis of MDR and XDR-TB [70].

1.6.3. Multiplex and real-time PCR

Several in-house multiplex and real-time PCR assays have been developed to detect resistance to first- and second-line drugs [71-73]. These assays are based on detection of the DNA amplified in each cycle by probes labelled with fluorophores, including Taqman probes [74], molecular beacons [75], or locked nucleic acid probes [76]. However, few tests are commercially available, such as Anyplex II MTB/MDR/XDR (Seegene, Korea), aimed to detect MDR/XDR-TB, and MeltPro TB (Zeesan Biotech, China), aimed to detect resistance to INH, STR, and MDR/XDR-TB [77-79].

1.6.4. GeneXpert MTB/RIF

GeneXpert MTB/RIF (Cepheid, USA) is an integrated micro-fluidic based system based on real-time PCR, aimed to diagnose TB and RIF resistance directly from the specimen (Figure 4) [80]. The specimen is treated with a reagent for 15 minutes and transferred into a cartridge, which contains all the necessary reagents for DNA extraction and real-time amplification inside the GeneXpert device. Real-time PCR is based on molecular beacons technology in which different fluorescent-labelled probes target wild-type sequences, and the absence of binding of any probe indicates RIF resistance [80]. The result is obtained within 2 hours, reporting whether the sample is negative or positive for *M. tuberculosis*, semiquantifying the bacillary load, and whether RIF resistance is present [80].

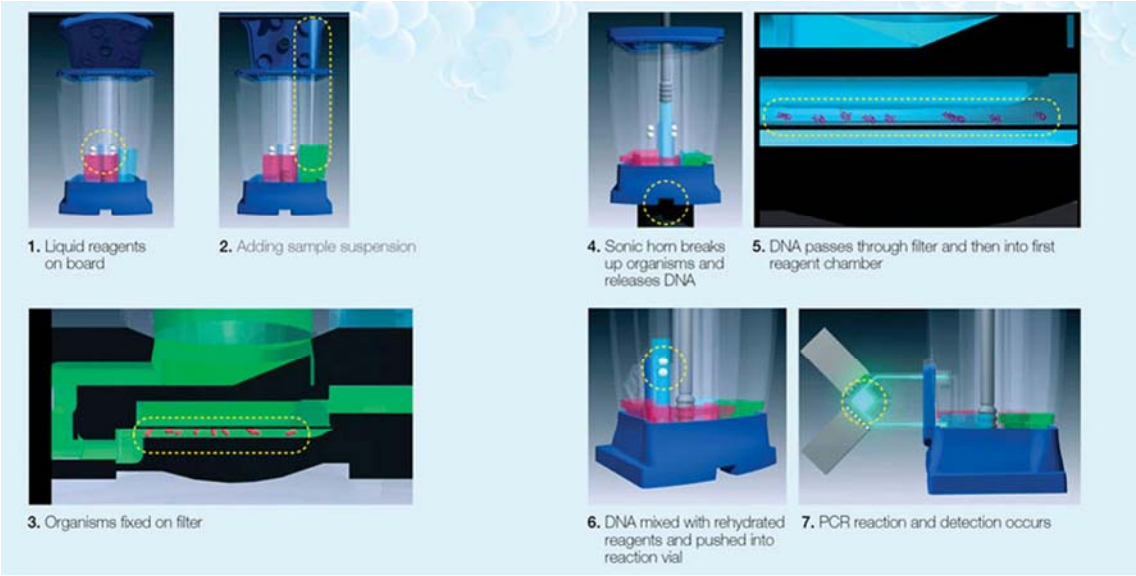


Figure 4. GeneXpert MTB/RIF reaction steps.
(http://www.cepheid.com/images/Cepheid-OnDemand/Fall-2010/cover_figures3.jpg)

GeneXpert MTB/RIF is recommended by the WHO as the initial diagnostic test for adults and children presumed to have MDR-TB or HIV-associated TB [80]. The pooled sensitivity and specificity of this test for detecting *M. tuberculosis* are 89% and 99%, respectively; whereas the pooled sensitivity and specificity for detecting RIF resistance are 95% and 98%, respectively [81]. In summary, GeneXpert MTB/RIF presents high accuracy and robustness, low risk and ease of use, and moderate requirements, but high economical cost [7].

1.6.5. Pyrosequencing

Pyrosequencing is based on DNA sequencing by synthesis [82]. After PCR, single-stranded DNA (ssDNA) is prepared to serve as the template where the pyrosequencing primer anneals. In each cycle of the pyrosequencing reaction, a nucleotide is added, and if it is complementary to the one present in the template, it is incorporated by the DNA polymerase. Upon incorporation of the nucleotide, a pyrophosphate (PPi) is released, which is quantitatively converted to ATP by sulfurylase, and ATP is used by luciferase to produce visible light that is measured in the pyrosequencer. At the end of each cycle, the unincorporated nucleotides are degraded by the apyrase. During the run, a pyrogram and the sequence are obtained (Figure 5).

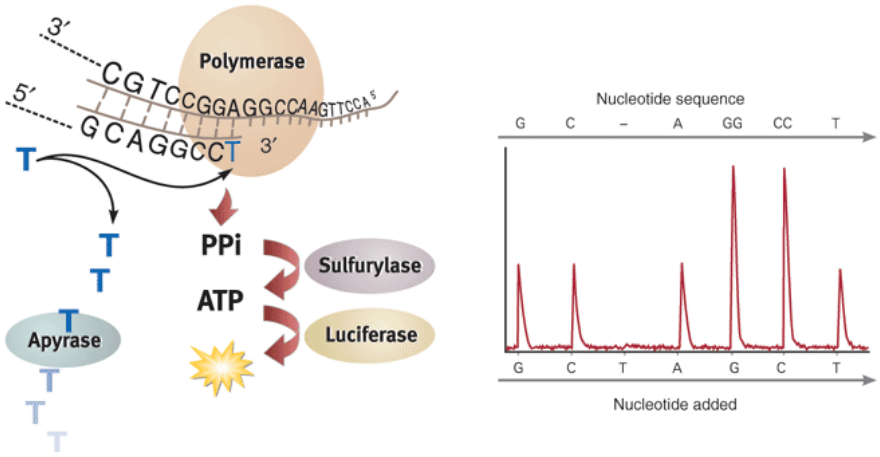


Figure 5. Principle of pyrosequencing.
(http://www.nature.com/app_notes/nmeth/2005/050929/full/nmeth800.html)

1.6.6. Multiplex PCR and microbead-based hybridization

This method consists on multiplex PCR amplification using the dual priming oligonucleotide (DPO) technology, and subsequent hybridization to probes attached to individually labelled microbeads. Afterwards, streptavidin-phycoerythrin is bound to the biotinylated DNA, and fluorescence is measured in the flow cytometry-based Luminex device, upon individual detection of the beads (Figure 6).

Figure 6. Principle of multiplex PCR (2), microbead-based hybridization (3), and detection by flow cytometry (4).
(https://www.luminexcorp.com/wp-content/uploads/sites/3/2013/04/fast.png)

1.6.7. Next generation sequencing

Next generation sequencing is the most up-to-date method, implemented in very few specialised centres, for whole genome sequencing (WGS) analysis. There are different next generation sequencing technologies, based on pyrosequencing (454 Life Sciences; Roche, France), sequencing by oligonucleotide ligation and detection (SOLiD; Applied Biosystems, USA), and sequencing by incorporation of fluorescently labelled nucleotides (Illumina, USA), which is the most used method [83].

The Illumina technology consists on preparing the genomic DNA by random fragmentation and ligation of adapters, and attaching the ssDNA fragments to a flow cell where bridge amplification takes place. After, in each cycle of the sequencing by synthesis process, the incorporated nucleotide is identified by the emitted fluorescence. After multiple cycles, the reads are checked and aligned to a reference genome, and the variants are called and filtered (Figure 7).

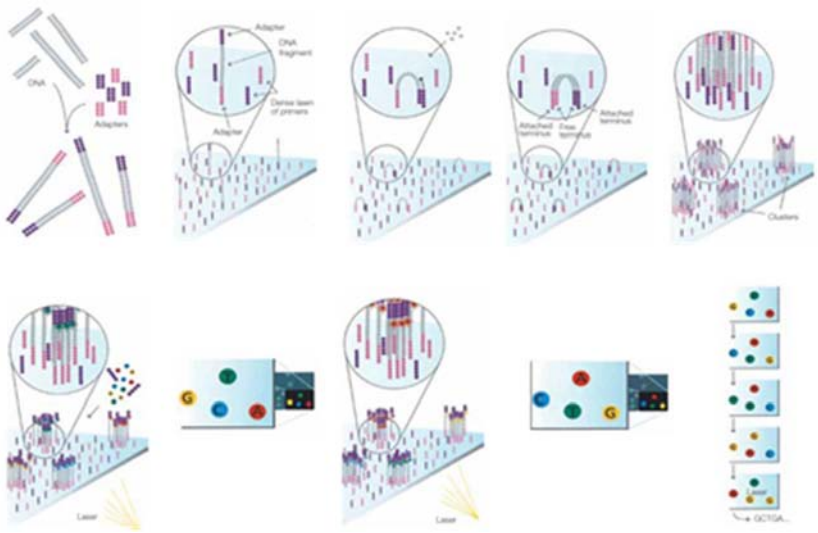


Figure 7. Principle of next generation sequencing by Illumina. Adapted from Technology Spotlight: Illumina® Sequencing.
(http://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf)

WGS has been used for the detection of drug resistance and for epidemiological investigations [84]. Data obtained by WGS provides useful information regarding the previously known mutations conferring drug resistance to all first- and second-line drugs [85,86]. In addition, WGS also allows identifying new genes and mutations of relevance to drug resistance [86].

1.7. Principles of molecular typing

Molecular typing or genotyping is aimed to assess the relatedness of the strains based on polymorphisms of different conserved regions of the genome. Genotyping of *M. tuberculosis* strains has been a valuable tool for TB control and has improved the knowledge about global TB epidemiology. Several different genotyping methods have been developed [87,88], and the selection of the method depends on the objective of the investigation [88,89]. A typing method based on a highly discriminatory and polymorphic but stable marker may serve to identify index cases, assess the genetic relatedness and the epidemiological links among TB cases, detect outbreaks, investigate possible laboratory cross-contaminations, and differentiate relapses from new infections. On the contrary, a typing method based on a phylogenetically robust marker may serve to characterize the evolution and global phylogeny of *M. tuberculosis*, define the phylogeographic specificity of circulating clades in population-based studies, and screen epidemiological links [87].

An optimal typing method for molecular epidemiology studies should be technically simple, reproducible, robust, cost effective, and rapid. In addition, it should be possible to use the method directly on clinical specimens, and the format of the results should be easily interpretable, standardized, and portable for database creation and interlaboratory comparison.

The most used methods are based on the polymorphism of the insertion sequence (IS) *6110*, the CRISPR (clustered regularly interspaced short palindromic repeats) locus (formerly known as direct repeat), and the mycobacterial interspersed repetitive units - variable number tandem repeats (MIRU-VNTR) (Figure 8). In addition, WGS is increasingly being used for epidemiological transmission studies, as it has a higher discriminatory power compared to the other molecular typing methods [90]: WGS is able to resolve false clusters defined by other genotyping methods [91], ruling out false transmission events and reducing unnecessary public health measures; also, WGS may identify transmission events missed by conventional epidemiological investigations [90].

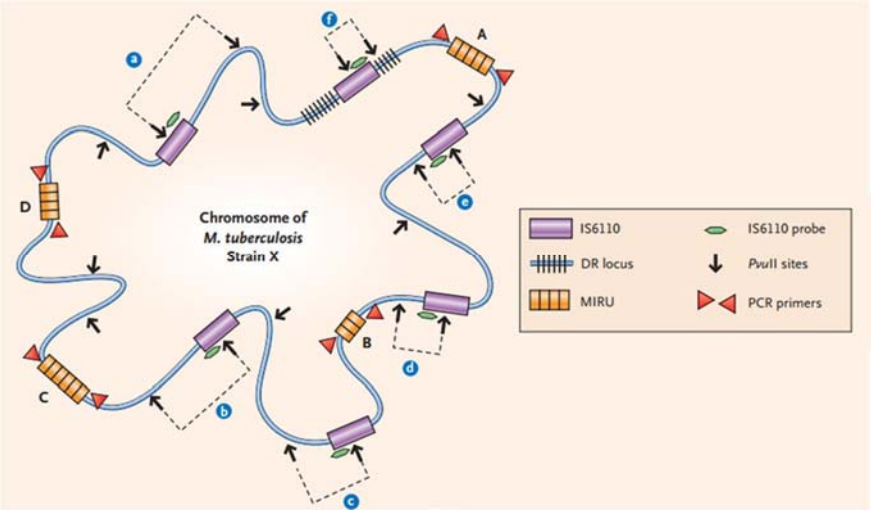


Figure 8. Different genotyping markers –IS*6110*, DR locus (CRISPR), and MIRU- depicted in the chromosome of a *M. tuberculosis* strain. Adapted from Barnes et al [92].

1.7.1. IS*6110* restriction fragment length polymorphism (RFLP)

IS*6110* restriction fragment length polymorphism (RFLP) is a genotyping method based on the polymorphism of copy number and location of the IS*6110*. Members of the *M. tuberculosis* complex usually harbour multiple copies of the IS*6110* (Figure 9), ranging from 0 to 25 copies, with the exception of *Mycobacterium bovis*, which usually harbours a single copy [93,94]. The IS*6110* copy number depends on the frequency of transposition, and the location is generally random, although there are integration hot spots where the frequency of transposition is higher [95]. The IS*6110*-RFLP procedure consists on DNA extraction of the genomic DNA, digestion with restriction endonucleases, separation of the restricted fragments by electrophoresis, hybridization with an IS*6110* probe by southern blot, and detection by autoradiography [96].

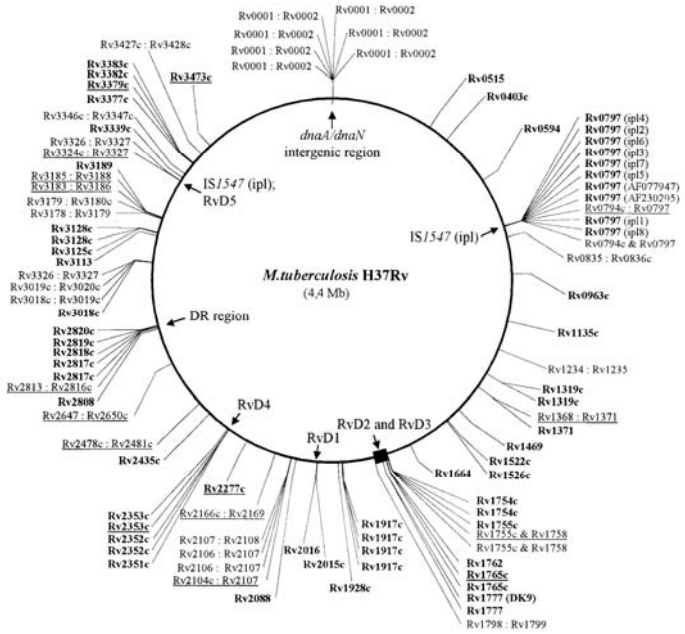


Figure 9. Distribution of the IS*6110* in the *M. tuberculosis* H37Rv genome. The insertion sites are underlined (Sampson et al) [97].

1.7.2. Spoligotyping

Spoligotyping (spacer oligonucleotide typing) is based on the polymorphism of the CRISPR locus, specific of *M. tuberculosis* complex [98]. This locus contains multiple, well-conserved direct repeats (DRs) of 36 base pairs (bp) interspaced with spacer sequences of 34-41 bp (Figure 10). Strains vary in the number of DRs and in the presence or absence of particular spacers. For example, the reference *M. tuberculosis* strain H37Rv is characterized by the absence of spacers 20, 21, and 33 to 36, whereas *M. bovis* BCG is characterized by the absence of spacers 3, 9, 16, and 39 to 43 [98]. The spoligotyping method consists on PCR amplification of the CRISPR locus and detection of the presence or absence of 43 specific spacers by reverse hybridization [98].

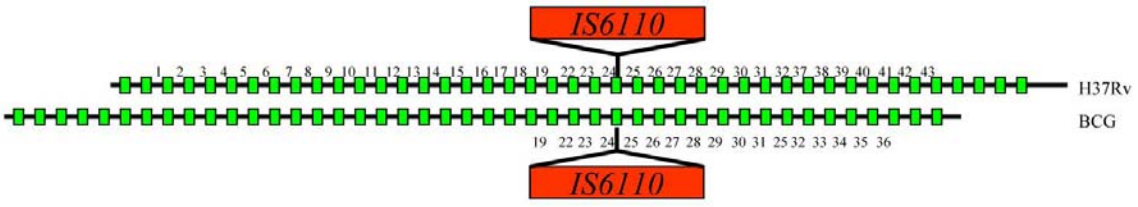


Figure 10. Structure of the CRISPR locus, with 36-bp direct repeats (green) interspaced with 34-41 bp spacer sequences (Spoligotyping kit manual, Isogen Lifescience).
(http://mycobactoscana.it/Manuale/PDF/Alleg12.1.pdf)

1.7.3. Mycobacterial interspersed repetitive units - variable number tandem repeats (MIRU-VNTR)

This method is based on the polymorphism of copy number of the repeats of the MIRU present in several loci of the *M. tuberculosis* genome. Several MIRU loci have been identified, and different sets of MIRU-VNTR have been described for high-discriminatory genotyping [99]. The procedure consists on PCR amplification of MIRU loci and determination of the size of the amplified fragments, indicative of the number of repetitive units, by gel electrophoresis [99], automated capillary sequencers, or the QIAxcel automated capillary electrophoresis system (Qiagen, The Netherlands) [100]. Results are expressed as numerical codes indicating the number of copies of each MIRU-VNTR analysed.

JUSTIFICATION

2. JUSTIFICATION

TB remains one of the major infectious diseases worldwide, with 9.6 million people estimated to have developed the disease in 2014. In addition, the emergence and spread of drug-resistant strains of *M. tuberculosis* is a public health threat. In order to improve TB control, rapid diagnosis of the disease and starting of an adequate treatment are required. Treatment regimens should be adjusted to the susceptibility pattern of the strain, but due to the slow growth rate of *M. tuberculosis*, availability of phenotypic DST results may take several weeks.

Molecular basis of *M. tuberculosis* resistance to some drugs has been elucidated, and genetic resistance has been mainly attributed to single nucleotide mutations in the chromosome. In order to rapidly detect these mutations and the associated drug resistance, different molecular methods, either commercial or in-house, have been developed. Diagnostic accuracy studies of molecular methods are needed in order to assess their usefulness in the clinical practice. Furthermore, in order to guide therapeutic decisions, it is essential to consider the impact that the mutations detected by the molecular methods may have on phenotypic drug resistance on the *M. tuberculosis* strains, and on the clinical outcome as well.

In addition to the rapid diagnosis and treatment, tracking the spread of *M. tuberculosis* strains is important for TB control, in order to detect outbreaks and epidemiological links among TB cases, differentiate relapse cases from new infections, and characterise the evolution and global migration of *M. tuberculosis*. The most widely used molecular typing methods present disadvantages: some methods are slow, laborious, time-consuming, and low-throughput, whereas other methods present low-discriminatory power. Therefore, rapid, technically simple, high-throughput, and discriminatory methods could be useful.

OBJECTIVES

3. OBJECTIVES

1. To develop, optimize, and evaluate molecular methods for detecting drug resistance in *M. tuberculosis* clinical strains and in patient specimens.
   1.1. To evaluate the usefulness of molecular methods for detecting resistance to first-line drugs (Articles I, II and II).
   1.2. To assess the usefulness of molecular methods for detecting resistance to second-line drugs (Articles II, III and IV).

2. To review the current molecular methods used for TB management and their impact on the clinical practice.
   2.1. To summarise the new molecular methods available for diagnosis, identification, detection of drug resistance, and epidemiology of TB (Article V).
   2.2. To discuss the relationship between molecular and phenotypic drug resistance, and clinical outcomes (Article VI).

3. To develop and evaluate novel molecular methods for genotyping *M. tuberculosis*.
   3.1. To evaluate a microbead-based spoligotyping method, TB-SPRINT (Article VII).
   3.2. To develop a pyrosequencing-based genotyping method, PyroTyping (Article VIII).

MATERIAL AND METHODS

4. MATERIAL AND METHODS

4.1. Specimens and clinical strains

Respiratory clinical specimens were retrospectively selected from a collection of specimens recovered in Hospital Universitari Germans Trias i Pujol (Articles I, II, and III). Specimens were digested and decontaminated using Kubica's N-acetyl-L-cysteine NaOH method [101,102]. After decontamination, auramine-rhodamine acid-fast staining was performed from the concentrated sediment. Smears were graded as follows: smear negative, absence of acid fast bacilli in 300 fields; smear 1+, one to ten bacilli in 100 fields; smear 2+, one to nine bacilli per field in 100 fields; and smear 3+, more than nine bacilli per field in 100 fields. The concentrated sediment was suspended and an aliquot was inoculated on Löwenstein-Jensen solid and BACTEC 460TB or MGIT 960 liquid media. The remaining decontaminated specimen was stored at -20ºC. Identification of *M. tuberculosis* in positive cultures was performed with Inno-Lipa Mycobacteria v2 assay (Innogenetics, Belgium).

M. tuberculosis clinical strains and specimens were retrospectively selected from collections in Hospital Universitari Germans Trias i Pujol (Badalona, Spain), National Tuberculosis and Infectious Diseases University Hospital (Vilnius, Lithuania), and Instituto Aragonés de Ciencias de la Salud (Zaragoza, Spain) (Articles I, II, III, IV, VII, and VIII).

Specimens and clinical strains were selected on the basis of previous characterization by means of phenotypic and molecular drug susceptibility testing for first- and/or second-line drugs (Articles I, II, III, and IV), or standard molecular genotyping methods (Articles VII and VIII). All the studies were approved by the institutional ethics committee.

4.2. Phenotypic detection of drug resistance

Phenotypic drug resistance was assessed by either the radiometric method BACTEC 460TB or the non-radiometric method MGIT 960, according to the period and laboratory of testing. For drug susceptibility testing, the test drugs were incorporated in the liquid medium bottles at the recommended critical concentrations (Articles I, II, III, and IV), and the clinical strain suspension was inoculated. Based on the proportion method, a 1:100 dilution of the strain suspension was inoculated in the drug-free control bottle.

In the radiometric method, the inoculated bottles were incubated at 37°C and the radioactivity was measured daily with the BACTEC 460TB instrument. If the daily increase of the growth index reported for the control tube was greater than the growth index for the drug-containing tube, the strain was susceptible to that drug; conversely, if the daily increase of growth index in the control tube was less than in the drug-containing tube, the strain was resistant to that drug [52].

In the non-radiometric method, the tubes were incubated in the MGIT 960 instrument and automatically read until the test concluded, when the control reached 400 growth units [103]. If the drug-free control registered growth before the drug-containing bottle, the strain was susceptible; conversely, if the drug-containing bottle registered growth before the drug-free control, the strain was resistant [104].

4.3. Molecular methods for detecting drug resistance

All the molecular methods were performed using either DNA extracted from clinical specimens with the commercial Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, USA) (Article III), or crude DNA extracts obtained from clinical specimens or clinical strains as previously described [62]. Briefly, few colonies of clinical strains cultured in solid media were resuspended in 300 µl of molecular biology-grade water; or a 1 ml aliquot of the decontaminated specimen was centrifuged at 10,000 x g for 15 min, the supernatant was discarded, and the pellet was resuspended in 100 µl of molecular biology-grade water. These preparations of clinical strains and specimens were then incubated at 95°C for 20 min, sonicated for 15 min, and centrifuged at 22,000 x g for 5 min, and the supernatant was collected. DNA extracts were stored at -20ºC until use.

4.3.1. Reverse hybridization array

The reverse hybridization array evaluated (GenoFlow DR-MTB Array Test, DiagCor Bioscience, Hong Kong) is aimed to detect mutations in *rpoB*, *katG*, and *inhA* promoter associated with resistance to RIF and INH (Article I). The method is based on PCR amplification and reverse hybridization on arrays using the FT-PRO flow-through system.

The test was performed following the manufacturer's instructions. Briefly, the PCR reaction was prepared with 19.6 µl of PCR Master Mix, 2 µl of Primer Mix, 0.1 µl of Internal Amplification Control, 0.4 µl of DNA Taq Polymerase, 0.9 µl of DNase-free water, and 2 µl of DNA template. PCR amplification conditions were the following: 95ºC for 9 min; 45 cycles of 95ºC for 20 s, 61ºC for 25 s, and 72ºC for 30 s; and 72ºC for 8 min.

After PCR, hybridization and detection were performed in the FT-PRO flow-through system (Figure 11). In short, pre-hybridization (5 min), hybridization of the heat-denaturated PCR product (5 min), and post-hybridization washes were performed at 46ºC; first blocking step (5 min) and enzyme conjugation (5 min) were performed at 25ºC; finally, post-conjugation washes, second blocking step, colour development (5 min) and washes, and reaction stop were performed at 36ºC. It is of note that after each incubation, the liquid was drained by

flow-through in the device. The results obtained were recorded and automatically interpreted by the DiagCor software (Figure 11), and were confirmed visually by the researcher.

Figure 11. Reverse hybridization array (A), FT-PRO flow-through hybridization system (B), and Capture-PRO image capture system (C).
(B. http://www.diagcor.com/en/mdx-products/detail/ft-pro-flow-through-hybridization-system)
(C. http://www.diagcor.com/en/mdx-products/detail/capture-pro-image-capture-system)

4.3.2. Multiplex PCR

The multiplex PCR test evaluated (Anyplex II MTB/MDR/XDR, Seegene, Korea) was aimed to detect mutations in *rpoB*, *katG*, *inhA* promoter, *gyrA*, *rrs*, and *eis* promoter associated with resistance to RIF, INH, FLQ, and second-line injectable drugs (Article II).

The method is based on (dual priming oligonucleotide) DPO and tagging oligonucleotide cleavage and extension (TOCE) technologies. DPO [105] consists on amplification primers composed by two fragments separated by a polydeoxyinosine linker: the longer 5' fragment allows stable priming, and the shorter 3' fragment allows target-specific extension. This technology enable specific multiplex amplification with longer primers without increasing the annealing temperature. TOCE technology consists on detection of target sequences by the use of pitcher probes, which hybridize to the target templates, and the catcher probes, which are the reporter molecules. The pitcher is composed of the targeting portion, which is specific for the target template, and the tagging portion, which does not anneal with the target template. Upon amplification of the template, the tagging portion is cleaved and released. The released tagging portion is complementary to the capturing portion of the catcher and serves as a primer for extension. Upon extension of the catcher, fluorescence signal is generated (Figure 12).

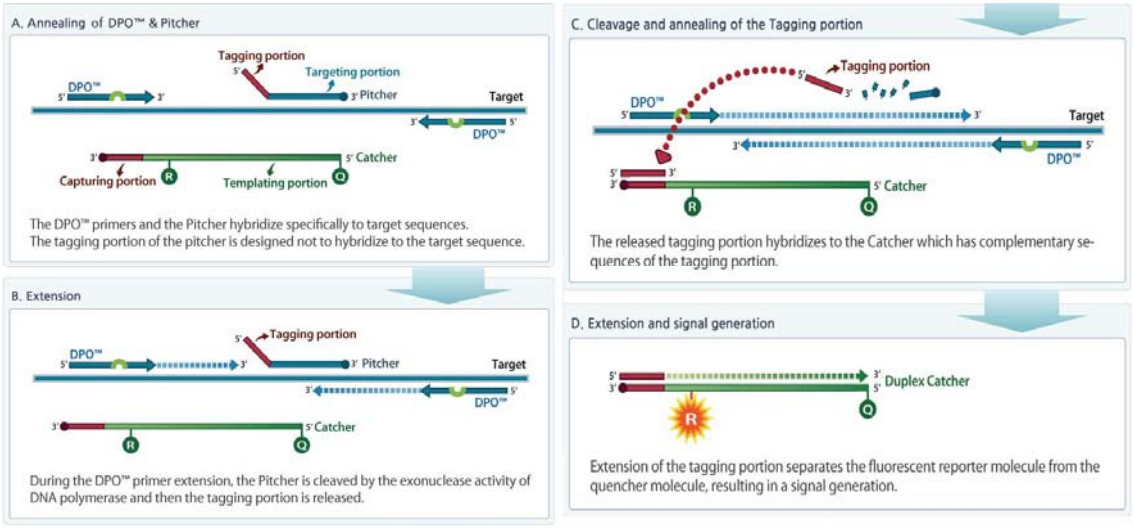


Figure 12. Principle of the Anyplex II MTB/MDR/XDR assay, based on dual priming oligonucleotide (DPO) and tagging oligonucleotide cleavage and extension (TOCE) technologies.
(www.seegene.com/neo/en/introduction/core_toce.php)

The multiplex PCR assay Anyplex II MTB/MDR/XDR includes a pitcher probe with a specific targeting portion for each of the mutations targeted (Article II). However, all the pitcher probes that target resistance to the same drug share a tagging portion that is complementary to a catcher probe with a specific fluorophore: HEX fluorophore (6-carboxy-2,4,4,5,7,7-hexachlorofluorescein succinimidyl ester) for RIF and FLQ resistance, and Cal Red 610 fluorophore for INH and injectable drugs resistance. Therefore, two PCR reactions were performed: one reaction for detecting resistance to INH and RIF (MTB/MDR), and another reaction for FLQ and injectable drugs (MTB/XDR) (Article II).

Following the manufacturer's instructions, PCR reactions were prepared with 5 µl 4X MTB/MDR or MTB/XDR oligo mix, 5 µl 4X Anyplex PCR Master Mix, 5 µl RNase-free water, and 5 µl of DNA template. PCR amplification conditions were the following: 95ºC for 15 min; 50 cycles of 95ºC for 30 s, 60ºC for 1 min, and 72ºC for 30 s; 55ºC for 30 s; and the melting curve detection step at 55-85ºC for 5s/0.5ºC. After amplification, the presence of a melting curve indicates resistance to the corresponding drug, whereas absence of a melting curve indicates susceptibility, and the Anyplex software yields a table reporting the results (Article II).

4.3.3. Reverse hybridization line probe assay

The reverse hybridization LPA evaluated (AID TB resistance, AID Diagnostika, Germany) was aimed to detect mutations in *rpoB*, *katG*, *inhA* promoter, *gyrA*, *embB*, *rrs*, and *rpsL* associated with resistance to RIF, INH, FLQ, EMB, KAN, AMK, CAP, and STR (Article III). The method is based on PCR amplification and reverse hybridization on three different nitrocellulose strips.

The test was performed following the manufacturer's instructions. Briefly, the PCR reaction was prepared with 15 µl of Primer Nucleotide Mix, 2.5 µl of 10X polymerase buffer, 1.5 µl of $MgCl_2$, 0.2 µl of GoTaq® Polymerase (Promega, USA), and 5.8 µl of DNA template. PCR amplification conditions were the following: 95ºC for 5 min; 14 cycles of 95ºC for 30 s and 63ºC for 2 min; 26 cycles of 95ºC for 10 s, 55ºC for 30 s, and 72ºC for 30 s; and 72ºC for 8 min.

Hybridization and detection were performed with AutoLipa (Fujirebio, Belgium), an automated washing and shaking device. In short, hybridization of the denaturated PCR product (30 min), and post-hybridization washes (15 min) were performed at 47ºC, and rinse, enzyme conjugation (30 min), post-conjugation washes, colour development (15 min), and reaction stop were performed at room temperature. The results obtained were interpreted by the researcher (Article III). For those cases with unspecific background bands after the hybridization step, DNA was diluted 1:10 and the assay was repeated from the PCR step.

4.3.4. Pyrosequencing

This pyrosequencing assay was aimed to detect mutations in *gyrA*, *rrs*, *eis* promoter, and *embB* associated with resistance to FLQ, KAN, AMK, CAP, and EMB (Article IV).

Individual PCR reactions for each genomic region were prepared in a final volume of 25µl with 15 µl of ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma Aldrich, USA), 0.4 µM of each forward and reverse primer [65] (Article IV), 7.8 µl of DNase-free water, and 2 µl of DNA template. PCR amplification conditions were the following: 95ºC for 12 min; 45 cycles of 94ºC for 30s, 60ºC for 1 min, and 72ºC for 2 min; and 72ºC for 7 min.

After PCR, ssDNA was prepared by serial washings with a vacuum preparation tool and deposited in the pyrosequencing 96-well plate, where the pyrosequencing primer [65] (Article

IV) was annealed. The pyrosequencing plate and the cartridge with the reagents (enzyme mix, substrate mix, and nucleotides) were loaded in the pyrosequencer, where the reaction took place (Figure 13). Pyrosequencing results were interpreted by analysing the pyrograms and the associated sequence.

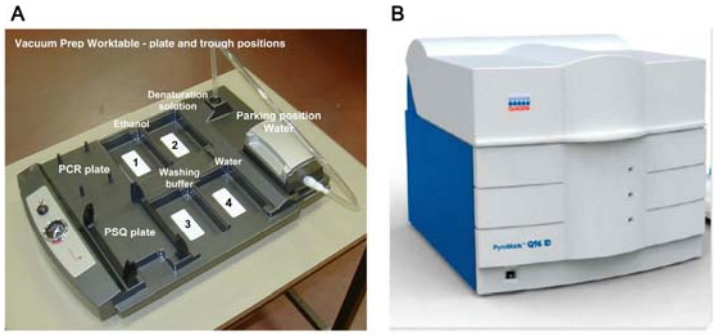


Figure 13. Vacuum preparation tool (A) and pyrosequencing device (B).
(A. Sample Preparation Guidelines for PSQ™96 and PSQ 96MA Systems. Biotage)
(B. EpiTect™ and PyroMark™. A novel Relation setting Standards for the reliable Detection and accurate Quantification of DNA-Methylation. Qiagen)

4.4. Methods for genotyping *Mycobacterium tuberculosis* strains

All genotyping methods were performed with DNA extracted from strains cultured on Löwenstein-Jensen following the cetyltrimethylammonium bromide protocol [106].

The genotyping results obtained for different strains by a given method were compared: if the genotyping patterns were identical, strains were considered to be clustered, whereas if the patterns were different, strains were considered to be unrelated (Article VIII).

4.4.1. IS*6110* restriction fragment length polymorphism (RFLP)

IS*6110*-RFLP was performed by southern blot as previously described [107]. Briefly, the procedure consists on digestion of the *M. tuberculosis* genomic DNA with the restriction endonuclease *PvuII*, separation of the fragments by agarose gel electrophoresis overnight, transference of the fragments from the gel to a positively charged nylon membrane, hybridization with an IS*6110* probe overnight, and development by autoradiography (Figure 14). The IS*6110*-RFLP result consists on a pattern of bands representing the copy number and location of the IS*6110* copies in the genome of the studied strain.

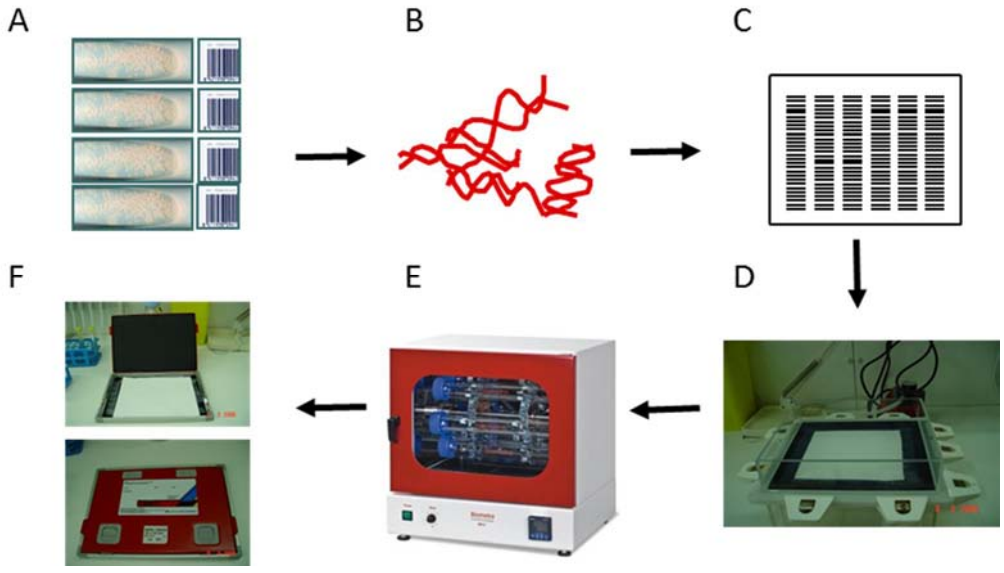


Figure 14. Procedure for RFLP analysis. Culture of the strains on solid media (A), DNA extraction and digestion (B), agarose gel electrophoresis (C), transference of the DNA to a membrane (D), hybridization with an IS*6110* probe (E), and development by autoradiography (F). Provided by Samper S et al (personal communication).

4.4.2. Spoligotyping

Spoligotyping was performed using the spoligokit (Ocimum Biosolutions, Hyderabad, India) following the adapted manufacturer's instructions. Briefly, the CRISPR locus was amplified by PCR (Figure 15). The PCR reaction was prepared with 10 µl of ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma Aldrich, USA), 2 µl of each Dra and Drb primers, 10 µl of DNase-free water, and 1 µl of DNA template. PCR amplification conditions were the following: 96ºC for 3 min; 20 cycles of 96ºC for 1 min, 55ºC for 1 min, and 72ºC for 30 s; and 72ºC for 5 min. After, PCR product was heat-denaturated and hybridized to a membrane at 60ºC for 60 min, streptavidin-peroxidase was bound to the biotinylated PCR product at 42ºC for 45 min, and the hybridization spots were detected by chemiluminescence. The spoligotyping result consists on a pattern of spots representing the presence or absence of the 43 spacers in the genome of the studied strain. Spoligotyping patterns were compared with those in the International Spoligotyping Database (SITVITWEB) (Articles VII and VIII).

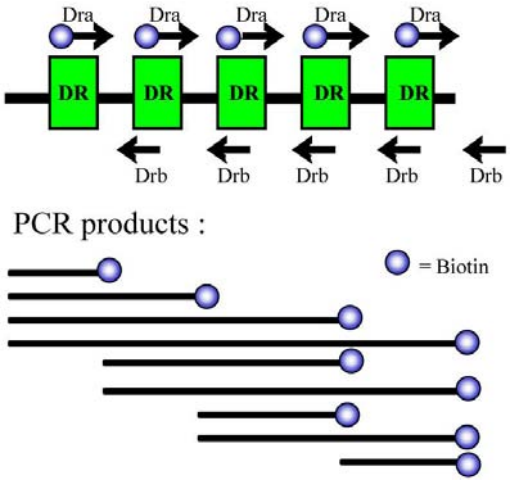


Figure 15. Principle of PCR amplification of the CRISPR locus with the use of primers Dra and Drb. (Spoligotyping kit manual, Isogen Lifescience).
(http://mycobactoscana.it/Manuale/PDF/Alleg12.1.pdf)

4.4.3. Microbead-based spoligotyping (TB-SPRINT)

The microbead-based hybridization assay evaluated (TB-SPRINT, Beamedex, France) is based on multiplex PCR using the dual priming oligonucleotide technology, followed by hybridization and detection by flow cytometry. This assay is aimed to perform spoligotyping, and to simultaneously detect mutations in *rpoB*, *katG*, and *inhA* promoter associated with resistance to RIF and INH (Article VII).

Briefly, the PCR reaction was prepared following the previously described specifications [108], in a total volume of 25 µl, with PCR buffer, 0.2 mM each dNTP, 1µM each primer, 1.0U of Taq polymerase, and 2 µl or 5 µl of DNA template (if the DNA concentration was superior or inferior to 10 ng/µl, respectively). PCR amplification conditions were the following: 95ºC for 3 min; 25 cycles of 95ºC for 30 s, 65ºC for 30 s, and 72ºC for 30 s; and 72ºC for 5 min.

Subsequently, PCR product was mixed with individually labelled microbeads with attached probes, and DNA denaturation and hybridization to the probes were performed at 95ºC for 10 min followed by 50°C for 20 min. Finally, streptavidin-phycoerythrin was added, and samples were loaded in the flow cytometry-based Luminex 200 system (Luminex Corp, USA) or BioPlex200 (Biorad, USA), where individual microbeads were identified and fluorescence was measured (Article VII).

Results obtained consisted on the value of relative fluorescence intensity for each probe. Raw values for spoligotyping and mutations associated with drug resistance were interpreted as previously described (Article VII) [108,109]. Spoligotyping patterns obtained with TB-SPRINT were compared with those in SITVITWEB, and a dendrogram was built with BioNumerics (Applied Maths, Belgium) (Article VII).

4.4.4. Pyrosequencing-based genotyping (PyroTyping)

PyroTyping is a genotyping method based on the polymorphism of the IS*6110* insertion site. It consisted on digestion of the *M. tuberculosis* genomic DNA with TaqI restriction enzyme, which cuts on a target located within the IS*6110* and in a target located in the 5' flanking region of the IS*6110*, followed by ligation of adaptors, touchdown PCR for amplification of the 5' IS*6110*-flanking region of all the IS*6110* copies present in the genome, and simultaneous pyrosequencing of the amplified fragments (Figure 16) (Article VIII).

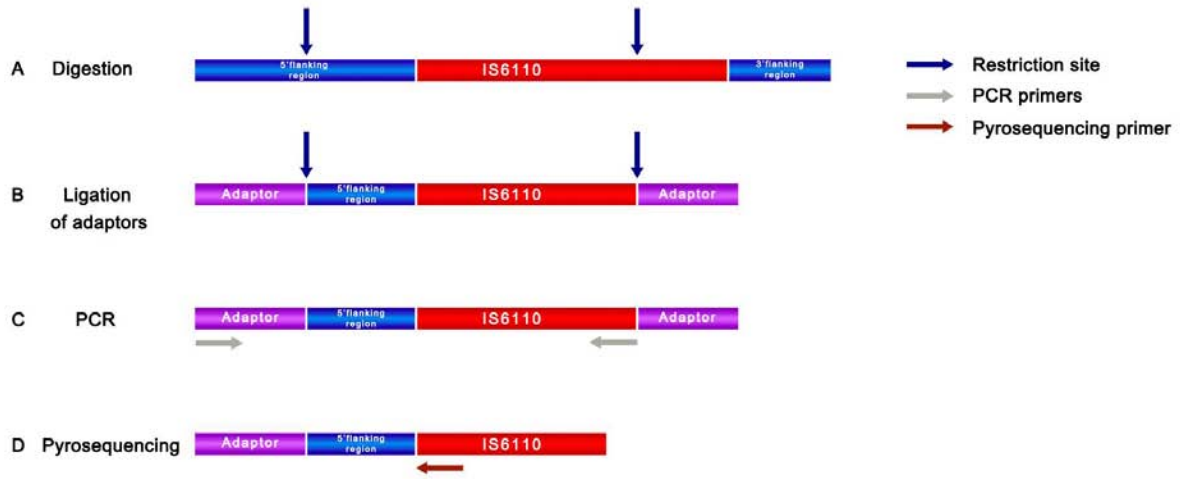


Figure 16. Procedure of the PyroTyping assay. Digestion of genomic DNA (A), ligation of adaptors (B), amplification by touchdown PCR (C), and pyrosequencing (D).

A first genomic digestion was performed in a final volume of 20 µl containing 5U Tru1I (MseI) (Thermo Fisher Scientific, , USA), 1X Tru1I buffer R (Thermo Fisher Scientific, USA), 0.1 mg/mL bovine serum albumin (BSA) (Hoffmann-La Roche, Switzerland), 0.5 mg/mL DNase-free RNase A (Hoffmann-La Roche, Switzerland), and 500 ng of genomic DNA. The Tru1I (MseI) digestion was carried out at 37ºC for at least 2 h. A second digestion was performed by addition of 10U TaqI (Thermo Fisher Scientific, USA), and incubation at 65ºC for 3 h, and 80ºC for 2 min. Subsequently, a 24.6 µl ligation mix containing 40U of T4 DNA ligase (New England Biolabs, USA), 2X T4 ligase buffer (Hoffmann-La Roche, Switzerland), and 0.2 µM of each adaptor (5'-CGGTCAGGACTCAT-3', 5'-CGATGAGTCCTGAC-3') (TIB MOLBIOL, Germany) was added to the digestion product. Ligation was carried out at 12ºC for 17 h, and 65ºC for 10 min.

Touchdown PCR [110] was performed in a final volume of 25 µl containing 1X HotStarTaq Master Mix (Qiagen, The Netherlands), 1 µM each primer (forward 5'biotin-ATGAGTCCTGACCGA-3, reverse 5'-CTGACATGACCCCATCCTTT-3') (TIB MOLBIOL, Germany), 1M betaine PCR reagent (Sigma-Aldrich, USA), and 2.5 µl of ligation product. Touchdown PCR was carried out with the Veriti thermal cycler (Applied Biosystems, USA) and the following amplification conditions: 94°C for 15 min; 10 cycles of 94°C for 20 s, 66-56ºC for 30 s (temperature decreasing 1°C every cycle from 66°C to 56ºC), and 72ºC for 2 min; 20 cycles of 94°C for 20 s, 56ºC for 30 s, and 72ºC for 2 min; and 72°C for 7 min. Finally, pyrosequencing of

the PCR product was performed using a pyrosequencing primer (5'-GGACATGCCGGGGCGGTT-3') (TIB MOLBIOL, Germany), and the nucleotide dispensation order 7x(ACTG).

The result for each strain consisted on a single pyrogram combining the simultaneous pyrosequencing of the 5' flanking regions of all the IS*6110* copies present in the genome (Article VIII).

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1. Development, optimization, and evaluation of molecular methods for detecting drug resistance in *Mycobacterium tuberculosis*

5.1.1. Usefulness of molecular methods for detecting resistance to first- and second-line drugs (Articles I, II, III, and IV)

The usefulness of different molecular methods for detecting resistance to first- and second-line anti-TB drugs in *M. tuberculosis* clinical strains and patient specimens was assessed by comparing the results of different molecular methods with the phenotypic results:

- A reverse hybridization array was evaluated to detect mutations in *rpoB*, *katG*, and *inhA* promoter associated with resistance to RIF and INH in clinical strains and specimens (Article I).
- A multiplex PCR was evaluated to detect mutations in *rpoB*, *katG*, *inhA* promoter, *gyrA*, *rrs*, and *eis* promoter associated with resistance to RIF, INH, FLQ, KAN, AMK, and CAP in clinical strains and specimens (Article II).
- A reverse hybridization LPA was evaluated to detect mutations in *rpoB*, *katG*, *inhA* promoter, *gyrA*, *embB*, *rrs*, and *rpsL* associated with resistance to RIF, INH, FLQ, EMB, KAN, AMK, CAP, and STR in patient specimens (Article III).
- A pyrosequencing assay was developed and used to detect mutations in *gyrA*, *rrs*, *eis* promoter, and *embB* associated with resistance to FLQ, KAN, AMK, CAP, and EMB in clinical strains (Article IV).

A critical aspect of any molecular method is the sensitivity and specificity for detecting resistance, which can vary according to the drug considered. Sensitivity and specificity values of the molecular methods evaluated in comparison with the phenotypic reference standard methods are shown in Table 3.

Table 3. Summary of the sensitivity and specificity values of the different molecular methods used to detect resistance to first- and second-line drugs in clinical strains and patient specimens (Articles I, II, III, and IV).

		Reverse hybridization array[a]		Multiplex PCR[b]		Reverse hybridization LPA[a]		Pyrosequencing	
		Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)
RIF	*Strains*	22/23 (95.7)	47/47 (100)	35/36 (97.2)	24/25 (96.0)	-	-	-	-
	Specimens	35/37 (94.6)	11/12 (91.7)	14/14 (100)	15/15 (100)	43/43 (100)	17/17 (100)	-	-
INH	*Strains*	41/59 (69.5)	11/11 (100)	39/51 (76.5)	10/10 (100)	-	-	-	-
	Specimens	38/39 (97.4)	10/10 (100)	14/15 (93.3)	14/14 (100)	45/46 (97.8)	14/14 (100)	-	-
FLQ	*Strains*	-	-	19/27 (70.4)	29/33 (87.9)	-	-	24/34 (70.6)	70/70 (100)
	Specimens	-	-	2/4 (50.0)	19/19 (100)	2/6 (33.3)	52/53 (98.1)	-	-
KAN	*Strains*	-	-	22/27 (81.5)	28/33 (84.8)	-	-	28/30 (93.3)	58/71 (81.7)
	Specimens	-	-	5/5 (100)	17/18 (94.4)	17/17 (100)	34/34 (100)	-	-
AMK	*Strains*	-	-	12/12 (100)	15/25 (60.0)	-	-	16/17 (94.1)	71/74 (95.9)
	Specimens	-	-	-	-	-	-	-	-
CAP	*Strains*	-	-	7/7 (100)	33/53 (62.3)	-	-	9/10 (90.0)	91/91 (100)
	Specimens	-	-	5/5 (100)	17/18 (94.4)	17/17 (100)	34/34 (100)	-	-
STR	*Strains*	-	-	-	-	-	-	-	-
	Specimens	-	-	-	-	22/22 (100)	28/29 (96.6)	-	-
EMB	*Strains*	-	-	-	-	-	-	35/54 (64.8)	43/49 (87.8)
	Specimens	-	-	-	-	21/35 (60.0)	22/24 (91.7)	-	-

Se: sensitivity; Sp: specificity.

[a] Sensitivity and specificity calculations were performed considering all the specimens included in the study.

[b] Sensitivity and specificity calculations were performed considering one specimen per patient.

The sensitivity of a molecular method depends mainly on the genes and mutations targeted by the assay, which in turn depends on the knowledge on the mechanisms of drug resistance and on the prevalence of each mutation in the different geographical settings. For some drugs, not all the mechanisms of resistance and mutations involved have been identified. On the contrary, the specificity of molecular methods is usually high, thus, when a mutation is detected the strain can be reported as drug-resistant. However, an important exception are mutations in *embB*, which have been detected in EMB-resistant strains and in EMB-susceptible strains as well. Therefore, the accuracy of detection of molecular resistance depends mostly on the strength of the association between the genetic mutation and the phenotypic drug susceptibility result.

In general, molecular methods explore the same most common genes associated with drug resistance, with some exceptions, such as the multiplex PCR (Article II) and the pyrosequencing assay (Article IV) that include the *eis* promoter, and the LPA that includes *rpsL* and *rrs*. Overall, the results of the different molecular methods were highly comparable. In fact, when a strain/specimen was analysed with different molecular methods, the results were concordant in most of the cases.

The sensitivity can also be affected by heteroresistance, defined as the presence of both drug-susceptible and drug-resistant subpopulations in a single strain or specimen. Heteroresistance may be due to the emergence and selection of a mutant subpopulation, or to a mixed infection of both wild-type and mutant populations [29]. Thus, heteroresistance is identified by molecular methods by simultaneous detection of wild-type and mutation sequences. Among the molecular methods evaluated, the reverse hybridization array and LPA and the pyrosequencing assay are able to detect heteroresistance, whereas the multiplex PCR is not, a drawback that may lead to a false drug susceptibility result. It is of note that in order to detect heteroresistance it is necessary to select more than one colony, in the case of cultures in solid media. The limit of detection of mutant DNA in heteroresistant samples has been studied: different LPAs detected of 5-50% mutant DNA, DNA sequencing detected 10-50% of mutant DNA, and pyrosequencing detected 35-50% of mutant DNA [111-113]. Heteroresistance occurs most commonly for FLQ, and it is identified in up to one-third of strains resistant to this drug [114]. Therefore, detection of heteroresistance is essential to

exclude the drug from the treatment and prevent the selection of further resistant bacilli. Furthermore, when molecular methods are performed with DNA from patient specimens, the sensitivity of detection is affected by the bacillary load. Several paucibacillary specimens (smear negative and smear 1+) were included for evaluating the reverse hybridization array and LPA and the multiplex PCR, and a variable number of invalid results were obtained with each method (Articles I, II, and III).

Given the limited sensitivity of molecular methods for detecting resistance to certain drugs and the impact of heteroresistance and the bacillary load, culture and subsequent phenotypic DST must be performed in order to confirm the molecular results, especially when wild-type patterns indicating drug susceptibility are obtained. The main advantage of all the molecular methods evaluated is that the drug susceptibility result can be obtained in one working day, although the exact turnaround time may vary from 3.5 h for multiplex PCR, to 5 h for the LPA. Also, the cost per sample analysed varies for each method, but is generally similar between them and significantly more expensive than the phenotypic methods. An important difference between the molecular methods evaluated is their throughput capacity: 16 reactions can be performed with the reverse hybridization array in each run, 30 reactions with the LPA, and 96 reactions with the multiplex PCR and pyrosequencing assays. Despite these differences in the throughput capacity, a lesser number of samples can be analysed, although the cost per sample might increase. Moreover, some methods are technically simple, such as the multiplex PCR, which only requires the preparation of the PCR mix, whereas the other methods, reverse hybridization array and LPA, and pyrosequencing, require a minimal training. This is basically due to the post-PCR steps, which also increase the assay time and the risk of cross-contamination, compared to the multiplex PCR that performs the detection in a closed system. The risk of cross-contamination has an important role in some settings, especially in low-income countries, where the volume of samples is higher and the laboratory capacity for molecular techniques is limited [115]. Another difference between the molecular methods is the requirement of specific equipment. In this sense, the reverse hybridization array must be performed in the FT-PRO device, which in turn shortened the hybridization time in comparison with the LPA, which can be performed either manually or with an automated washing and shaking device. In addition, the multiplex PCR evaluated requires a specific real-time PCR device, and for the pyrosequencing assay a pyrosequencer is necessary.

Moreover, the distinct molecular methods differed in the interpretation of the results. For the reverse hybridization array and LPA, the purple spots (Figure 17) or bands developed (Figure 18) show the presence of the wild-type sequences or the mutations according to the specific probes of each test. According to the presence and/or absence of the bands, the sample is reported as resistant or susceptible to the considered drug. It is of note that the array test included a software for the interpretation, report, and storage of results images. With the array test it is possible to identify the exact mutation (for example *rpoB* H526D), whereas with the LPA it is only possible to know the location of the mutation (*rpoB* 526).

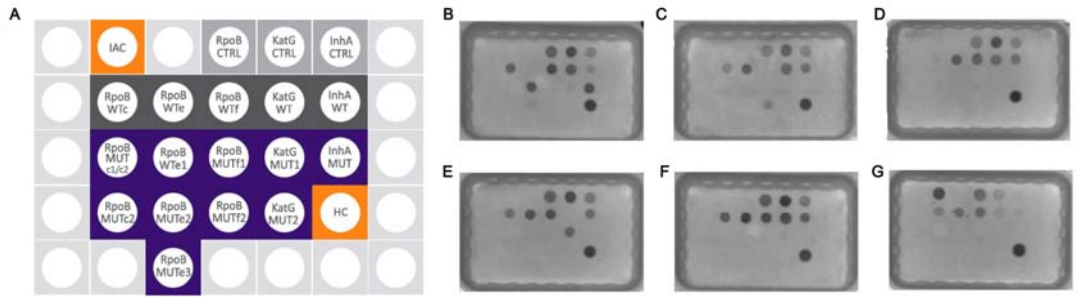


Figure 17. Examples of results obtained by the reverse hybridization GenoFlow DR-MTB Array. Location of the different probes corresponding to controls, wild-type sequences, and mutations in the array (A). Strain RIF^R/INH^R with mutations *rpoB* H526D and *inhA* C-15T (B). Strain RIF^R/INH^S with mutation *rpoB* S531W (C). Strain RIF^R/INH^S by absence of hybridization to the probe targeting *rpoB* codon 516 wild-type (D). Strain RIF^S/INH^R with mutation *katG* S315T1 (E). Strain RIF^S/INH^S (F). Invalid result due to the absence of *inhA* control (G). IAC: internal amplification control; CTL: *rpoB*, *katG* and *inhA* control; WT: wild-type; MUT: mutation; HC: hybridization control.

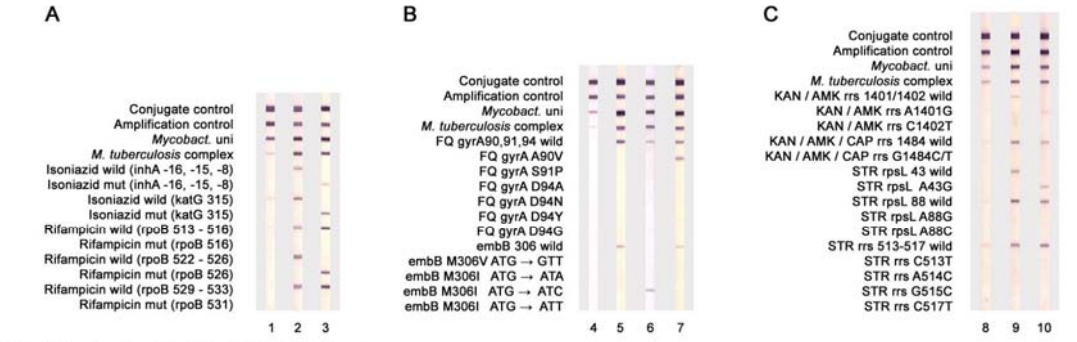


Figure 1. Examples of results obtained by AID TB Resistance assay.

A: Results obtained for INH/RIF module. Lane 1: example of an uninterpretable result. Lane 2: example of a pattern of INH^S and RIF^S. Lane 3: example of a pattern of INH^R and RIF^R.
B: Results obtained for FLQ/EMB module. Lane 4: example of an uninterpretable result. Lane 5: example of a pattern of FLQ^S and EMB^S. Lane 6: example of a pattern of FLQ^S and EMB^R. Lane 7: example of a pattern of $FLQ^{R/S}$ and EMB^S.
C: Results obtained for AG/STR module. Lane 8: example of an uninterpretable result. Lane 9: example of a pattern of $KAN^S/AMK^S/CAP^S$and STR^S. Lane 10: example of a pattern of $KAN^R/AMK^R/CAP^S$ and STR^R

Figure 18. Examples of results obtained by the reverse hybridization LPA AID TB Resistance (Article III). Results obtained for INH/RIF module (A). Results obtained for FQ/EMB module (B). Results obtained for AG module (C).

Results for the multiplex PCR consist on the presence or absence of melting curves (Figure 19) and are interpreted by a software, which reports any drug resistance detected and the location (genomic region) of the mutation, but it is not possible to identify the exact sequence. Furthermore, a drawback of this method is that the melting curve is present when a mutation is detected and absent when the sequence is wild-type. In addition, this multiplex PCR assay does not discriminate resistance among the second-line injectable drugs KAN, AMK, and CAP.

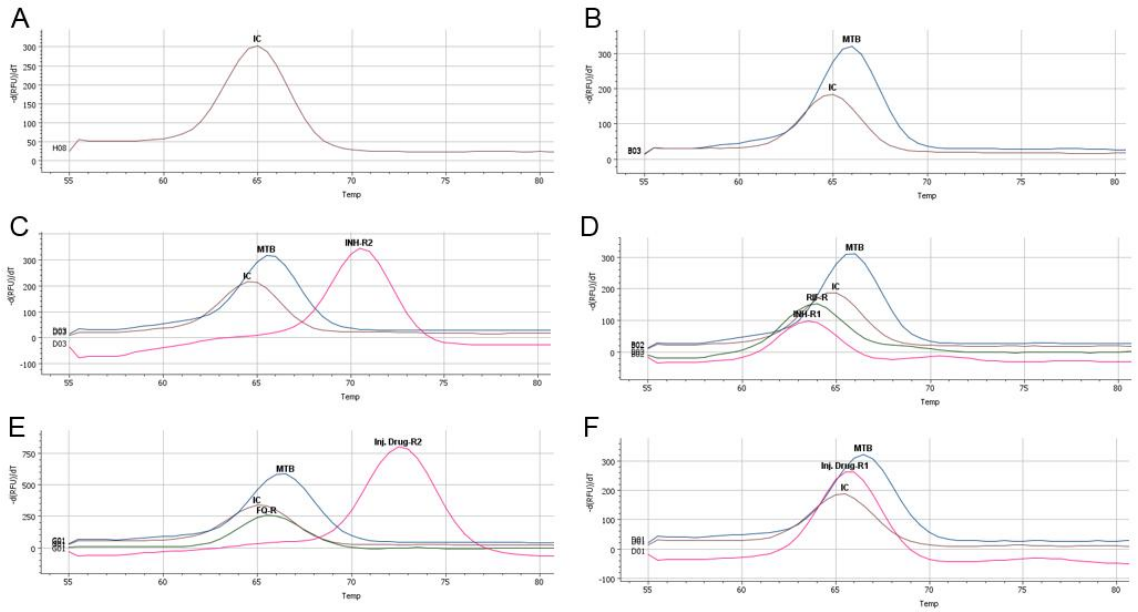


Figure 19. Examples of results obtained by the multiplex PCR Anyplex II MTB/MDR/XDR (Article II). Example of an excluded result due to absence of MTB melting curve (A). Example of a strain sensitive to both INH and RIF (B). Example of a strain resistant to INH and sensitive to RIF (C). Example of a strain resistant to both INH and RIF (D). Example of a strain resistant to both FLQ and injectable drugs (E). Example of a strain sensitive to FLQ and resistant to injectable drugs (F).

Finally, interpretation of pyrosequencing results relies on the pyrogram and the sequence generated (Figure 20), hence, the exact mutation can be identified. In addition, it is possible to detect missense mutations, silent mutations, and new mutations in the targeted regions.

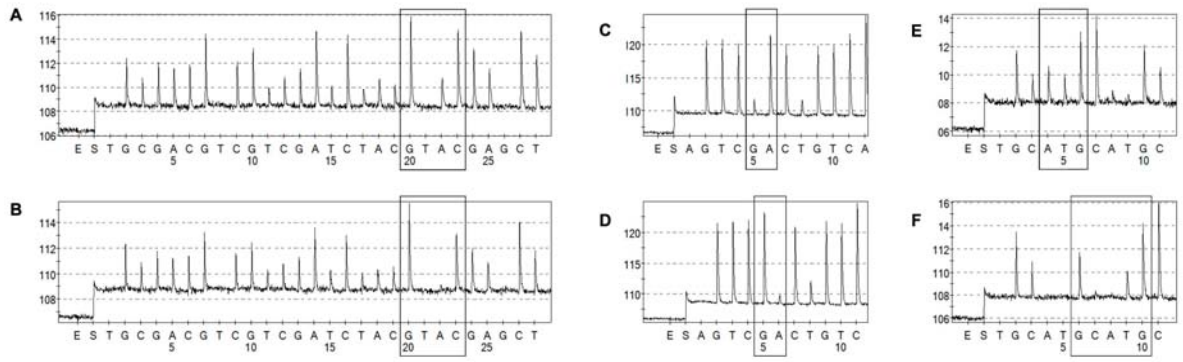


Figure 20. Examples of pyrograms: *gyrA* codon 94 GAC (wild-type) (A), *gyrA* codon 94 GGC (mutation) (B), *rrs* 1401 A (wild-type) (C), *rrs* 1401 G (mutation) (D), *embB* codon 306 ATG (wild-type) (E), and *embB* codon 306 GTG (mutation) (F).

The potential of a molecular method to identify a specific mutation is essential in order to tailor an adequate treatment regimen, and thus reduce the risk of selecting drug resistance and increase the rates of treatment success [116,117]. In addition, although standard treatment regimens can be useful in some settings, specific criteria to prescribe them may not be met by patients from other settings. This is the case of the short-course MDR-TB treatment regimen [15]: in the European region of the WHO, only 4% of the MDR-TB cases could benefit from this regimen [37]. Therefore, it is of outmost importance to perform DST for first- and second-line drugs. Furthermore, the timely detection of any resistance by molecular methods can guide the prescription of a tailored treatment regimen. In this line, a recent study compared GeneXpert MTB/RIF, LPA, and WGS with phenotypic DST, assessing the treatment regimen that would be prescribed according to the results obtained with each method (C Lange, personal communication). The highest concordance regarding the predictable treatment prescription was found between LPA and DST. In contrast, the lowest concordance was obtained for GeneXpert, which only reports RIF resistance and does not give enough detailed information about the specific mutations detected.

Molecular detection of resistance to first-line drugs, especially RIF and INH, should be performed for patients with suspicion of MDR-TB. In addition, it is recommended over conventional testing or no testing at the time of diagnosis of TB, and it also allows the early identification of patients on inappropriate first-line treatment regimens [48]. Subsequently, when a mutation in *rpoB* has been detected or MDR-TB has been phenotypically confirmed,

molecular detection of resistance to second-line drugs, especially FLQ and injectable agents, is recommended (Article VI) [15]. Initial treatment should be guided by molecular results while phenotypic DST results are pending (Article VI).

Despite all the advantages that molecular methods can offer, conventional cultures and phenotypic DST are still needed. Due to the limitations of molecular methods for detecting resistance to some drugs, molecular results should be confirmed by phenotypic DST. Moreover, DST may also be necessary to assess the level of resistance, for example, high-level or low-level resistance to INH, and thus, consider if the drug may be still used for treatment or not. Finally, commercially available molecular methods do not detect resistance to all drugs available for treatment of drug-resistant TB. In view of this, despite the relationship between phenotypic DST results and clinical outcomes is not always clear, phenotypic methods are to date considered the gold standard for DST (Article VI) [48]. Nevertheless, results of molecular methods can help design an initial tailored treatment.

5.2. Review of the molecular methods used for tuberculosis management and impact on the clinical practice

Different methods commercially available can be used for diagnosing active TB, identifying strains, detecting drug resistance, and studying the molecular epidemiology. The knowledge of the relationships between molecular and phenotypic drug resistance and clinical outcomes for the most important drugs used for the treatment of drug-susceptible and drug-resistant TB is reviewed in the following section.

5.2.1. New molecular methods for diagnosis, identification, detection of drug resistance, and epidemiology of tuberculosis (Article V)

Several real-time PCR tests that can be used to detect *M. tuberculosis* DNA and diagnose active TB are GeneXpert MTB/RIF (Cepheid, USA), FluoroType MTB (Hain Lifescience, Germany), and Genedrive (Epistem, United Kingdom), which present similar performance characteristics [81,118-120]. Another method is loop-mediated isothermal amplification (TB-LAMP, Eiken Chemical, Japan) [121]. In addition, GeneXpert MTB/RIF and Genedrive can be also used to detect RIF resistance.

Moreover, several LPA kits can be used to diagnose the disease (GenoType Mycobacteria Direct and GenoQuick MTB (Hain Lifescience, Germany), identify the species of mycobacteria (Genotype MTBC and GenoType CM/AS, Hain Lifescience, Germany; INNO-LiPA Mycobacteria, Fujirebio, Belgium), and detect drug resistance (GenoType MTBDRplus and GenoType MTBDRsl, Hain Lifescience, Germany; INNO LiPA Rif.TB, Fujirebio, Belgium).

Multiplex real-time PCR assays have been applied for diagnosis of active TB, identification of the species, and detection of drug resistance. Pyrosequencing has also been used to identify the species, detect mutations associated with drug resistance, and detect specific single nucleotide polymorphisms for molecular epidemiological purposes. Mass spectrometry has been adapted for identification of strains [122] and detection of drug resistance [123,124]. Hybridization on microbeads has also been used to identify the species, detect resistance, and

study the molecular epidemiology. Finally, the ultimate methodology is WGS, which has been used to detect drug resistance and perform molecular epidemiological investigations [84]. However, the technology and expertise to analyse the results are not widely available, therefore, the impact in the clinical practice is still limited.

5.2.2. Relationship between molecular and phenotypic drug resistance and clinical outcomes (Article VI)

5.2.2.1. First-line drugs

5.2.2.1.1. Isoniazid

The mutations targeted by commercial methods, mainly LPAs, for detecting INH resistance are *katG* S315T and *inhA* promoter A-16G, C-15T, and T-8C/A. Mutations in *katG*315 are present in a highly variable percentage of INH-resistant clinical strains, from 5 to 95%, depending on the setting (Article VI) [4,125]. Mutations in *katG*315 result in a very high level of resistance (minimum inhibitory concentrations higher than 1µg/ml), and indirect evidence strongly suggests that high-level resistance affects clinical outcomes. Limited data on direct association between *katG* S315T and clinical outcome suggest increased risk of first-line treatment failure, relapse, and death. In addition, *katG* S315T is associated with multidrug resistance. Hence, if this mutation is detected by any molecular method, INH should be excluded from the treatment (Figure 21).

Mutations in *inhA* promoter are present in 10–42% of INH-resistant clinical strains, depending on the setting, and are usually associated with low-level resistance (minimum inhibitory concentrations of 0.2–1 µg/ml) (Figure 21) [4]. However, additional mutations in the coding region of *inhA*, together with promoter mutations, result in minimum inhibitory concentrations higher than 1 µg/ml. Limited direct and indirect data suggest no effect of *inhA* mutations on cure rates for standard first-line treatment. In case of detecting an *inhA* mutation, the level of resistance should be confirmed by phenotypic methods, but a higher dose of INH may be administered in combination with other drugs. Conversely to *katG* S315T, *inhA* promoter mutations have not been associated with MDR-TB, but with XDR-TB in South Africa [126]. Since mutations in *inhA* confer cross-resistance between INH and ETO/PTO, low-level INH resistant strains with *inhA* mutations are usually resistant to ETO, but susceptible to

high doses of INH [7]. Finally, mutations associated with INH resistance have been identified with WGS in more than 40 genes [19], but more studies are needed to assess their effect on the clinical outcome.

5.2.2.1.2. Rifampicin

The specific mutations targeted for detecting RIF resistance are located in *rpoB* codons 516, 526, and 531. Mutations in codons 531 and 526 are present in 40-65% and 10-40% of RIF-resistant strains, respectively [58,127,128]. Mutations S531L and H526Y/D confer high-level resistance to all rifamycins [4], whereas H526L (and possibly H526N/S) confer low-level RIF resistance. There is a strong direct and indirect evidence of association of these mutations with clinical resistance (Figure 21). Mutations in codon 516 are present in 5–32% of RIF-resistant strains. Different mutations in codon 516, along with F514F, and S522L, are associated with resistance to RIF but susceptibility to RBT [4]. Nevertheless, the relationship between RBT phenotypic susceptibility and clinical outcomes of patients with RIF-resistant TB has not been studied [7]. Different mutations in L533 have been detected in 2–5% of RIF-resistant strains, and have a slight effect on susceptibility to all rifamycins. Mutation I572F, despite being outside the *rpoB* RIF resistance-determining region, has been described in strains resistant to RIF and RBT, and may have a role in RIF resistance. Finally, some mutations in *rpoB*, such as E510H, L511P, D516Y, N518D, H526N and L533P, are found in strains detected as RIF-susceptible by some phenotypic methods, but they can cause treatment failure [4].

5.2.2.1.3. Pyrazinamide

Most of the mutations associated with PZA resistance are scattered along the *pncA* gene, but non-synonymous mutations have also been detected in some PZA-susceptible strains [4]. The only commercially available method is an LPA (Nipro Corporation, Japan) that covers a long region of the *pncA* gene, which had a sensitivity of 89.7-100% and a specificity of 96-100% [67]. In addition, mutations in *rpsA* are usually associated with low-level PZA resistance [4], and mutations in *panD* have been detected in PZA-resistant MDR-TB strains [4].

Some studies show a very high agreement between genotypic and phenotypic PZA resistance [4], whereas others do not. It is of note that PZA phenotypic DST is technically difficult and

unreliable: mycobacterial growth leading to false resistance results [4] may be due to the difficulty in achieving the low pH required for drug activity, whereas growth inhibition leading to false susceptibility results may also be due to the low pH. In addition, the critical concentration recommended may not be appropriate. As for the clinical resistance, patients with PZA-resistant TB or MDR-TB have worse outcomes than those with PZA-susceptible disease [4].

5.2.2.1.4. Ethambutol

The most common mutations targeted for detecting EMB resistance are located in *embB* codon 306, which are present in 20-88% of EMB-resistant strains [4]. The different amino acid substitutions described cause different levels of phenotypic EMB resistance, from low to moderate [4]. However, these mutations have been also detected in EMB-susceptible MDR-TB strains. Finally, mutations in *ubiA* in conjunction with *embB* cause high-level EMB resistance [4,26]. Nevertheless, there is uncertain reliability of the EMB phenotypic DST, since results using current critical concentrations are poorly accurate and reproducible [7]. In addition, the clinical implications of phenotypic and genotypic EMB resistance are unclear (Figure 21).

5.2.2.2. Second-line drugs

5.2.2.2.1. Fluoroquinolones

Among FLQ-resistant strains, 64% and 3% of strains have mutations in *gyrA* and *gyrB*, respectively [28]. Among the *gyrA* mutations, 81% are inside the quinolone-resistance-determining region (codons 85 to 96) and 19% are outside [28]. Commercial assays usually target *gyrA* codons 88 to 94, and more specifically mutations in codons 90, 91, and 94, which are present in 54% of the FLQ-resistant strains [28]. Mutations in codon 94 have been detected in 40-58% of FLQ-resistant strains, and confer clinical resistance to ofloxacin (Figure 21). Mutations in codon 90 have been detected in 20-30% of FLQ-resistant strains. Mutations in codons 90 and 91 are discussed controversially, available data is limited, and the clinical implications are thus unclear. Mutations in codons 74 and 88 are less frequent [4]. Mutations in codons 94, 90 and 88 confer high-level FLQ resistance, but those in codon 80 might not confer FLQ resistance [4].

Among the *gyrB* mutations, 44% are detected inside the quinolone-resistance-determining region (codons 500 to 540) and 56% are detected outside [28]. Mutations in *gyrB* are generally associated with lower levels of FLQ resistance [4]; however, a combination of *gyrA* and *gyrB* mutations could result in a higher level of resistance, and cross-resistance between FLQs [4]. The recently available GenoType MTBDRsl version 2.0 (Hain Lifescience, Germany) targets *gyrB* wild-type codons 536 to 541 and the mutations N538D and E540V. However, given that these mutations have a low prevalence and that other mutations are not targeted by the assay, detection of *gyrB* mutations may have limited utility [129,130].

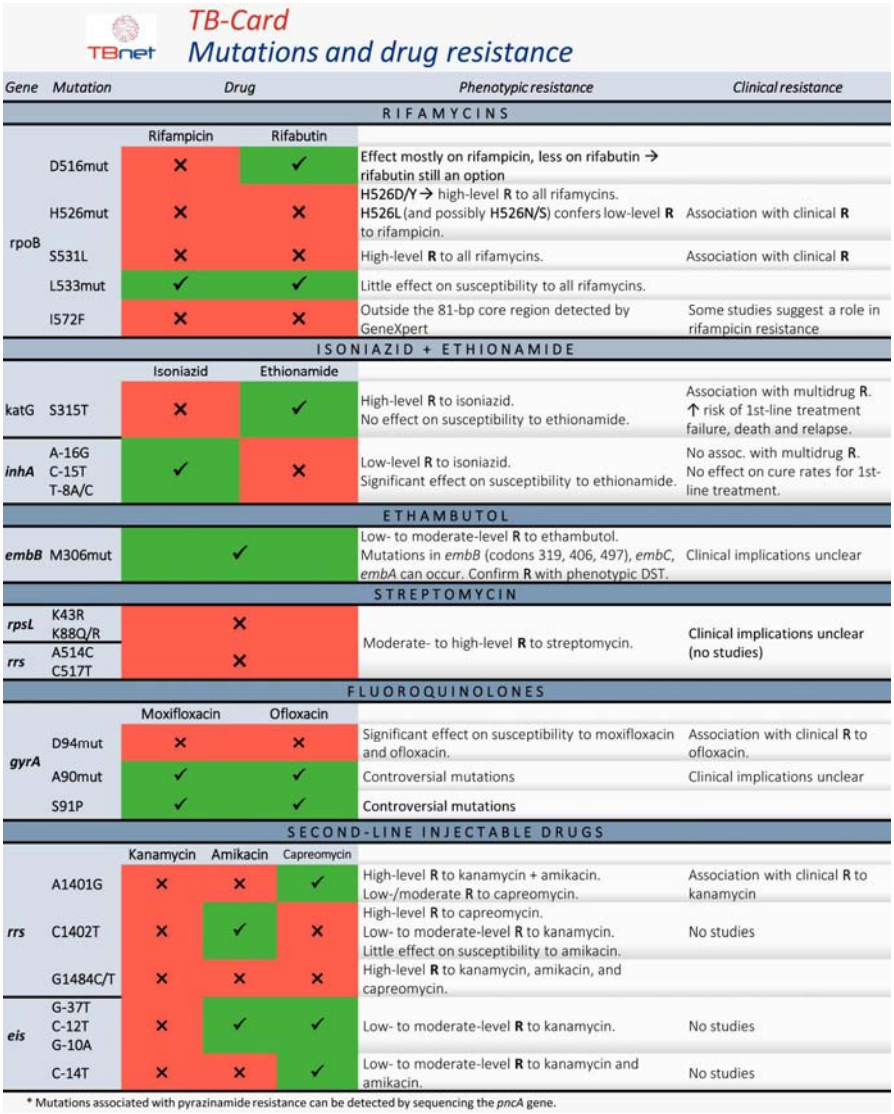
5.2.2.2.2. Injectable drugs

Regarding STR resistance, the main mutations are *rpsL* K43R and *rpsL* K88Q/R, and *rrs* A523C and C526T, which are found in 75-90% of STR-resistant strains and are very specific of STR resistance (Article VI). Prevalence of mutations varies according to the geographical setting: 24-89% of STR-resistant strains have *rpsL*43 mutations (Article VI), 5-27% have *rpsL*88 mutations (Article VI), and 20% have *rrs* mutations [4]. These mutations confer moderate- to high-level phenotypic STR resistance (Figure 21) (Article VI). Conversely, mutations in *gidB* confer low-level STR resistance (Article VI) [4]. In addition, mutations in the promoter region of *whiB7*, which lead to an increased expression of the tap efflux gene and *eis*, have been detected in strains presenting cross-resistance between STR and KAN [4,7]. Nevertheless, studies assessing the direct effects of any of these mutations on clinical resistance to STR have not been performed yet (Article VI).

Regarding KAN, AMK, and CAP, different mutations in *rrs* are associated with partial cross-resistance between these drugs (Figure 21) (Article VI) [4]. The *rrs* A1401G mutation is the most frequent one, and confers high-level resistance to both KAN and AMK, but only low-level to CAP (Article VI) [4]. In a systematic review, the A1401G mutation was present in 56% of KAN-resistant strains, 78% of AMK-resistant strains, and in 76% of CAP-resistant strains [32]. It is of note that 7% of CAP-susceptible strains carried the A1401G mutation [32]. The *rrs* A1401G mutation is associated with clinical resistance to KAN (Article VI). The *rrs* C1402T mutation confers high-level CAP resistance and low- to intermediate-level KAN resistance, but it slightly affects AMK susceptibility (Article VI). The *rrs* G1484C/T mutations confer high-level KAN, AMK, and CAP resistance (Article VI). The mutations *rrs* C1402T and G1484T were rare

(0-2% each) among strains resistant to any of the injectable drugs [32]. Nevertheless, studies assessing the direct effects of *rrs* mutations on clinical outcomes have not been performed yet (Article VI).

In addition, mutations in the promoter region of *eis* confer low-level KAN resistance [31]. The most common mutations are G-37T, C-14T, C-12T, and G-10A, which were detected in 5%, 11%, 3%, and 22% of KAN-resistant strains, respectively [32]. In addition, some mutations may also confer low-level resistance to AMK (Article VI). Mutations G-37T, C-14T, and C-12T were detected in 2%, 9%, and 6% of AMK-resistant strains, respectively, whereas G-10A was detected also in AMK-susceptible strains [32]. Nevertheless, studies assessing the direct effects of *eis* mutations on clinical outcomes have not been performed yet (Article VI).



TB-Card
Mutations and drug resistance

Gene	Mutation	Drug			Phenotypic resistance	Clinical resistance
RIFAMYCINS						
		Rifampicin	Rifabutin			
rpoB	D516mut	✗	✓		Effect mostly on rifampicin, less on rifabutin → rifabutin still an option	
	H526mut	✗	✗		**H526D/Y** → high-level **R** to all rifamycins. **H526L** (and possibly **H526N/S**) confers low-level **R** to rifampicin.	Association with clinical **R**
	S531L	✗	✗		High-level **R** to all rifamycins.	Association with clinical **R**
	L533mut	✓	✓		Little effect on susceptibility to all rifamycins.	
	I572F	✗	✗		Outside the 81-bp core region detected by GeneXpert	Some studies suggest a role in rifampicin resistance
ISONIAZID + ETHIONAMIDE						
		Isoniazid	Ethionamide			
katG	S315T	✗	✓		High-level **R** to isoniazid. No effect on susceptibility to ethionamide.	Association with multidrug **R**. ↑ risk of 1st-line treatment failure, death and relapse.
inhA	A-16G C-15T T-8A/C	✓	✗		Low-level **R** to isoniazid. Significant effect on susceptibility to ethionamide.	No assoc. with multidrug **R**. No effect on cure rates for 1st-line treatment.
ETHAMBUTOL						
embB	M306mut		✓		Low- to moderate-level **R** to ethambutol. Mutations in *embB* (codons 319, 406, 497), *embC*, *embA* can occur. Confirm **R** with phenotypic DST.	Clinical implications unclear
STREPTOMYCIN						
rpsL	K43R K88Q/R		✗		Moderate- to high-level **R** to streptomycin.	**Clinical implications unclear (no studies)**
rrs	A514C C517T		✗			
FLUOROQUINOLONES						
		Moxifloxacin	Ofloxacin			
gyrA	D94mut	✗	✗		Significant effect on susceptibility to moxifloxacin and ofloxacin.	Association with clinical **R** to ofloxacin.
	A90mut	✓	✓		Controversial mutations	Clinical implications unclear
	S91P	✓	✓		Controversial mutations	
SECOND-LINE INJECTABLE DRUGS						
		Kanamycin	Amikacin	Capreomycin		
rrs	A1401G	✗	✗	✓	High-level **R** to kanamycin + amikacin. Low-/moderate **R** to capreomycin.	Association with clinical **R** to kanamycin
	C1402T	✗	✓	✗	High-level **R** to capreomycin. Low- to moderate-level **R** to kanamycin. Little effect on susceptibility to amikacin.	No studies
	G1484C/T	✗	✗	✗	High-level **R** to kanamycin, amikacin, and capreomycin.	
eis	G-37T C-12T G-10A	✗	✓	✓	Low- to moderate-level **R** to kanamycin.	No studies
	C-14T	✗	✗	✓	Low- to moderate-level **R** to kanamycin and amikacin.	No studies

* Mutations associated with pyrazinamide resistance can be detected by sequencing the *pncA* gene.

Figure 21. TBnet Card of mutations and drug resistance, aimed to help in the design of a tailored regimen (Article VI).

5.3. Development, optimization, and evaluation of molecular methods for genotyping *Mycobacterium tuberculosis*

5.3.1. Usefulness of a microbead-based spoligotyping method, TB-SPRINT (Article VII), and a pyrosequencing-based genotyping method, PyroTyping (Article VIII)

The usefulness of TB-SPRINT and PyroTyping was assessed by comparing the results of clustering or discrimination of strains obtained with these two methods with the results obtained with membrane-based spoligotyping and IS*6110*-RFLP.

For the evaluation of TB-SPRINT, 67 strains were included, and an interpretable result was obtained for 65 of them. The concordance between membrane-based and microbead-based spoligotyping was 99.6% (2785/2795 spoligotype data points). Both spoligotyping methods assigned the same lineage for 63 of the 65 strains (96.9%). For one of the two remaining strains, typing of lineage-specific single nucleotide polymorphisms (SNP typing) was in agreement with microbead-based spoligotyping. For the other strain, no result was obtained by SNP typing (Article VII). According to microbead-based spoligotyping, the strains were classified as Haarlem, Latin-America and Mediterranean, T, Africanum, East African Indian, X, Beijing, Bovis, Central Asian, or the family could not be assigned (Article VII). Interestingly, for one strain the microbead-based spoligotyping result suggested the presence of two different populations, which was confirmed by MIRU analysis. The TB-SPRINT assay was also evaluated for detecting mutations in *rpoB*, *katG*, and *inhA*. TB-SPRINT was highly sensitive and specific in comparison with phenotypic DST and DNA sequencing/pyrosequencing, although the number of drug-resistant strains was low to draw a robust conclusion (Article VII).

The concordance between PyroTyping, IS*6110*-RFLP, and spoligotyping was 100% for the 94 strains analysed: all three methods agreed on clustering and discriminating the strains. According to spoligotyping, the strains were classified as Latin-America and Mediterranean, T, Haarlem, Africanum, or the family could not be assigned (Article VIII).

Among the genotyping methods studied, spoligotyping (either the membrane- or the microbead-based method), is the one regarded as the least discriminatory [131]. In fact, among the strains analysed in the evaluation of TB-SPRINT, the six spoligotyping clusters detected were discriminated by IS*6110*-RFLP. On the contrary, in the set of strains analysed with PyroTyping, results of clustering and discrimination of IS*6110*-RFLP, spoligotyping, and PyroTyping were concordant in all the cases. On the other hand, IS*6110*-RFLP is less discriminatory than spoligotyping for low IS*6110* copy number strains (considered as six or few bands in the RFLP pattern [88]) [132]. In this line, among the nine low IS*6110* copy number strains analysed with TB-SPRINT, two IS*6110*-RFLP clusters were detected, but the nine strains were discriminated by spoligotyping. Conversely, among the 11 low IS*6110* copy number strains analysed with PyroTyping, different IS*6110*-RFLP clusters were detected, but results of IS*6110*-RFLP, spoligotyping, and PyroTyping were concordant (Article VIII).

As previously mentioned, the selection of the genotyping method depends on the objective of the investigation [88,89]. Methods based on the polymorphism of the IS*6110* insertion site (i.e. IS*6110*-RFLP and PyroTyping), which are highly discriminatory, may serve to assess the genetic relatedness and the epidemiological links among TB cases, detect outbreaks, investigate possible laboratory cross-contaminations, and differentiate relapses from new infections. On the contrary, spoligotyping, which is based on a phylogenetically robust marker may serve to characterize the evolution and global phylogeny of *M. tuberculosis*, to define the phylogeographic specificity of circulating clades in population-based studies, and to screen epidemiological links as a first-line method [87].

TB-SPRINT and PyroTyping are rapid methods, with a turnaround time of one and two days, respectively, and they are easier to perform and less labour-intensive than IS*6110*-RFLP genotyping. However, both methods require specific equipment: the Luminex analyser for TB-SPRINT, and a pyrosequencer for PyroTyping. Also, both methods can be performed in 96-well plates for high-throughput analysis. Although interpretation of the results by TB-SPRINT and PyroTyping is completely different (Articles VII and VIII), it does not require a complex bioinformatics analysis. In addition, both technologies, microbead-based hybridization and pyrosequencing, have been used for detecting mutations associated with drug resistance [109,133,134] (Article IV). Thus, genotyping and detection of drug resistance performed in a

single combined assay further increases the clinical value of the technologies for patient management. It is of note that for the time being, WGS is the ultimate method for discrimination of isolates and simultaneous determination of the drug resistance profile [86,135]. Nevertheless, given the still high cost, and the complexity of the analysis, many laboratories are not yet able to carry out WGS. In addition, for optimal cost-effectiveness, it may be necessary to analyse a relatively high number of strains at the same time, which may not be readily available in low incidence settings, thus delaying the results. In these cases, TB-SPRINT or PyroTyping combined with detection of drug resistance by pyrosequencing may be more affordable and rapid local solutions.

CONCLUSIONS

6. CONCLUSIONS

Development, optimization, and evaluation of molecular methods for detecting first- and second-line drug resistance in *Mycobacterium tuberculosis* clinical strains and patient specimens.

1. The molecular methods evaluated present variable sensitivity and specificity, depending on the drug considered, when compared with phenotypic drug susceptibility testing. The sensitivity and specificity values of these molecular methods are comparable between them.
2. Sensitivity is high for detecting resistance to rifampicin, amikacin, capreomycin, and streptomycin. Hence, detection of wild-type sequences indicative of susceptibility is valuable to rule in the corresponding drug for treatment until phenotypic drug susceptibility testing results are available.
3. Sensitivity is moderate or low for detecting resistance to isoniazid, fluoroquinolones, kanamycin, and ethambutol. Hence, a susceptibility result should be considered with caution and phenotypic resistance should not be ruled out until it is confirmed by phenotypic methods.
4. Specificity is high for most of the drugs, hence, detection of mutations indicative of resistance is valuable to rule out the corresponding drug for treatment until phenotypic drug susceptibility testing results are available.
5. In general, the different molecular methods evaluated show good results when tested either on clinical strains or patient specimens. Nevertheless, sensitivity of detection of molecular methods can be affected by the bacillary load of the patient specimen.
6. The main advantage of all these molecular methods is the short turnaround time of less than one working day. Nevertheless, due to particular differences between them in terms of throughput capacity, equipment and training required, and interpretation of the results, a specific method may be more convenient in a given setting.

Review of new molecular methods for diagnosis, identification, detection of drug resistance, and epidemiology studies of tuberculosis. Relationship between molecular and phenotypic drug resistance and clinical outcomes.

1. Several molecular methods are commercially available for tuberculosis management, and each methodology can be aimed for more than one objective.
2. Current available methods vary in terms of throughput capacity, equipment and training required. In addition, interpretation of the results differs considerably between methods.
3. Current knowledge on the genes and mutations involved in resistance varies depending on the drug considered. The molecular mechanisms of resistance are known for rifampicin, and, to a lesser extent, for the injectable drugs. On the contrary, the molecular mechanisms of resistance for isoniazid, fluoroquinolones, and ethambutol remain to be completely elucidated.
4. The relationship between mutations, phenotypic drug susceptibility testing, and clinical outcomes has been studied for some of the most common mutations in *rpoB* for rifampicin resistance, *katG* and *inhA* for isoniazid resistance, *gyrA* for ofloxacin, and *rrs* for kanamycin. However, studies assessing the effect of other mutations for these and other drugs on clinical outcomes have not been performed yet.
5. Large studies are needed to establish these relationships and to identify the subset of mutations predictive of treatment failure. Once these mutations have been identified, results of molecular methods can be used to design a tailored treatment, in order to use the limited drugs available adequately. In this line, the effective drugs can be used, and non-effective drugs that in turn cause adverse effects can be ruled out, improving the management of TB patients.

Development, optimization, and evaluation of a microbead-based spoligotyping method and a pyrosequencing-based method for genotyping *Mycobacterium tuberculosis* strains.

1. TB-SPRINT and PyroTyping present differences in terms of discriminatory power, hence, the selection of the genotyping method depends on the objective of the investigation.
2. These methods are rapid and easy to perform, have high-throughput capacity, and interpretation of the results does not require a complex bioinformatics analysis, but they require specific equipment.
3. Microbead-based hybridization and pyrosequencing can be also used for detecting drug resistance simultaneously, increasing the clinical value for patient management.

FUTURE PERSPECTIVES

7. FUTURE PERSPECTIVES

Despite the availability of several molecular methods for TB diagnosis, identification of the *Mycobacterium* species, detection of drug resistance, and epidemiology studies of *M. tuberculosis*, TB remains one of the major infectious diseases worldwide. In order to improve TB control, rapid diagnosis of the disease, determination of the susceptibility pattern for an adequate treatment, and detection of epidemiological links among TB cases can be potentially accomplished by molecular methods. Ideally, a single method could be developed for all these objectives, but it has to be highly sensitive and specific, to be able to detect *M. tuberculosis* in patient specimens with low bacillary load, high discriminatory for molecular epidemiology studies, high-throughput, easy to perform, and affordable for low-income countries. Moreover, in order to determine the susceptibility pattern of the strain with molecular methods, it is essential to have complete knowledge on the molecular mechanisms involved in drug resistance, and the mutations conferring drug resistance and drug susceptibility as well. These mutations will have to be correlated with both phenotypic drug resistance in the *M. tuberculosis* strains and the clinical outcome.

In addition, several new or repurposed anti-TB drugs are in advanced phases of clinical development. Ideally, a new drug should have a validated safety profile, be more potent than existing drugs to reduce the treatment duration, have new targets in order to treat multidrug and extensively drug-resistant TB, and be compatible with anti-retroviral therapy. Furthermore, drugs that can eliminate *M. tuberculosis* bacilli in different replication states are needed. Ideally, three new drugs with no antagonism between them should be available, in order to design a new regimen and increase the probability of treatment success and prevent selection of additional drug resistance. Moreover, in order to guarantee a successful treatment, adherence, adequate follow-up, and support of patients should be assured.

In conclusion, although the diagnosis and treatment of TB has significantly improved in the last years, new diagnostics, drugs, and knowledge on the *M. tuberculosis* resistance mechanisms, among other TB control strategies, are needed to achieve TB elimination.

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ARTICLES ORIGINATED FROM THIS THESIS

Article I

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Evaluation of GenoFlow DR-MTB Array Test for Detection of Rifampin and Isoniazid Resistance in *Mycobacterium tuberculosis*

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The aim of this study was to evaluate the GenoFlow DR-MTB array test (DiagCor Bioscience, Hong Kong) on 70 cultured isolates and 50 sputum specimens. The GenoFlow array test showed good sensitivity and specificity compared to the phenotypic Bactec 460TB. This array accurately detected mutations in *rpoB*, *katG*, and *inhA* associated with resistance to rifampin and isoniazid.

Rapid detection and drug susceptibility testing of *Mycobacterium tuberculosis* are hampered by the slow growth of mycobacteria (1). The transmission of strains resistant to both rifampin (RIF) and isoniazid (INH), i.e., multidrug-resistant (MDR) strains, remains a public health problem. These strains may harbor mutations in *rpoB* (2, 3), *katG*, and *inhA*, among other genomic regions (4, 5). The aim of this study was to evaluate the diagnostic accuracy of the GenoFlow DR-MTB array test (DiagCor Bioscience, Hong Kong) for the detection of *M. tuberculosis* molecular resistance to RIF and INH.

A total of 70 *M. tuberculosis* isolates from 70 patients and 50 sputum specimens from 25 patients (more than one specimen was obtained from nine patients) were retrospectively selected from a collection of cultured isolates and specimens recovered from the Hospital Universitari Germans Trias i Pujol (Badalona, Spain), the Instituto Aragonés de Ciencias de la Salud (Zaragoza, Spain), and Serveis Clínics (Barcelona, Spain). The isolates and specimens were selected to represent different resistance profiles. The study was approved by the institutional ethics committee at Hospital Universitari Germans Trias i Pujol.

Specimens were decontaminated using Kubica's *N*-acetyl-L-cysteine NaOH method (6, 7), stained by auramine-rhodamine, graded on a scale from 0 to 3+, and cultured on Lowenstein-Jensen and Bactec 460TB (Becton Dickinson, Sparks, MD, USA). The remaining decontaminated specimens were stored at −20°C (8). The INNO-LiPA mycobacteria version 2 assay (Innogenetics, Ghent, Belgium) was used to identify *M. tuberculosis* complex organisms for all the isolates and cultures from the specimens. Drug susceptibility testing (DST) was performed with Bactec 460TB (Bactec) using 2 μg/ml RIF and 0.1 μg/ml INH as critical concentrations (9).

For molecular drug resistance detection, DNA from isolates and specimens was extracted, as previously described (10). The GenoFlow array test consists of PCR amplification and hybridization in the FT^{PRO} flowthrough system. The mutations targeted are *rpoB* D516V, D516G, H526D, H526Y, H526L1, S531L, and S531W; *katG* S315T1 and S315T2; and *inhA* C-15T. An internal amplification control, hybridization control, and *rpoB*, *katG*, and *inhA* controls were included in each reaction. The results obtained by the array were recorded, automatically interpreted by the DiagCor software, and confirmed visually by the researcher. These

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TABLE 1 Distribution of GenoFlow DR-MTB array results according to Bactec 460TB for 70 clinical isolates and 50 sputum specimens

	Bactec 460TB result (%) for (*n*)[b]:											
	Clinical isolates (70)						Sputum specimens (50)					
	RIF		INH		MDR (23)		RIF		INH		MDR (37)	
GenoFlow result[a]	R (23)	S (47)	R (59)	S (11)	RIF	INH	R (37)	S (13)	R (40)	S (10)	RIF	INH
R	22		41		22	17	35	1	38		35	36
S	1	47	18	11	1	6	2	11	1	10	2	1
I								1[c]	1[c]			

[a] R, resistant; S, sensitive; I, invalid.
[b] RIF, rifampin; INH, isoniazid; MDR, multidrug resistant (resistant to both rifampin and isoniazid).
[c] Invalid GenoFlow results for both RIF and INH were obtained for the same specimen.

results were compared to those obtained by the Bactec. Discordant results between the array and the Bactec were compared to those obtained by alternative molecular methods. DNA sequencing targeted mutations in the *katG* gene, *oxyR-ahpC*, *mabA-inhA*, and the 81-bp core region of *rpoB* (11); the GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) targeted mutations in *rpoB* (codons 516, 526, and 531), *katG* (codon 315), and *inhA* (positions −8, −15, and −16) (10); and pyrosequencing targeted mutations in *rpoB* (codons 516 and 526 to 531), *katG* (codon 315), and *inhA* (positions −16 to −5) (12). This diagnostic accuracy study was reported in accordance with the Standards for Reporting of Diagnostic Accuracy (STARD) statement guidelines (13).

The distribution of GenoFlow results, according to the Bactec results for clinical isolates and sputum specimens, is presented in Table 1. The sensitivity, specificity, and agreement between the GenoFlow and Bactec tests were >90% for detecting RIF resistance in cultured isolates and sputum specimens and for INH resistance in sputum specimens; however, the sensitivity of the array for INH resistance in clinical isolates was 69.5% (Table 2). A total of 23 discordant results were obtained between Bactec and GenoFlow tests for 22 isolates/specimens (for one isolate, discrepant results were obtained for both drugs) (Table 3). At least one of the results obtained by DNA sequencing, GenoType MTBDR*plus*, or pyrosequencing was in agreement with the array in 82.6% (19/23) of the cases.

Of the 50 sputum specimens selected, two were smear negative, and 48 were smear positive; eight specimens were smear 1+ (1 to 10 acid-fast bacilli [AFB] per 100 fields), nine specimens were smear 2+ (1 to 9 AFB per field), and 31 specimens were smear 3+ (>9 AFB per field). An invalid GenoFlow test result (absence of *katG* and *inhA* controls) was obtained for one specimen, which was 3+ and rifampin sensitive/isoniazid resistant. For four specimens, discordant results between the Bactec and GenoFlow tests were obtained: one specimen was smear negative, one specimen was smear 1+, and two specimens were smear 3+ (Table 3). Furthermore, for two of the specimens with a discordant result between the Bactec and GenoFlow tests, consecutive samples collected during the treatment were available, and a concordant result was obtained for those specimens. Thus, the molecular result did not appear to be affected by potential changes in the DST profile or in the different resistant/susceptible subpopulations in the sample during the treatment of the patients.

The sensitivity and specificity values of the GenoFlow test for detecting RIF resistance were comparable to those of GenoType MTBDR*plus* and INNO-LiPA Rif. TB assays (14). These high values were expected, since >95% of rifampin-resistant isolates harbor mutations in the targeted region of *rpoB* (15). Regarding INH resistance, the lower sensitivity of the GenoFlow test was partially in contrast with that of the GenoType MTBDR*plus* assay (16). The data presented here, despite the bias introduced in the selection of isolates, was more in accordance with those of another systematic review that reported a combined cumulative frequency of 79.9% for *katG* codon 315 and *inhA* position −15 mutations worldwide, which reached 83.9% when additional mutations in *inhA* and *ahpC* were included (17).

Nowadays, several molecular tests are available (18–21), but more studies are still needed to assess their clinical value. For instance, an evaluation has demonstrated the noninferiority of the GenoType MTBDR*plus* version 2.0 and Nipro line probe assays in comparison to the WHO-endorsed first version of the GenoType

TABLE 2 Sensitivity and specificity of GenoFlow DR-MTB array for detecting drug resistance, and agreement values between GenoFlow DR-MTB array and Bactec 460TB[a]

Resistance	Clinical isolates					Sputum specimens				
	Sensitivity (no. detected/total no. [%] [95% CI])	Specificity (no. detected/total no. [%] [95% CI])[b]	Agreement (no. detected/ total no. [%])	Kappa[c]	SE	Sensitivity (no. detected/total no. [%] [95% CI])	Specificity (no. detected/total no. [%] [95% CI])[b]	Agreement (no. detected/ total no. [%])	Kappa[c]	SE
RIF	22/23 (95.7) (76.0–99.8)	47/47 (100) (90.6–100)	69/70 (98.6)	0.967	0.032	35/37 (94.6) (80.5–99.1)	11/12 (91.7) (59.8–99.6)	46/49 (93.9)	0.839	0.090
INH	41/59 (69.5) (56.0–80.5)	11/11 (100) (67.9–100)	52/70 (74.3)	0.417	0.096	38/39 (97.4) (84.9–99.9)	10/10 (100) (65.5–100)	48/49 (98.0)	0.939	0.060
MDR	17/23 (73.9) (51.3–88.9)	47/47 (100) (90.6–100)[b]	64/70 (91.4)	0.792	0.079	34/37 (91.9) (77.0–97.9)	13/13 (100) (71.7–100)[b]	47/50 (94.0)	0.847	0.084

[a] RIF, rifampin; INH, isoniazid; MDR, multidrug resistance (resistance to both rifampin and isoniazid); CI, confidence interval; SE, standard error.
[b] For specificity calculations of MDR detection, we considered isolates/specimens sensitive to either RIF or INH or both.
[c] Kappa values of >0.6 and kappa values between 0.4 and 0.6 indicate a strong and moderate agreement, respectively.

TABLE 3 Results obtained by molecular methods for the cultured isolates and sputum specimens with a discordant result between Bactec 460TB and GenoFlow DR-MTB array[a]

Isolate or specimen	Bactec 460TB		GenoFlow DR-MTB array		DNA sequencing		GenoType MTBDR*plus*		Pyrosequencing	
	RIF	INH	RIF	INH	RIF	INH	RIF	INH	RIF	INH
Isolates	R	R	516 WTØ[b]	WT	516 TAC	WT	WT	WT	516 TAC	WT
	R	R	531 TGG	WT	531 TGG	WT	WT	WT	531 TTG	WT
	S	R	WT	WT	NP	*oxyR-aphC* G-12A	WT	WT	WT	WT
	S	R	WT	WT	NP	WT	WT	WT	WT	WT
	S	R	WT	WT	NP	*inhA* T-8C	WT	*inhA* T-8C	WT	*inhA* T-8C
	S	R	WT	WT	NP	WT (*katG* NP)	WT	WT	WT (531 NR)	WT
	R	R	WT	WT	531 TTG	WT	531 TGG	WT	WT	WT
	S	R	WT	WT	NP	WT	WT	WT	WT	WT
	S	R	WT	WT	NP	WT	WT	WT	WT	WT
	S	R	WT	WT	NP	WT	WT	WT	WT	WT
	S	R	WT	WT	NP	*inhA* C-15T	WT	WT	WT	WT
	S	R	WT	WT	NP	WT	WT	WT	WT	WT
	S	R	WT	WT	NP	WT	WT	WT	WT	WT
	S	R	WT	WT	WT	WT	WT	*katG* S315T1	WT	WT
	S	R	WT	WT	NP	*katG* S315T1	WT	WT	WT	*katG* S315T1
	R	R	531 TTG	WT	531 TTG	WT (*inhA*, *oxyR-aphC* NP)	531 TTG	WT	531 TTG	WT
	R	R	531 TTG	WT	531 TTG	WT (*inhA*, *oxyR-aphC* NP)	531 TTG	WT	531 TTG	WT
	R	R	516 GGT	WT	516 GGT	WT (*inhA*, *oxyR-aphC* NP)	516 GGT	WT	516 GGT	WT
Specimens	R[c]	R	WT	*inhA* C-15T	NP	NP	NP	NP	NR	NP
	R[d]	R	WT	*katG* S315T1	NP	NP	NP	NP	WT	NP
	S[e]	S	531 TTG	WT	NP	NP	WT	WT	WT	WT
	R[e]	R	531 TTG	WT	NP	NP	531 TTG	WT	531 TTG	WT

[a] RIF, rifampin; INH, isoniazid; WT, wild type; NP, not performed; NR, no result obtained.
[b] 516 WTØ, the GenoFlow probe targeting *rpoB* 516 wild type was absent.
[c] This specimen was smear negative.
[d] This specimen was smear 1+.
[e] This specimen was smear 3+.

MTBDR*plus* assay for the rapid detection of multidrug-resistant tuberculosis (MDR-TB) (22). Moreover, in order to improve patient management, it is important to consider not only the molecular result (presence/absence of mutation) but also the mutation detected and its correlation with the phenotypic result and clinical outcome (23).

The main advantages of the GenoFlow assay were the use of the FT^{PRO} hybridization device, which shortens the hybridization protocol to 45 min (that of the GenoType MTBDR*plus* assay is 2 h), and the specific software that facilitates the interpretation, report, and storage of the results. In addition, an automated hybridization device is under development, which may reduce the hands-on-time of the hybridization step. Another aspect that could also be improved is the low-throughput capacity.

In conclusion, the GenoFlow assay may be useful for rapid, sensitive, and specific screening of resistance to RIF and INH in isolates and specimens, and its performance is comparable to that of other molecular methods. Although molecular results should be confirmed by phenotypic testing, the identification of resistance can be helpful to rule out drugs and improve the management of tuberculosis patients.

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Article II

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Diagnostic accuracy study of multiplex PCR for detecting tuberculosis drug resistance

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Summary *Objective:* To study the diagnostic accuracy of a multiplex real-time PCR (Anyplex II MTB/MDR/XDR, Seegene, Corea) that detects *Mycobacterium tuberculosis* resistant to isoniazid (INH), rifampicin (RIF), fluoroquinolones (FLQ) and injectable drugs (kanamycin [KAN], amikacin [AMK] and capreomycin [CAP]) in isolates and specimens.
Methods: One hundred fourteen cultured isolates and 73 sputum specimens were retrospectively selected. Results obtained with multiplex PCR were compared with those obtained with BACTEC. Discordant results between multiplex PCR and BACTEC were tested by alternative molecular methods.
Results: Sensitivity and specificity of multiplex PCR for detecting drug resistance in isolates were 76.5% and 100%, respectively, for INH; 97.2% and 96.0%, respectively, for RIF; 70.4% and 87.9%, respectively, for FLQ; 81.5% and 84.8%, respectively, for KAN; 100% and 60%, respectively, for AMK, and 100% and 72.3%, respectively, for CAP. Sensitivity and specificity of Anyplex for detecting drug resistance in specimens were 93.3% and 100%, respectively, for INH; 100% and 100%, respectively, for RIF; 50.0% and 100%, respectively, for FLQ; and

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100% and 94.4%, respectively, for both KAN and CAP. Among the discordant results, 87.7% (71/81) of results obtained with the multiplex PCR were concordant with at least one of the alternative molecular methods.
Conclusions: This multiplex PCR may be a useful tool for the rapid identification of drug resistant tuberculosis in isolates and specimens, thus allowing an initial therapeutic approach. Nevertheless, for a correct management of patients, results should be confirmed by a phenotypic method.

Introduction

Tuberculosis (TB) is a global public health threat due to the emergence and spread of drug resistant *Mycobacterium tuberculosis* strains. Strategies for TB control are based on rapid diagnosis of the disease and implementation of an effective treatment based on drug susceptibility testing (DST) results. However, obtaining phenotypic results may take from weeks to months due to the slow growing rate of mycobacteria.

The most efficient drugs for TB treatment are isoniazid (INH) and rifampicin (RIF). *M. tuberculosis* isolates resistant to both drugs are denominated multidrug-resistant (MDR). Furthermore, MDR-TB isolates resistant to fluoroquinolones (FLQ) and any of the second-line injectable drugs [kanamycin (KAN), amikacin (AMK) and capreomycin (CAP)] are known as extensively drug-resistant (XDR). In 2012, WHO reported 84,000 confirmed MDR-TB cases worldwide, and 9.6% of these cases were XDR-TB.[1] Treatment failure and mortality rates among these patients are higher than that of drug-sensitive TB.[1] Thus, it is crucial to have rapid methods to detect specifically MDR and XDR *M. tuberculosis* and adjust the treatment.

The molecular basis of drug resistance in *M. tuberculosis* consists in the stepwise acquisition of genetic mutations in genes coding for drug targets or drug-converting enzymes. The most common mutations associated with INH resistance are located in codon 315 of *katG*, encoding the catalase peroxidase involved in the activation of INH prodrug, and also in positions −8, −15 and −16 in the regulatory region of *inhA*, a gene encoding an enoyl-acil carrier protein reductase.[2–4] As for RIF, 95–99% of resistant isolates harbor mutations in the 81-bp core region of *rpoB*, encoding the β subunit of RNA polymerase, and more frequently in codons 531, 526 and 516.[5,6] With respect to FLQ, amino acid changes in codons 90, 91 and 94 in the quinolone resistance determining region (QRDR) of *gyrA*, coding for the α subunit of DNA gyrase, have been detected in resistant isolates.[7,8] Concerning second-line injectable drugs, cross-resistance between KAN, AMK and CAP has been reported.[9] Mutations in the *rrs* gene at positions 1401 and 1484 have been associated with resistance to the three drugs, while mutations at 1402 have been detected in isolates resistant to CAP.[10] In addition, resistance to KAN has also been associated with mutations at positions −10, −14 and −37 in the promoter region of *eis*.[10,11]

In order to rapidly diagnose TB and detect drug resistance, different molecular methods have been developed.[12] The gold standard is DNA sequencing but it is expensive and requires technical expertise. Alternative molecular methods such as line probe assays, real-time PCR or pyrosequencing have been developed to improve the molecular detection of drug resistance.[13–17] These tests can be easily implemented in the clinical laboratory routine protocols to detect resistance-associated mutations in cultured isolates or directly in clinical specimens, reducing the diagnostic time. Regarding real-time PCR, several studies have assessed the performance of the commercial GeneXpert MTB/RIF (Cepheid, USA), aimed to detect *M. tuberculosis* and RIF resistance in clinical specimens, obtaining good results.[18] This method however does not detect INH resistance and the implementation in the clinical routine is not affordable in some settings. Other in-house real-time PCR methods have also been developed, specially to detect resistance to first-line drugs.[19,20] Nevertheless, to our knowledge there are not published studies evaluating commercially available real-time PCR tests to detect both first- and second-line drug resistance.

In the present study we determined the diagnostic accuracy of the commercial multiplex real-time PCR assay Anyplex II MTB/MDR/XDR (Seegene, Corea) to detect *M. tuberculosis* complex and mutations associated with resistance to INH, RIF, FLQ and injectable drugs in cultured isolates and sputum specimens.

Materials and methods

Cultured isolates

A total of 114 *M. tuberculosis* isolates were retrospectively selected from a collection of cultured isolates in Hospital Universitari Germans Trias i Pujol (Badalona, Spain) and National Tuberculosis and Infectious Diseases University Hospital (Vilnius, Lithuania). Eighty-five of the 114 isolates were initially isolated in Spain and the remaining 29 in Lithuania. For this study we included isolates that were previously characterized by means of both phenotypic and molecular drug susceptibility testing for first- and/or second-line drugs. Moreover, these isolates were selected in order to have different and representative resistance profiles for each drug. The study was approved by the institutional ethics committees of both sites. Each isolate corresponded to one patient and no epidemiological connection between patients was suspected. Isolates were identified as *M. tuberculosis* by Inno-Lipa Mycobacteria v2 assay (Innogenetics, Belgium).

Sputum specimens

A total of 73 sputum specimens corresponding to 34 patients were retrospectively selected from a collection

of specimens recovered in Hospital Universitari Germans Trias i Pujol. As done for the isolates, specimens were selected on the basis of previous characterization by means of both phenotypic and molecular drug susceptibility testing for first- and/or second-line drugs, in order to have different drug resistance profiles. All sputum specimens were collected directly from patients, they were not obtained by split, and therefore, there was no repeat testing of a single specimen. Sputum specimens were processed as follows. First, they were digested and decontaminated using Kubica's *N*-acetyl-L-cysteine NaOH method.[21,22] After decontamination, auramine-rhodamine acid-fast staining was performed from the concentrated sediment. Specimens that were positive by fluorochrome staining were confirmed with Ziehl-Neelsen staining. The auramine-rhodamine smears were graded on a scale from 0 to 3+. The concentrated sediment was suspended in 2 ml sterile phosphate buffer (pH 7.0) and an aliquot was cultured on Lowenstein-Jensen solid and BACTEC 460TB liquid media (Becton Dickinson, USA). After inoculation for growth detection, the remaining decontaminated specimen was stored at −20 °C.[23] *M. tuberculosis* complex was isolated in all specimens included in this evaluation. Identification of *M. tuberculosis* in cultures was confirmed by Inno-Lipa Mycobacteria v2 assay (Innogenetics, Belgium).

Drug susceptibility testing

First- and second-line DST for the cultured isolates and clinical specimens selected in Spain was performed by the radiometric method BACTEC 460TB. First and second-line DST for the isolates from Lithuania was performed with the non-radiometric BACTEC MGIT. BACTEC 460TB critical concentrations for INH, RIF, moxifloxacin (MOX), KAN and CAP were 0.1 μg/ml, 2 μg/ml, 0.5 μg/ml, 5 μg/ml and 1.25 μg/ml, respectively.[24,25] BACTEC MGIT critical concentrations for INH, RIF, ofloxacin (OFX), levofloxacin (LVX), KAN, CAP and AMK were 0.1 μg/ml, 1.0 μg/ml, 2 μg/ml, 1.5 μg/ml, 2.5 μg/ml, 3 μg/ml and 1.5 μg/ml, respectively.[25–27] In this study, BACTEC (460 TB or MGIT) was considered the reference standard method.

Characterization of molecular drug resistance

Molecular drug resistance of cultured isolates and sputum specimens included in this study was previously characterized by pyrosequencing,[13,14] DNA sequencing,[28] GenoType MTBDR*plus*[15] and/or GenoType MTBDR*sl* (Hain Lifescience GmbH, Germany).[14] Briefly, pyrosequencing was used to detect mutations in *katG* codon 315, *inhA* promoter positions −16 to −5, *rpoB* codons 516 and 526 to 531, *gyrA* codons 80 and 88 to 95, *rrs* positions 1401, 1402, and 1484, and *eis* promoter positions −37, −14, −12 and −10. For a set of isolates, the entire *katG* gene, specific regions of the *oxyRahpC* located upstream of the gene, the *mabA-inhA* regulatory region, and the 81-bp core region of *rpoB* were sequenced. In addition, GenoType MTBDR*plus* and/or GenoType MTBDR*sl* tests were performed following manufacturer's instructions. The mutations that can be detected by these tests and that are relevant for this study are the following ones: *katG* codon 315; *inhA* positions −8, −15 and −16; *rpoB* codons 516, 526, and 531; *gyrA* codons 90, 91 and 94; and *rrs* positions 1401, 1402 and 1484.

Anyplex II MTB/MDR/XDR

DNA from cultured isolates and sputum specimens was extracted as previously described.[15] Anyplex II MTB/MDR/XDR was performed following the instructions of the manufacturer. This is a multiplex real-time PCR based on dual priming oligonucleotide (DPO) and tagging oligonucleotide cleavage and extension (TOCE) technologies. Two PCR reactions in independent tubes are performed simultaneously: a PCR reaction detects *M. tuberculosis* (MTB melting curve) and resistance to INH and RIF (MTB/MDR), and the other PCR reaction detects *M. tuberculosis* and resistance to FLQ and injectable drugs (MTB/XDR). An internal control (IC) is included in each of these reactions. Cultured isolates and sputum specimens with previous results of first-line molecular DST were tested for MTB/MDR, whereas isolates and specimens with previous results of second-line molecular DST were tested for MTB/XDR. Among the 114 cultured isolates, 61 were analyzed by the MTB/MDR test and 60 were analyzed by the MTB/XDR test (seven of these isolates were analyzed by both MDR and XDR tests) (Fig. 1). Among the 73 sputum specimens, 60 were analyzed by the MTB/MDR test and 60 were analyzed by the MTB/XDR test (47 specimens were analyzed by both MDR and XDR tests) (Fig. 1).

This assay detects 25 MDR-associated mutations (7 INH-resistance mutations and 18 RIF-resistance mutations) and 13 XDR-associated mutations (7 FLQ-resistance mutations and 6 injectable drug-resistance mutations). The assay covers the following mutations: *katG* S315I (ATC), S315N (AAC), S315T

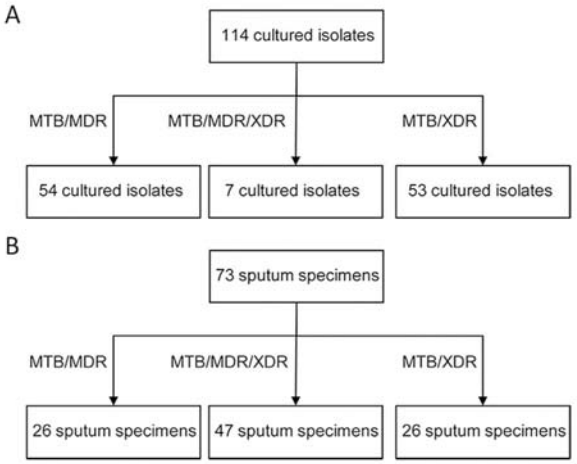

Figure 1 Number of cultured isolates and sputum specimens retrospectively selected for the study and analyzed by the MTB/MDR, MTB/XDR, or both MTB/MDR/XDR tests. A: a total of 61 cultured isolates were analyzed by the MTB/MDR test and 60 were analyzed by the MTB/XDR test. B: among the sputum specimens, 60 were analyzed by the MTB/MDR test and 60 were analyzed by the MTB/XDR test.

(ACC) and S315T (ACA); *inhA* promoter −15(T), −8(A) and −8(C); *rpoB* L511P(CCG), Q513K(AAA), Q513L(CTA), Q513P (CCA), 3 amino acid deletion in 513–516, D516V(GTC), D516Y(TAC), S522L(TTC), S522Q(CAG), H526C(TGC), H526D (GAC), H526L(CTC), H526N(AAC), H526R(CGC), H526Y(TAC), S531L(TTG), S531W(TGG) and L533P(CCG); *gyrA* A90V(GTG), S91P(CCG), D94A(GCC), D94G(GGC), D94H(CAC), D94N (AAC), D94Y(TAC); *rrs* 1401(G), 1402(T) and 1484(T); *eis* promoter −37(T), −14(T) and −10(A).

In each run, an independent reaction with wild-type DNA was performed, as the result of the tested sample is interpreted by comparing it with the result of the wild-type control. In order to consider a result as valid, IC melting curve must be detected. MTB melting curve is present when *M. tuberculosis* complex DNA is detected. Melting curves referring to drug resistance are only present when a mutation is detected. Therefore, the absence of a drug resistance-melting curve indicates that the isolate is sensitive to the respective drug, while its presence indicates resistance. Regarding INH-resistance detection, two melting peaks can be detected: a peak at a lower melting temperature if the mutation is in *katG*, and a peak at a higher melting temperature if the mutation is in *inhA* promoter. Concerning detection of RIF and FLQ resistance, the corresponding melting curves show a single peak, as only one genomic region is targeted for each drug. As for injectable drugs, this test does not discriminate between KAN, AMK and CAP resistance. However, three melting peaks can be detected: a first peak at the lowest melting temperature is present when any of the *eis* promoter mutations are detected; a second melting peak indicates the presence of *rrs* A1401G and/or G1484T mutations; and a third peak at the highest melting temperature is present when the *rrs* C1402T mutation is detected. In addition to the melting curves, Anyplex software yields a table reporting an auto interpretation stating if the result is invalid, if there is only presence of *M. tuberculosis* DNA (in case of a drug susceptible isolate), or if any drug resistance is detected along with the location of the mutation. Results obtained with Anyplex were compared with those obtained with BACTEC. Discordant results between Anyplex and BACTEC were compared with results obtained with pyrosequencing, DNA sequencing, GenoType MTBDR*plus* and/or GenoType MTBDR*sl*. Researchers who read and recorded Anyplex results were blind to both BACTEC and molecular drug susceptibility results.

Statistical analysis

Sensitivity and specificity values of Anyplex II MTB/MDR/XDR, with their corresponding 95% confidence intervals (CI), were calculated considering BACTEC as reference method. Agreement values and kappa coefficients were also calculated comparing both methods. Kappa (k) values below 0.40 indicate weak correlation, values between 0.41 and 0.60 indicate good agreement and values above 0.60 indicate strong agreement. The diagnostic accuracy was calculated and reported following the STARD statement guidelines.[29]

The commercial statistical software package used was SPSS 15.0 (SPSS Inc, USA).

Results

Cultured isolates

The resistance patterns obtained with BACTEC for all drugs tested are presented in Table 1. Results obtained with Anyplex were compared with those obtained with BACTEC and are shown in Table 2. Examples of results reported by Anyplex software are presented in Fig. 2.

MTB/MDR detection

With regard to detection of INH resistance, for the 12 phenotypically resistant isolates that were identified as susceptible by Anyplex, the molecular result was concordant with both pyrosequencing and GenoType MTBDR*plus*.

Concerning RIF, one phenotypically resistant isolate was identified as susceptible by Anyplex, and the result was concordant with pyrosequencing. However, MTBDR*plus* and DNA sequencing detected the *rpoB* 531(TTG) mutation. One RIF^S isolate, identified as resistant by Anyplex, was found to harbor the *rpoB* 516(TAC) mutation by pyrosequencing. However, MTBDR*plus* identified this isolate as sensitive, and it was in agreement with BACTEC.

Globally, among the 36 MDR cultured isolates, 30 (83.3%) were correctly identified as MDR by Anyplex. Regarding the six remaining isolates, five were identified as RIF^R/INH^S by real-time PCR, a result that was concordant with pyrosequencing and MTBDR*plus*. The last isolate was identified as RIF^S/INH^S by Anyplex and also by pyrosequencing, but both MTBDR*plus* and DNA sequencing detected a mutation in *rpoB* codon 531.

MTB/XDR detection

Regarding FLQ resistance detection, eight phenotypically resistant isolates were identified as susceptible by Anyplex. These molecular results were in complete agreement with those obtained with both pyrosequencing and GenoType MTBDR*sl*, with the exception of a single isolate identified as heteroresistant by MTBDR*sl*. For the four FLQ^S isolates that were identified as resistant by Anyplex, this molecular result was concordant with that obtained with both pyrosequencing and MTBDR*sl*, with the exception of an isolate identified as FLQ^S by pyrosequencing.

Concerning KAN, for the five phenotypically resistant isolates identified as susceptible by the multiplex PCR, both MTBDR*sl* and pyrosequencing detected the *rrs* positions 1401, 1402 and 1484 as wild-type. Nonetheless, for three out of these five isolates, pyrosequencing detected the -C12T mutation in *eis* promoter, a position that is not explored by Anyplex test. The other two isolates harbored wild-type sequences in *eis* promoter. Among the five KAN^S isolates identified as resistant by multiplex PCR, two had a mutation in *rrs* detected by both pyrosequencing and MTBDR*sl*. For the three remaining isolates, all carrying a mutation in the *eis* promoter, Anyplex result was concordant with pyrosequencing for two of them. Susceptibility profiles for KAN, AMK and CAP and the location of the

Table 1 Resistance pattern obtained with BACTEC for INH, RIF, FQ, KAN, AMK and CAP for cultured isolates and sputum specimens.

Drug	Cultured isolates						Sputum specimens				
	INH	RIF	FQ	KAN	AMK	CAP	INH	RIF	FQ	KAN	CAP
Resistant (%)	51 (83.6)	36 (59.0)	27 (45.0)	27 (45.0)	12 (32.4)	7 (11.7)	45 (75.0)	44 (73.3)	10 (16.7)	24 (40.0)	24 (40.0)
Sensitive (%)	10 (16.4)	25 (41.0)	33 (55.0)	33 (55.0)	25 (67.6)	53 (88.3)	15 (25.0)	16 (26.7)	50 (83.3)	36 (60.0)	36 (60.0)
Total	61	61	60	60	37[a]	60	60	60	60	60	60

[a] DST for AMK was not performed for 23 isolates.

mutations detected by Anyplex are presented in Table 3. As for AMK, ten phenotypically sensitive isolates were identified as resistant by multiplex PCR, detecting a mutation in *eis* promoter. This result was concordant with pyrosequencing for nine isolates. Finally, among the 20 CAP^S isolates identified as resistant by Anyplex, two isolates carried a mutation in *rrs* 1401/1484 and the remaining 18 isolates in *eis* promoter. Mutations in *rrs* were also detected by both MTBDR*sl* and pyrosequencing for the two isolates, and mutations in *eis* promoter were detected by pyrosequencing for 17 of the 18 isolates.

Globally, among the 17 isolates resistant to FLQ and at least one of KAN/AMK/CAP included in the study, 12 were correctly identified by Anyplex. Four isolates were identified as sensitive to both FLQ and injectable drugs by the assay as well as by the alternative molecular methods. The last isolate was identified as resistant to second-line injectable drugs with a mutation in the *eis* promoter, and sensitive to FLQ. The alternative molecular methods also detected this isolate as FLQ^S.

Sputum specimens

Among the 73 sputum specimens tested, six were smear negative and 67 were smear positive. A total of 12 specimens had an acid fast bacillus count of one to ten per 100 fields (smear 1+), 11 specimens had one to nine bacilli per field (smear 2+), and 44 specimens had more than nine bacilli per field (smear 3+). A valid result was obtained for all the sputum specimens. However, for three specimens IC was positive but *M. tuberculosis* was not

Table 2 Distribution of Anyplex II MTB/MDR/XDR results according to BACTEC result for the cultured isolates.

BACTEC result (no. of isolates)	No. of isolates with the following Anyplex II MTB/MDR/XDR result	
	Resistant (%)	Sensitive (%)
INH		
Resistant (n = 51)	39 (76.5)	12 (23.5)
Sensitive (n = 10)	0 (0)	10 (100)
Total (n = 61)	39 (63.9)	22 (36.1)
RIF		
Resistant (n = 36)	35 (97.2)	1 (2.8)
Sensitive (n = 25)	1 (4.0)	24 (96.0)
Total (n = 61)	36 (59.0)	25 (41.0)
FQ		
Resistant (n = 27)	19 (70.4)	8 (29.6)
Sensitive (n = 33)	4 (12.1)	29 (87.9)
Total (n = 60)	23 (38.3)	37 (61.7)
KAN		
Resistant (n = 27)	22 (81.5)	5 (18.5)
Sensitive (n = 33)	5 (15.2)	28 (84.8)
Total (n = 60)	27 (45.0)	33 (55.0)
AMK		
Resistant (n = 12)	12 (100)	0 (0)
Sensitive (n = 25)	10 (40.0)	15 (60.0)
Total (n = 37)	22 (59.5)	15 (40.5)
CAP		
Resistant (n = 7)	7 (100)	0 (0)
Sensitive (n = 53)	20 (37.7)	33 (62.3)
Total (n = 60)	27 (45.0)	33 (55.0)

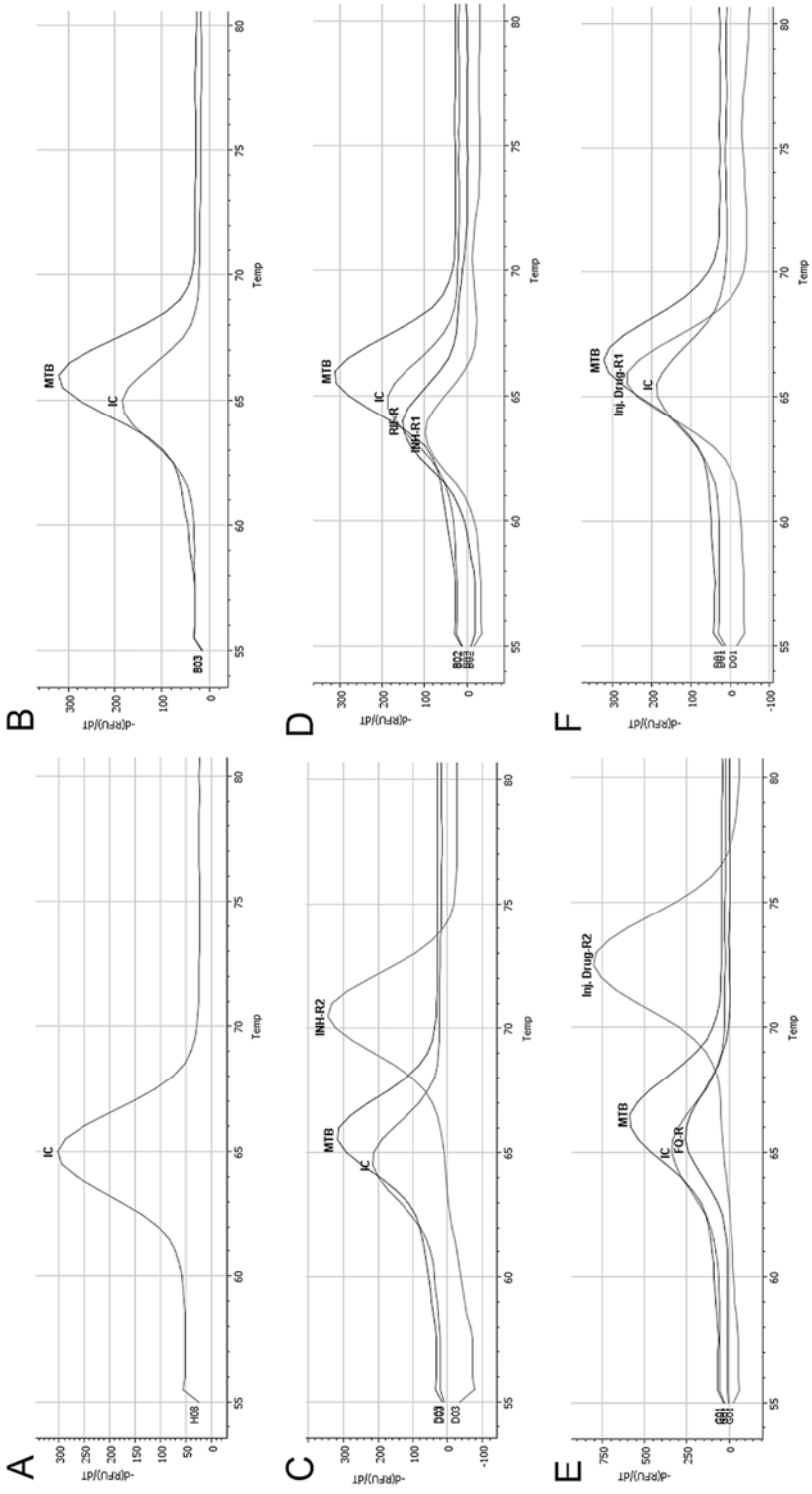

Figure 2 Examples of results obtained with Anyplex II MTB/MDR/XDR. Plot of the rate of change of the relative fluorescence units with time (-d(RFU)/dT) versus the temperature (Temp, °C). A: example of an excluded result due to absence of MTB melting curve. B: example of a INH^S and RIF^S isolate. C: example of a INH^R isolate, with a mutation in *inhA* promoter, and RIF^S. D: example of an isolate resistant to both INH and RIF, with mutations in *katG* and *rpoB*, respectively. E: example of an isolate resistant to both FQ and injectable drugs, with mutations in *gyrA* and *rrs*, respectively. F: example of an isolate FQ^S and resistant to injectable drugs, with a mutation in *eis* promoter.

Table 3 Relationship between profiles of resistance to KAN, AMK and CAP obtained with BACTEC and mutations in *rrs* and *eis* promoter detected by Anyplex II MTB/MDR/XDR for the cultured isolates.

Susceptibility to:			No. of isolates with the following mutation				Total
			rrs		*eis* promoter	NM	
KAN	AMK	CAP	A1401G/G1484T	C1402T			
R	R	R	3	1[a]	1	–	4
R	R	S	–	–	8	–	8
R	S	S	–	–	7	5	12
R	ND[b]	R	3	1[a]	–	–	3
S	S	S	–	–	3	10	13
S	ND[b]	S	2	–	–	18	20

R: resistant; S: susceptible; ND: not done; NM: no mutation in the explored positions.
[a] This isolate harbored mutations at both 1401/1484 and 1402.
[b] DST for AMK was not performed for 23 isolates.

detected and therefore they were excluded for the analysis. One of these excluded specimens, analyzed by the MTB/MDR test, was smear negative. The remaining two excluded specimens, analyzed by the MTB/XDR test, were smear negative and smear 1+, respectively. Therefore, overall sensitivity of Anyplex to detect *M. tuberculosis* was 95.9% (70/73): 98.5% (66/67) in smear positive sputum specimens and 66.7% (4/6) in smear negative specimens.

Resistance patterns obtained with BACTEC for all the drugs tested are presented in Table 1. Results obtained with Anyplex regarding drug resistance were compared with those obtained with BACTEC and are shown in Table 4.

MTB/MDR detection

Among the four INH^R sputum specimens identified as sensitive by multiplex PCR, two derived from the same patient and were found to be sensitive by both GenoType MTBDR*plus* and pyrosequencing. However, for the remaining two specimens, obtained from two different patients, both alternative molecular methods detected the mutation *katG*315 (ACC). These specimens were smear 3+. Moreover, other specimens from the same patients were tested, and the results obtained with Anyplex were in concordance with phenotypic DST.

With regard to RIF, among the three phenotypically resistant sputum specimens identified as sensitive by the real-time PCR, two were from the same patient and were identified as resistant by both MTBDR*plus* and pyrosequencing. The last specimen was tested by the line probe assay and the result was in agreement with that obtained with real-time PCR.

Globally, among the 44 MDR sputum specimens included in this study, obtained from 14 different patients, Anyplex correctly identified 38 as resistant to both INH and RIF. Considering only one specimen per patient, Anyplex

Table 4 Distribution of Anyplex II MTB/MDR/XDR results according to BACTEC result for the sputum specimens.

BACTEC result (no. of specimens)	No. of specimens with the following Anyplex II MTB/MDR/XDR result		
	Resistant (%)	Sensitive (%)	Invalid (%)
INH			
Resistant (n = 45)	41 (91.1)	4 (8.9)	–
Sensitive (n = 15)	0 (0)	14 (93.3)	1 (6.7)
Total (n = 60)	41 (68.3)	18 (30.0)	1 (1.7)
RIF			
Resistant (n = 44)	41 (93.2)	3 (6.8)	–
Sensitive (n = 16)	0 (0)	15 (93.8)	1 (6.3)
Total (n = 60)	41 (68.3)	18 (30.0)	1 (1.7)
FQ			
Resistant (n = 10)	6 (60.0)	4 (40.0)	–
Sensitive (n = 50)	0 (0)	48 (96.0)	2 (4.0)
Total (n = 60)	6 (10.0)	52 (86.7)	2 (3.3)
KAN/CAP			
Resistant[a] (n = 24)	20 (83.3)	3 (12.5)	1 (4.2)
Sensitive (n = 36)	1 (2.8)	34 (94.4)	1 (2.8)
Total (n = 60)	21 (35.0)	37 (61.7)	2 (3.3)

[a] These 24 sputum specimens were resistant to both KAN and CAP.

detected 13 of the 14 MDR specimens. The remaining specimen was detected as INH^S/RIF^R by Anyplex, and this result was concordant with both GenoType MTBDR*plus* and pyrosequencing.

MTB/XDR detection

Regarding the detection of FLQ resistance, four phenotypically resistant specimens were identified as susceptible by the multiplex PCR as well as by GenoType MTBDR*sl* and pyrosequencing.

Regarding the injectable drugs, all specimens were phenotypically resistant to both KAN and CAP. Three resistant specimens were identified as sensitive by Anyplex, but both pyrosequencing and GenoType MTBDR*sl* detected the *rrs* mutation A1401G. One of these three specimens was smear negative, and the remaining two were smear 1+. For one KAN^S/CAP^S specimen, a mutation was detected in the *eis* promoter by Anyplex and by pyrosequencing as well.

Overall performance

Sensitivity and specificity values of Anyplex for detecting resistance to INH, RIF, FLQ, KAN, AMK and CAP in cultured isolates and sputum specimens are shown in Table 5. Agreement values between the multiplex real-time PCR and BACTEC according to each drug considered are also shown in Table 5.

Overall, there was a total of 81 discordant results between Anyplex and BACTEC. GenoType tests and/or pyrosequencing were concordant with the multiplex PCR result in 100% (12/12), 100% (2/2), 100% (12/12), 90% (9/10), 90% (9/10) and 95% (19/20) of the isolates regarding the detection of INH, RIF, FLQ, KAN, AMK and CAP resistance, respectively. Likewise, the result obtained with at least one of the alternative molecular methods was concordant with the multiplex PCR result in 50% (2/4), 33.3% (1/3), 100% (4/4), and 25.0% (1/4), of the sputum specimens with regard to the detection of INH, RIF, FLQ and KAN/CAP resistance, respectively. In summary, considering together isolates and specimens and all the drugs tested, Anyplex results were concordant with at least one of the alternative molecular methods in 87.7% (71/81) of the discordant cases.

Discussion

Results obtained in this study show that Anyplex II MTB/MDR/XDR, a multiplex real-time PCR test, may be a useful method to detect resistance to the most important first- and second-line drugs in cultured isolates and in decontaminated sputum specimens.

Overall sensitivity and specificity values of Anyplex to detect INH resistance considering isolates and specimens were 80.3% and 100%, respectively. The agreement values between cultured isolates and sputum specimens are not comparable between them because these isolates are not obtained from these specimens. Hence, these cultured isolates and sputum specimens were obtained from different patients and no epidemiological connection is suspected. The higher agreement between multiplex PCR and BACTEC for this drug and also for other ones is due to

Table 5 Sensitivity and specificity of Anyplex II MTB/MDR/XDR for detecting drug resistance considering one isolate and one specimen per patient and agreement values between Anyplex II MTB/MDR/XDR and BACTEC.

Drug	Cultured isolates					Sputum specimens					
	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Agreement (%)	Kappa	SE	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Agreement (%)	Kappa	SE
INH	39/51 (76.5) (62.2–86.8)	10/10 (100) (65.5–100)	49/61 (80.3)	0.516	0.110	14/15 (93.3) (66.0–99.7)[a]	14/14 (100) (73.2–100)[a]	55/59 (93.2)	0.829	0.081
RIF	35/36 (97.2) (83.8–99.9)	24/25 (96.0) (77.7–99.8)	59/61 (96.7)	0.932	0.047	14/14 (100) (73.2–100)[a]	15/15 (100) (74.7–100)[a]	56/59 (94.9)	0.874	0.070
FLQ	19/27 (70.4) (49.7–85.5)	29/33 (87.9) (70.9–96.0)	48/60 (80.0)	0.590	0.105	2/4 (50.0) (0.09–90.8)[b]	19/19 (100) (79.1–100)[b]	54/58 (93.1)	0.713	0.133
KAN	22/27 (81.5) (61.3–93.0)	28/33 (84.8) (67.3–94.3)	50/60 (83.3)	0.663	0.097	5/5 (100) (46.3–100)[b]	17/18 (94.4) (70.6–99.7)[b]	54/58 (93.1)	0.854	0.070
AMK	12/12 (100) (69.9–100)	15/25 (60.0) (38.9–78.2)	27/37 (73.0)	0.493	0.118	–[c]	–[c]	–	–	–
CAP	7/7 (100) (56.1–100)	33/53 (62.3) (47.9–74.9)	40/60 (66.7)	0.278	0.091	5/5 (100) (46.3–100)[b]	17/18 (94.4) (70.6–99.7)[b]	54/58 (93.1)	0.854	0.070

CI, confidence interval.
[a] Twenty-nine sputum specimens were included for INH and RIF sensitivity and specificity calculations.
[b] Twenty-three sputum specimens were included for FLQ and KAN/CAP calculations.
[c] AMK phenotypic DST was not performed for the sputum specimens.

the presence or absence of the mutations detected in the cultured isolates and sputum specimens. Sensitivity of in-house real-time PCR assays ranged from 49.1% to 100%, while specificity remained 100%.[19,20,30] It is of note that in some of these studies, mutations in *inhA* promoter were not explored. In the present study, INH^R isolates/specimens identified as sensitive by Anyplex, GenoType MTBDR*plus* and pyrosequencing may harbor mutations in the *ahpC-oxyR* intergenic region, in *kasA*, or in other genomic regions.[31] Regarding the detection of RIF resistance, overall sensitivity and specificity of Anyplex was 98.0% and 97.5%, respectively, values that are in accordance with the reported in other studies.

Concerning FLQ resistance and taking into account all the isolates and specimens tested in this study, Anyplex had a sensitivity of 67.7% and specificity of 92.3%. Other real-time PCR assays that mainly explored mutations in codons 90, 91 and 94 of *gyrA* showed a sensitivity ranging from 71% to 82.7%, a specificity of 100% and concordance of 94–100% with DNA sequencing.[32–34] In our study, cultured isolates and sputum specimens phenotypically FLQ^R but identified as sensitive by Anyplex may harbor mutations in other regions of *gyrA* or in *gyrB*. In addition, phenotypic resistance may be due to the effect of active efflux pumps.

As for second-line injectable drugs, Anyplex indicates resistance without discriminating between KAN, AMK and CAP. This is a drawback of the test, as it reports resistance to the three injectable drugs in cases where the mutation does not confer resistance to one of the drugs, e.g. an isolate phenotypically sensitive to CAP harboring a mutation in *eis* promoter. Nonetheless, since the peak of the melting curve indicates if the mutation is in *eis* promoter, *rrs* 1401/1484 or *rrs* 1402, it is possible to interpret if the isolate is resistant to KAN, AMK and/or CAP. Thus, it is important to have in mind which mutations are associated with resistance to each of these drugs. The mutations *rrs* A1401G and G1484T are associated with resistance to the three drugs while C1402T mutation has been detected in isolates resistant to CAP.[10,35,36] Mutations in *eis* promoter are not associated with CAP resistance, but which of the mutations confer resistance to KAN and/or AMK is controversial. In a systematic review, Georghiou et al. showed that both G-10A and C-14T mutations at *eis* promoter but not the C-12T change are associated with KAN resistance.[10] In contrast, Rodwell et al. found that the three mutations conferred KAN resistance.[37] Moreover, in another study both G-10A and C-12T changes were more frequently detected in KAN^S isolates, and only C-14T was considered to be associated with KAN resistance.[38] Regarding AMK resistance, in the aforementioned systematic review, both C-12T and C-14T mutations were highly specific.[10] In contrast, Rodwell and colleagues reported that the C-12T mutation is not specific.[37] This variability of results may be due to the different geographical origin of isolates included in those studies. Our set of isolates was previously characterized by pyrosequencing and we found that G-10A, C-12T and C-14T mutations were more common in KAN^R isolates, and that C-14T was also more frequent in AMK^R isolates than in KAN^S and AMK^S isolates (unpublished results). Considering that it is not possible to identify the exact mutation by the Anyplex test, and that *rrs* 1402 mutation was always detected together with *rrs* 1401/1484, we can assume that isolates with mutations in *eis* promoter are KAN^R, and isolates with mutations in *rrs* 1401/1484 are $KAN^R/AMK^R/CAP^R$. In this case, sensitivity and specificity of Anyplex to detect AMK resistance would be 25% and 100%, respectively, whereas these values for CAP resistance would be 85.7% and 96.2%. This way, the test would present lower sensitivity for AMK resistance because the isolates with the C-14T mutation in *eis* promoter are being considered as AMK^S. Excepting these cases, sensitivity and specificity values of Anyplex for detecting resistance to KAN, AMK and CAP are similar to those obtained with other real-time PCR methods exploring mutations in *rrs* or *rrs* together with *eis* promoter.[33,39]

Heteroresistance, defined as the presence of both drug-sensitive and drug-resistant populations, and identified by molecular methods by simultaneous detection of wild-type and mutation sequences, is frequent among *M. tuberculosis* isolates.[40] Anyplex is not able to detect heteroresistance, while other molecular methods such as sequencing or line probe assays can identify them.[14] In our study, one FLQ^R specimen by BACTEC was detected as heteroresistant by line probe assay but sensitive by Anyplex. Therefore, it is possible that some resistant isolates or specimens may be incorrectly identified as sensitive by Anyplex because the ratio of mutated/wild-type DNA is not high enough to be detected. However, there are other in-house real-time PCR-based methods that are able to detect heteroresistance.[30,32–34,39]

In this study, Anyplex detected *M. tuberculosis* DNA in 98.5% of the smear positive sputum specimens and in 66.7% of the smear negative ones. These values are in concordance with the sensitivity of GeneXpert MTB/RIF on smear positive and smear negative specimens.[41] Nevertheless, the number of smear negative specimens included in our study was low.

Anyplex assay indicates drug resistance by the presence of a melting curve only when a mutation is detected, whereas when the sequence is wild-type the drug resistance-melting curve is absent. Consequently, results obtained with this method are more easily readable that the ones obtained with previously described methods, such as high resolution melting analysis or other real-time PCR methods. An important drawback of the evaluated method is that it is not possible to identify the specific mutation involved. In addition, since the melting curve is only detected when there is a mutation, its absence indicates that the sequence is wild-type. Nonetheless, melting curve may be missing due to a fail in amplifying the sequence target, and therefore a false susceptibility result may be obtained. A possible explanation can be that the DNA recovery during the extraction process is not optimal, considering that we obtained different results when testing different specimens from the same patient.

The gold standard molecular method to detect mutations associated with drug resistance is DNA sequencing, although it is more laborious and expensive in comparison to other available methods.[42] Some alternative molecular methods, such as line probe assays or arrays, are also technically challenging, with more risk of cross-contamination and requiring additional post PCR steps that may increase assay time. Conversely, the evaluated multiplex PCR is a fast and simple method. The complete protocol lasts 3.5 h: 30 min for DNA extraction, and 3 h for PCR and interpretation of the molecular drug susceptibility result

provided by the software. On the other hand, a significant drawback for molecular diagnostic methods in general is that not all genes or mechanisms of resistance have been identified. Hence the maximum sensitivity that can be achieved depends on the targets explored and the prevalence of each mutation in different geographical settings. However, since the specificity of molecular methods is high, when a mutation is detected the isolate can be confidently reported as drug resistant. In cases of suspected drug resistance, and especially for INH and FLQ, it may be rapidly detected by molecular methods if any of the mutations targeted is present, although it will be necessary to wait for the phenotypic DST to confirm the result. Furthermore, results obtained in our study suggest that RIF resistance can be used as a useful marker of MDR-TB, even though INH resistance was not genotypically detected in some cases. On the contrary, when resistance to FLQ or to any of the second-line injectable drugs is detected the possibility of an XDR-TB must be confirmed by additional testing. Studies performing whole genome sequencing, especially for those drug-resistant isolates lacking known mutations may shed light on new mutations associated with drug resistance.[31,43]

In conclusion, this is the first study assessing the clinical accuracy of the Anyplex II MTB/MDR/XDR test. This multiplex real-time PCR based on DPO and TOCE technologies has been proven to be useful to detect resistance to INH, RIF, FLQ and, with some considerations, to second-line injectable drugs on cultured isolates and sputum specimens. Nevertheless, phenotypic DST is still required, especially for confirming susceptibility results.

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Conflicts of interest

None of the investigators has any financial interest or financial conflict with the subject matter or materials discussed in this report.

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Article III

Molina-Moya B, Lacoma A, Prat C, Diaz J, Dudnyk A, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Ausina V, Dominguez J

AID TB resistance line probe assay for rapid detection of resistant *Mycobacterium tuberculosis* in clinical samples

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AID TB resistance line probe assay for rapid detection of resistant *Mycobacterium tuberculosis* in clinical samples

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Summary *Objectives:* To determine the sensitivity and specificity of AID TB Resistance line probe assay (AID Diagnostika, Germany) to detect *Mycobacterium tuberculosis* and its resistance to first- and second-line drugs in clinical samples using BACTEC 460TB as the reference standard.
Methods: The test consists on three strips to detect resistance to isoniazid/rifampicin, fluoroquinolones/ethambutol, and kanamycin/amikacin/capreomycin/streptomycin, respectively. This test was performed on 65 retrospectively selected clinical samples corresponding to 32 patients.
Results: A valid result was obtained for 92.3% (60/65), 90.8% (59/65) and 78.5% (51/65) of the samples tested, considering the three strips, respectively. Global concordance rates between AID and BACTEC for detecting resistance to isoniazid, rifampicin, fluoroquinolones, ethambutol, kanamycin/capreomycin and streptomycin were 98.3% (59/60), 100% (60/60), 91.5% (54/59), 72.9% (43/59), 100% (51/51) and 98.0% (50/51), respectively. Regarding the discordant

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results obtained between AID and BACTEC, the alternative molecular methods performed (GenoType MTBDR*plus*, GenoType MTBDR*sl* [Hain Lifescience, Germany] and/or pyrosequencing) confirmed the genotypic result in 90.9% (20/22) of the cases.
Conclusions: AID line probe assay is a useful tool for the rapid detection of drug resistance in clinical samples enabling an initial therapeutic approach. Nevertheless, for a correct management of drug resistant tuberculosis patients, molecular results should be confirmed by a phenotypic method.

Introduction

In 2012, World Health Organization (WHO) reported 8.6 million new tuberculosis (TB) cases and 1.3 million deaths.[1] According to the resistance pattern, multidrug-resistant (MDR) TB is defined as *Mycobacterium tuberculosis* resistant to isoniazid (INH) and rifampicin (RIF), and extensively drug-resistant (XDR) TB strains are those additionally resistant to fluoroquinolones (FQ) and at least one of the second-line injectable drugs kanamycin (KAN), amikacin (AMK) or capreomycin (CM).[2,3] The long time to achieve a diagnosis and the incorrect treatment regimens have led to the emergence and spread of drug-resistant TB.[4] In 2012, WHO reported 84,000 confirmed MDR-TB cases worldwide, and 9.6% of these cases were XDR-TB.[1]

Drug resistance in *M.tuberculosis* emerges by the stepwise acquisition of genetic mutations in genes coding for drug targets or drug-converting enzymes.[5,6] Regarding first-line drug resistance, INH resistant strains may harbor mutations in *katG* codon 315 and positions −8, −15 and −16 of the *inhA* promoter.[7] Nevertheless, there is a significant percentage of strains that may carry mutations in other still unknown genomic regions. As for RIF, mutations in the 81-bp core region of *rpoB* have been detected in 95–99% of resistant isolates.[7–9] The most common mutations are located in codons 531, 526 and 516.[10,11] Considering ethambutol (EMB), mutations in *embCAB* have been detected in resistant strains, being the *embB*306 the codon most commonly affected.[12,13] Finally, streptomycin (STR) resistant strains usually harbor mutations in *rpsL* codons 43 and 88 or in *rrs* gene regions 530 and 915.[7,14,15]

With regard to second-line drugs, most of the FQ-resistant strains harbor mutations in *gyrA* and *gyrB*. The most common mutations are located in codons 90, 91 and 94, in the quinolone resistance determining region (QRDR) of *gyrA*.[16,17] Concerning injectable drugs, cross-resistance between KAN, AMK and CM has been reported.[18] Mutations in the *rrs* gene at positions 1401 and 1484 have been associated with resistance to the three drugs, while mutations at 1402 are associated with resistance to KAN and CM.[7,19] In addition, resistance to KAN has also been associated with mutations at positions −10, −14 and −37 in the promoter region of *eis*.[20]

One of the most evaluated methods aimed to detect resistance to first- and second-line drugs are the line probe assays (LPA).[21] This method is based on PCR amplification of specific gene regions associated with drug resistance and the subsequent detection of genetic mutations by hybridization of the PCR product to specific probes immobilized to a nitrocellulose membrane. LPA can be performed not only in clinical isolates, but also directly in clinical samples. In 2008 GenoType MTBDR*plus*, aimed to detect resistance to INH and RIF, was endorsed by the WHO for use in smear positive samples.[22] Later on, GenoType MTBDR*sl* was developed for detecting resistance to FQ, KAN/AMK/CM and EMB. During the last years, these tests have been the only commercially available LPA for the molecular detection of first- and second-line drug resistance in *M.tuberculosis*. Recently, a new commercial LPA named AID TB Resistance (AID Diagnostika, Germany) has been developed.

The present study was undertaken to determine the diagnostic accuracy of AID TB Resistance for the detection of *M.tuberculosis* and its resistance to INH, RIF, FQ, EMB, KAN, CM and STR directly in clinical samples comparing the results with those obtained by the reference phenotypic method BACTEC 460TB.

Materials and methods

Clinical samples

A total of 65 respiratory clinical samples (62 sputum samples, 1 bronchoalveolar lavage sample, 1 bronchial aspirate sample, 1 gastric fluid sample) from 32 patients were retrospectively selected. The study was approved by the institutional ethics committee. From 10 patients, more than one sample were obtained. All samples had been collected at the time of diagnosis or during a maximum period of 2 months; they were not obtained by split, and therefore, there was no repeat testing of a single sample. Samples were processed as follows. First, they were digested and decontaminated using Kubica's *N*-acetyl-L-cysteine NaOH method.[23,24] After decontamination, auramine-rhodamine acid-fast staining was performed from the concentrated sediment. Specimens that were positive by fluorochrome staining were confirmed with Ziehl-Neelsen staining. The auramine-rhodamine smears were graded on a scale from 0 to 3+. Three samples were smear negative and 62 were smear positive. A total of 10 samples had an acid fast bacillus count of one to ten per 100 fields (smear 1+), 10 samples had one to nine bacilli per field (smear 2+), and 42 samples had more than nine bacilli per field (smear 3+). The concentrated sediment was suspended in 2 ml sterile phosphate buffer (pH 7.0) and an aliquot was cultured on Lowenstein-Jensen solid and BACTEC 460TB liquid media (Becton Dickinson, USA), on the basis of laboratory testing practices at the time of diagnosis. After inoculation for growth detection, the remaining

decontaminated specimen was stored at −20 °C.[25] Identification of *M. tuberculosis* in cultures was confirmed by Inno-Lipa Mycobacteria v2 assay (Innogenetics, Belgium).

Drug susceptibility

First- and second-line phenotypic DST was performed at the time of diagnosis with the radiometric method BACTEC 460TB. Critical concentrations for INH, RIF, EMB, STR, moxifloxacin (MOX), KAN and CM were 0.1 μg/ml, 2 μg/ml, 7.5 μg/ml, 6 μg/ml, 0.5 μg/ml, 5 μg/ml and 1.25 μg/ml, respectively. In this study, BACTEC 460TB was considered the gold standard method.

Genotypic drug resistance characterization

Clinical specimens were incubated at 95 °C during 30 min for *M.tuberculosis* inactivation before DNA extraction. DNA extraction was performed using Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promega, USA). Immediately after DNA extraction, AID TB Resistance assay was performed following manufacturer's instructions. Hybridization and detection were performed with AutoLipa (Innogenetics, Belgium), an automated washing and shaking device. For those cases with unspecific background bands after the hybridization step, DNA was diluted 1:10 and the assay was repeated from the PCR step. This test is a LPA consisting of three modules termed INH/RIF, FQ/EMB and AG. This last module detects mutations related to resistance to the following aminoglycosides (AG): KAN, AMK and STR; and to the cyclic peptide CM. Four control probes (Conjugate control, Amplification control, *Mycobacterium* genus control and *M. tuberculosis* complex control) are present in each strip to verify the test procedures. In order to consider a result valid, all four control bands should be present; otherwise, the result is considered invalid. INH/RIF module consists of 14 reaction zones and detects mutations in *inhA* positions −16, −15 and −8, *katG* codon 315 and *rpoB* codons 516, 526 and 531. FQ/EMB module consists of 17 reaction zones and detects mutations in *gyrA* codons A90V, S91P, D94A, D94N, D94Y and D94G, and *embB* codons M306V and M306I. AG module consists of 19 reaction zones and detects mutations in *rrs* positions A1401G, C1402T and G1484 C/T, *rrs* positions C513T, A514C, G515C and C517T and *rpsL* codons A43G, A88G and A88C. The presence of all wild-type hybridization bands in combination with the absence of mutation bands indicates that *M.tuberculosis* is susceptible to the drug considered. The absence of at least one wild-type hybridization band and/or the presence of any mutation bands indicates resistance to the considered drug. The presence of all wild-type hybridization bands, in combination with a mutation band in a target gene indicates heteroresistance, a combination of both susceptible and resistant *M. tuberculosis*. Researchers who read and recorded AID results were blind to the BACTEC 460TB result. Discordant results between AID and BACTEC 460TB were analyzed by GenoType MTBDR*plus*, GenoType MTBDR*sl* (Hain Lifescience GmbH, Germany) and/or pyrosequencing. Both commercial assays were performed following the manufacturer's instructions and pyrosequencing was performed as previously described.[26–28] Briefly, the pyrosequencing method consists of a PCR amplification followed by the pyrosequencing reaction. PCR and pyrosequencing primers for *rpoB*, *katG*, *inhA*, *gyrA*, *rrs*, and *embB* were previously described. Mutations detected by pyrosequencing are located in codons 516, 526 and 531 of *rpoB*, codon 315 of *katG*, positions −5, −8, −15 and −16 of *inhA*, codons 80–81 and 88 to 95 of *gyrA*, positions 1401, 1402, and 1484 of 16S *rrs*, and codon 306 of *embB*. Pyrosequencing reaction and data analysis were performed as recommended by the PSQ96MA and SQA software manufacturer (Qiagen GmbH, Hilden, Germany).

Statistical analysis

AID values of sensitivity and specificity, with their corresponding 95% confidence intervals (CI), agreement values and kappa coefficients were calculated considering as reference method BACTEC 460TB. Kappa (*k*) values below 0.40 indicate weak correlation, values between 0.41–0.60 indicate good agreement and values above 0.60 indicate strong agreement. The commercial statistical software package used was SPSS 15.0 (SPSS Inc, USA).

Results and discussion

Sixty-five clinical samples were tested by the three AID modules. Phenotypic resistance profiles to INH, RIF, FQ, EMB, KAN, CM and STR according to BACTEC are presented in Table 1. Forty-five samples corresponding to 13 patients were MDR, and there were no XDR samples. A valid result was obtained for 60 out of 65 (92.3%), 59 out of 65 (90.8%) and 51 out of 65 (78.5%) samples, considering the three separate modules (INH/RIF, FQ/EMB and AG), respectively. The 25 invalid results corresponded to strips in which conjugate, amplification and *Mycobacterium* genus controls were present, but *M.tuberculosis* complex control and bands referring to resistance were missing or extremely faint. As shown in Table 2, the smear result seems to affect the valid result rate, specially for the AG module, since the number of valid results tends to increase when samples are smear 2+ or 3+. Nevertheless, the number of smear negative samples included in this study is low. Previous studies evaluating the performance of GenoType line probe assays

Table 1 First- and second-line drugs resistance pattern obtained by BACTEC 460TB for the clinical samples.

	Drug						
	INH	RIF	EMB	STR	FQ	KAN	CM
Resistant (%)	49 (75.4)	45 (69.2)	37 (56.9)	31 (47.7)	7 (10.8)	20 (30.8)	20 (30.8)
Susceptible (%)	16 (24.6)	20 (30.8)	28 (43.1)	34 (52.3)	58 (86.2)	45 (69.2)	45 (69.2)

Table 2 Number of samples with a valid result according to the module considered and the smear result.

Module tested	Smear result				Total
	Negative (%)	Smear 1+ (%)	Smear 2+ (%)	Smear 3+ (%)	
INH/RIF	1/3 (33.3)	8/10 (80.0)	9/10 (90.0)	42/42 (100)	60/65 (92.3)
FQ/EMB	1/3 (33.3)	8/10 (80.0)	10/10 (100)	40/42 (95.2)	59/65 (90.8)
AG	0/3 (0)	6/10 (60.0)	6/10 (60.0)	39/42 (92.9)	51/65 (78.5)

directly in clinical samples reported overall rates of valid results that ranged from 78.5% to 100%,[27–32] including smear positive and smear negative samples. Considering only smear negative samples, this rate ranged from 46.2% to 100%.[27,28,33]

The distribution of AID results, according to BACTEC 460TB susceptibility pattern is shown in Table 3. Examples of strip results obtained by the molecular test are shown in Fig. 1.

INH and RIF resistance detection

With respect to INH, 45 out of the 46 resistant samples were correctly identified as resistant by AID. For the remaining sample identified as sensitive by the assay, this result was in agreement with the result obtained by both GenoType MTBDR*plus* and pyrosequencing. All 14 samples identified as INH^S by BACTEC 460TB were correctly identified as sensitive by AID. Sensitivity and specificity values were 97.8% and 100%, respectively (Table 4). These data are in concordance with the previously reported in other studies evaluating the performance of GenoType MTBDR*plus* directly in clinical samples.[28–31,34,35] In this study, none of the molecular methods detected one INH-resistant sample by exploring the most common mutations at *katG* and *inhA*. However, mutations in the *ahpC-oxyR* intergenic region or in *kasA* have been found in INH resistant isolates.[8,15,36] Finally, our sample may harbor a mutation or in other yet unknown genomic regions.

Table 3 Distribution of AID results according to BACTEC 460TB result for the 65 clinical samples.

Drug and BACTEC result (no. of clinical samples)	No. of samples (%) with the following AID result			
	Resistant	Sensitive	Mix R/S[a]	Invalid
INH				
Resistant (49)	45 (91.8)	1 (2.1)	–	3 (6.1)
Sensitive (16)	0 (0)	14 (87.5)	–	2 (12.5)
Total (65)	45 (69.2)	15 (23.1)	–	5 (7.7)
RIF				
Resistant (45)	43 (95.6)	0 (0)	–	2 (4.4)
Sensitive (20)	0 (0)	17 (85.0)	–	3 (15.0)
Total (65)	43 (66.2)	17 (26.1)	–	5 (7.7)
FQ				
Resistant (7)	2 (28.6)	4 (57.1)	–	1 (14.3)
Sensitive (58)	0 (0)	52 (89.7)	1 (1.7)	5 (8.6)
Total (65)	2 (3.1)	56 (86.2)	1 (1.5)	6 (9.2)
EMB				
Resistant (37)	21 (56.8)	14 (37.8)	–	2 (5.4)
Sensitive (28)	2 (7.1)	22 (78.6)	–	4 (14.3)
Total (65)	23 (35.4)	36 (55.4)	–	6 (9.2)
KAN/CM				
Resistant (20)	17 (85.0)	0 (0)	–	3 (15.0)
Sensitive (45)	0 (0)	34 (75.6)	–	11 (24.4)
Total (65)	17 (26.2)	34 (52.3)	–	14 (21.5)
STR				
Resistant (31)	22 (71.0)	0 (0)	–	9 (29.0)
Sensitive (34)	1 (2.9)	28 (82.4)	–	5 (14.7)
Total (65)	23 (35.4)	28 (43.1)	–	14 (21.5)

[a] Samples identified as heteroresistant.

As for RIF, the results between the test and BACTEC 460TB were in complete agreement for all the 43 RIF^R and the 17 RIF^S samples. Hence, sensitivity and specificity of AID were 100% (Table 4). In a recent meta-analysis Bwanga et al. reported values of 99% of sensitivity and specificity for the detection of resistance to RIF by GenoType MTBDR*plus* considering both clinical isolates and samples.[37] This high level of agreement between molecular and phenotypic DST is due to the fact that mutations associated with RIF resistance are mainly located in the 81bp-core region of *rpoB*, and mutations outside this region are uncommon.[38]

FQ and EMB resistance detection

Concerning FQ, 2 out of 6 phenotypically resistant samples were correctly identified by AID. The remaining 4 samples identified as FQ sensitive by the assay were also identified as FQ^S by both GenoType MTBDR*sl* and pyrosequencing. Fifty-two out of the 53 samples identified as FQ^S by BACTEC 460TB were identified as sensitive by AID. The remaining sample was identified as heteroresistant by the test, as defined by the presence of both *gyrA* wild-type and a mutation band with the same intensity (Fig. 1). The mutation detected by AID was *gyrA* A90V. Results obtained by GenoType MTBDR*sl* and pyrosequencing for this sample were in agreement with the phenotypic result, thereby they did not confirm the AID result. Sensitivity and specificity values of AID were 33.3% and 98.1%, respectively (Table 4). It is of note that the number of FQ^R samples included in this study is small. These samples corresponded to two patients and mutation in *gyrA* was detected in only one of them. In previous studies, the sensitivity of GenoType MTBDR*sl* for the detection of FQ resistance directly in clinical specimens ranged from 33.3% to 100%, and specificity ranged from 84.6% to 100%.[27,32,33,39–41] In a meta-analysis evaluating this same test, Feng et al. reported a sensitivity of 87% and a specificity of 97% considering strains and specimens altogether.[42] FQ^R samples/strains not detected by any

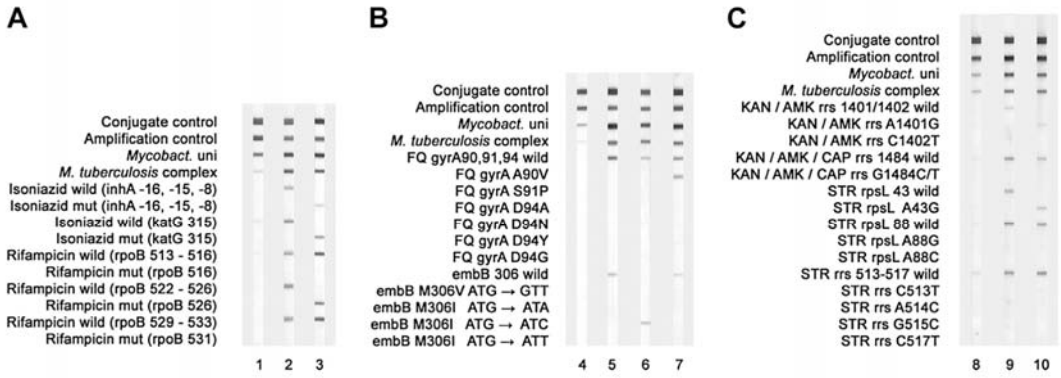

Figure 1 Examples of results obtained by AID TB Resistance assay. A: results obtained for INH/RIF module. Lane 1: example of an invalid result. Lane 2: example of a pattern of INH^S and RIF^S. Lane 3: example of a pattern of INH^R and RIF^R. B: results obtained for FQ/EMB module. Lane 4: example of an invalid result. Lane 5: example of a pattern of FQ^S and EMB^S. Lane 6: example of a pattern of FQ^S and EMB^R. Lane 7: example of a pattern of $FQ^{R/S}$ and EMB^S. C: results obtained for AG module. Lane 8: example of an invalid result. Lane 9: example of a pattern of $KAN^S/AMK^S/CM^S$ and STR^S. Lane 10: example of a pattern of $KAN^R/AMK^R/CM^S$ and STR^R.

molecular assays exploring the QRDR of *gyrA* may harbor a mutation in other *gyrA* gene regions or in *gyrB*.[16] In addition, although there is cross-resistance within fluoroquinolone drugs, MOX minimum inhibitory concentrations are usually lower than those of other FQ.[16] Thus, if a genotypic test indicates FQ resistance whereas the phenotypic DST reveals susceptibility, this discordance might be due to the fluoroquinolone considered and the critical concentration recommended for the DST.

Regarding EMB, 21 out of the 35 phenotypically resistant samples were identified as resistant by the line probe assay. For the remaining 14 samples identified as sensitive, this result was in complete agreement with the results obtained by GenoType MTBDR*sl* and pyrosequencing. Twenty-two out of the 24 samples identified as sensitive by BACTEC 460TB were correctly identified as sensitive by AID. The remaining 2 samples, identified as resistant by the molecular assay, were also detected as resistant by GenoType MTBDR*sl* and pyrosequencing. These two samples harbored the mutation *embB* M306I (codon ATA), that was confirmed by pyrosequencing. In this study, AID assay was 60.0% sensitive and 91.7% specific for the detection of EMB resistance (Table 4). These results are in concordance with those obtained in other studies assessing the yield of GenoType MTBDR*sl* in clinical samples, which reported sensitivities that ranged from 33.3% to 72.2% and specificities that oscillated from 52.6% to 100%.[27,32,33,40] GenoType MTBDR*sl* sensitivity and specificity values estimated in a meta-analysis considering strains and specimens together were 68.0% and 80.0%, respectively.[42] These low sensitivity values may be due to the presence of mutations in codons other than *embB* 306, that are neither explored by AID nor GenoType MTBDR*sl*. These mutations have been located in *embB* codons 319 and 497, and also in *embC* and *embA* genes.[43,44] On the other hand, several studies reported different specificity values, that have been attributed to

Table 4 Sensitivity, specificity and agreement values between AID assay and BACTEC 460TB for detecting resistance to first- and second-line drugs in clinical samples.[a]

Drug	AID TB resistance		Agreement between AID TB resistance and BACTEC		
	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Agreement (%)	Kappa	SE
INH	45/46 (97.8) (87.0—99.9)	14/14 (100) (73.2—100)	59/60 (98.3)	0.955	0.045
RIF	43/43 (100) (89.8—100)	17/17 (100) (77.1—100)	60/60 (100)	1.000	0.000
FQ	2/6 (33.3) (6.0—75.9)	52/53 (98.1) (88.6—99.9)	54/59 (91.5)	0.473	0.216
EMB	21/35 (60.0) (42.2—75.6)	22/24 (91.7) (71.5—98.5)	33/59 (72.9)	0.479	0.103
KAN/CM	17/17 (100) (77.1—100)	34/34 (100) (87.4—100)	51/51 (100)	1.000	0.000
STR	22/22 (100) (81.5—100)	28/29 (96.6) (80.4—99.8)	50/51 (98.0)	0.963	0.036

CI, confidence interval. SE, standard error.
[a] Invalid results obtained were excluded for these calculations.

the complexity of the phenotypic methods to determine resistance to EMB.[32,40]

KAN/CM and STR resistance detection

All the samples included in our study were resistant to both KAN and CM. The results between AID and BACTEC 460TB were in complete agreement for all the 17 resistant and the 34 sensitive samples. Therefore, AID sensitivity and specificity values to detect resistance to both injectable drugs were 100% (Table 4). Feng et al. estimated the sensitivity (44% and 82% for KAN and CM, respectively) and specificity (99% and 97% for KAN and CM, respectively) of GenoType MTBDR*sl* considering together strains and specimens.[42] Sensitivity and specificity values reported differ considerably between studies.[27,40,41] This may be due to the presence of cross-resistance between KAN, AMK and CM.[18] The presence of A1401G mutation in *rrs* has been detected in isolates resistant to the three injectable drugs, but also in isolates resistant to both KAN and AMK but susceptible to CM.[40] That may decrease the specificity of the molecular test used to detect resistance to CM. On the other hand, isolates that are resistant to KAN only, or resistant to both KAN and AMK, may harbor a mutation in *eis* promoter region.[20] In these cases, a molecular test that does not explore this region would present a decreased sensitivity for detecting resistance to these drugs.

As for STR, all 22 samples identified as resistant by BACTEC 460TB were correctly identified as resistant by the test. Of the 29 phenotypically sensitive samples, AID identified 28 as sensitive. For the remaining sample, the mutation *rrs* C516T was detected, thus this sample was identified as resistant by the assay. STR resistance was not analyzed by GenoType MTBDR*sl* or pyrosequencing. Sensitivity and specificity values of AID were 100% and 96.6%, respectively (Table 4). Sensitivity of other molecular methods, such as sequencing, high resolution melting and array, to detect STR resistance in clinical isolates by exploring mutations in *rpsL* and *rrs* genes varies between 36.6 and 87.5%, according to the different settings considered.[36,45–47] In our study, the frequency of STR resistant isolates with identified mutations is higher than the previously reported in our geographical area.[45,48] However, it is of note that in our study patients with STR resistant isolates were born in eastern Europe (5/6) or in India (1/6), where the frequency of STR resistant isolates with detected mutations is 85%.[49] The specificity of AID for detecting STR resistance is in agreement with the values reported in other studies evaluating rapid tests.[36,45–47]

Overall results

Agreement values between AID and BACTEC 460TB results according to the drug considered are shown in Table 4. The highest kappa values were obtained in the detection of resistance to INH, RIF, KAN, CM and STR, which were above 0.95, while the lowest values were obtained for FQ and EMB, which were below 0.5.

Altogether, there were a total of 22 discordant results between AID and BACTEC 460TB, considering each drug individually: one for INH, 4 for FQ, 16 for EMB and 1 for STR. These discordant results corresponded to 22 samples obtained from 7 patients. Results obtained with the alternative molecular methods were in agreement with those obtained with AID in all cases, with two exceptions: the sample identified as FQ heteroresistant by the tested line probe assay and the STR-sensitive sample with the *rrs* C516T mutation detected, because STR resistance was not analyzed by GenoType MTBDR*sl* or pyrosequencing.

Final considerations

This study demonstrates the usefulness of AID assay to detect resistance to first- and second-line drugs in clinical specimens. This is a rapid molecular method that allows obtaining results in one working day, from the DNA extraction to the final report of the susceptibility pattern. The other tests used in this study to analyze the samples with discordant results between AID and BACTEC were GenoType MTBDR*plus*/MTBDR*sl* and pyrosequencing. These are also rapid methods with a similar turnaround time. In general terms, AID and GenoType are LPA that explore the same genes associated with drug resistance. Nonetheless, GenoType tests include more wild-type probes for *rpoB* and *gyrA* than AID, and the loss of hybridization signal for any of these additional wild-type probes theoretically increases the likelihood to detect drug resistance. On the contrary, AID explores mutations associated with STR resistance that are not included in GenoType tests. On the other hand, pyrosequencing is a more flexible method that allows the analysis of different targets of interest. Furthermore, is possible to investigate by this method other regions associated with drug resistance. Regarding the equipment required and the cost of the three tests presented, LPA reverse hybridization steps can be performed manually or with an automated washing and shaking device, whereas pyrosequencing requires more specific and expensive equipment. Similarly, the current WHO recommended method (Cepheid Xpert MTB/RIF System)[50] utilizes expensive cartridge and equipment than AID test, but handling is easier than the multiple steps of LPA.

This is the second study reporting the performance of AID in clinical samples. Ritter et al. recently reported a good agreement between AID and the reference methods (DNA sequencing and/or phenotyical DST) although most of the samples were clinical isolates.[51] Smear positive clinical specimens were also tested, with a rate of valid results higher than 95%, and the LPA showed complete agreement with DNA sequencing and phenotypic DST results. Nevertheless, the low number of resistant samples included was a limitation to obtain definite conclusions about the real clinical usefulness of AID line probe assay.

It is of note that the sensitivity of any molecular method depends on the knowledge about the genes and mutations involved in drug resistance and, additionally, on the prevalence of these mutations in each geographical setting. The sensitivity is also subject to the proportion of wild-type and mutant DNA: molecular methods are able to detect resistance if the ratio of mutant DNA is 10% or more, while phenotypical DST reports resistance if 1% of the bacilli in the population grows in presence of the tested drug.[7]

Moreover, the possibility of obtaining a valid result with a genotypic method when testing clinical samples further depends on the amount of mycobacteria present, considering that is more difficult to detect DNA in smear negative or smear 1+ samples. In consequence, culture for *M.tuberculosis* isolation and subsequent phenotypical DST must be performed in order to confirm the molecular results, especially when wild type patterns indicating drug susceptibility are obtained. Finally, new insights on the molecular mechanisms involved in drug resistance are needed, as the sensitivity of molecular methods, especially to detect resistance to FQ and EMB, is usually lower.

In conclusion, AID line probe assay shows a good performance and can be a useful tool to detect resistance to first- and second-line drugs directly in clinical samples in a short turnaround time. The rapid identification of the susceptibility/resistance pattern would facilitate adjusting treatment and consequently improving the clinical management of TB patients.

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Article IV

Lacoma A*, Molina-Moya B*, Prat C, Pimkina E, Diaz J, Dudnyk A, García-Sierra N, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Ausina V, Dominguez J

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Mycobacteriology

Pyrosequencing for rapid detection of *Mycobacterium tuberculosis* second-line drugs and ethambutol resistance

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ABSTRACT

The aim of this work was to study the diagnostic accuracy of pyrosequencing to detect resistance to fluoroquinolones, kanamycin, amikacin, capreomycin, and ethambutol (EMB) in *Mycobacterium tuberculosis* clinical strains. One hundred four clinical isolates previously characterized by BACTEC 460TB/MGIT 960 were included. Specific mutations were targeted in *gyrA*, *rrs*, *eis* promoter, and *embB*. When there was a discordant result between BACTEC and pyrosequencing, Genotype MTBDR*sl* (Hain Lifescience, Nehren, Germany) was performed. Sensitivity and specificity of pyrosequencing were 70.6% and 100%, respectively, for fluoroquinolones; 93.3% and 81.7%, respectively, for kanamycin; 94.1% and 95.9%, respectively, for amikacin; 90.0% and 100%, respectively, for capreomycin; and 64.8% and 87.8%, respectively, for EMB. This study shows that pyrosequencing may be a useful tool for making early decisions regarding second-line drugs and EMB resistance. However, for a correct management of patients with suspected extensively drug-resistant tuberculosis, susceptibility results obtained by molecular methods should be confirmed by a phenotypic method.

1. Introduction

Tuberculosis (TB) remains one of the most threatening infectious diseases worldwide: the World Health Organization estimated 9.0 million new cases and 1.5 million deaths in 2013 (WHO, 2014). The main strategy for TB control relies on rapid diagnosis and implementation of an adequate treatment based on drug susceptibility testing (DST). However, due to the slow growing rate of *Mycobacterium tuberculosis*, culture and DST may take several weeks, thus facilitating the emergence and spread of drug-resistant strains.

Multidrug-resistant (MDR) TB is defined as *M. tuberculosis* resistant to the first-line drugs isoniazid (INH) and rifampicin (RIF). Additionally to RIF and INH, ethambutol (EMB) is another important antituberculous first-line drug that can be useful for the treatment of MDR-TB. Once resistance to first-line drugs is detected, susceptibility to second-line drugs should be assessed, thus increasing the period while the patient is not correctly treated. MDR-TB strains additionally resistant to fluoroquinolones (FLQ) and at least one of the three second-line injectable drugs, kanamycin (KAN), amikacin (AMK), and capreomycin (CAP), have been defined as extensively drug-resistant (XDR). Globally and on average, the proportion of MDR-TB patients with XDR-TB was 9.0% in 2013 (WHO, 2014).

Drug resistance emerges due to the stepwise acquisition of genetic mutations in genes and also in promoters, coding for drug targets or drug-converting enzymes. Resistance to FLQ is associated with mutations in the quinolone resistance–determining region (QRDR) in *gyrA* and, to a lesser extent, in *gyrB*. The most frequent mutations in *gyrA* are located at codons 94, 90, 91, and 88 (Takiff et al., 1994; Zhang and Yew, 2009; Maruri et al., 2012). Regarding the injectable drugs, cross-resistance between KAN, AMK, and CAP has been reported (Jugheli et al., 2009; Maus et al., 2005). The mutations A1401G and G1484T at *rrs* are associated with resistance to the three injectable drugs (Georghiou et al., 2012), while C1402T leads to resistance to CAP. In addition, resistance to KAN has been associated with mutations at positions −10, −14, and −37 in the promoter region of *eis* (Zaunbrecher et al., 2009), whereas C-12T and C-14T changes have been associated with

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AMK resistance (Georghiou et al., 2012). Finally, mutations related to EMB resistance have been mainly located in *embB* codons 306 and 497 (Zhang and Yew, 2009; Plinke et al., 2006; Shi et al., 2011; Jin et al., 2013).

The reference molecular method to detect these mutations is sequencing, but different methods based on reverse hybridization (Lacoma et al., 2012, 2008; Molina-Moya et al., 2015), real-time PCR (Molina-Moya et al., 2015), or pyrosequencing (Richter et al., 2009) have also been developed (Garcia-Sierra et al., 2011; Arnold et al., 2005; Engstrom et al., 2012; Lin et al., 2014; Molina-Moya et al., 2014). In a previous work, we evaluated GenoType MTBDR*sl* line probe assay (LPA) for 34 *M. tuberculosis* isolates and 54 clinical samples from Spain, and we used pyrosequencing to check only discordant results between LPA and the phenotypic DST BACTEC. For clinical strains, pyrosequencing confirmed LPA results in 90% of the cases, while for clinical specimens, pyrosequencing confirmed 81% of the cases (Lacoma et al., 2012).

The objective of our study was to determine the accuracy of pyrosequencing for detecting FLQ, KAN, AMK, CAP, and EMB resistance in *M. tuberculosis* clinical strains by exploring the most frequent mutations in *gyrA*, *rrs*, *eis* promoter, and *embB*.

2. Materials and methods

2.1. Clinical strains

A total of 104 *M. tuberculosis* strains were retrospectively selected. Sixty-seven strains were isolated in the Infectious Diseases and Tuberculosis Hospital, Vilnius, Lithuania, and 37 strains were isolated in Hospital Universitari Germans Trias i Pujol, Badalona, Spain. The study was approved by the institutional ethics committee. Each strain corresponded to one patient, and no epidemiological connection of these patients was suspected. Strains were identified as *M. tuberculosis* by Inno-Lipa Mycobacteria v2 assay (Innogenetics, Ghent, Belgium).

2.2. Drug susceptibility

First- and second-line phenotypic DST was performed with the radiometric method BACTEC 460TB for 21 strains isolated in Spain and with the nonradiometric method BACTEC MGIT 960 for the remaining 16 ones, based on the time of testing. Critical concentrations for BACTEC 460TB for moxifloxacin (MOX), KAN, CAP, and EMB were 0.5 mg/L, 5 mg/L, 1.25 mg/L, and 7.5 mg/L, respectively (Heifets, 1988; Pfyffer et al., 1999; WHO, 2008). For the 67 strains isolated in Lithuania, second-line phenotypic DST was performed with BACTEC MGIT 960. Critical concentrations for BACTEC MGIT 960 for ofloxacin (OFX), levofloxacin (LVX), KAN, CAP, AMK, and EMB were 2 mg/L, 1.5 mg/L, 2.5 mg/L, 3 mg/L, 1.5 mg/L, and 5 mg/L, respectively (WHO, 2008; Lin et al., 2009; Rodrigues et al., 2008). In this study, BACTEC 460TB/MGIT 960 was considered the gold standard method.

2.3. Characterization of genotypic drug resistance

DNA extraction was performed by suspending a loopful of *M. tuberculosis* colonies in a screw-cap tube containing 400-μL 1X TRIS-EDTA and incubating at 100 °C for 10 min. After, the sample was centrifuged at 22,000×*g* for 5 min, and the supernatant was collected. DNA was stored at −20 °C until use. The pyrosequencing method consists of a PCR amplification followed by the pyrosequencing reaction. PCR and pyrosequencing primers for *gyrA*, *rrs*, and *embB* were previously described (Lacoma et al., 2012). Primers for *eis* promoter were adapted from Engstrom et al. (2012). Mutations explored by pyrosequencing are located in codons 80–81 and 88–95 of *gyrA*; positions 1401, 1402, and 1484 of *rrs*; positions −37, −14, −12, and −10 of *eis* promoter; and codon 306 of *embB*. A total of six pyrosequencing reactions per clinical strain were performed to analyze these positions. Pyrosequencing reaction and data analysis were performed as recommended by the PSQ96MA and SQA software manufacturer (Qiagen, Hilden, Germany). An invalid result was defined as absence of interpretable/readable pyrogram peaks after repeating the test; therefore, no sequence was obtained. Due to lack of enough DNA, pyrosequencing of *rrs* was not performed for one strain, and for this strain and four additional ones, *eis* promoter was neither analyzed by pyrosequencing. Pyrosequencing results were compared with those obtained with the phenotypic methods. Discordant results between pyrosequencing and BACTEC were analyzed by Genotype MTBDR*sl* (Hain Lifescience, Germany). This test is a commercial LPA that identifies the same mutations in *gyrA*, *rrs*, and *embB* and can further detect drug resistance by loss of hybridization of wild type probes. However, this LPA does not detect mutations in *eis* promoter. Researchers who read and interpreted both pyrosequencing and Genotype results were blind to the BACTEC results.

2.4. Statistical analysis

Pyrosequencing values of sensitivity and specificity, with the corresponding 95% confidence interval (CI), were calculated considering BACTEC 460TB/MGIT 960 as reference method. Agreement values and kappa coefficients were also calculated comparing both methods. Kappa (k) values below 0.40 indicate weak correlation, values of 0.41–0.60 indicate good agreement, and values above 0.60 indicate strong agreement. The commercial statistical software package used was SPSS 15.0 (SPSS, Chicago, Illinois, USA).

3. Results

One hundred four clinical strains were included in the study. Resistance profiles to MOX, LVX, OFX, KAN, AMK, CAP, and EMB are shown in Table 1. A comparison between pyrosequencing and BACTEC results is shown in Table 2. In Table 3, the mutations detected in the different loci analyzed are presented.

3.1. FLQ resistance

For three of the 34 phenotypical resistant strains, pyrosequencing showed both wild-type and mutation sequences, which corresponded to examples of heteroresistant strains (Table 3). According to the time and setting, different fluoroquinolone drugs were used for phenotypic DST. Pyrosequencing correctly detected 2/5 (40%) MOX^R, 5/5 (100%) LVX^R, and 17/24 (70.8%) OFX^R strains. Of the ten FLQ^R strains identified as sensitive by pyrosequencing, LPA detected wild-type sequence for eight strains; for one strain, the result suggested heteroresistance; and for the remaining one, the result was invalid. Finally, the distribution of the *gyrA* S95T polymorphism among strains included in our study was as follows: 29 of the 31 (93.5%) FLQ^R and 68 of the 74 (91.9%) FLQ^S strains.

3.2. KAN resistance

Two KAN^R strains were identified as sensitive by pyrosequencing as well as by LPA. For 13 KAN^S strains, pyrosequencing detected a mutation in the *eis* promoter. In our set of strains, *eis* promoter mutations identified by pyrosequencing were as follows: G-10A in 26.7% (8/30)

Table 1
Resistance pattern obtained by BACTEC 460TB/MGIT 960 for FLQ, KAN, AMK, CAP, and EMB for the 104 clinical strains.

	Drug				
	FLQ	KAN	AMK	CAP	EMB
Resistant (%)	34 (32.7)	30 (29.1)	17 (18.3)	10 (9.8)	54 (52.4)
Sensitive (%)	70 (67.3)	73 (70.9)	76 (81.7)	92 (90.2)	49 (47.6)
Not performed (%)		1 (1.0)	11 (10.6)	2 (1.9)	1 (1.0)
Total performed	104	103	93	102	103

Table 2
Distribution of pyrosequencing results according to BACTEC 460TB/MGIT 960 result for the 104 clinical strains.

Drug and BACTEC result (no. of strains)	No. of isolates (%) with the following pyrosequencing result		
	Resistant	Sensitive	Not performed
FLQ			
Resistant (n = 34)	24 (70.6)	10 (29.4)	
Sensitive (n = 70)		70 (100)	
Total (n = 104)	24 (23.1)	80 (76.9)	
MOX			
Resistant (n = 5)	2 (40.0)	3 (60.0)	
Sensitive (n = 17)		17 (100)	
Total (n = 22)	2 (9.1)	20 (90.9)	
LVX			
Resistant (n = 5)	5 (100)		
Sensitive (n = 7)		7 (100)	
Total (n = 12)	5 (41.7)	7 (58.3)	
OFX			
Resistant (n = 24)	17 (70.8)	7 (29.2)	
Sensitive (n = 46)		46 (100)	
Total (n = 70)	17 (24.3)	53 (75.7)	
KAN			
Resistant (n = 30)	28 (93.3)	2 (6.7)	
Sensitive (n = 73)	13 (17.8)	58 (79.5)	2 (2.7)
Total (n = 103)	41 (39.8)	60 (58.3)	2 (1.9)
AMK			
Resistant (n = 17)	16 (94.1)	1 (5.9)	
Sensitive (n = 76)	3 (3.9)	71 (93.5)	2 (10.6)
Total (n = 93)	19 (20.4)	72 (77.4)	2 (2.2)
CAP			
Resistant (n = 10)	9 (90.0)	1 (10.0)	
Sensitive (n = 92)		91 (98.9)	1 (1.1)
Total (n = 102)	9 (8.8)	92 (90.2)	1 (1.0)
EMB			
Resistant (n = 54)	35 (64.8)	19 (35.2)	
Sensitive (n = 49)	6 (12.2)	43 (87.8)	
Total (n = 103)	41 (39.8)	62 (60.2)	

KAN^R and in 9.9% (7/71) KAN^S strains; C-12T in 10.0% (3/30) KAN^R and in 2.8% (2/71) KAN^S strains; and C-14T in 26.7% (8/30) KAN^R and in 4.2% (3/71) KAN^S strains (Table 3). In light of these results, we considered that the three mutations G-10A, C-12T, and C-14T were associated with KAN resistance.

3.3. AMK resistance

One of the phenotypical resistant strains was identified as sensitive by pyrosequencing and also by LPA. For this strain, the G-10A mutation in *eis* promoter was detected by pyrosequencing. Interestingly, this mutation has been detected in AMK^R as well as in AMK^S strains in this study (Table 3). For three of the AMK^S strains, the C-14T mutation was detected by pyrosequencing. In our set of strains, the *eis* promoter mutation G-10A was detected by pyrosequencing in 5.9% (1/17) of AMK^R and in 18.9% (14/74) of AMK^S strains; C-12T was detected in none (0/17) of AMK^R and in 6.8% (5/74) of AMK^S strains; and C-14T was detected in 47.1% (8/17) of AMK^R and in 4.1% (3/74) of AMK^S strains (Table 3). Therefore, we considered that only C-14T was associated with resistance to AMK.

3.4. CAP resistance

For a CAP^R strain, both pyrosequencing and LPA detected wild-type *rrs* positions. However, it is of note that this strain presented cross-resistance to KAN and AMK and pyrosequencing detected the C-14T mutation in *eis* promoter.

3.5. KAN, AMK, and CAP cross-resistance

In Table 4, the different profiles of resistance to KAN, AMK, and CAP determined by BACTEC 460TB/MGIT 960 and the location of mutations detected are shown.

Table 3
Distribution of mutations identified in the loci associated with resistance to second-line drugs and EMB in clinical strains.

Drug	Locus	Amino acid change	Codon change	Nucleotide change	No. of strains with phenotypic result	
					Resistant	Sensitive
MOX	*gyrA*	S91P	TCG/CCG		1	
		D94N	GAC/AAC		1	
		wt			3	17
LVX	*gyrA*	A90V	GCG/GTG		1	
		D94G	GAC/GGC		3[a]	
		D94Y	GAC/TAC		1	
		wt				7
OFX	*gyrA*	A90V	GCG/GTG		1	
		A90V + D94V	GCG/GTG + GAC/GTC		2[b]	
		S91P	TCG/CCG		4	
		D94A	GAC/GCC		1	
		D94G	GAC/GGC		9	
		wt			7	46
KAN	*rrs*			A1401G	8	1
				G1484T	1	
	eis			C-14T	8	3
				C-12T	3	2
				G-10A	8	7
	wt				2	58
AMK	*rrs*			A1401G	8	
	eis			C-14T	8	3
				C-12T		5
				G-10A	1	14
	wt					52
CAP	*rrs*			A1401G	8	
				G1484T	1	
				wt	1	91
EMB	*embB*	M306I	ATG/ATA		6	1
		M306I	ATG/ATC		1	1
		M306I	ATG/ATT			1
		M306V	ATG/GTG		27	3
		M306V	ATG/GTG-GCG		1	
			wt		19	43

wt = wild-type.
[a] One strain was heteroresistant.
[b] Heteroresistance in codon 94 for these 2 strains.

3.6. EMB resistance

Of the 54 phenotypical resistant strains, 19 were identified as sensitive by pyrosequencing as well as by LPA. For six of the 49 strains EMB^S by BACTEC, pyrosequencing detected a mutation in *embB* codon 306. For three strains, the sequence mutation was GTG (M306V), and for three strains, the sequences were ATT, ATA, and ATC (M306I), respectively. LPA results were in complete agreement with pyrosequencing.

Table 4
Relationship between profiles of resistance to KAN, AMK, and CAP determined by BACTEC 460TB/MGIT 960 and mutations identified in *rrs* and *eis* promoter.

Susceptibility to			No. of strains with the following mutation							
			rrs		*eis* promoter			NM	ND	Total
KAN	AMK	CAP	A1401G	G1484T	C-14T	C-12T	G-10A			
R	R	R	5		1					6
R	R	S			7		1			8
R	R	ND	1							1
R	S	S				3	7	2		12
R	ND	R	2	1						3
S	R	R	1							1
S	S	S			3	2	7	50	2	64
S	ND	S							8	8
ND	R	ND	1							1

R = resistant; S = sensitive; ND = not done; NM = no mutation in the explored positions.

3.7. Summary of the results

Overall, there were a total of 55 discordant results between pyrosequencing and BACTEC. The results obtained with Genotype MTBDR*sl* were in agreement with those obtained by pyrosequencing in 36/55 cases (65.5%). Regarding the remaining 19 cases, for two FLQ^R strains, LPA result was invalid or indicated heteroresistance, respectively, and for 17 strains, pyrosequencing detected mutations in *eis* promoter that are not explored by Genotype MTBDR*sl*.

Specificity and sensitivity values of pyrosequencing for detecting FLQ, KAN, AMK, CAP, and EMB resistance in clinical strains are shown in Table 5. Agreement values between BACTEC and pyrosequencing results according to the drug considered are shown in Table 5.

4. Discussion

This study presents additional evidence regarding the utility of pyrosequencing for detecting genetic mutations associated with resistance to second-line drugs and EMB in clinical strains of *M. tuberculosis* by exploring specific codons or positions in *gyrA*, *rrs*, *eis* promoter, and *embB*. Here, we also highlight the relevance of mutations in *rrs* and *eis* promoter in isolates with different patterns of cross-resistance to KAN, AMK, and CAP.

4.1. FLQ resistance

In our set of strains, pyrosequencing presented a moderate sensitivity and good specificity. Two published papers, by Engstrom et al. (2012) and Lin et al. (2014), reported a sensitivity of pyrosequencing of 87.1% and 87.0%, respectively, exploring only *gyrA*. However, our results are in accordance with the data reported by Maruri et al. (2012) in a systematic review, where 64% of FLQ^R isolates had a mutation in the QRDR of *gyrA*. For the phenotypical FLQ^R strains that did not present any mutation, we cannot exclude the presence of mutations in other locus not explored in the present study, such as *gyrB* or other regions of *gyrA* (Takiff et al., 1994; Aubry et al., 2006; Wang et al., 2007).

The kappa value obtained for detecting resistance to all the FLQ is above 0.6, indicating a strong agreement between pyrosequencing and BACTEC. However, and despite the low number of isolates MOX^R or LVX^R in comparison with those OFX^R, the kappa values tend to differ when considering the three drugs separately. A strong agreement is observed for detecting LVX and OFX resistance, whereas a weak agreement is observed for MOX resistance. The weak correlation for MOX may be due to the recommended critical concentration we used, which may be close to the MIC for some isolates. Despite cross-resistance between MOX, LVX, and OFX, *gyrA* mutations can distinctively affect the susceptibility to these drugs, and the MICs may differ. In this line, *gyrA* mutant isolates have been found to exhibit different MICs when comparing the different FLQ (Sirgel et al., 2012; Cheng et al., 2004). In addition, mutations in *gyrA* have been more frequently detected in isolates with high-level OFX resistance (Chernyaeva et al., 2013), but they were absent in isolates with low-level OFX resistance (Cheng et al., 2004). In another work, all mutations in *gyrA* were associated with phenotypic resistance to all the FLQ tested (Nosova et al., 2013).

There was a single isolate OFX^R by BACTEC and sensitive by pyrosequencing that was identified as heteroresistant by Genotype MTBDR*sl*. The difference between pyrosequencing and LPA for detecting mixed genotypes may be due to a higher sensitivity of the latter to detect a lower amount of mutant DNA. Moreover, LPA is based on hybridization on separate and independent wild-type and mutant probes, which allows a clear detection of simultaneous hybridization on both probes. In contrast, interpretation of pyrosequencing results relies on a single pyrogram and the sequence associated. Therefore, pyrograms should be carefully interpreted to detect possible heteroresistance.

Finally, we detected the *gyrA* S95T change in most of the FLQ^R as well as the FLQ^S strains. This is a frequent polymorphism not associated with resistance to FLQ; instead, this change, together with *katG* 463, is used to assign the strains to three genotypic groups (Sreevatsan et al., 1997). Considering the *gyrA* S95T polymorphism detected in the present study, most of the strains belong to group 1 or group 2.

4.2. KAN resistance

In our study, pyrosequencing showed good sensitivity, but specificity was moderate. Indeed, the kappa value was slightly above 0.6, indicating a strong agreement between pyrosequencing and BACTEC. In two published works, sensitivity of pyrosequencing was 83.7% when exploring both *rrs* and *eis* promoter and 85.7% when exploring only *rrs* (Engstrom et al., 2012; Lin et al., 2014). Remarkably, in our study, analysis of *eis* promoter allowed us to detect more resistant isolates, although some phenotypical sensitive strains were incorrectly identified. In fact, the impact of each *eis* promoter mutation remains controversial. In a systematic review, it was shown that both G-10A and C-14T mutations at *eis* promoter but not the C-12T change are associated with KAN resistance (Georghiou et al., 2012). In another study, Engstrom et al. (2012) considered that only C-14T conferred KAN resistance because G-10A and C-12T were more frequently detected in KAN^S isolates. In contrast, Rodwell et al. (2014) found that the three mutations were associated with KAN resistance. It is of note that the frequency of the G-37T mutation is low (Georghiou et al., 2012). Indeed, we did not detect it in any strain.

Table 5
Pyrosequencing sensitivity and specificity for detecting FLQ, KAN, AMK, CAP, and EMB resistance and agreement values between pyrosequencing and BACTEC 460TB/MGIT 960.

	Pyrosequencing		Agreement between pyrosequencing and BACTEC		
Drug	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Agreement (%)	Kappa	SE
FLQ	24/34 (70.6) (53.7–83.3)	70/70 (100) (93.8–100)	94/104 (90.4)	0.764	0.069
MOX	2/5 (40) (11.6–77.1)	17/17 (100) (78.4–100)	19/22 (86.4)	0.397	0.240
LVX	5/5 (100) (51.1–100)	7/7 (100) (59.6–100)	12/12 (100)	1.000	0.000
OFX	17/24 (70.8) (50.6–85.3)	46/46 (100) (90.8–100)	63/70 (90.0)	0.761	0.083
KAN	28/30 (93.3) (77.6–99.2)	58/71 (81.7) (71.0–89.1)	86/101 (85.1)	0.678	0.074
AMK	16/17 (94.1) (71.1–100)	71/74 (95.9) (88.3–99.1)	87/91 (95.6)	0.862	0.067
CAP	9/10 (90.0) (57.4–100)	91/91 (100) (95.1–100)	100/101 (99.0)	0.942	0.058
EMB	35/54 (64.8) (51.5–76.2)	43/49 (87.8) (75.4–94.6)	78/103 (75.7)	0.519	0.081

Mutations in *eis* promoter, especially at positions −10 and −12, have been detected in isolates with a slightly increase the MIC (Zaunbrecher et al., 2009; Campbell et al., 2011). Discordances found between studies may be due to the fact that phenotypic DST for second-line drugs was not fully standardized and the critical concentrations used were close to the MICs. As a consequence, isolates harboring mutations in *eis* promoter could be misclassified, and discordant results between phenotypic and molecular methods may be found. Regarding the resistant strains with wild-type sequences, mutations conferring KAN resistance could be located in the 5′-UTR of *whiB7* (Reeves et al., 2013). In addition, an isolate susceptible to KAN but resistant to AMK and CAP harbored the *rrs* A1401G mutation. The discrepancy for KAN may be due to the critical concentration used, to a false result of the DST, or to other mechanisms that restore the susceptibility to KAN. For the 13 KAN^S strains with a mutation in *eis* promoter, the phenotypic result could be mistaken due to the recommended critical concentration used, since previous studies reported a high specificity of these mutations (Georghiou et al., 2012; Rodwell et al., 2014).

4.3. AMK resistance

In our set of strains, pyrosequencing showed a good sensitivity and specificity, and the high kappa value for detecting AMK resistance indicated a strong agreement between pyrosequencing and BACTEC. It is of note that the AMK^R strains presented different patterns of cross-resistance with KAN and CAP and that they harbored mutations in *rrs* or *eis* promoter. In other evaluations of pyrosequencing, a sensitivity of 82.1% was reported exploring both *rrs* and *eis* promoter (Engstrom et al., 2012), whereas a sensitivity of 100% was achieved exploring only *rrs* (Lin et al., 2014). The differences between studies may be due to the number of resistant isolates tested, the geographic origin, and the pattern of cross-resistance with KAN and CAP.

It was previously shown that mutations C-12T and C-14T were specific of AMK resistance (Georghiou et al., 2012). However, the present study supports another work reporting that the C-12T change is not that specific (Rodwell et al., 2014). Furthermore, in our set of strains and considering only *rrs* mutations to detect AMK resistance, sensitivity and specificity of pyrosequencing would be 47.1% and 98.6%, respectively, whereas considering also the C-14T mutation in *eis* promoter, these values are 94.1% and 95.9%, respectively (Rodwell et al., 2014). Finally, the G-10A mutation in *eis* promoter was detected by pyrosequencing in a phenotypical resistant strain. However, this isolate was identified as sensitive by pyrosequencing, since this mutation has been detected in AMK^R as well as in AMK^S strains in this study and also in previous works (Georghiou et al., 2012; Rodwell et al., 2014).

4.4. CAP resistance

In the present study, we obtained good sensitivity and specificity for the detection of CAP^R by pyrosequencing. Accordingly, the kappa value is above 0.6, which indicated a strong agreement between pyrosequencing and BACTEC. It is of note that this high value is influenced by the low number of CAP^R isolates included as well as by the high specificity of the detected mutation. Sensitivity values of pyrosequencing reported in other studies were 79.5% and 100%, respectively (Engstrom et al., 2012; Lin et al., 2014). In our study, the only CAP^R strain detected as sensitive by pyrosequencing was also KAN^R and AMK^R and harbored the C-14T mutation in *eis* promoter. It is possible that this strain may also carry a mutation in *tlyA* leading to CAP resistance, although these mutations are rare among clinical isolates (Georghiou et al., 2012; Wang et al., 2014; Engstrom et al., 2011).

4.5. KAN, AMK, and CAP cross-resistance

Cross-resistance between the three second-line injectable drugs has been evidenced. Several studies reported that mutations in *rrs*, especially A1401G, are commonly found in KAN^R/AMK^R/CAP^R or KAN^R/AMK^R isolates (Jugheli et al., 2009; Campbell et al., 2011; Gikalo et al., 2012). This is in contrast with our results, since most of the strains resistant to both KAN and AMK harbored a mutation only in *eis* promoter (Gikalo et al., 2012). In addition, although no epidemiological relation between these patients was suspected, strains included in this study may be geographically related. Finally, phenotypic DST for second-line drugs was not fully standardized; hence, the discrepancy between phenotypic and genotypic tests may be due to the critical concentrations used. In this study, we used the recommended critical concentrations, but the discordances we found, especially those related to *rrs* A1401 mutation, may be due because the critical concentrations are close to the MIC.

4.6. EMB resistance

The significance of mutations in *embB*, particularly at codon 306, and its direct relationship with phenotypical resistance to EMB have been controversial because some mutations have been detected in both sensitive and resistant clinical strains (Mokrousov et al., 2002; Hazbon et al., 2005). This could be explained by the fact that MIC could be near the critical concentration (Sirgel et al., 2012). Due to the low sensitivity and specificity, the kappa value for detecting EMB resistance indicated a moderate agreement between pyrosequencing and BACTEC. Engstrom et al. (2012) found similar values of sensitivity (61.2%) and specificity (84.8%), despite they also covered codons 313 and 315. Isola et al. (2005) also evaluated pyrosequencing for detecting EMB resistance, but the number of strains tested was lower. Mutations in different codons and genes, such as *embB* 319 and 497 and *embC* and *embA*, may also be implicated in EMB resistance (Ramaswamy et al., 2000). In a recent study, Jin et al. (2013) found that 26.8% of the EMB-resistant clinical isolates harbored a mutation in *embB* codon 497. In our experience, specificity of molecular methods was moderate. It is of note that these strains were MDR, and the relationship between the *embB* 306 mutations in isolates resistant to both INH and RIF but sensitive to EMB has been already described (Hazbon et al., 2005). Moreover, the M306I mutation has been found in isolates with a modest increase in the MIC (Engstrom et al., 2012). This suggests that the significance of mutations in this codon is at the moment somehow limited and highlights the need for a better understanding of the molecular basis of EMB resistance. In addition, it would be convenient to have better knowledge about treatment outcomes and treatment failure in patients harboring strains with *embB* mutations. These mutations could be clinically relevant; thus, molecular tests may be more reliable than phenotypic tests in guiding treatment options.

4.7. Overall considerations

Pyrosequencing has a short turnaround time, and results can be obtained within one working day. Despite its simplicity, cheapness per sample analyzed, and relative rapidity, the methodology requires trained staff and specific equipment. An advantage of pyrosequencing is the possibility of detecting missense mutations, silent mutations, and new mutations in the targeted regions. In fact, pyrosequencing method offers sensitivity and specificity values comparable to Sanger sequencing (Engstrom et al., 2012; Campbell et al., 2011). In addition, pyrosequencing is a flexible method, and different genomic regions can be targeted depending on the drug or the geographical area. For instance, we detected mutations in *eis* promoter region, which are more common in Eastern Europe, and other methods such as LPA do not currently target it. This is a limitation of our study, since *eis* promoter mutations detected in isolates with discordant results between BACTEC and pyrosequencing could not be checked by GenoType MTBDR*sl*.

On the other hand, the main limitation of pyrosequencing and the molecular tests in general are the suboptimal sensitivity for detecting resistance to some of the drugs, due to the lack of complete knowledge

of the mutations involved in phenotypical resistance. In addition, the accuracy of molecular resistance detection depends largely on the strength of the association between the genetic mutation and the phenotypic drug susceptibility result. Finally, sensitivity of pyrosequencing and other molecular methods also depends on the proportion of resistant mycobacteria that harbor a mutation in the mycobacterial population. For instance, Engstrom et al. (2013) detected mutations by pyrosequencing when at least 35–50% of the DNA was mutant, whereas phenotypic DST reports resistance when there are 1% of resistant mycobacteria in the whole population (Richter et al., 2009).

In conclusion, this study shows that pyrosequencing may be a useful tool for making early decisions regarding second-line drugs and EMB resistance and adjusting the treatment. However, for a correct management of patients with suspected XDR-TB, results must be confirmed by a phenotypical method.

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Article V

Molina-Moya B, Latorre I, Lacoma A, Prat C, Dominguez J

Recent advances in tuberculosis diagnosis: IGRAs and molecular biology

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Mycobacterial Infections (J Esteban, Section Editor)

Recent Advances in Tuberculosis Diagnosis: IGRAs and Molecular Biology

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Opinion statement

Tuberculosis (TB) is one of the most deadly, curable infectious diseases. In 2012, the World Health Organization (WHO) reported 8.6 million new TB cases and 1.3 million deaths. Despite intensive research to improve tuberculosis diagnosis and drug susceptibility testing, TB still remains one of the most threatening curable infectious diseases. Effective control of TB remains in prevention, the immediate detection of *Mycobacterium tuberculosis* and rapid detection of drug-resistant strains, followed by prompt implementation of an adequate anti-tuberculous therapy. Providing rapid antibiotic resistance detection systems is essential to treat accurately the patients, especially with the emergence and spread of drug-resistant TB. In 2012, WHO reported 84,000 confirmed multi-drug-resistant TB cases worldwide, and 9.6 % of these cases were extensively drug-resistant TB. However, conventional techniques for culture isolation and antibiotic susceptibility testing are slow. In recent years, the molecular basis of resistance to anti-tuberculous drugs has been elucidated. Molecular methods based on sequencing have been used to detect the main mutations involved in resistance from isolated strains and clinical samples. New molecular methods have been developed to detect the most common mutations conferring *M. tuberculosis* resistance. Furthermore, the diagnosis of the latently infected people allows measures to prevent them from developing the active disease, and thus help break the chain of transmission of the microorganism. The tuberculin skin test,

the classical method for diagnosing latent TB infection, has several drawbacks: low specificity, and also a low sensitivity in immunosuppresed patients. The development of the IFN-γ release assays (IGRAs), with a recognized higher specificity, has improved the diagnosis of latent TB infection. However, for the correct use and interpretation a close cooperation between physicians and expert microbiologists is required.

Introduction

Tuberculosis (TB) remains a serious public health problem. TB control is based on prevention, early diagnosis and rapid identification of drug resistance, followed by prompt implementation of an adequate antituberculous therapy [1, 2••]. However, conventional methods have some limitations: lack of specificity of the tuberculin skin test (TST) in diagnosing latent TB infection (LTBI), and lack of sensitivity and specificity and slow methods in detecting *Mycobacterium tuberculosis* and its resistance pattern in active TB patients [3]. In this sense, in recent years, a number of new diagnostic methods have been developed, improving the diagnosis and management of the patients. The introduction of IFN-γ release assays (IGRAs) for measuring in vitro the T cell-mediated immune response after specific antigen stimulation has offered a high specific diagnostic of the LTBI. Similarly, the inclusion of the molecular biology technology in the diagnosis algorithm has supposed a rapid detection of the presence of the bacilli in the clinical samples, and also in the resistance detection.

IGRAS

Diagnosis of latent tuberculosis infection

TST has been during the last 100 years the classical method for diagnosing LTBI. Unfortunately, this in vivo assay has a compromised specificity that leads to false-positive results caused by cross-reaction with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine strain and non-tuberculous mycobacteria (NTM) [4]. Furthermore, false-negative results can occur due to low sensitivity on high-risk individuals with impaired cellular immunity [5].

CD4 T cells producing IFN-γ are major players in the protection against TB. Therefore, an immunodiagnostic assay focused on in vitro detection of this key cytokine could be an alternative to TST for LTBI diagnosis. A decade ago, IGRAs were developed as an aid for diagnosing LTBI. IGRAs are based on in vitro IFN-γ detection released by sensitized T cells after a specific stimulation with *M. tuberculosis* antigens (ESAT-6, CFP-10 and TB7.7) [6, 7]. Currently, there are two U.S. Food and Drug Administration (FDA) and CE (for use in Europe) commercially available formats: QuantiFERON-TB Gold In-Tube (QFN-G-IT, QIAGEN, Düsseldorf, Germany) and T-SPOT.TB (Oxford Immunotec, Abingdon, UK). The QFN-G-IT test stimulates whole blood with ESAT-6, CFP-10 and TB7.7 antigens in the same tube, and measures the concentration of IFN-γ in supernatants with an enzyme-linked immunosorbent assay. In contrast, the T-SPOT.TB assay stimulates isolated peripheral blood mononuclear cells (PBMCs) with ESAT-6 and CFP-10 separately, and detects the number of IFN-γ producing T cells by means of an enzyme-linked immunospot assay (ELISPOT). Both in vitro assays include a positive control using phytohemagglutinin for detecting T cells' capacity to produce IFN-γ. This control is very useful in immunosuppressed patients for detecting lack of immune response.

IGRA accuracy and performance on immunocompetent individuals

There is no gold standard for LTBI diagnosis. This is the major drawback for assessing diagnostic accuracy of IGRAs. One possible approach is to evaluate their sensitivity in active TB patients (as LTBI is a major requirement for TB disease) and their specificity in low-risk healthy individuals with no TB exposure. According to a recent published review [8], specificity of IGRAs for LTBI diagnosis is around 95 % in low TB endemic countries. In addition, their sensitivity is reported to be approximately around 80 % [8, 9••]. This sensitivity could be reduced in some immunocompromised patients groups such as HIV individuals, children aged below 5 years old or patients with immune-mediated inflammatory diseases (IMIDs) in treatment with TNF-α agents (see section below: IGRAs in immunosuppressed patients and children). Both assays are reported to avoid false-positive results in BCG vaccinated patients and most NTM sensitizations and infections. Thus, they avoid unnecessary preventive treatment prescriptions being more cost-efficient than TST [10].

Risk of transmission is related to the degree and duration of *M. tuberculosis* exposure to index case; therefore, this indicator has been also assessed to determine IGRA accuracy. In this context, several studies indicate that IGRAs correlate better than TST in *M. tuberculosis* exposed individuals and with environmental risk factors of infection [11–13, 14••, 15, 16]. An alternative approach for measuring IGRA accuracy is to follow-up disease progression in LTBI close-contacts with an index TB case. In this sense, there are some meta-analyses suggesting that IGRAs have a modest predictive value. A pioneer study by Diel et al. observed that six out of 41 positive QFN-G-IT close contacts developed TB over time, finding a positive predictive value of 14.6 % [17]. As a consequence, only a low proportion of LTBI individuals diagnosed by IGRA progress to active TB, meaning that there is still an unsatisfactory high proportion of persons that have to be treated. However, this predictive value could be especially useful when performed in high-risk individuals. For example, Aichelburg et al. screened 830 HIV + individuals with QFN-G-IT. A total of 36 patients were positive and followed during 19 months. Three of them developed active TB, resulting in a positive predictive value of 8.3 % [18]. Conversely, negative predictive value of IGRAs is very high, indicating that the two in vitro assays classify correctly those healthy individuals that have no risk of developing TB [14••, 19, 20•].

IFN-γ assays seem to be a good alternative to TST for LTBI diagnosis in individuals that undergo serial testing such as health care workers (HCWs) [21]. Serial TST testing could induce a boosting effect making very difficult its interpretation. This boost could be caused by a remote LTBI infection, NTM sensitization, or a prior BCG-vaccination. In contrast, IGRAs permit performing consecutive testing for detecting recent LTBI because they do not induce boosting phenomenon. Even though reversion and conversion of IGRAs during serial testing is still an unclear frequent phenomenon. This has become more evident since two recent longitudinal studies conducted in a big cohort of HCWs from the United States have observed high rate of conversion and reversions during serial-testing with QFN-G-IT and/or T-SPOT.TB [22]. As a consequence, a consensus about results interpretation near the threshold and a clear definition of test conversion/reversion has to be addressed. Moreover, it is important to take into account that when using a two-step approach for LTBI diagnosis (initial TST testing followed by an IGRAs test confirmation) TST can interfere in IFN-γ results interpretation if it is administered more than three days prior to the IGRA [23].

IGRAs in immunosuppressed patients and children

Patients with an altered immune response including HIV + individuals, patients with IMIDs, solid-organ transplant recipients, patients under hemodialysis, patients with cancer, or children aged <5 years old are very complex groups because in some situations their immune system is not enough for guaranteeing an optimal test performance. On the basis of available data, TST and IGRAs performance differs according to the etiology of immunodeficiency. However, in specific clinical situations in vitro tests are advantageous over TST [24].

HIV-infected individuals

A compromised IGRA performance in HIV + individuals seems to be in agreement with CD4 T cells counts. Nevertheless, some studies and recent reviews have reported that in vitro tests are more robust (or at least equally) than TST in such population. In particular, T-SPOT.TB may be less affected by the level of immunosuppression than QFN-G-IT [20•, 25–28]. Interestingly, a poor agreement between IGRAs has been observed in the HIV + population when tested simultaneously. These discordances could be explained by differences in the two tests methodologies [28–30]. In any case, given that there is no existence of a LTBI gold standard diagnostic method, it is not possible to know which IFN-γ assay (or TST) is giving the true result. Probably, a strategy that comprises the combination of both in vitro assays increases sensitivity in severe immunosuppressed patients. Another important issue in HIV + individuals is the increase of indeterminate IGRA results. A recent clinical review [24] estimated that the proportion of these indeterminate results oscillate around 1.5 % to 16 % for QFN-G-IT and 0 to 33 % for T-SPOT.TB. Furthermore, indeterminate results are related to a decreased number of CD4 cells.

Patients with immune-mediated inflammatory diseases (IMIDs)

The use of biologic therapy requires prior evaluation and monitoring of patients because a clear association between LTBI reactivation and anti-TNF-α treatment has been established [31, 32]. Several studies have evaluated IGRAs for LTBI diagnosis in patients with inflammatory diseases, suggesting that in vitro assays are less influenced than TST by immunosuppression. However, it is still difficult to assess their accuracy in such individuals because studied groups tend to be quite heterogeneous (mixture of different inflammatory diseases) and with different degrees of drug-immunosuppression [33–35]. Moreover, some results indicate that corticosteroid treatment leads to high indeterminate IGRA results [36–38]. At this moment, a two-pronged approach (TST combined with IGRA) seems to be the most prudent option for LTBI screening in this setting. However, current available data suggest that IGRAs might be used alone in a near future.

Paediatric population

Diagnosis of LTBI in children is still a challenge due to an immature immune system. As a consequence, performance of IFN-γ assays, especially in children younger than 5 years old, is still questionable [39]. A pTBnet (paediatric branch of TBnet) collaborative study, observed that age correlated with a positive QFN-

G-IT and/or T-SPOT.TB result. Furthermore, percentages of indeterminate results were low and they were associated with young ages. Discordant results between IGRAs and TST have also been observed in non-BCG vaccinated children. This factor has been attributable to the high percentage of asymptomatic NTM sensitizations in children [40].

In summary, investigations in high-risk individuals are still limited and it is essential to improve their potential diagnostic increasing sensitivity and reducing indeterminate results. Longitudinal studies that monitor patients for detecting newly LTBI acquisition or reactivation would be of great interest. In addition, further investigations are required to assess the utility of IGRAs in poorly studied populations such as transplant recipients or patients with cancer. The indications for use of IGRAs are shown in Table 1.

IGRAs in active TB

IGRAs have 80 % sensitivity for active TB. Subsequently, they are suboptimal for TB diagnosis when used alone. In most cases, patients with disease have a low frequency of IFN-γ-producing $CD4^+$ T cells on peripheral blood [8, 9••]. These findings could be attributable to a possible role of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) as players in dampening the effector immune response, or to a sequestration of effector-specific T cells at site of infection. Moreover, IGRAs are not designed for distinguishing between LTBI and disease; as a consequence, they should be used as complementary tools for diagnosis. In this regard, a multicentre TBnet study showed that IGRAs combined with TST might help TB exclusion [41]. An alternative approach for TB diagnosis using IGRAs is the detection of sensitized T cells from site of infection. Therefore, the use of samples such as bronchoalveolar lavage, pleural effusion, or cerebrospinal fluid is a great challenge for pulmonary and extrapulmonary TB diagnosis [42, 43].

It is thought that IFN-γ response is related to bacillary burden and antigen load. Cells detected by IGRAs are effector T cells that have been in contact with TB antigens in vivo, and consequently, they can release IFN-γ when they are re-

Table 1. The main indications for the use of the IGRA methods

IGRA indications	
Contact tracing studies:	Adults[1]
	Children[2]
LTBI screening:	School screening[1]
	Community screening[1]
	Health care workers serial testing[3]
	HIV patients
	Anti-TNF-α treatment candidates
Active TB diagnosis:	Complementary tool[4]
	Diagnostic tool[5]

[1]Especially in BCG-vaccinated individuals
[2]The tests could have a reduced sensitivity in children under 5 years old
[3]Special attention with reversions and conversions are needed
[4]When used in blood samples
[5]When used in non-blood samples: BAL, pleural effusion, and cerebrospinal fluid

exposed in vitro to the specific antigens, reflecting a dynamic process of antigen load. Thus, IGRAs are supposed to monitor treatment response and to predict TB relapse. In this sense, it has been reported that IFN-γ production decreases with treatment. However, this response varies between individuals, therefore it is very difficult to assess their usefulness for monitoring therapy [44].

Molecular biology

New molecular methods for diagnosing active TB

The molecular diagnostic method with more impact in the history of TB is GeneXpert MTB/RIF (Cepheid, USA), an automated and closed system able to perform DNA extraction and real-time PCR directly from respiratory and non-respiratory samples and detect *M. tuberculosis* and RIF resistance in 2 hours with minimal hands-on technical time. In a recent review conducted to assess the diagnostic accuracy of GeneXpert MTB/RIF for pulmonary TB, the reported pooled sensitivity and pooled specificity were 89 % and 99 %, respectively. Specifically, in smear-negative samples pooled sensitivity was 67 % and pooled specificity was 99 %. Furthermore, for people with HIV infection, GeneXpert pooled sensitivity was 79 % [45].

FluoroType MTB (FT MTB, Hain Lifescience, Germany) and Genedrive (Epistem, United Kingdom) are other recently developed real-time PCR tests that present performance characteristics similar to that of GeneXpert. Specifically, sensitivity and specificity of FluoroType MTB reported in two studies were 88.1 – 95.1 % and 96.4 – 98.9 %, and sensitivity rates for smear-positive and smear-negative TB specimens were 100 % and 56.3 – 84.6 %, respectively [46, 47]. Regarding performance of Genedrive, authors used model samples spiked with *M. tuberculosis* and reported 90.8 % sensitivity and 100 % specificity for *M. tuberculosis* detection [48]. This study also included samples from smear-negative or scanty TB patients, and sensitivity of Genedrive was 100 %, compared to 93.5 % of the GeneXpert assay. The characteristics of these platforms make them particularly useful for detecting *M. tuberculosis* in low-resource and high-incidence settings, although this must be addressed in further studies.

Another molecular method recently developed to detect *M. tuberculosis* is based on loop-mediated isothermal amplification (TB-LAMP, Eiken Chemical, Japan). Average sensitivities and specificities obtained range from 77.7 % to 88.2 % and 93.9 to 96.3 %, respectively [49]. This method is also fast (15 – 40 min), it requires only a heat block, it is robust and it generates a result that can be detected with the naked eye, therefore, making it attractive as a diagnostic platform for resource-poor settings.

Additionally, reverse hybridization-based line probe assays are also able to detect *M. tuberculosis* in decontaminated pulmonary and extra-pulmonary samples. One of these tests is GenoType Mycobacteria Direct (Hain Lifescience, Germany). Overall sensitivity and specificity values obtained in different studies were 62.4 – 93.7 % and 100 %, respectively [50, 51].

GenoQuick MTB (Hain Lifescience) is a similar method, but amplicon-probe-complex is detected on a lateral-flow dipstick. Moure et al evaluated this method and reported 85.4 % sensitivity, including 78.1 % smear negative samples [52].

The sensitivity and specificity of the more common methods for detecting *M. tuberculosis* in the clinical samples are presented in Table 2.

Table 2. Sensitivity and specificity of the more common molecular methods for detecting *M. tuberculosis* in clinical samples

Molecular method	Sensitivity (%)	Specificity (%)	Studies
GeneXpert	89	99	[45]
FluoroType	88-95.1	96.4-88.9	[46, 55]
TB-LAMP	77.7-88.2	93.9-96.3	[49]
GenoType Mycobacteria Direct	62.4-93.7	100	[50, 51]
GenoQuick MTB	85.4	100	[52]

New molecular methods for identifying strains

Identification of the species is important for detecting clinically significant mycobacteria that are associated with disease and for implementing an effective antibiotic therapy as well.

One of the first commercial and widely used tests was AccuProbe (GenProbe, USA), based on acridinium-ester-labeled DNA probes [53]. The next most used molecular method has been the line probe assay (LPA). This method is based on the reverse hybridization of the amplified mycobacterium 16S-23S rDNA spacer region on oligonucleotide probes arranged on a membrane strip, and the subsequent detection with a colorimetric system. Some of these LPA tests are Genotype MTBC and GenoType CM/AS (Hain Lifescience, Germany), and INNO-LiPA Mycobacteria (Innogenetics, Belgium), and in general they present good accuracy [54]. Another commercial test is based on the previously mentioned lateral-flow dipstick, Speed-oligo Mycobacteria (Vircell, Spain) that presents good performance as well [55].

In some studies, pyrosequencing of short fragments of the hypervariable region A of the 16S rRNA gene has been used to identify isolates as *M. tuberculosis* and different NTM [56]. The reported concordance between pyrosequencing and diverse reference methods was >90 % for most of the studies. The approach to discriminate between members of the *M. tuberculosis* complex at the species level has been based on the detection of specific SNPs in *gyrB*, *glpK*, and *pykA* genes [57, 58].

Multiplex real-time PCR is another useful method to differentiate mycobacteria at the species level. Commercial tests that have been recently evaluated are AdvanSure TB/NTM assay (LG Life Sciences, Korea) [59], and Anyplex MTB/NTM (Seegene, Korea) [60], both showing accurate results.

Recently, mass spectrometry (MS) has also been useful for the rapid identification and classification of mycobacteria by means of protein and/or nucleic acid detection and profile characterization. The most widespread used MS technologies are the matrix-assisted laser desorption/ionization (MALDI) and the electrospray ionization (ESI) coupled with a time-of-flight (TOF) analyzer, i.e. MALDI-TOF and ESI-TOF, respectively. Proteomic analysis is focused on mycobacteria identification, and it can be based on mycolic acid or protein profiles. Several protocols have been set up, showing in general high reproducibility and percentage of correct identification [61, 62•]. MS has also been demonstrated to be a useful and accurate method for identification based on the analysis of 16S rDNA signature sequences [63].

Finally, Bergval and colleagues developed an assay based on multiplex ligation-dependent probe amplification (MLPA) followed by hybridization on probe-coated microbeads and analysis with a liquid array reader [64]. They applied this method to identify the most clinically relevant NTM species and discriminate the species within the *M. tuberculosis* complex.

New molecular methods for detecting resistance

Drug resistance in *M. tuberculosis* emerges by the stepwise acquisition of genetic mutations in genes coding for drug targets or drug-converting enzymes. Resistance mechanisms for some drugs are well known, but for other important drugs there is a lack of knowledge about the location of all mutations conferring drug resistance.

As previously mentioned, GeneXpert MTB/RIF detects resistance to RIF, with a pooled sensitivity of 95 % and a pooled specificity of 98 %, according to the most recent review [45]. Regarding performance of Genedrive, 72.3 % sensitivity and 100 % specificity have been reported [48].

Numerous studies have assessed the performance of line probe assays (GenoType MTBDR*plus* and GenoType MTBDR*sl*) [65, 66•] and pyrosequencing to detect mutations associated with drug resistance. The ranges of sensitivity and specificity values of these molecular methods are presented in Table 3.

Since 2009, whole-genomic sequencing (WGS) has been used in order to detect drug resistance in several studies. In general, resistance genotypes showed a strong correlation with drug susceptibility phenotypes, since the mutations detected are known to confer drug resistance [67, 68]. WGS has also been useful to show that several mutations occur during the development of drug resistance and also to identify potential compensatory mutations [69, 70••].

Several groups developed and evaluated in-house, multiplex, real-time PCR tests to detect drug resistant *M. tuberculosis*. Nevertheless, there are some commercial tests whose performance has not yet been assessed and published studies are required. A recently evaluated test is MeltPro TB/INH assay (Zeesan Biotech, China), aimed to detect INH resistance mutations in *katG*, *inhA* promoter, and *ahpC* promoter, with a clinical sensitivity and specificity of 90.8 % and 96.4 %, respectively, and 99.1 % concordance with DNA sequencing [71].

Table 3. Sensitivity and specificity of GenoType and pyrosequencing for detecting drug resistance

Drug	GenoType[a]		Studies	Pyrosequencing		Studies
	Se (%)	Sp (%)		Se (%)	Sp (%)	
INH (*katG*, *inhA*, *ahpC*)	70.6-96.0	87.1-100	[90–92]	63.8-94.4	100	[93–96]
RIF (*rpoB*)	95.9	98.0	[97]	94.0	98.0	[98]
STR (*rpsL*)	-	-		78.7	95.9	[96]
EMB (*embB*)	67.9	79.9	[99]	31.3-66.7	87.0-100	[93, 96]
FLQ (*gyrA*)	87.4	97.1	[99]	70-87.1	98.9-100	[93, 95, 96, 100]
KAN (*rrs*, *eis*)	44.4	99.3	[99]	83.7-85.7	98.4-100	[93, 95]
AMK (*rrs*)	82.6	99.5	[99]	77.3-100	99.0-100	[93, 95, 96]
CAP (*rrs*)	82.0	97.3	[99]	79.5-100	97.2-99.0	[93, 95]

INH: isoniazid; RIF: rifampicin; STR: streptomycin; EMB: ethambutol; FLQ: fluoroquinolones; KAN: kanamycin; AMK: amikacin; CAP: capreomycin; Se: sensitivity; Sp: specificity

[a]GenoType MTBDR*plus* for INH and RIF; GenoType MTBDR*sl* for FLQ, KAN, AMK, CAP and EMB

Genomic profile characterization by mass spectrometry has also been used to detect drug resistance. Massire et al and Simner et al developed assays which consisted on a multiplex PCR followed by ESI-TOF, which enabled the characterization of drug resistance to RIF, INH, EMB, and FLQ, presenting sensitivies and specificities comparable to the other molecular methods [72, 73].

Finally, different assays based on microbeads have been developed and used to obtain information regarding *M. tuberculosis* drug resistance to RIF and INH [74•, 75, 76]. This method has been reported to present good sensitivity and specificity values compared to both DNA sequencing and phenotypic drug susceptibility testing results.

New methods in molecular epidemiology

There are different ways to classify *M. tuberculosis* isolates into main lineages for evolutionary studies, establish chains of transmission during suspected outbreaks, differentiate between reinfection and relapse and assess possible laboratory cross-contaminations.

The most used and referenced molecular methods for epidemiological studies have been IS6110 restriction fragment length polymorphism (RFLP), spoligotyping (clustered regularly interspaced short palindromic repeat [CRISPR]-based genotyping) and mycobacterial interspersed repetitive units - variable number tandem repeat (MIRU-VNTR) [77–79], but other molecular approaches have started to take place.

Pyrosequencing assays have been developed to detect SNPs associated with the three principal genetic groups (PGGs), classify strains into SNP cluster groups (SCGs) and also identify W-Beijing strains [57, 80•].

The aforementioned microbead-based multiplex method has been also used to classify isolates epidemiologically by spoligotyping, showing near 100 % correlation with classical spoligotyping results [75, 76, 81]. This same method also served to detect lineage-specific SNPs willing to improve the taxonomic, evolutionary, and epidemiological classification [82].

WGS has also been used in molecular epidemiology studies, and shown to be a useful tool to study the transmissibility of *M. tuberculosis* isolates, differentiate between relapse and re-infection, identify micro-evolutionary events during transmission, identify new epidemiologic links in the chain of transmission, and predict the existence of undiagnosed cases, demonstrating that WGS is superior to conventional genotyping [67, 83••, 84, 85].

Future directions

Immunodiagnostic assays detecting IFN-γ in vitro have induced the development of next generation assays based on new antigens and multiple testing cytokines. It has been described that IP-10 cytokine can be expressed at higher amounts than IFN-γ, suggesting it as a novel diagnostic marker for diagnosing active TB and LTBI [86]. Although IFN-γ and IP-10 are promising cytokines for active TB and LTBI diagnosis, their single quantification in blood cannot distinguish between disease and infection. In this sense, potential host biomarkers are needed to diagnose TB. The ideal TB biomarker should provide

correlates of risk to TB, protection against active disease and success of treatment. Flow cytometry is currently being used for measuring multiple cytokines and cell surface marker expression (immunophenotyping) in order to find a TB-signature. In addition, immune response to novel TB antigens like RD1 selected peptides, DosR-regulon encoded proteins, or resuscitation-promoting factors is being also explored [87]. Today, global "omics" approaches are also attractive options to follow and the initial studies in this direction provide encouraging results [88]. Further investigations are required to develop novel state-of-the-art techniques that could be transformed into point-of-care assays for TB diagnosis.

Regarding molecular methods in the management of active TB patients, one of the main advantages in general is the short turnaround time and the rapid generation of results, that can be available several weeks earlier in comparison with conventional methods. Other advantages are the high throughput and the flexibility, that open the door for individualized monitoring of patients.

On the other hand, molecular methods present drawbacks as well. Some methods can be relatively complex, the cost of instrumentation may be high, and they require trained staff. Moreover, molecular methods depend on the bacillary load; therefore, a false-negative result may be obtained. Another special drawback is the sensitivity of detection of drug resistance, since not all the genes and mutations are known and included in all the methods, with exception of WGS. At the moment, only GeneXpert and the LPA for detecting drug resistance are endorsed by WHO [89].

In conclusion, molecular methods may be useful for many purposes, and during recent years, they have started to take place, facilitating TB control worldwide. Nevertheless, current molecular tests cannot fully replace the conventional smear microscopy, culture, and phenotypic drug susceptibility testing. At the moment, the rational use of molecular methods and the obtainment of rapid results make them good complementary tests for an initial guidance in the managment of suspected or diagnosed tuberculosis patients.

Compliance with Ethics Guidelines

Conflict of Interest

The authors are members of the European Tuberculosis Network (TBnet) Group. J. Domínguez is a researcher founded from the *Miguel Servet* programme of the *Instituto de Salud Carlos III* (Spain). None of the researchers have any financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This work was supported by a grant from the *Sociedad Española de Neumología y Cirugía Torácica* (SEPAR); the *Fundació Catalana de Pneumologia* (FUCAP); and the *Fondo de Investigación Sanitaria* (PI13/1546). None of the scientific societies, and neither reagent companies had a role in the study design, conduct, collection, management, analysis, or interpretation of the data, or preparation, review, or approval of the manuscript.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by the authors.

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Article VI

Domínguez J, Boettger EC, Cirillo D, Cobelens F, Eisenach KD, Gagneux S, Hilleman D, Horsburgh R, Molina-Moya B, Niemann S, Tortoli E, Whitelaw A, Lange C; TBNET; RESIST-TB networks

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REVIEW ARTICLE

Clinical implications of molecular drug resistance testing for *Mycobacterium tuberculosis*: a TBNET/RESIST-TB consensus statement

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SUMMARY

The emergence of drug-resistant strains of *Mycobacterium tuberculosis* is a challenge to global tuberculosis (TB) control. Although culture-based methods have been regarded as the gold standard for drug susceptibility testing (DST), molecular methods provide rapid information on mutations in the *M. tuberculosis* genome associated with resistance to anti-tuberculosis drugs. We ascertained consensus on the use of the results of molecular DST for clinical treatment decisions in TB patients. This document has been developed by TBNET and RESIST-TB groups to reach a consensus about reporting standards in the clinical use of molecular DST results. Review of the available literature and the search for evidence included hand-searching journals and searching electronic databases. The panel identified single nucleotide mutations in genomic regions of *M. tuberculosis* coding for *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L and *gyr*A that are likely related to drug resistance in vivo. Identification of any of these mutations in clinical isolates of *M. tuberculosis* has implications for the management of TB patients, pending the results of in vitro DST. However, false-positive and false-negative results in detecting resistance-associated mutations in drugs for which there is poor or unproven correlation between phenotypic and clinical drug resistance complicate the interpretation. Reports of molecular DST results should therefore include specific information on the mutations identified and provide guidance for clinicians on interpretation and on the choice of the appropriate initial drug regimen.

KEY WORDS: clinician guidance; interpretation; molecular methods

WHILE THE GLOBAL INCIDENCE of tuberculosis (TB) has declined in recent years, the emergence of drug-resistant strains of *Mycobacterium tuberculosis* is a challenge to TB control in many parts of the world.[1] Treatment for *M. tuberculosis* has been available for over 60 years. During this time, we have observed the emergence of multidrug-resistant TB (MDR-TB), which is formally defined as resistance to at least isoniazid (INH, H) and rifampicin (RMP, R). We have also observed the development of extensively drug-resistant TB (XDR-TB), defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) and one of the second-line injectable drugs, kanamycin (KM), amikacin (AMK) or capreomycin (CPM), and, most recently, the development of so-called totally drug-resistant strains.[2,3] According to the World Health Organization (WHO), 136 412 patients were notified with MDR-TB in 2013 worldwide. The average proportion of MDR-TB cases with XDR-TB was 9.0%.[1] Estimated

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numbers of patients with MDR-TB are 3.5 times higher, at approximately 480 000 (credibility range 350 000–610 000).[1]

Effective TB control depends upon rapid case detection and initiation of adequate treatment. Conventional procedures for the isolation of *M. tuberculosis* and DST are slow, causing substantial delays until patients with drug-resistant TB receive adequate treatment. Important aspects regarding the molecular basis of anti-tuberculosis drug resistance have recently been elucidated. Molecular methods based on genomic DNA sequencing have been used to detect the main mutations involved in drug resistance. New molecular methods, such as polymerase chain reaction (PCR) based solid-phase reverse hybridisation line-probe assays (LPAs), have been developed to detect the most common mutations conferring *M. tuberculosis* drug resistance.[4] In addition, technologies for sequencing and analysing the whole genome of *M. tuberculosis* have become available to guide physicians on the treatment selection for patients with drug-resistant TB.[5] These methods are sensitive and specific when detecting resistance mutations in bacterial isolates and also in clinical samples.[6,7]

Although the WHO recommends LPAs for rapid molecular diagnosis of RMP and INH resistance, these tests are not currently recommended by the WHO for rapid second-line DST in *M. tuberculosis*. A recent Cochrane review found that in adults with TB,[8] a positive LPA result (GenoType® MTBDR*sl*, Hain Lifescience, Nehren, Germany) for FQ resistance, second-line injectable drug resistance or XDR-TB can be treated with confidence. However, current generations of LPAs cannot detect approximately one in four cases of second-line injectable resistant TB, and will miss between one in four and one in three cases of XDR-TB. However, despite the absence of an official WHO recommendation, LPAs are frequently used in clinical practice for the initial diagnosis of second-line drug resistance patterns of *M. tuberculosis* in patients with MDR-/XDR-TB. In very few specialised centres, whole genome sequencing (WGS) is already implemented for molecular DST in TB.[9]

To guide clinicians in the initial treatment of MDR-/XDR-TB patients, we summarise current knowledge on the ability of molecular methods to predict in vitro drug resistance of first- and second-line anti-tuberculosis drugs, and present a minimal consensus on which information obtained from molecular DST should influence initial treatment decisions in such patients.

METHODOLOGY

This document has been developed by physicians, microbiologists, molecular biologists and clinical epidemiologists of the TBNET (www.tb-net.org) and RESIST-TB (www.resisttb-org) groups to reach a consensus about reporting standards in the clinical use of *M. tuberculosis* molecular DST results. Review of the available literature and the search for evidence included hand-searching journals and searching electronic databases including MEDLINE and PubMed. Consensus statements were developed in a stepwise approach:[10]

Step 1: Preliminary proposals for key recommendations were drafted by the coordinating author (JD). All co-authors were asked to provide alternative statements.
Step 2: Alternative statements were collected from co-authors.
Step 3: Co-authors were asked to select one preferred statement among the alternative statements. The co-authors were blinded to the vote.
Step 4: For each recommendation, the statement that received most votes was selected for inclusion in the manuscript.
Step 5: All co-authors were asked to indicate their agreement, disagreement or whether they preferred to abstain from a decision.

TREATMENT OF TUBERCULOSIS PATIENTS

Anti-tuberculosis drugs are categorised by the WHO in groups, from the most effective, most commonly used drugs (Group 1) to those that are rarely used and have unclear effectiveness (Group 5). These drugs work through a variety of mechanisms, as outlined in the Appendix (Appendix Table A.1).* More detailed information on these mechanisms can be found in several excellent recent reviews.[11]

Recommendations for treatment regimens for drug-susceptible TB were developed following a series of clinical trials over a 20-year period, culminating in the currently recommended 'standard regimen' consisting of INH, RMP, pyrazinamide (PZA, Z) and ethambutol (EMB, E) (HRZE) for 2 months, followed by INH and RMP for 4 months.[12] The WHO currently recommends the following strategy:[13]

1 In the treatment of MDR-TB patients, an FQ should be used (strong recommendation, very low-quality evidence).
2 In the treatment of MDR-TB patients, a later-generation FQ rather than an earlier-generation FQ should be used (conditional recommendation, very low-quality evidence).

*The appendix is available in the online version of this article, at http://www.ingentaconnect.com/content/iuatld/ijtld/2015/00000020/00000001/art00007

3 In the treatment of MDR-TB patients, ethionamide (ETH) (or prothionamide [PTH]) should be used (strong recommendation, very low-quality evidence).
4 In the treatment of MDR-TB patients, four second-line anti-tuberculous drugs that are likely to be effective (including a parenteral agent), as well as PZA, should be included in the intensive phase (conditional recommendation, very low-quality evidence).
5 In the treatment of MDR-TB patients, regimens should include at least PZA, a FQ, a parenteral agent, ETH (or PTH), and either cycloserine (CS) or para-aminosalicylic acid (PAS) if CS cannot be used (conditional recommendation, very low-quality evidence).

The abovementioned WHO recommendations result largely from the paucity of drugs that are effective and well tolerated, and from the fact that many patients with drug-resistant disease have isolates that are resistant to considerably more drugs than merely INH and RMP. These regimens have substantial toxicity,[14–18] and effectiveness was estimated at 54–66% in two meta-analyses.[19,20] Programmatic data suggest that fewer than 50% of patients successfully complete treatment.[1]

Ideally, treatment regimens should be based on in vitro DST of the patient's *M. tuberculosis* isolates, but phenotypic testing can take 6–8 weeks. In addition, in many settings phenotypic testing of first- and/or second-line drugs is not possible and empirical treatment is prescribed. Many patients are therefore treated with suboptimal regimens for prolonged periods. The advent of DNA-based diagnostics offers the potential for rapid assessment of susceptibility and prompt administration of the optimal regimen, within the context of current guidelines.[10]

In the past few years, several new agents have been developed that offer new hope for improved MDR-TB treatment regimens. These agents comprise either new drug classes (diarylquinolines, nitroimidazole derivatives) or new agents in classes in which current agents are relatively toxic (oxazolidinones).[21] Two new drugs, bedaquiline, the first approved agent in the diarlyquinolone class, and delamanid, the first approved agent in the nitroimidazole derivative class, have recently become available for clinical use. Clinical trials are urgently needed to determine which companion drugs will lead to the best clinical outcomes for MDR-TB patients. Until recently, there were limited possibilities of determining if a patient's isolate was susceptible to these agents, because neither bedaquiline nor dalamanid were available from the manufacturer for laboratory in vitro DST. In addition, standardised methodologies suitable for widespread adoption by reference laboratories have not been fully developed or established. [22,23]

In conclusion

1 Until recently, there were no systematic trials of regimens for the treatment of drug-resistant TB.
2 Most experts think that regimens should be designed to include at least four drugs to which isolates are susceptible in vitro.
3 Phenotypic testing takes 6–8 weeks, resulting in substantial delays in optimising MDR-TB treatment regimens.
4 Phenotypic DST against second-line drugs is not available in many areas.

EVOLUTION OF DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

M. tuberculosis complex develops drug resistance as a result of spontaneous mutations in genes encoded on the chromosome. These mutations include single nucleotide changes, small insertions and deletions (*indels*) or larger deletions, and either modify the drug target itself, silence drug activating enzymes in the case of pro-drugs, or circumvent drug action by increasing the gene product targeted by the drug.[11] The bacterial cells carrying such mutations are selected during periods of ineffective patient treatment and increase in frequency, eventually replacing the drug-susceptible bacterial population.[24] The probability of acquiring resistance through spontaneous mutations varies by drug, ranging from approximately 1 in 10^8 bacilli for RMP, to approximately 1 in 10^6 bacilli for INH, streptomycin (SM) and EMB.[25] Moreover, *M. tuberculosis* comprises various phylogenetically distinct lineages,[26] and recent studies indicate that the rate of mutation towards drug resistance might be influenced by the lineage to which a particular strain belongs.[27,28]

Drug-resistant subpopulations of bacteria may be selected in patients treated with only one effective drug.[29–31] The main reasons for phases of monotherapy include improper prescription of treatment regimens, addition of single drugs to failing treatment regimens, inadequate drug supply, patient non-adherence, quality of the anti-tuberculosis drugs (a very relevant reason in many settings), differences in pharmacogenomics, and the pharmacodynamic and kinetic properties of the drugs administered.[32] The development of multidrug resistance results from several periods of 'sequential monotherapy' during which resistance to other drugs is acquired, a phenomenon referred to as amplification of drug resistance. Recent data indicate that *M. tuberculosis* strains evolve within individual patients during treatment, and that this micro-evolution is dynamic, leading to the presence of different subpopulations of bacteria with divergent sets of drug resistance mutations.[29,33] This intra-patient diversity is likely

to influence the performance of molecular and phenotypic DST, and needs to be considered when interpreting routine diagnostic results.

An important factor driving the current MDR-TB epidemic is the direct transmission of MDR-TB strains, leading to high proportions of MDR-TB strains in patients who have not been treated previously.[34] Because of the frequent delays in diagnosing MDR-TB, strains from these patients with primary MDR-TB are at a high risk for developing further drug resistance.[35,36] Undetected MDR-TB and the less effective second-line treatment regimens also contribute to prolonged periods of sputum smear positivity among MDR-TB patients, enhancing the role of transmission of MDR-TB strains.[32,35] The overall impact of transmission as opposed to de novo acquisition of drug resistance on the global MDR-TB epidemic has been a subject of controversy for a long time due to a postulated lower fitness of MDR-TB strains of *M. tuberculosis*.[37,38] Early data posited that the genetic mutations conferring drug resistance in *M. tuberculosis* resulted in reduced bacterial fitness,[39] leading to the assumption that drug-resistant strains would not disseminate widely in the community.[37,40] However, it has become clear that resistance mutations with no or low fitness impact exist that facilitate the spread and amplification of resistance.[41–43] In addition, more recent studies have indicated that some drug-resistant *M. tuberculosis* strains have acquired compensatory mechanisms that restore the fitness cost associated with resistance mutations,[44–46] leading to the expansion of particular highly transmissible MDR-TB clones in different areas of the world.[47] The interaction between a specific drug resistance-conferring mutation and a compensatory mutation is an example of epistasis, which occurs when the phenotypic effect of one mutation is modified by the presence of a second mutation.[48]

In conclusion

1 Large studies are necessary to establish the relationship between the mutations detected and the phenotype finally expressed by *M. tuberculosis*.
2 The correlation with clinical outcome has not been investigated.
3 An international database with validated drug resistance mutations should be established.
4 Clinical trials are necessary to demonstrate the usefulness of individualised treatment regimens based on multi-analyte molecular assays.

PRINCIPLES OF PHENOTYPIC DRUG SUSCEPTIBILITY TESTING

Phenotypic DST of mycobacteria assesses the ability of the organism to grow in the presence of the antibiotic using either solid or liquid medium. Various methods have been described (absolute concentration, resistance ratio, proportion methods, in a variety of commercial and non-commercial systems),[49–53] but only the commonly used proportion method will be outlined, as it is often used as a reference standard.

The proportion method is based on the premise that if <1% of the organisms in a given population are resistant to a drug at a given concentration (the so-called critical concentration), the population as a whole is susceptible, and conversely, if >1% of the organisms are resistant, the population as a whole is resistant. The critical concentration represents the lowest concentration of the agent that inhibits >95% of wild-type (wt) (susceptible) strains.[54] Thus, the critical concentration basically corresponds to what is known as epidemiological cut-off (ECOFF).[55] The organism is inoculated onto drug-free and drug-containing medium, and following incubation, the number of colonies is compared to calculate the proportion of resistant colonies. When performed in liquid culture, growth in an antibiotic-containing medium (using a critical concentration) is compared to growth in an antibiotic-free medium. If the drug-free medium registers growth before the drug-containing medium, the isolate is regarded as susceptible, and vice versa for the determination of resistance. In many commercial systems, the inoculum in the antibiotic-free medium is a 1:100 dilution of the inoculum in the antibiotic-containing medium reflecting the proportion method. It is important to note that resistance as defined here is a technical term: it does not correspond to clinical resistance and it is not to be confused with mutational resistance, the driver of acquired drug resistance in *M. tuberculosis*.[56] In combination with the critical concentration, the critical proportion is a laboratory term used in in vitro DST to define the epidemiological cut-off.

The critical concentrations of many antibiotics were published by the WHO in 2008, with updates suggested at a meeting in 2012 (these updates have not yet been formally published by the WHO) (Appendix Table A.2).[57,58] Results using current critical concentrations are generally accurate and reproducible for RMP and INH, but less so for EMB and SM.[59,60] Although critical concentrations have been recommended for PZA testing, phenotypic testing for this drug is technically difficult, given the drug's activity at a low pH, which inhibits mycobacterial growth.[61] Concerns have also been raised about the appropriateness of the critical concentration for PZA as well as the reliability of the current methods compared to molecular detection of resistance mutations.[62,63] Regarding second-line agents, the critical concentrations for FQs and injectable agents (AMK, KM and CPM) are currently appropriate,[60,64,65]

although the evidence is not as strong as for RMP and INH.

A drawback to the use of critical concentrations is that it assumes that there are two clearly defined populations of organisms (resistant and susceptible), and that the minimum inhibitory concentration (MIC) distributions for these two populations can be easily separated. If the MICs of resistant and susceptible bacilli are close together or form more of an MIC continuum, then the use of critical concentration to separate resistant from susceptible is problematic[66] and may lead to the variability in results discussed above. The relative proportions of strains with different MICs circulating in the community will also affect the accuracy of phenotypic DST. For example, if there is a relatively high prevalence of strains with low-level resistance, phenotypic testing may not correlate as well with the clinical outcome as it would when strains with 'high-level' resistance are more common and the current critical concentrations would separate susceptible strains more easily from resistant strains.[67] The other problem associated with the use of critical concentrations is that, in some instances, the critical concentration is close to the serum levels attained using standard dosing regimens; this is especially true for second-line agents.[66,68]

The use of two critical concentrations (low and high) has been advocated when testing certain antibiotics[54,57] such as INH, EMB and SM. Resistance at the high concentration indicates resistance, while an isolate resistant at the low concentration, but susceptible at the high concentration, suggests low-level resistance, and higher doses of the respective drug may still be clinically effective. However, there is a need to critically re-evaluate many of the current critical concentrations as well as testing methodologies to better standardise phenotypic resistance testing for *M. tuberculosis*, and to better understand the correlation between the phenotypic DST result and treatment outcomes. It is most likely that quantitative measures for drug susceptibility need to be implemented in diagnostic mycobacteriology.[66,69–71]

In conclusion

1 While phenotypic DST is still commonly regarded as the gold standard for determining the susceptibility of *M. tuberculosis* to various drugs, it has a number of limitations.
2 The DST results for some drugs (such as INH and RMP) are more reliable than for other drugs (such as EMB).
3 There is a lack of good clinical outcome data to correlate with the phenotypic DST results for some agents.
4 A good understanding of local epidemiology and molecular resistance mechanisms is important to appropriately interpret phenotypic DST results.

PRINCIPLE OF GENOTYPIC DRUG SUSCEPTIBILITY TESTING

Drug resistance in *M. tuberculosis* is mainly due to single nucleotide mutations (SNMs) that accumulate over time on specific genes. For some antibiotics, the association between the mechanisms of resistance and the responsible genes are very well known, whereas for others we still have incomplete knowledge. Not all of the SNMs detectable in strains showing a resistant phenotype are responsible for drug resistance: some are phylogenetic markers or cause silent mutations.[66,69,70] The frequency of the mutations is also different for the different genes associated with drug resistance.

Molecular detection of the SNMs associated with drug resistance is the fastest way to design a personalised treatment regimen, and it also has the potential to become a bedside technology. WHO-endorsed commercial methods for drug resistance detection include LPAs and the Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA).[72,73] The specificity and sensitivity of these tests have been evaluated against liquid culture and phenotypic DST as gold standard.[74] The use of 'phenotypic DST' as gold standard for the evaluation of molecular tests was recently challenged.[75] In the future, the use of multiple standards based on sequencing, quantitative DST and clinical outcomes should be considered.

Current molecular techniques detect both live and dead bacteria, and a positive result does not imply the viability of the pathogen. These methods cannot therefore be used for monitoring treatment response.[76]

Line-probe assay

Commercial systems based on DNA probe assays are available for the detection of the most frequent mutations responsible for resistance to RMP, INH, FQs, second-line injectable drugs and EMB.[77–83] The regions of interest are investigated with wt probes, which, in the presence of mutations, fail to hybridise. Most of the systems include confirmatory probes designed to detect the more frequent mutations.

Different commercial assays detect RMP resistance by targeting the hot spot of the *rpo*B gene, known as the RMP resistance-determining region, which harbours more than 95% of mutations responsible for RMP resistance.[84,85] This region is covered in the available LPAs by a number of overlapping wt probes. Five probes are present in INNO LiPA Rif.TB® (Fujirebio, Ghent, Belgium), eight in GenoType® MTBDR*plus* (Hain) and three in the AID TB Resistance assay (Autoimmun Diagnostika GMBH,

Strassberg, Germany) to detect possible mutations in almost the same *rpo*B region using missed hybridisation. INNO LiPA[86] and GenoType MTBDR*plus*[87] include four mutated probes specific to the mutations Asp→Val at codon 516, Ser→Leu at codon 531, and His→Tyr or His→Asp at codon 526 (Appendix Table A.3). The AID TB Resistance assay includes a total of three probes to detect mutations Asp→Val or Asp→Tyr at codon 516, Ser→Leu or Ser→Trp at codon 531, and His→Tyr, His→Asp or His→Arg at codon 526.[88]

Mutations confirmed by mutated probes are most frequently detected in RMP-resistant strains. Mutations at codons 516, 526 and 531, other than those recognised by mutated probes, may also be associated with high-level RMP resistance. Certain mutations have been reported not to be detected by phenotypic susceptibility testing,[89–91] particularly codons that are prone to silent mutations and do not affect drug susceptibility (Appendix Table A.3).

GenoType MTBDR*plus* includes the detection of mutations responsible for INH resistance. In the *kat*G gene, the codon 315 is investigated with a wt probe and two mutated probes specific for the ACC or ACA mutations, both of which are responsible for the change Ser→Thr. In the promoter region of the *inh*A gene, three positions are monitored: the upstream position –16 is targeted by a wt and a mutated probe aiming to recognise the A to G mutation; in the upstream –15 position, a wt and a mutated probe can identify the C to T mutation; while in the upstream position –8, a wt and two mutated probes can discriminate between the T to C or T to A mutations. The AID TB Resistance assay includes two wt probes targeting the *inh*A (positions –16, –15 and –8) and *kat*G codon 315, respectively, and two mutated probes that detect mutations –16G, –15T, –8A and –8C in *inh*A, and S315T in *kat*G, respectively.

Mutations at codon 315 are detected in 60–80% of high-level INH-resistant strains.[92] Mutations in the *inh*A gene promotor are present in 10–20% of resistant strains and are frequently responsible for low-level resistance. The *M. tuberculosis* lineage can influence the level of INH resistance conferred by *inh*A or *kat*G mutations.[93] These mutations also affect susceptibility to ETH.

FQ resistance is investigated by GenoType MTBDR*sl* using a set of three wt probes covering codons 85–96 of the *gyr*A gene and by six mutated probes specific for mutations at codon 90, 91 and (four probes) 94. The AID TB Resistance assay includes one wt probe and six probes to detect mutations in codons 90, 91 and 94 of *gyr*A. Mutations in the *gyr*A gene are present in about 70% of the strains resistant to FQs,[6] although they may not be as useful for reflecting resistance to the later generations of FQs, such as moxifloxacin (MFX).

The detection of resistance to second-line injectable drugs in GenoType MTBDR*sl* is focused on two specific mutations, A1401G and A1484T, in the *rrs* gene. For this purpose, two wt and two mutated probes are used. In the AID TB Resistance test, two wt probes target *rrs* positions 1401/1402 and 1484, respectively, while there are three mutated probes to detect changes in A1401G, C1402T and G1484C/T. According to the review published by Georghiou et al., most of the strains with A1401G, C1402T or G1484T mutations were resistant to second-line injectable drugs. It is of note that 7% of CPM-susceptible isolates carried the A1401G mutation.[94]

SM resistance is only assessed by the AID TB Resistance assay. This test includes a total of three wt probes covering codons 43 and 88 of the *rps*L gene, and *rrs* positions 513 to 517. Most of the strains harbouring the targeted mutations in *rps*L and *rrs* are resistant to SM.[95–97] Seven mutations are targeted by the assay: *rps*L K43R, K88R and K88Q, and *rrs* C513T, A514C, G515C and C517T. Resistance to EMB is investigated at the level of codon 306 of the *emb*B gene with a wt and two mutated probes discriminating the mutations Met→Ile and Met→Val. The AID TB Resistance test includes one wt probe and four mutation probes: M306V, M306I ATA, M306I ATC and M306I ATT. Mutations at codon 306 are present in about 55% of EMB-resistant strains.[6] The clinical significance of strains with a mutation in the presence of a susceptible phenotypic result is as yet unclear.[98,99]

A second generation GenoType MTBDR*sl* is currently under evaluation: the main differences consist in the addition of the mutation in the *eis* gene associated with resistance to KM and in the absence of codon 306 of *emb*B.

A new LPA that has recently been commercialised (Nipro Corporation, Osaka, Japan) is designed to detect *pnc*A mutations associated with PZA resistance. In the only publication available to date,[100] the agreement between LPA and the phenotypic method was low. As the MICs of these PZase-positive PZA-resistant isolates with wt *pnc*A were very low using the BACTEC™ 460™ (BD, Sparks, MD, USA) method, they may have shown false resistance due to the acidity of the medium used for PZA DST, which inhibited *M. tuberculosis* growth.

Xpert MTB/RIF

The Xpert assay is an integrated micro-fluidic based system comprising a GeneXpert instrument and Xpert test cartridges. The system uses an automated protocol with simultaneous DNA amplification, and is based on molecular beacons technology in which each probe is labelled with a different fluorescent dye, permitting simultaneous detection with in-built controls. The PCR target for RMP resistance is the 81 bp region of the *rpo*B gene. The assay flags the presence

of resistance in the absence of binding of wt probes to the target sequence. The pooled sensitivity and specificity of Xpert for RMP detection are respectively 95% (95% confidence interval [CI] 90–97) and 98% (95%CI 97–99). The test is recommended by the WHO as the initial diagnostic test for adults and children presumed to have MDR-TB or human immunodeficiency virus (HIV) associated TB.[101]

Multiplex polymerase chain reaction

Another approach for the detection of mutations is multiplex PCR. Several in-house assays have been developed to detect resistance to first-line and, to a lesser extent, second-line drugs. Depending on the assay, the presence of mutations associated with drug resistance is detected by the presence or absence of an amplification curve. To our knowledge, only one multiplex PCR test is commercially available, the Anyplex™ II MTB/DR/XDR detection kit (Seegene, Seoul, Korea), but only one study has been published.[102] An important drawback of this method is that it is not possible to identify the specific mutation involved.

Platforms for simultaneous detection of multiple mutations

Knowledge about mutations associated with a resistant phenotype is increasing, with the wide accessibility of Next Generation Sequencing (NGS) technology that allows the collection of a large amount of data in a short time at a relatively low cost. Most studies perform WGS from cultured isolates.[9,103] Brown et al. reported the successful and accurate sequencing of *M. tuberculosis* genomes directly from uncultured sputum samples.[104] This alternative could further reduce delays, allowing more personalised treatment.

Microarray-based platforms will allow the comprehensive detection of resistance mutations for first- and second-line drugs, overcoming the limitations of current rapid molecular tests, which can only analyse a few genetic targets. Similar to LPAs, but on a larger scale, these platforms will allow both the detection of the wt sequence and the identification of the specific substitution.

NGS can be considered as the approach of the future for drug resistance detection. Several NGS platforms with different technical characteristics and throughput are available. Different approaches may be considered, from multiplexing several target genes to full genome sequencing. Because of the depth of the information obtained, raw sequence data will be of little use without a highly developed user-friendly software package to interpret results, and it will take enormous efforts to correlate the genomic findings with clinical data. The lack of clinical correlation is a huge problem, and much more so for genomic sequencing data than for phenotypic results.

In conclusion

1 Molecular tests targeting mutations associated with drug resistance have high specificity and sensitivity when compared to DNA sequencing as gold standard.
2 Multiple molecular platforms with different levels of automation are available (and more will be in the future) for the detection of mutations in *M. tuberculosis*.
3 Uncertainty about the correlation between single nucleotide polymorphisms and phenotypic DST, and lack of data correlating mutations to clinical outcomes, is delaying our capacity to use genotypic results to guide personalised patient management.
4 Only full genome sequencing on an extremely large number of strains collected worldwide, coupled with phenotypic DST results, drug treatment and clinical outcomes data, can provide the appropriate statistical power to identify the subset of mutations predictive of treatment failure to any given drug.

RELATIONSHIP BETWEEN THE RESULTS OF GENOTYPIC AND PHENOTYPIC DRUG SUSCEPTIBILITY TESTING AND CLINICAL OUTCOME

Molecular tests may show discordant results when compared to phenotypic DST based on critical concentration testing.[74,98,105–112] The clinical consequences of the limitations in the accuracy of molecular resistance assays depend on the drug, but follow a general pattern. False-positive test results will lead to drug-susceptible TB being treated with one or more second-line drugs, i.e., treatment that is generally more toxic, less effective, prolonged and more expensive. Furthermore, salvage treatment tends to be less effective than standard treatment, and there is often a greater risk of default.

Sensitivity

False-negative results of molecular resistance assays can be due to platform characteristics. For example, the previous generation GenoType MTBDR*plus* assay had limited sensitivity for detecting *M. tuberculosis* when used directly on smear-negative, culture-positive sputum.[113] More importantly, test sensitivity depends on the proportion of relevant mutations that are targeted by the assay, which generally declines with the increasing number of different genes and intergenic regions involved in resistance to the drug of interest, either known or unknown.[114] Test sensitivity may also show geographic variations if the proportion of relevant mutations covered by the assay varies between regions.[115] This can be due to associations with the genetic background of the strain,[6,93,116,117] and possibly to differential consequences of muta-

tions for bacterial fitness over time, leading to the predominance of the mutation with the least fitness cost.[118]

Finally, studies reporting frequencies of mutations among phenotypically resistant strains may differ in their selection of isolates and DST used. Together, these factors may cause the sensitivity of specific mutations for phenotypic DST to vary widely (Appendix Table A.4).

Specificity

False-positive results of molecular resistance assays can also be due to platform characteristics.[182] In addition, they may occur due to silent mutations picked up by wt probes included in the assay to cover resistance-conferring mutations that are scattered across a larger genomic region, such as with the *pnc*A gene for resistance to PZA.[61] False-positive results in molecular resistance testing may in fact be truly positive if the reference standard (phenotypic DST) has incomplete sensitivity when identifying resistance.[55]

This indicates a more general problem in the interpretation of genotypic DST results. Resistance mutations have almost always been identified based on comparison with phenotypic DST rather than with clinical outcomes. However, phenotypic DST based on critical concentration testing may correlate poorly with clinical resistance. As mentioned before, DST for EMB, SM and ETH at recommended critical concentrations show poor discrimination between clinically resistant and clinically susceptible isolates.[50] Direct evidence of clinical outcomes is available for only a few resistance mutations (Table).

Prediction of a positive or negative test result

Prediction of test results is generally expressed as positive (PPV) and negative (NPV) predictive values. However, as these predictive values depend not only on sensitivity and specificity, but also on the proportion of patients who have true resistance, PPV and NPV can only be meaningfully interpreted for a given pre-test probability of resistance. For mutations that have 100% specificity, the PPV equals 1 (i.e., a positive test result always means growth at the critical concentration), while the NPV equals 1–sensitivity (e.g., for a test sensitivity of 80%, the NPV for growth at the critical concentration will be 20%). In LPA, the PPV is high for RMP, INH, AMK, KM and SM, as all *rpo*B mutations, the *kat*GS315T mutation, all *inh*A promoter mutations, the *rrs* A1408G mutation, and the *rrs* and *rpsl* mutations covered by LPAs have a specificity of practically 100%. For other drugs, the specificity is less than 100%, and the PPV and NPV will differ. An alternative approach is to express this prediction as positive (LR+) and negative likelihood ratios (LR–). Appendix Table A.4 shows these likelihood ratios for EMB, FQs and injectables based on a meta-analysis of studies that evaluated the Genotype MTBDR*sl* assay.[108] A Cochrane review analysing the diagnostic accuracy of the GenoType MTBDR*sl* assay in detecting second-line anti-tuberculosis drug resistance has recently been published.[8] The pooled sensitivity of the test for the detection of FQ resistance was 83.1% and the pooled specificity was 97.7%; the pooled sensitivity and pooled specificity of the test for injectables were respectively 76.9% and 99.5%.

In conclusion

1 Reported sensitivity and specificity estimates for certain mutations are difficult to interpret for drugs for which there is poor or unproven correlation between phenotypic and clinical resistance (e.g., EMB, Group 4 and 5 drugs).

CONSENSUS RECOMMENDATIONS

1. Should molecular testing for M. tuberculosis *rifampicin resistance using currently available methods be the reference for the diagnostic evaluation of patients with presumptive MDR-/XDR-TB?*

Currently available LPA methods detect mutations in *rpo*B codons 516, 526 and 531. There is a high level of agreement between molecular and phenotypic DST. This is due to the fact that mutations associated with RMP resistance are mainly located in the 81 base-pair (bp) core region of *rpo*B, and mutations outside this region are uncommon. However, clinicians need to be aware that strains carrying rare mutations or mutations outside the conventional hot spots targeted by commercial assays may spread and become prevalent in some settings.

Although they do not cover all mutations involved in RMP resistance, molecular methods for RMP could be considered a standard for the diagnostic evaluation of patients with presumptive MDR-TB. In low MDR-TB prevalence countries, physicians should be aware of possible false-positive resistance results of molecular tests, and RMP resistance should be confirmed by a second molecular test on a different sample or by phenotypic tests.

Agreed: 12; disagreed: 0; abstained: 1.

2. Is there value in molecular testing for M. tuberculosis *isoniazid resistance using currently available methods for the diagnostic evaluation and selection of drug regimen of patients with presumptive MDR-/XDR-TB?*

Although >90% of RMP-resistant strains are also resistant to INH, molecular testing for INH drug resistance is important. First, it offers the possibility to add INH to a second-line drug regimen in the absence of a *kat*G*315* mutation. Second, the implications of RMP resistance are different if accompanied by INH resistance.

Table Clinical implications of mutations detected by molecular methods
A)

Mutation	Drug*		Association with in vitro phenotypic resistance	Association with clinical resistance	Frequency among strains categorised as resistant on the basis of critical concentration testing‡
	INH	ETH			
katG S315T	–	+	S315T confers high-level INH resistance (MIC >1 mg/l), but does not affect susceptibility to ETH[69,70,119–121] Note: there are additional mutations in *inh*A or *eth*A, which confer ETH resistance[122]	Indirect evidence strongly suggests that high-level resistance affects clinical outcomes. *katG* S315T mutations are associated with multidrug resistance (see e.g.[119]). Limited data on direct association between *katG* S315T mutation and clinical outcome suggest increased risk of first-line treatment failure, death and relapse[123,124]	In a systematic review of 52 studies, 5–98% of INH-resistant isolates showed *katG* S315T mutations (median 64%, interquartile range 54–79) (Hooijer et al. unpublished)
*inh*A –16G –15T –8A/C	+	–	*inh*A promoter mutations confer low-level INH resistance (MIC <1 mg/l), but significantly affect ETH susceptibility[69,70,119–121,123] Note: there are additional mutations in the structural *inh*A gene, which together with *inh*A promoter mutations result in INH MIC levels >1 mg/l[125]	Limited direct and indirect data, suggesting no effect on cure rates for standard first-line treatment.[123,124] One study showed increased relapse rates with INH-EMB (6 months) in the continuation phase;[138] *inh*A promoter mutations are not associated with multidrug resistance when compared to the *katG* S315T mutation,[119] but have been associated with XDR-TB in South Africa[126]	In various studies, 12–42% of INH-resistant isolates had *inh*A promoter region mutations[113,119,127–130]
	RMP	RBT			
*rpo*B S531L H526mut	–	–	S531L and H526D/Y confer high-level resistance to all rifamycins.[131–136] In contrast, mutation H526L (and possibly H526N/S) only confer low-level resistance to RMP	Strong direct and indirect evidence for association with clinical resistance[128]	More than 95% of RMP-resistant isolates have mutations in the 81-bp core region of the *rpo*B gene. Most studies showed mutations in codons 531 and 526 in 40–65% and 10–40% of RMP-resistant isolates, respectively[127,129,137]
D516mut	–	+	D516mut predominantly affects RMP, but much less so RBT; RBT is still an option for combination chemotherapy[132–134,136,138]		Most studies showed codon 516 and 533 mutations in 5–32% and 2–5% of RMP-resistant isolates, respectively.[127,129,137] Their frequencies are probably underestimated, as low-level resistant isolates may be tested as phenotypically susceptible[67]
L533mut	+	+	L533mut affects susceptibility to all rifamycin only slightly; RMP and RBT are still an option for combination chemotherapy[134,139–141]		
I572F	–	–	I572F mutations are outside the 81-bp core region[142,143–145]	Some studies suggested a role for this mutation in RMP resistance[146,147]	Among the isolates obtained from patients who did not respond to the anti-tuberculosis treatment, some isolates showed mutation at codon 572.[148] Cross-resistance to RBT has been described in one study[149]
	EMB				
*emb*B M306mut	+		M306mut mostly confers low- to moderate-levels of drug resistance, the clinical implications of which are not clear[69,70,150–154]	There have been no studies of the direct effect of *emb*B306 mutations on clinical resistance	In various studies, 20–88% of EMB-resistant isolates had *emb*B306 mutation[108,127,155–159]

Table (continued)
B)

	Drug*			Association with in vitro phenotypic resistance	Association with clinical resistance	Frequency among strains categorised as resistant on the basis of critical concentration testing‡
	KM	AMK	CPM			
rrs A1408G† (1401)	–	–	+	A1408G confers high-level resistance to both KM and AMK, but only low-level CPM resistance; CPM is still an option for combination chemotherapy[46,152,160-163] Note: cave additional mutations in *tlyA* which in conjunction with A1408G confer high-level CPM resistance (unpublished data)	The *rrs* A1401G mutation was associated with clinical resistance to KM[164]	In a systematic review of 22 studies, the A1401G mutation was present in 78% of AMK-resistant and in 76% of CPM-resistant isolates, but in only 56% of KM-resistant isolates[94]
C1409T† (1402)	–	+	–	C1409T confers high-level CPM resistance and low- to intermediate-level KM resistance, but has little effect on AMK susceptibility; AMK is still an option for combination chemotherapy[46,165,166]	There have been no studies of the direct effect of *rrs* 1402 or *rrs*1484 mutations on clinical resistance	In a systematic review of 22 studies, *rrs* C1402T and G1484T mutations were rare (0–2% each) among isolates resistant to any of the injectables[94]
G1491C/T† (1484)	–	–	–	G1491C/T confers high-level AMK, KM and CPM resistance[46,165,166]		
eis G-37T C-12T G-10A	–	+	+	*eis* mutations confer low-level KM resistance.[167] C-14T may confer low-level resistance to both KM and AMK[129]	There have been no studies of the direct effect of *eis* mutations on clinical resistance	In a systematic review of 22 studies, 22% of the KM-resistant isolates harboured the G-10A mutation, 11% the C-14T and 5% the G-37T mutation.[35] In another study, the C-12T mutation was present in 13% of the KM-resistant isolates.[129] C-14T mutation was also associated with AMK resistance[129]
C-14T	–	–	+			
	SM					
rpsL K43R	–			*rpsL* K43R, *rpsL* K88Q/R and *rrs* A523C and C526T confer moderate- to high-level SM resistance[95,168,169]	There have been no studies of the direct effect of *rpsL or rrs* mutations on clinical resistance to SM	In various studies, 24–89% of SM-resistant isolates had *rpsL*43 mutations.[95,116,159,170] *rpsL*88 mutations have been found in 5–27% of SM-resistant isolates, but this prevalence may be lower depending on the geographical setting.[171–173] Together and on average, *rpsL* and *rrs* mutations are found in from 75% to over 90% of SM-resistant isolates[128]
K88Q/R	–					
rrs A523C† (514) C526T† (517)	–					
	MFX	OFX				
gyrA D94mut	–	–		Mutations in *gyrA* affect MFX and OFX susceptibility. Mutations of residue D94 confer clinical resistance; mutations affecting codon A90 are discussed controversially[174–179] For mutations of codon S91, few data are available, most likely similar to mutations of residue A90[45]	Strong indirect and some direct evidence for association of *gyrA* codon 94 mutations with clinical resistance to OFX[128,164]	D94mut and A90mut in 40–58% and 20–30%, respectively, of OFX- or MFX-resistant isolates[127,129,180,181]
A90mut	+	+				

*– = high-level resistance; the drug should not be given; + = drug susceptibility is not affected or low-level resistance, the drug is an option for combination chemotherapy, in particular when other options are limited due to scarce availability of active compounds.
† *Escherichia coli rrs* nomenclature; the homologous *M. tuberculosis* position is given in brackets.
‡ Note that critical concentration testing uses the ECOFF value to categorise clinical isolates as susceptible. Growth at the critical concentration does not necessarily imply clinical resistance, as it does not define the quantitative level of resistance, i.e., it does not differentiate between low- and high-level 'resistance'.
INH = isoniazid; ETH = ethionamide; MIC = minimum inhibitory concentration; EMB = ethambutol; XDR-TB = extensively drug-resistant tuberculosis; RMP = rifampicin; RBT = rifabutin; bp = base pair; KM = kanamycin; AMK = amikacin; CPM = capreomycin; MFX = moxifloxacin; OFX = ofloxacin; ECOFF = epidemiological cut-off.

Agreed: 13; disagreed: 0; abstained: 0.

3. When should the evaluation for the presence of second-line drug resistance by molecular methods be considered in patients with a presumptive or confirmed diagnosis of tuberculosis?

In all patients with evidence of *M. tuberculosis* with an *rpo*B mutation in a direct specimen or when DST indicates MDR-TB, molecular testing for second-line resistance should be undertaken to guide treatment and to reduce the time to diagnose XDR-TB.

Agreed: 13; disagreed: 0; abstained: 0.

4. What molecular resistance testing results on rifampicin should influence treatment decisions?

More than 95% of RMP-resistant isolates have mutations in the 81-bp core region of the *rpo*B gene. S531L and H526Y/D confer high-level resistance to all rifamycins, with strong direct and indirect evidence of association with clinical resistance. In contrast, D516mut predominantly affects RMP, but much less rifabutin (RBT). RBT could still therefore be considered as an option for combination chemotherapy, although clinical data for the use of RBT in this setting are lacking. As L533mut has only a slight effect on susceptibility to all rifamycins, RMP and RBT are an option for combination chemotherapy for corresponding isolates.

Agreed: 13; disagreed: 0; abstained: 0.

5. What molecular resistance testing results on isoniazid should influence treatment decisions?

The currently available LPA methods detect mutations in *inh*A positions –16, –15 and –8, and *kat*G codon 315. Mutation S315T confers high-level INH resistance (MIC > 1 mg/l), but does not affect susceptibility to ETH. Indirect evidence strongly suggests that high-level resistance affects clinical outcomes. The limited data on the direct association between *kat*G S315T mutation and clinical outcome suggest increased risk of first-line treatment failure, death and relapse. In the case of *katG* S315T mutation, INH should therefore be excluded from treatment.

Compared to *kat*G S315T, *inh*A promoter mutations confer low-level INH resistance (MIC < 1 mg/l), but significantly affect ETH susceptibility. Limited direct and indirect data suggest no effect on cure rates for standard first-line treatment. In the case of *inh*A promoter mutations, INH—preferably in high doses (15–20 mg/kg body weight)—may be administered in combination with other drugs. In the case of *inh*A promoter mutation, the level of resistance should be confirmed by phenotypic methods.

Agreed: 13; disagreed: 0; abstained: 0.

6. What molecular resistance testing results on pyrazinamide should influence treatment decisions?

Unfortunately, until recently, no commercial molecular methods have been able to detect PZA mutations. Mutations associated with resistance can be detected by sequencing the *pnc*A gene.

Agreed: 13; disagreed: 0; abstained: 0.

7. What molecular resistance testing results on ethambutol should influence treatment decisions?

Mutations in *emb*CAB have been detected in resistant strains, with *emb*B306 the codon most commonly affected. M306mut mostly confers low to moderate levels of drug resistance; 20–88% of EMB-resistant isolates had *emb*B306 mutations. These low sensitivity values may be due to the presence of mutations in codons other than *emb*B306, which are not explored by LPAs. These mutations have been located in *emb*B codons 319, 406 and 497, and also in the *emb*C and *emb*A genes. Physicians must be aware of possible false-negative results of molecular tests; in addition, EMB resistance should be confirmed by phenotypic methods. Furthermore, as *emb*B306 mutations have been detected in MDR-TB isolates that are susceptible to EMB, 'false' EMB resistance results may be obtained by molecular tests.

The clinical implications of EMB resistance, which is mostly low or moderate, are not clear at present, nor are those of *emb*B mutations.

Agreed: 13; disagreed: 0; abstained: 0.

8. What molecular resistance testing results on aminoglycosides/polypeptides should influence treatment decisions?

Partial cross-resistance between KM, AMK and CPM has been reported. The *rrs* A1401G mutation is most frequent, and confers high-level resistance to both KM and AMK, but only low-level CPM resistance; CPM may still be an option for combination chemotherapy. C1402T confers high-level CPM resistance and low- to intermediate-level KM resistance, but there is little effect on AMK susceptibility; AMK is therefore still an option for combination chemotherapy. G1484C/T confers high-level AMK, KM and CPM resistance. *rrs* C1402T and G1484T mutations are rare (0–2% each) among isolates resistant to any of the injectables.

Mutations in the *eis* promoter region confer low levels of resistance to KM, and possibly AMK. In these cases, LPA tests that do not explore this region present reduced sensitivity in detecting resistance to these drugs. The clinical significance of these low-level resistance mutations is unclear.

Agreed: 13; disagreed: 0; abstained: 0.

9. What molecular resistance testing results on fluoroquinolones should influence treatment decisions?

LPAs are relatively specific; however, their unsatisfactory sensitivity affects molecular testing for resistance to FQs. Mutations in *gyr*A affect MFX

and ofloxacin (OFX) susceptibility. D94mut and A90mut have been detected in respectively 40–58% and 20–30% of OFX- or MFX-resistant isolates. Treatment with FQs should be excluded when *gyr*A D94mut is detected. For mutations affecting codon A90, the clinical implications are less clear; for mutations in codon S91, few data are available, but these are most likely similar to mutations of residue A90. Current LPA methods detect mutations in codons 80–81 and 88–95 of *gyr*A. Discordance between LPA and phenotypic DST results may therefore be due to mutations in other *gyr*A gene regions or in *gyr*B. Clinicians should be aware of possible false-negative results of molecular tests, and FQ susceptibility should be confirmed by phenotypic methods.

Agreed: 13; disagreed: 0; abstained: 0.

10. What should be the consequences of the evaluation for the presence of second-line drug resistance by molecular methods in patients with a presumptive or confirmed diagnosis of tuberculosis pending the results of drug susceptibility testing in solid or liquid culture media?

While the results of phenotypic second-line drug resistance testing are pending, physicians should be guided by the principles of investigating molecular DST results for RMP, INH, EMB, FQs and injectable agents (as outlined in this statement) in their initial choice of a second-line anti-tuberculosis drug regimen.

Agreed: 12; disagreed: 1; abstained: 0.

11. Can treatment recommendations be provided based on the molecular drug susceptibility testing results of any other available drugs (delamanid, bedaquiline, prothionamide/ethionamide, cycloserine/terizidone, PAS, meropenem/imipenem, clofazimine, linezolid)?

Current molecular methods do not detect mutations related to resistance of these drugs. However, *inhA* promoter mutations significantly affect ETH/PTH (see answer to 'What molecular resistance testing results on isoniazid should influence treatment decisions?')

Agreed: 13; disagreed: 0; abstained: 0.

12. Should molecular testing for M. tuberculosis *drug resistance be performed by targeted diagnoses (LPAs, Xpert) or by whole genome sequencing)?*

While LPAs and other technologies (e.g., Xpert) are apparently limited in their ability to provide comprehensive information on genomic mutations that confer bacterial drug resistance, WGS provides the complete sequence information of the bacterial genome. However, due to the lack of correlation with in vitro (phenotypic DST) and in vivo (treatment outcome) data at present, it is not possible to interpret the clinical value of the vast majority of mutations or polymorphisms detected. Systematic data collection and correlation of WGS data with in vitro DST and clinical outcomes will be required to assess the added clinical value of this method over existing technologies.

Agreed: 13; disagreed: 0; abstained: 0.

13. If the results of molecular and culture-based drug susceptibility testing differ, what is the gold standard?

The level of discordance between molecular and culture-based DST depends on the drug and the genomic region evaluated. Despite the fact that results of phenotypic methods do not always correspond to response to clinical treatment, culture-based methods are still regarded by most experts involved in this document as the gold standard for DST.

Agreed: 13; disagreed: 0; abstained: 0.

14. How should the results of molecular drug susceptibility testing be reported by the laboratory to the clinicians?

Whenever molecular testing allows, results should always be reported with the specific mutation detected and a description of the clinical implications of the presence of the mutation, as outlined in the Table.

Agreed: 13; disagreed: 0; abstained: 0.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Due to the slow growth rate of *M. tuberculosis*, culture-based DST results are not readily available to treating physicians to guide the initial decision regarding the choice of treatment for MDR-TB patients. Molecular methods are revolutionising the management of drug-resistant TB patients. For several years, the WHO has strongly recommended the use of molecular DST using Xpert for the detection of RMP resistance in individuals presumed to have MDR-TB or HIV-associated TB,[73,101] and recommended the use of commercial LPAs for the rapid diagnosis of MDR-TB.[72] Xpert and LPA results form the basis of clinical decision-making where these technologies have become available.[74,183] This is especially important for the identification of mutations occurring in the *rpo*B gene, resulting in RMP resistance, as RMP is currently the most effective drug for the treatment of TB.[184,185]

Rapid second-line *M. tuberculosis* DST is necessary to tailor anti-tuberculosis treatment regimens for individual patients early after the diagnosis of TB. It was recently identified that 60% of MDR-TB strains of *M. tuberculosis* in the European Region are also resistant to EMB and PZA by phenotypic testing, >30% are resistant to ETH/PTH, >25% are resistant to any WHO Group 2 second-line injectable

drug, and >17% are resistant to any WHO Group 3 FQ.[186] Almost all the XDR-TB strains of *M. tuberculosis* in Europe are also resistant to PZA and EMB.[187]

At present, the WHO does not advocate the use of molecular DST for second-line anti-tuberculosis drugs by either LPAs or WGS. However, there is growing evidence that molecular DST can be a reliable method for the rapid identification of genomic mutations in *M. tuberculosis*, e.g., to detect mutations that lead to drug resistance to WHO Group 2 drugs (second-line injectable drugs) or WHO Group 3 drugs (FQs), providing the potential for individualising anti-tuberculosis treatment at the start of treatment. There is still a caveat not to ignore phenotypic DST, as LPAs still frequently miss drug resistance mutations. False-positive results are very uncommon in molecular DST.[8] Novel technologies such as NGS allow rapid identification of clinical relevant mutations not yet detected by Xpert or LPAs.[142,188]

For the first time, this TBNET/RESIST-TB statement provides a consensus of clinicians, molecular biologists and microbiologists on the interpretation and reporting of the specific genetic results of molecular DST to guide the management of patients with drug-resistant TB. Basing treatment decisions on the results of molecular DST has been common practice for physicians caring for HIV-infected patients for more than a decade,[189,190] and this is now becoming important for physicians caring for patients with TB as well.

In this rapidly evolving field, the present consensus recommendations from this document are only a snapshot in time, and such recommendations will need to be updated on a regular basis. In the future, reporting molecular DST results by laboratories should go beyond 'susceptible' and 'resistant' and list identified mutations to provide guidance for physicians according to the best available evidence. As molecular technologies are further developed, it will be important to match information about molecular DST results, quantitative measures of phenotypic drug resistance and clinical outcome in quality controlled databases.[191] This will be important not only for mutations with known clinical relevance, but also for the great majority of mutations identified by WGS with unknown significance.[192–195]

Synergistic analysis of mutations in the *M. tuberculosis* genome, phenotypic DST results and information on clinical outcome will substantially improve the treatment of patients with drug-resistant TB. If quality-assured data can be collected systematically and the results are reliable and reproducible, the growing evidence on the significance of specific mutations in the *M. tuberculosis* genome may ultimately allow molecular diagnostics to replace culture-based anti-tuberculosis DST.

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APPENDIX

Table A.1 Anti-tuberculosis drugs, their mechanism of action, resistance mechanism and function

Drug	Mechanism of drug action	Resistance mechanism	Function of gene product	Comments
Group 1 drugs				
EMB	Interferes with cell wall synthesis	*emb*A, *emb*B	Arabinosyl transferase	
INH	Interferes with mycolic acid synthesis	*kat*G, *inh*A	Catalase/peroxidase (katG), enoyl reductase (inhA)	*inh*A mutations confer low grade phenotypic resistance
PZA	Unclear	*pcn*A	Pyrazinamidase	
RMP/RBT/RPT	Inhibits RNA polymerase	*rpo*B	RNA polymerase (beta subunit)	Cross-resistance between members of this family
Group 2 drugs				
AMK	Inhibits protein synthesis	*rrs, eis*	16S rRNA (*rrs*), aminoglycosidase acetyltransferase (*eis*)	KM, AMK and CPM partly show cross-resistance depending on the gene and mutation involved
CPM/viomycin	Inhibits protein synthesis	*rrs, tly*A	16S rRNA (*rrs*), rRNA methyltransferase (*tly*A)	
KM	Inhibits protein synthesis	*rrs, eis*	16 rRNA (*rrs*), aminoglycosidase acetyltransferase (*eis*)	*eis* mutations confer low grade resistance towards KM
SM	Inhibits protein synthesis	*rps*L, *rrs, gid*B	S12 ribosomal protein (*rps*L), 16S rRNA (*rrs*), guanosine methyltransferase (*gid*B)	*gid*B mutations confer low grade resistance towards SM
Group 3 drugs				
LFX/OFX/MFX	Interferes with mycobacterial topoisomerase	*gyr*A, *gyr*B	DNA gyrase	Other mechanisms are thought to exist but have not been identified
Group 4 drugs				
CS/terizidone	Inhibits peptidoglycan synthesis (presumably interferes with synthesis of D-ala-D-ala)	Unknown	Unknown	Unknown
ETH/PTH	Inhibits mycolic acid synthesis	*inh*A *eth*A	Enoyl reductase	Cross-resistance with INH (*inh*A)
PAS	Interferes with folate metabolism	*thy*A *ribD* *fol*C	Thymidylate synthase A Dihydrofolat reductase Dihydrofolat synthase	Other mechanisms of resistance may exist
Group 5 drugs				
Amoxicillin plus clavulanate	Interfers with peptidoglycan synthesis	Unknown	Unknown	
CLM	Inhibits protein synthesis	*erm*	23S rRNA methylase	*M. tuberculosis* has inducible *erm* methylase
CFZ	Unknown	Rv 0678	Transcriptional repressor of MmpS5-MmpL5 efflux pump	Mutations confer cross-resistance to bedaquiline
Linezolid	Inhibits protein synthesis	*rpl*C, *rrl*	Ribosomal L3 protein, 23S rRNA	
Meropenem plus clavulanate	Interferes with peptidoglycan synthesis	Unknown	Unknown	
Thioacetazone	Unknown	Unknown	Unknown	
New drugs				
Apramycin	Inhibits protein synthesis	*rrs*	16S rRNA (*rrs*)	
Bedaquiline	Inhibition of ATP synthase	*atp*E, Rv0678	ATP synthase, transcriptional repressor (Rv0678) of MmpS5-MmpL5 efflux pump	Mutations of Rv0678 mediate cross-resistance to CFZ
Delamanid/PA-824	Inhibits cell wall synthesis	*ddn*	Deazaflavin-dependent nitroreductase	
		fdG1	Glucose-6-phosphate dehydrogenase	
		*fbi*A, *fbi*B, *fbi*C	Synthesis of deazaflavin cofactor F420	
Spectinamides	Inhibits protein synthesis	*rrs*	16S rRNA (*rrs*)	
Sutezolid/AZD-5847	Inhibits protein synthesis	*rrl*	23S rRNA	

EMB = ethambutol; INH = isoniazid; PZA = pyrazinamide; RMP = rifampicin; RBT = rifabutin; RPT = rifapentine; AMK = amikacin; KM = kanamycin; CPM = capreomycin; SM = streptomycin; LFX = levofloxacin; OFX = ofloxacin; MFX = moxifloxacin; ETH = ethionamide; PTH = prothionamide; PAS = para-aminosalicylic acid; CLM = clarithromycin; CFZ = clofazimine; ATP = adenosine triphosphate.

Table A.2 Updated WHO critical concentrations for elected first- and second-line agents for the treatment of tuberculosis[57,58]

Drug	Löwenstein-Jensen µg/ml	Middlebrook 7H10 µg/ml	Middlebrook 7H11 µg/ml	MGIT 960 µg/ml
Rifampicin	40.0	1.0	1.0	1.0
Isoniazid	0.2	0.2	0.2	0.1
Pyrazinamide	—	—	—	100.0
Ethambutol	2.0	5.0	7.5	5.0
Streptomycin	4.0	2.0	2.0	1.0
Kanamycin	30	5.0	6.0	2.5*
Amikacin	30*	4.0*	—	1.0
Capreomycin	40	4.0*	—	2.5
Ofloxacin	4.0*	2.0	2.0	2.0
Moxifloxacin	—	0.5*	—	0.5*†
		2.0*		2.0*

* Suggested updates from reference 58; not yet formally published by the WHO.
† Proxy for ofloxacin in case ofloxacin is not tested.
WHO = World Health Organization; MGIT = Mycobacteria Growth Indicator Tube.

Table A.3 Hot spot of *rpo*B gene: result of commercial LPA tests in the presence of mutations in specific codons, codons known to host silent mutations, mutations associated with susceptible RMP result in the phenotypic MGIT DST*

Codon	INNO–LiPA®	GenoType®	AID TB Resistance	Silent mutation	MGIT-S†
505	—	W1–	—		
506	—	W1–	—		
507	—	W1–	—		
508	W1–	W1–	—	T508	
509	W1–	W1–	—		
510	W1–	W2–	—	Q510	
511	W1–	W2–	—	L511	Gln/Pro
512	W2–	W2–	—		Arg
513	W2–	W2– W3–	W1–	Q513	
514	W2–	W3–	W1–	F514	
515	W2–	W3–	W1–		
516 Val	W2– M2+	W3– W4– M1+	W1– M1+		Val
516 Tyr	—	—	W1– M1+		
516 other	W2–	W3– W4–	W1– M1+		Phe
517	W2–	W4–	W1–		
518	W3–	W4– W5–	—		
519	W3–	W4– W4–	—		
520	W3–	W4–	—		
521	W3–	W4–	—		
522	W3–	W4– W6–	—		Gln
523	W4–	W6–	W2–		
524	W4–	W6–	W2–	T524	
525	W4–	W6–	W2–		
526 Tyr	W4– M4a+	W7– M2a+	W2–		
526 Asp	W4– M4b+	W7– M2b+	W2– M2+		
526 Arg	—	—	W2– M2+		
526 other	W4–	W7–	W2– M2+		Asn/Cys/ Leu/Ser
527	W4–	W7–	W2– M2+		
528	W5–	W7–	W2–		
529	W5–	W7–	—		
530	W5–	W8–	—		
531 Leu	W5– M5+	W8– M3	W3–		
531 Trp	—	—	W3– M3+		
531 other	W5–	W8–	W3–		Tyr
532	W5–	W8–	W3– M3+	A532	
533	W5–	W8–	W3– M3+	L533	Arg/Pro‡
534	—	W8–	W3–		

* W = wild type probe; M = mutated probe; S = codon in which silent mutations have been reported. Some of the codons that could be clinically relevant, such as V146F and I572F, are not included in LPAs.
† Mutations reported associated to susceptible RMP result in the phenotypic MGIT DST.
‡ 533P can be missed by LPA.
LPA = line-probe assay; MGIT = Mycobacteria Growth Indicator Tube; DST = drug susceptibility testing; RMP = rifampicin.

Table A.4 LR+ and LR– for resistance to EMB, FQs and injectables (GenoType® MTBDRs*l* assay)*

Drug	Sensitivity % (95%CI)	Specificity % (95%CI)	LR+ % (95%CI)	LR– % (95%CI)
EMB	67.9 (65.2–70.6)	79.9 (77.3–82.3)	4.879 (2.250–10.581)	0.498 (0.383–0.648)
FQs	87.4 (84.5–89.9)	97.1 (96.1–98.0)	26.368 (12.851–54.102)	0.182 (0.109–0.303)
Amikacin	82.6 (77.7–86.9)	99.5 (98.7–99.8)	68.851 (7.845–604.234)	0.192 (0.150–0.245)
Kanamycin	44.4 (39.6–49.2)	99.3 (98.5–99.7)	48.693 (7.289–325.260)	0.561 (0.430–0.732)
Capreomycin	82.0 (77.2–86.2)	97.3 (96.3–98.1)	18.211 (9.964–33.285)	0.151 (0.037–0.609)

* Reproduced from reference 108.
LR = likelihood ratio; + = positive; – = negative; EMB = ethambutol; FQ = fluoroquinolone; CI = confidence interval.

RESUME

L'émergence de souches de *Mycobacterium tuberculosis* pharmacorésistantes défie la lutte contre la tuberculose (TB) dans le monde. Bien que les méthodes basées sur la culture aient été considérées comme l'étalon or des tests de pharmacosensibilité (DST), les méthodes moléculaires fournissent des informations rapides sur les mutations du génome de *M. tuberculosis* associé à la résistance aux médicaments antituberculeux. Nous avons obtenu un consensus sur l'utilisation des résultats des DST moléculaires pour les décisions relatives au traitement clinique des patients tuberculeux. Ce document a été élaboré par TBNET et RESIST-TB afin d'atteindre un consensus sur les standards de rapports de l'utilisation clinique des résultats des DST moléculaires. La revue de la littérature disponible et le recherche de preuves a inclus la recherche manuelle de revues médicales et la recherche dans les bases de données électroniques. Le panel a identifié des mutations isolées d'un seul nucléotide dans les régions génomiques de *M. tuberculosis* codant pour *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L et *gyr*A, qui sont probablement liées à la pharmacorésistance in vivo. L'identification de l'une quelconque de ces mutations dans des isolats cliniques de *M. tuberculosis* a des implications en termes de prise en charge des patients tuberculeux, dans l'attente des résultats des DST in vitro. Cependant, l'interprétation est compliquée par des résultats faussement positifs et négatifs dans la détection des mutations associées à la résistance aux médicaments. En effet, il y a une corrélation médiocre ou non démontrée entre la pharmacorésistance phénotypique et clinique. En conséquence, les rapports relatifs aux résultats des DST moléculaires devraient inclure des informations spécifiques sur les mutations identifiées et fournir une guidance aux cliniciens dans l'interprétation et le choix du protocole thérapeutique initial approprié.

RESUMEN

La aparición de cepas de *Mycobacterium tuberculosis* resistentes a los fármacos anti-tuberculosos representa un reto para el control global de la tuberculosis (TB). Aunque los métodos basados en los cultivos han sido considerados como el método de referencia para el estudio de la susceptibilidad a los fármacos (DST), los métodos moleculares proveen de una información rápida de la presencia de las mutaciones asociadas a resistencia a estos fármacos. TBNET y RESIST-TB han elaborado este documento de consenso para la interpretación de los resultados moleculares de detección de resistencias en la toma de decisiones terapéuticas en los pacientes con TB. La revisión de la bibliografía disponible y la búsqueda de evidencia se ha realizado mediante búsqueda manual en las publicaciones científicas y búsqueda electrónica en las bases de datos. El grupo de trabajo ha identificado mutaciones puntuales en regiones genómicas de *M. tuberculosis* en *kat*G, *inh*A, *rpo*B, *emb*B, *rrs*, *rps*L y *gyr*A, que están relacionadas con resistencia in vivo a los fármacos antituberculosos. Mientras se dispone de los resultados fenotípicos, la detección de estas mutaciones en los aislados clínicos de *M. tuberculosis* tiene implicaciones en el manejo de los pacientes con TB. Sin embargo, la existencia de resultados falsos positivos y negativos al detectar mutaciones con muy poca o sin una demostrada correlación entre resistencia clínica y fenotípica, complica la interpretación. Como consecuencia de ello, los resultados de las técnicas de detección molecular de resistencias deben incluir información específica de las mutaciones identificadas y proveer pautas para los clínicos en la interpretación y en la elección del régimen antibiótico inicial apropiado.

Article VII

Molina-Moya B, Gomgnimbou MK, Lafoz C, Lacoma A, Prat C, Refrégier G, Samper S, Domínguez J, Sola C

Molecular characterization of *Mycobacterium tuberculosis* strains with TB-SPRINT

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Molecular Characterization of *Mycobacterium tuberculosis* Strains by TB-SPRINT

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The objective was to evaluate TB-SPRINT, a microbead-based method for simultaneous spoligotyping and detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis*. For that, 67 *M. tuberculosis* complex strains with different lineages and IS*6110*-restriction fragment length polymorphism (RFLP) patterns isolated in Spain were retrospectively selected. Five strains were phenotypically resistant to both rifampicin and isoniazid and two strains were isoniazid monoresistant. Membrane-based spoligotyping, RFLP, DNA sequencing/pyrosequencing of *rpoB*, *katG*, and *inhA* promoter, TB-SPRINT, and 3R SNP typing were performed. Among the 65 strains with an interpretable TB-SPRINT result, concordance between spoligotyping methods was 99.6% (2785/2795 spoligotype data points). For most of the discordant cases, either the same lineage was assigned by both methods, or 3R SNP typing was in agreement with TB-SPRINT. Concordance between phenotypic drug susceptibility testing and TB-SPRINT for detecting rifampicin and isoniazid resistance was 98.4% (63/64) and 93.8% (60/64), respectively. Concordance between DNA sequencing/pyrosequencing and TB-SPRINT for detecting mutations in *rpoB*, *katG*, and *inhA* were 98.4% (60/61), 100% (64/64), and 96.9% (62/64), respectively. In conclusion, TB-SPRINT is a rapid, easy to perform, and affordable assay for genotyping and detecting drug resistance in a single tube, therefore it may be a useful tool to improve epidemiological surveillance.

Keywords: Tuberculosis; molecular epidemiology; spoligotyping; multidrug resistance; IS*6110*-RFLP; DNA sequencing.

INTRODUCTION

During the last decades, emergence and spread of drug resistant strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), have posed challenges to the control of this disease (1). The worldwide dissemination of *M. tuberculosis* strains has led to the study of the genetic diversity. It has been proposed that different *M. tuberculosis* lineages present differences in their virulence and transmissibility, in the capacity of acquiring drug resistance conferring mutations, and possibly in the outcome of the disease (2, 3). From a local perspective, the study of the genetic diversity is a valuable tool for contact tracing and outbreak management that can help interrupting transmission chains and preventing the spread of TB (2).

The first and most widely used method for *M. tuberculosis* genotyping was IS*6110* restriction fragment length polymorphism (RFLP), which presents a high discriminatory power for differentiating strains (4). However, this method requires large amounts of DNA, and is slow and laborious. Nowadays, this method has almost been replaced by the more rapid methods mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR), and spoligotyping (*spacer oligonucleotide* typing) (5). Spoligotyping is based on a PCR amplification of the CRISPR (clustered regularly interspaced short palindromic repeats) locus in the *M. tuberculosis* genome and subsequent detection of the presence of the different spacers between the repeats by reverse hybridization on a nylon membrane. In 2010, Zhang et al adapted spoligotyping to hybridization on microbeads, increasing the throughput capacity of the method (6).

In addition to preventing the dissemination of resistant *M. tuberculosis* strains, the rapid detection of drug resistance is essential to tailor an adequate treatment (1). Strains resistant to rifampicin (RIF) and isoniazid (INH), have been defined as multidrug resistant (MDR). After *M. tuberculosis* isolation, phenotypic drug susceptibility testing (DST) should be performed. However, this may take weeks and there is a risk of contamination. Drug resistance in *M. tuberculosis* arises through the stepwise accumulation of chromosomal mutations (7). The most common mutations are located in codons referred to as 531, 526, and 516 of *rpoB* regarding RIF resistance, and codon 315 of *katG* and position -15 of *inhA* regarding INH resistance (7, 8). Some of the most used rapid methods for detecting molecular drug resistance are the WHO-endorsed GeneXpert MTB/RIF (Cepheid, Sunnyvale, California, USA) (9), line probe assays (10, 11), multiplex PCR (12), DNA sequencing (13), and pyrosequencing (14).

Recently, the previously mentioned microbead-based spoligotyping implemented by Zhang and colleagues was upgraded to Tuberculosis-Spoligo-Rifampicin-Isoniazid Typing (TB-SPRINT) in order to additionally detect mutations associated with RIF and INH resistance (15). The objective of the present study was to evaluate the TB-SPRINT assay on DNA extracted from cultured isolates For that purpose we compared TB-SPRINT results with conventional membrane-based spoligotyping, IS*6110*-RFLP, 3R SNP typing, phenotypic DST, and DNA sequencing/pyrosequencing of *rpoB*, *katG*, and *inhA*.

MATERIALS AND METHODS

Clinical strains

A total of 67 *M. tuberculosis* complex strains isolated in Spain were retrospectively selected from the collection in Instituto de Investigación Sanitaria de Aragón, Zaragoza, Spain. The strains were selected by including different lineages with different IS*6110*-RFLP patterns. Five of the 67 strains were MDR and two strains were INH monoresistant.

Phenotypic drug susceptibility testing

Drug susceptibility testing was performed either with VersaTREK Myco Susceptibility Kit (Trek Diagnostics, Cleveland, OH, USA), or Bactec MGIT960 (Becton Dickinson, Sparks, USA). Critical concentrations used were 1 µg/ml for RIF, and 0.4 µg/ml and 0.1 µg/ml for INH (16).

Spoligotyping and IS*6110*-RFLP

Genomic DNA was extracted from strains cultured on solid medium following the cetyltrimethylammonium bromide protocol. Spoligotyping on membrane and IS*6110*-RFLP were performed as described previously (4, 17). The individual spoligotyping patterns were compared with those in the International Spoligotyping Database (SITVITWEB) of the Pasteur Institute of Guadeloupe, (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Spoligotyping International Types (SIT) were assigned according to spoligotype patterns signatures provided in SITVITWEB.

DNA sequencing and pyrosequencing

DNA sequencing for detecting mutations in *rpoB* was performed as previously described (18). Only mutations within the RIF resistance-determining region (RRDR) were reported. Pyrosequencing for detecting mutations in *katG* codon 315 and *inhA* positions -5, -8, -15, and -16 was performed as previously described (14). Briefly, this method is based on PCR amplification followed by the pyrosequencing reaction. Reaction and data analysis were performed as recommended by the PSQ96MA and SQA software manufacturer (Biotage AB, Uppsala, Sweden, and Qiagen GmbH, Hilden, Germany).

TB-SPRINT

Strains were blindly analyzed by the TB-SPRINT assay (Beamedex SAS, Orsay, France; www.beamedex.com) (15). This is a 59-plex assay: 43 probes target the oligonucleotide spacers for spoligotyping, 10 probes target RIF resistance [two *rpoB* spanning probes covering the 81-bp RRDR; *rpoB* 516 GAC (wild-type) and GTC; *rpoB* 526 CAC (wild-type), GAC, and TAC; and *rpoB* 531 TCG (wild-type), TTG and TGG], and 6 probes target INH resistance [*katG* 315 AGC (wild-type), ACC, and ACA; *inhA* -15C (wild-type), -15T, and -8A]. Briefly, CRISPR region, *rpoB*, *katG*, and the promoter region of *inhA* were simultaneously amplified by PCR using dual-priming oligonucleotide (DPO) primers. Subsequently, PCR product was hybridized to oligonucleotide-precoupled microbeads, and detection was performed either with the flow cytometry-based Luminex 200 system (Luminex Corp, Austin, TX, USA) and XPONENT software for LX100/LX200 (version 3.1.871.0), or BioPlex200 (Biorad, Hercules, CA, USA) running under Bio-Plex Manager 5.0.

Results obtained consist on the value of relative fluorescence intensity (RFI) for each probe. Spoligotyping raw values were interpreted as previously described (19). Regarding the SNPs associated with drug resistance, the wild-type/mutated result was inferred by paired comparison of the RFI values obtained for wild-type and mutant probes and by comparison of those values with their corresponding cut-offs, as previously described (15). Numerical data were combined in a Microsoft Excel spreadsheet file and uploaded to BioNumerics version 6.1 software (Applied Maths, Kortrijk, Belgium). As for patterns obtained with membrane-based spoligotyping, patterns obtained with TB-SPRINT were compared with those in SITVITWEB, and SIT labels and clades were assigned.

3R SNP typing

Strains were also subjected to typing of seven lineage-specific SNPs: *alkA* 807, specific of Bovis; *ligB* 1212, of Latin-America and Mediterranean (LAM); *ligC* 809, of TUR-T3-Osaka; *mgtC* 545, of Haarlem (H); *recC* 1491, of X; *recO* 606, of East African Indian (EAI); and *recR* 94, T2-related. SNP typing was performed by multiplex PCR, microbead-based hybridization and detection with Luminex 200 system (20).

RESULTS

TB-SPRINT and membrane-based spoligotyping

A total of 67 *M. tuberculosis* complex strains were included in this study. Fig 1 shows genotyping results obtained for the 67 strains.

Among all the strains tested, TB-SPRINT result could not be obtained for one strain (strain 76) likely

because of DNA degradation. For an additional strain (strain 49), TB-SPRINT spoligotyping result suggested the presence of two different populations since RFI values for different spacers were far above the statistical cutoff but not as high as for other strains. This strain (#49) was analyzed by multiplex PCR of MIRU24 and MIRU31, and agarose gel electrophoresis revealed four bands at around 450bp, 500bp, 550bp, and 650bp, respectively. The presence of more than the two copies expected, one for each MIRU loci, confirmed that strain 49 was composed of two populations.

According to TB-SPRINT results, distribution of families among the 65 remaining strains was as follows: H, n=18 (27.7%); LAM, n=13 (20.0%); T super-family (T), n=13 (20.0%); AFRI, n=2 (3.1%); EAI, n=2 (3.1%); X, n=2 (3.1%); Beijing, n=1 (1.5%); Bovis (BOV-1), n=1 (1.5%); Central Asian (CAS), n=1 (1.5%); S, n=1 (1.5%); for 9 strains (13.8%) a SIT number could be assigned but the family to which they belonged was unknown; finally, the remaining 2 strains (3.1%) did not match any pattern of the SITVITWEB (Fig 1). Considering the 65 strains, the concordance between membrane-based spoligotyping and TB-SPRINT was 99.6% (2785/2795 spoligotype data points). For 8 of the 65 strains, discordant results between membrane-based spoligotyping and TB-SPRINT were obtained for some spacers (Table 1): for five strains the same lineage was assigned by both methods, and for two strains different lineages were assigned. For one of the latter two strains, 3R SNP typing assignation comforted microbead-based method (Table 1).

TB-SPRINT and IS*6110*-RFLP

All strains found in the six clusters identified by TB-SPRINT, were discriminated by IS*6110* RFLP (Fig 1). On the other hand, the single IS*6110*-RFLP cluster, which grouped two strains carrying a single IS*6110* copy (strains 49 and 68), was discriminated by membrane-based spoligotyping (Fig 1). In addition, although strain 49 was excluded from the TB-SPRINT analysis, the different microbead-based spoligotyping results allowed discriminating these strains.

Table 1. Membrane-based spoligotyping, TB-SPRINT, and 3R SNP typing results of the strains with discordant results between spoligotyping methods.

Strain	Spacer	Membrane-based spoligotyping			TB-SPRINT			3R SNP typing
		Spacer	SIT	Lineage	Spacer	SIT	Lineage	
2	37	Present	93	LAM5	Possibly absent[a]	Orphan	LAM5	LAM
6	23	Absent	740	H3	Present[b]	631	H3	Haarlem
19	9, 10	Absent	383	H1	Present[b]	47	H1	Haarlem
29	10	Absent	1243	H3	Present[b]	50	H3	Haarlem
36	31	Present	53	T1	Absent[c]	50	H3	Haarlem
50	16	Absent	1105	T1	Present[b]	53	T1	NR[d]
63	22, 37	Present	794	CAS1-Delhi	Possibly absent[a]	Orphan	Orphan	NR[e]
77	16	Absent	Orphan	Orphan	Present[b]	106	Orphan	LAM

TB-SPRINT: Tuberculosis-Spoligo-Rifampicin-Isoniazid Typing; 3R SNP typing: typing assay based on detection of lineage-specific single nucleotide polymorphisms, most of them located in genes involved in replication, repair and recombination (3R) functions of *M. tuberculosis*. SIT: Spoligo-International Type. NR: no result.

[a] Relative fluorescence intensity (RFI) values for the discordant spacers were near the cutoff.

[b] RFI values for the discordant spacers were significantly high.

[c] RFI values for the discordant spacers were significantly low.

[d] No T1-specific SNP was included in this analysis.

[e] No CAS-specific SNP was included in this analysis.

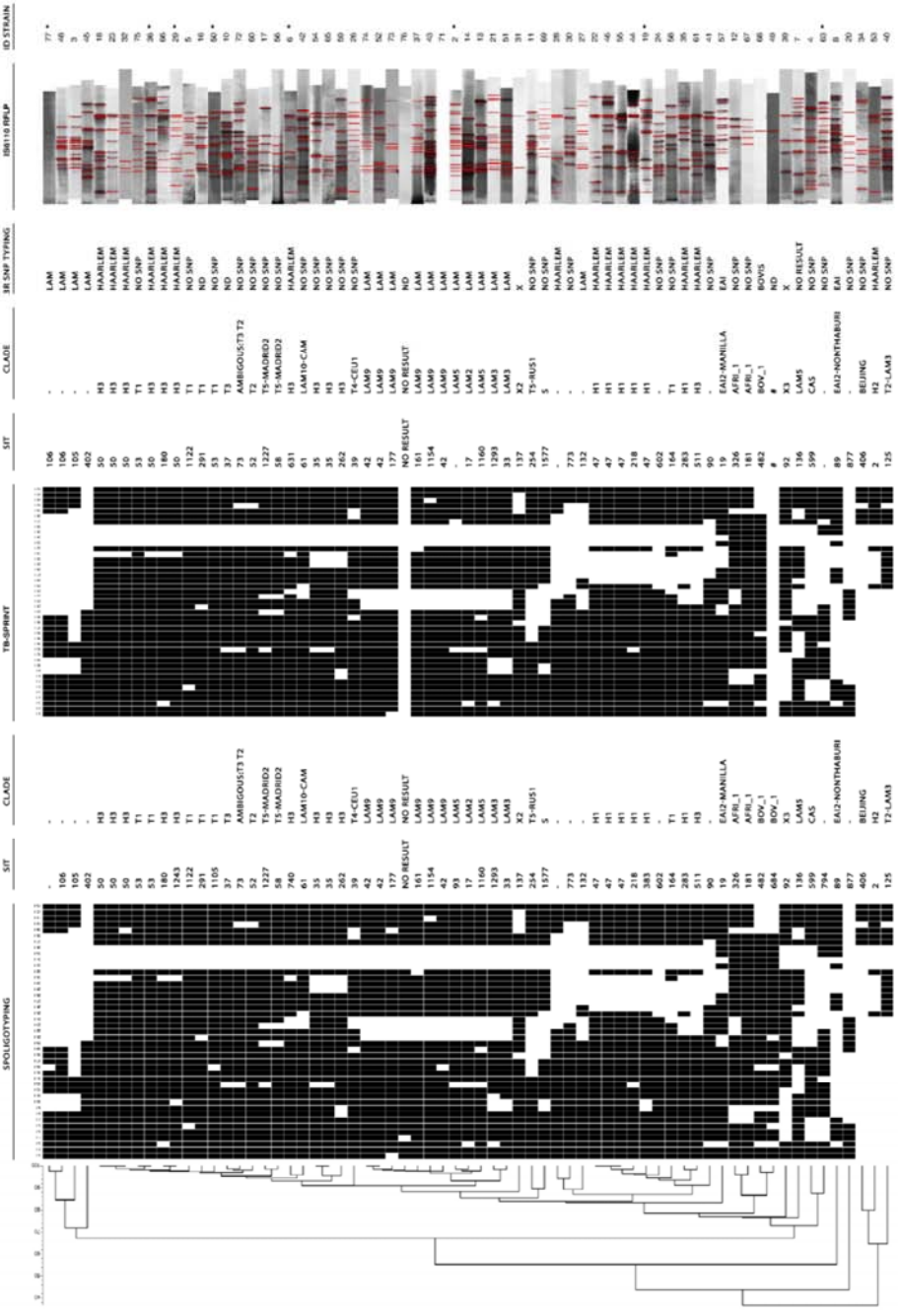

Figure 1. Genotyping results obtained for the 67 *M. tuberculosis* complex strains included in this study. From left to right are shown the dendrogram, spoligotypes, SIT, and clade obtained by membrane-based spoligotyping; spoligotypes, SIT, and clade obtained by TB-SPRINT; clade identified by 3R SNP typing, IS*6110*-RFLP pattern, and strain ID. SIT and clades were identified according to SITVITWEB Strains with any discordance between membrane-based spoligotyping and TB-SPRINT are marked with an asterisk. TB-SPRINT spoligotyping result for strain 49 (marked with #) suggested the presence of two different populations and it is not shown. TB-SPRINT: Tuberculosis-Spoligo-Rifampicin-Isoniazid Typing. 3R SNP typing: typing of lineage-specific single nucleotide polymorphisms in genes involved in replication, repair and recombination functions of *M. tuberculosis*; RFLP: restriction fragment length polymorphism. ND: not done. Dendrogram built with BioNumerics version 6.1.

3R SNP typing

Of the total 67 strains included in the study, 3R SNP typing was performed for 63 strains, for which appropriate DNA concentration and absence of mixed contamination had been confirmed. Of the 63 strains, 37 strains were classified by 3R SNP typing, and for the remaining 26 strains no lineage-specific SNPs were detected (Fig 1).

For one strain classified as LAM by TB-SPRINT (strain 7), the LAM-specific SNP in *ligB* 1212 was clearly absent. Interestingly, no clear result was obtained for *alkA* (specific of Bovis) and *recC* (specific of X). Regarding the 11 strains for which no clade was identified according to TB-SPRINT and SITVITWEB, 3R SNP typing classified five strains as LAM and one strain as Haarlem, while for the remaining five strains no specific SNP was detected and they were not classified (Fig 1).

Detection of drug resistance

Regarding phenotypic DST, among the 67 strains, 61 (91.0%) strains were sensitive to RIF, 5 (7.5%) strains were resistant (Table 2), and for one (1.5%) strain RIF DST result was not available; 59 (88.1%) strains were sensitive to INH, 7 (10.4%) strains were resistant (Table 2), and for one (1.5%) strain INH DST result was not available. As for DNA sequencing/pyrosequencing, 57 (85.0%) strains presented a wild-type *rpoB* sequence, 5 (7.5%) strains harbored a mutation (Table 2), and for 5 (7.5%) strains *rpoB* results were not available; 64 (95.5%) strains presented a wild-type *katG* sequence, 2 (3.0%) strains harbored a mutation (Table 2), and for one (1.5%) strain *katG* results were not available; finally, 60 (89.5%) strains presented a wild-type *inhA* sequence, 6 (9.0%) strains harbored a mutation (Table 2), and for one (1.5%) strain *inhA* results were not available. TB-SPRINT results were excluded for two strains (strains 49 and 74) as previously mentioned. Table 3 shows the sensitivity, specificity, and concordance of TB-SPRINT using phenotypic DST or DNA sequencing/pyrosequencing as reference methods.

Table 2. Phenotypic and molecular drug susceptibility result for rifampicin and isoniazid for the strains with any resistance or mutation detected by phenotypic drug susceptibility testing, DNA sequencing / pyrosequencing, or TB-SPRINT.

Strain	Phenotypic DST		DNA sequencing / pyrosequencing			TB-SPRINT		
	RIF	INH	*rpoB*	*katG*	*inhA*	*rpoB*	*katG*	*inhA*
5	S	S	wt	wt	C-15T	wt	wt	C-15T
7	S	S	wt	wt	wt	No 531 wt[a]	wt	wt
16	S	S	NR	wt	wt	wt	wt	No -15 wt[b]
21	S	R	wt	wt	C-15T	wt	wt	C-15T
39	S	R	wt	wt	wt	wt	wt	wt
73	R	R	S531L	wt	C-15T	S531L	wt	C-15T
74	R	R	H526D	wt	wt	H526D	wt	wt
75	R	R	H526Y	S315T	C-15T	No 516 wt[a]	S315T	wt
76	R	R	S531L	S315T	C-15T	NR	NR	NR
77	R	R	S531L	wt	C-15T	S531L	wt	C-15T

DST: drug susceptibility testing; RIF: rifampicin; INH: isoniazid: TB-SPRINT: Tuberculosis-Spoligo-Rifampicin-Isoniazid Typing; R: resistant; S: susceptible; wt: wild-type; NR: no result.

[a] The result of the specified probe was considered negative since the RFI value was low, and the strain was regarded as RIF resistant by TB-SPRINT.

[b] The result of the specified probe was considered negative since the RFI value was low, and the strain was regarded as INH resistant by TB-SPRINT.

Table 3. Sensitivity, specificity, and concordance of TB-SPRINT for detecting drug resistance using phenotypic drug susceptibility testing or DNA sequencing/pyrosequencing as reference methods.

	Sensitivity (no. detected/ total no. [%], [95% CI])	Specificity (no. detected/ total no. [%] [95% CI])	Concordance (no. detected/total no. [%])
RIF[a]	4/4 (100) (39.6-100)	59/60 (98.3) (89.9-99.9)	63/64 (98.4)
INH[a]	4/6 (66.7) (24.1-94.0)	56/58 (96.6) (87.0-99.4)	60/64 (93.8)
rpoB[b]	4/4 (100) (39.6-100)	56/57 (98.2) (89.4-99.9)	60/61 (98.4)
katG[b]	1/1 (100) (0.05-100)	63/63 (100) (92.8-100)	64/64 (100)
inhA[b]	4/5 (80.0) (29.9-98.9)	98.3 (58/59) (89.7-99.9)	62/64 (96.9)

[a] Phenotypic drug susceptibility testing was used as reference method for sensitivity, specificity, and concordance calculations.
[b] DNA sequencing/pyrosequencing were used as reference methods for sensitivity, specificity, and concordance calculations.
CI: confidence interval.

DISCUSSION

Current technologies to diagnose, treat, and prevent dissemination of drug resistant TB have limitations, and there is an increased need for more rapid, simple, sensitive, and affordable methods. In this study we present additional evidence of the utility of TB-SPRINT, a microbead-based assay for simultaneous spoligotyping and detection of mutations in *rpoB*, *katG*, and *inhA*, associated with resistance to RIF and INH. A reference method for genotyping *M. tuberculosis* strains has been IS*6110*-RFLP typing, which presents a high discriminatory power (4). In the present study, strains clustered by spoligotyping presented different IS*6110*-RFLP patterns; on the contrary, strains clustered by IS*6110*-RFLP were differentiated by both membrane-based spoligotyping and TB-SPRINT. It is of note that the only IS*6110*-RFLP cluster comprised two strains with one IS*6110* copy, and that these strains were identified as *Mycobacterium bovis* by both membrane-based spoligotyping and TB-SPRINT. The higher discriminatory power of spoligotyping for strains with less than five IS*6110* copies has been previously reported (21). In addition, IS*6110*-RFLP requires 2-3 µg of good quality DNA, and it is a laborious and time-consuming method.

Another important aspect in the management of TB patients is to detect the presence of mixed infections. In this study, TB-SPRINT suggested the presence of two different *M. tuberculosis* strains in one sample, which could be suspected since the microbead hybridization method yields semi-quantitative measures. This result was confirmed by a rapid 2-MIRU-VNTR analysis. MIRU-VNTR typing has shown utility for detecting mixed infections and has become another widespread used rapid molecular method for epidemiological purposes (22). If run on real-time, TB-SPRINT could provide spoligotyping fingerprints for a highly reliable first broad picture. After, since the discriminatory power of spoligotyping is moderate, partial MIRU-VNTR genotyping could be sufficient to achieve adequate discrimination of *M. tuberculosis* strains and define epidemiologically linked clusters (23). In addition to spoligotyping and MIRU-VNTR, 3R SNP typing may also be useful to classify strains with undefined spoligotype signatures in research studies interested in phylogenetic relationships, as shown in the present study and in others (20, 23). Furthermore, whole genome sequencing (WGS) may be another convenient method (24). However, since WGS requires significant resources, TB-SPRINT used as first-line and high-throughput method could constitute a screening procedure to minimize the time and cost for TB transmission studies.

Either TB-SPRINT or microbead-based spoligotyping has been successfully used in previous works (6, 23, 25, 26). The present study represents another report of a high agreement between the membrane-based and the microbead-based spoligotyping methods (6). TB-SPRINT has recently been used in the field in a collaborative study with Nigeria and Ethiopia, using DNA extracted from Ziehl-Neelsen-stained microscopy slides and from sputum specimens. In these low-

quality samples, the rate of interpretable results for spoligotyping was 60% and 90%, respectively (27, 28). In addition, TB-SPRINT results obtained from sputum specimens showed good agreement with both GeneXpert MTB/RIF and GenoType MTBDR*plus* (28).

Regarding the detection of drug resistance, and although the number of resistant strains in the present study was low, TB-SPRINT showed a good concordance with both phenotypic DST and DNA sequencing/pyrosequencing results. Gomgnimbou and colleagues previously obtained a perfect concordance with DNA sequencing for detecting resistance to both RIF and INH, and with phenotypic DST for RIF (15). Concordance with phenotypic DST for INH was lower (90.4%) since some isolates lacked mutations in the targeted regions. In fact, *katG*315 and *inhA*-15 mutations only explain 83% of global phenotypic INH resistance, and the prevalence of these mutations is highly dependent on the geographic region (8).

For the time being, the most promising method for both molecular epidemiological studies and detection of drug resistance is WGS (24, 29). Nevertheless, given the complexity of the analysis and the still high cost, one of the prior goals of this method should be to discover new mutations in drug resistant isolates lacking the known ones. Once these mutations have been identified, lower-cost, flexible, multiplex, and high-throughput assays could target all the known mutations to increase the sensitivity of molecular resistance detection. Furthermore, with the simultaneous spoligotyping analysis by TB-SPRINT providing first-line screening of potential epidemiological links, this approach could be more cost-effective and attractive for low-resource countries with a high burden of MDR-TB (6, 15). Although genotypic testing cannot replace phenotypic DST for detecting MDR-TB due to the limited sensitivity of molecular tests, particularly for INH, molecular testing may be valuable as a complementary tool, especially when drug resistance is detected, to rule out the considered drug for treatment (30).

In conclusion, TB-SPRINT is a rapid and high-throughput assay for simultaneous genotyping and molecular detection of resistance to RIF and INH in a single tube. Implementation of this method would be useful to improve epidemiological surveillance, and to obtain a preliminary drug susceptibility profile before phenotypic results are available, especially in high-burden TB settings, thus improving the management of TB patients and preventing further spread of drug resistant *M. tuberculosis* strains.

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Article VIII

Molina-Moya B, Lacoma A, Garcia-Sierra N, Blanco S, Haba L, Maldonado J, Samper S, Ruiz-Manzano J, Prat C, Arnold C, Domínguez J

PyroTyping, a novel pyrosequencing-based assay for *Mycobacterium tuberculosis* genotyping

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PyroTyping, a novel pyrosequencing-based assay for *Mycobacterium tuberculosis* genotyping

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We developed a novel method, PyroTyping, for discrimination of *Mycobacterium tuberculosis* isolates combining pyrosequencing and IS*6110* polymorphism. We analized 94 isolates by IS*6110*-restriction fragment length polymorphism (RFLP), spoligotyping, and PyroTyping. PyroTyping results regarding clustering or discrimination of the isolates were completely concordant with RFLP and spoligotyping. PyroTyping is more rapid than RFLP and presents the same discriminatory power, thus, it may be useful for taking timely decisions for tuberculosis control.

Keywords: Molecular epidemiology; pyrosequencing; PyroTyping; IS*6110*; restriction fragment length polymorphism; spoligotyping.

Tuberculosis (TB) is one of the leading causes of death among curable infectious diseases, and worldwide spread of *Mycobacterium tuberculosis* isolates, the causative agent, poses a threat to the global control of TB (1). In order to improve TB control, it is important to track the spread of *M. tuberculosis* isolates, identify index cases, and detect outbreaks. For these purposes, several genotyping methods have been developed (2). Some of these methods are based on the polymorphism of copy number and location of the insertion sequence (IS) *6110* (3). The most used IS*6110*-based genotyping method is restriction fragment length polymorphism (RFLP) (4). However, RFLP is laborious and time-consuming, thus, the time until results are available may be too long for decision-making. Another extensively used genotyping method is spoligotyping (5), based on the polymorphism of the clustered regularly interspersed short palindromic repeats (CRISPR) locus in the *M. tuberculosis* genome. Although spoligotyping is more rapid and simple than RFLP, it occasionally presents a lower discriminatory power (3), hence, new rapid, simple, and discriminatory methods for molecular epidemiology studies could be useful. The aim of the present study was to develop a novel molecular method based on PCR amplification and pyrosequencing (PyroTyping) for discrimination of isolates based on the polymorphism of the IS*6110* insertion site. In addition, PyroTyping results regarding clustering or

discrimination of the isolates were compared with RFLP and spoligotyping results.

The PyroTyping assay (Figure 1) consists of digestion of the *M. tuberculosis* genomic DNA with TaqI restriction enzyme, which cuts on a target located within the IS*6110* and in a target located 5' to the IS*6110*, dependent on insertion point; ligation of adaptors; touchdown PCR for amplification of the 5' IS*6110*-flanking region of all the IS*6110* copies present in the genome; and simultaneous pyrosequencing of the amplified fragments. When two isolates share the same RFLP pattern, the IS*6110* copies are located in the same position in the genome, and thus, the sequence of the IS*6110*-5' flanking regions would be identical. In this case, pyrosequencing profiles would also be identical. On the contrary, when two isolates exhibit different RFLP patterns, pyrosequencing profiles would also be different. The PyroTyping assay was shown to be reproducible in the three independent reactions performed using DNA extracted from the *M. tuberculosis* reference strain H37Rv. In addition, the limit of detection was set at 100ng of DNA.

The PyroTyping assay was tested in a set of 94 *M. tuberculosis* isolates corresponding to 29 molecular epidemiology case studies that were part of standard investigations of contact tracing or suspected cases of laboratory cross-contamination (Table 1). Results between RFLP, spoligotyping, and PyroTyping for the 94 *M. tuberculosis* isolates were concordant: the three methods agreed on clustering for 74 isolates (Figure 2a), and on discrimination for 20 isolates (Figure 2b) (Table 1). Interestingly, in two case studies, the isolates were clustered by RFLP, but the patterns showed one- and two-band differences, respectively. In one case study, both isolates presented the same RFLP pattern with 15 bands, but one of the isolates had two additional bands. Moreover, the same PyroTyping profile and spoligotyping pattern (not described in SITVITWEB) were obtained. In another case study, both isolates presented the same RFLP pattern with nine bands, but one of the isolates had one additional band. As before, the same PyroTyping profile and spoligotyping pattern (SIT53/T1) were obtained.

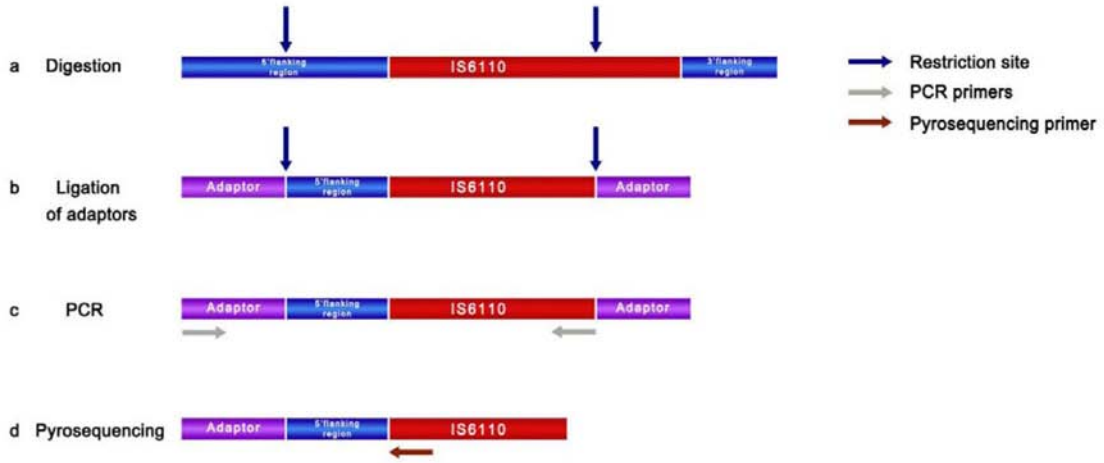

Figure 1. Schematic diagram of the PyroTyping assay. Genomic DNA of *M. tuberculosis* isolates grown in Löwenstein-Jensen solid medium was extracted by the cetyltrimethylammonium bromide protocol. After, DNA was digested with Tru1I (MseI) and TaqI restriction enzymes (a); adaptors were ligated to the TaqI restricted fragments (b); the fragments were amplified by PCR using a biotinylated primer complementary to the adaptor and a primer complementary to the IS*6110* (c); finally, the amplification product was subjected pyrosequencing of the 5' IS*6110*-flanking regions by using a primer complementary to the 5' end of the IS*6110* (d).

Table 1. Case studies of molecular epidemiology investigations.

Type of investigation	No. of case studies (no. of isolates)	No. of patients in each case study (no. of patient isolates clustered by RFLP)
Contact tracing	22 (72)	
Family members	9 (23)	3 (3), 2 (2), 3 (3), 2 (2)[a], 2 (2), 4 (4), 3 (3), 2 (2), 2 (2)
Spatio-temporal proximity	5 (32)	15 (4, 2, 2, 2), 5 (5), 2 (0), 8 (8), 2 (2)[b]
Cohabitants	3 (7)	3 (2), 2 (2), 2 (0)
Workplace	4 (8)	2 (0), 2 (0), 2 (2), 2 (2)
Friends	1 (2)	2 (0)
Laboratory cross-contamination	7 (22)	3 (3), 3 (2), 6 (4), 2 (2), 3 (2), 3 (3), 2 (2)

a These isolates shared RFLP patterns that differed in only two bands.
b These isolates shared RFLP patterns that differed in only one band.

Among the 94 isolates, 11 isolates had a low IS*6110* copy number (considered as six or fewer bands in the RFLP pattern (6)). These 11 isolates corresponded to four case studies comprising 5, 3, 2 and 2 isolates, respectively (one isolate had a high IS*6110* copy number). Regarding the five-isolate case study, four isolates were clustered by RFLP, spoligotyping (orphan pattern), and PyroTyping, whereas one isolate presented a different RFLP, spoligotyping (SIT326, AFRI_1) and PyroTyping profile. The isolates of the three-isolate case study were clustered by RFLP, spoligotyping (SIT334, T1), and PyroTyping. The isolates of one of the two-isolate case study were clustered by RFLP, spoligotyping (SIT41, LAM7-TUR) and PyroTyping. The two isolates of the remaining case study presented different RFLP (one isolate had a high IS*6110* copy number), spoligotyping (SIT42, LAM9 and SIT160, clade not assigned) and PyroTyping profiles. Results regarding characterization of the 94 isolates by spoligotyping is available as Supplemental Material.

Genotyping of *M. tuberculosis* isolates has been a valuable tool for TB control and has improved knowledge about TB epidemiology (6, 7). The most widely used method based on IS*6110* has been RFLP, but it presents limitations for taking rapid decisions for TB control. To overcome the disadvantages, different rapid genotyping assays based on PCR and the polymorphism of IS*6110* have been developed. Some of these methods are based on DNA digestion with restriction enzymes, ligation of adaptors, PCR amplification, and analysis of the amplified fragments. The most used and improved assays have been ligation-mediated PCR (8, 9) and mixed-linker PCR (10, 11). Most of these methods perform a gel electrophoresis after PCR, and the fingerprint patterns obtained may display a low number of bands, which limits the level of discrimination that may be achieved. In contrast, in the PyroTyping assay we carried out a touchdown PCR for specific and efficient amplification of the IS*6110* 5'-flanking regions, followed by pyrosequencing, which yields a pyrogram with a variable number of nucleotide peaks, allowing better discrimination compared to the limited number of bands of a gel electrophoresis. In fact, there was complete concordance between PyroTyping and RFLP, hence, when PyroTyping profiles are the same, the isolates can be certainly clustered, and vice versa. Furthermore, direct analysis of patient specimens with PyroTyping may be considered, although the protocol may have to be optimized in order to obtain enough double stranded high-quality DNA required for digestion.

Interestingly, there were two clusters of isolates that presented RFLP patterns that differed only in one or two bands, which were present in one isolate but absent in the other one. There has been some controversy on considering or not as being part of the same cluster isolates with these slightly different RFLP patterns (6), since RFLP profiles of isolates

from a single source may differ in the presence or absence of at least one band (12). In the present study, we considered that these isolates were clustered because the patients within case studies

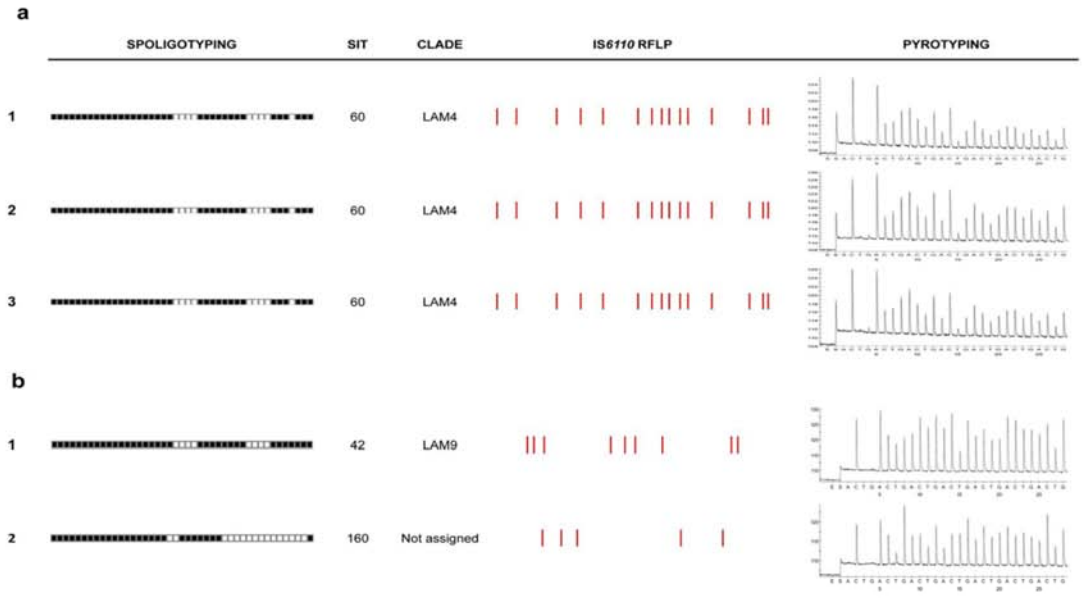

Figure 2. Examples of spoligotyping, IS*6110*-RFLP, and PyroTyping results for some of the *M. tuberculosis* isolates included in this study. a) Results for isolates sharing the same spoligotyping, IS*6110*-RFLP, and PyroTyping profiles. b) Results for isolates showing different spoligotyping, IS*6110*-RFLP, and PyroTyping profiles.
RFLP: restriction fragment length polymorphism.

were epidemiologically related, isolates from related patients may present these minor changes (13), and the rate of transposition increases with the number of copies of IS*6110* (14). Therefore, the molecular relatedness of the isolates within case studies was supported by RFLP, and also by spoligotyping and PyroTyping, despite slightly more information was obtained by RFLP.

A drawback of IS*6110*-based genotyping methods is the reduced discriminatory power for low IS*6110* copy number isolates. RFLP clusters involving these isolates should be discriminated by another genotyping method not based on IS*6110*. Since PyroTyping is based on IS*6110*, it will not be informative for these isolates. However, in the present study, RFLP results regarding low IS*6110* copy number isolates were concordant with spoligotyping, and also with PyroTyping results. Variations of the method based on other repetitive elements present would be easily developed along the same lines reported here.

PyroTyping assay can be performed in two days, the first day for digestion and ligation procedures, and the second one for PCR and pyrosequencing reaction. In addition, PyroTyping requires specific equipment (pyrosequencer) and can be performed in 96-well plates, for high-throughput analysis. Furthermore, PyroTyping profiles of the isolates within each case study were compared by the naked eye, therefore, the interpretation does not require a complex bioinformatic analysis, but for now it is restricted to a limited number of isolates. However, with a comprehensive software tool for storage and comparison of the PyroTyping profiles, a database for *M. tuberculosis* isolates from a setting could be created, for clinical as well as for epidemiological

purposes. Moreover, since pyrosequencing has been used for detecting mutations associated with drug resistance (15, 16), genotyping and detection of drug resistance could be performed in a combined assay, further increasing the clinical value of the technology for patient management. Lastly, the PyroTyping assay is potentially applicable for genotyping other bacterial species presenting polymorphism of insertion sequences, such as *Salmonella typhimurium* or *Staphylococcus aureus* (17, 18).

In conclusion, we have developed PyroTyping, a novel, rapid, and highly discriminatory assay that offers a promising alternative for *M. tuberculosis* genotyping for epidemiological studies in local laboratories. The introduction of the touchdown PCR and pyrosequencing improved the performance over other methods analysing the variability on the flanking regions of the IS*6110* elements.

METHODS

During the period from 2006 to 2014, a total of 94 *M. tuberculosis* isolates were retrospectively selected. The isolates were received from local clinical laboratories or were isolated in Hospital Universitari Germans Trias i Pujol, and corresponded to 29 molecular epidemiology case studies that were part of standard contact tracing investigations or suspected cases of laboratory cross-contamination (Table 1). The isolates were subcultured on Löwenstein-Jensen (LJ) solid medium for at least four weeks or until colonies were well grown.

DNA was extracted from isolates cultured on LJ following the cetyltrimethylammonium bromide protocol (19). RFLP was performed as previously described (4). Table 1 shows the molecular epidemiology investigation case studies, with the number of patients in each case study and the number of patient isolates clustered by RFLP.

Spoligotyping was performed using the spoligokit (Ocimum Biosolutions, Hyderabad, India) following the manufacturer's instructions. The individual spoligotyping patterns were compared with those in the International Spoligotyping Database (SITVITWEB) of the Pasteur Institute of Guadeloupe, (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Spoligotyping International Types (SIT) were assigned according to spoligotype pattern signatures provided in SITVITWEB. When RFLP and spoligotyping patterns were identical, isolates were considered to be clustered, whereas when patterns of RFLP and/or spoligotyping were different, isolates were considered to be unrelated. It is of note that RFLP patterns with a high number of bands that differed only in one or two bands were considered to be clustered (6, 13).

PyroTyping is a genotyping method based on the polymorphism of IS*6110* (Figure 1). It consists of digestion of the *M. tuberculosis* genomic DNA with TaqI restriction enzyme, which cuts on a target located within the IS*6110* and in a target located 5' to the IS*6110*, dependent on insertion point; ligation of adaptors; touchdown PCR for amplification of the 5' IS*6110*-flanking region of all the IS*6110* copies present in the genome; and simultaneous pyrosequencing of the amplified fragments. When two isolates share the same RFLP pattern, the IS*6110* copies are located in the same position in the genome, and thus, the sequence of the IS*6110*-5' flanking regions would be identical. In this case, pyrosequencing profiles should be identical. On the contrary, when two isolates exhibit different RFLP patterns, pyrosequencing profiles should be different.

A first genomic digestion was performed in a final volume of 20 µl containing 5U Tru1I (MseI) (Thermo Fisher Scientific, Waltham, MA, USA), 1X Tru1I buffer R (Thermo Fisher Scientific, Waltham, MA, USA), 0.1 mg/mL bovine serum albumin (BSA) (Hoffmann-La Roche, Basel, Switzerland), 0.5 mg/mL DNase-free RNase A (Hoffmann-La Roche, Basel, Switzerland), and 500 ng of genomic DNA. The Tru1I (MseI) digestion was carried out at 37ºC for at least 2 h. A second digestion was performed by addition of 10U TaqI (Thermo Fisher Scientific, Waltham, MA, USA), and incubation at 65ºC for 3 h, and 80ºC for 2 min. Subsequently, a 24.6 µl ligation mix containing 40U of T4 DNA ligase (New England Biolabs, Ipswich, MA, USA), 2X T4 ligase buffer (Hoffmann-La Roche, Basel, Switzerland), and 0.2

µM of each adaptor (5'-CGGTCAGGACTCAT-3', 5'-CGATGAGTCCTGAC-3') (TIB MOLBIOL, Berlin, Germany) was added to the digestion product. Ligation was carried out at 12ºC for 17 h, and 65ºC for 10 min.

Touchdown PCR (20) was performed in a final volume of 25 µl containing 1X HotStarTaq Master Mix (Qiagen, Venlo, The Netherlands), 1 µM each primer (forward 5'biotin-ATGAGTCCTGACCGA-3, reverse 5'-CTGACATGACCCCATCCTTT-3') (TIB MOLBIOL, Berlin, Germany), 1M betaine PCR reagent (Sigma-Aldrich, St. Louis, MO, USA), and 2.5 µl of ligation product. Touchdown PCR was carried out with the Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) and the following amplification conditions: 94°C for 15 min; 10 cycles of 94°C for 20 s, 66-56ºC for 30 s (temperature decreasing 1°C every cycle from 66°C to 56ºC), and 72ºC for 2 min; 20 cycles of 94°C for 20 s, 56ºC for 30 s, and 72ºC for 2 min; and 72°C for 7 min. Finally, pyrosequencing of the PCR product was performed using a PSQ 96MA and SQA software as recommended by the manufacturer (Qiagen, Venlo, The Netherlands). Briefly, the protocol consisted on the preparation of the single-stranded DNA with a vacuum preparation tool, annealing of the sequencing primer (5'-GGACATGCCGGGGCGGTT-3') (TIB MOLBIOL, Berlin, Germany), and real-time pyrosequencing. In this protocol, the nucleotide dispensation order was 7x(ACTG), thus, the pyrograms presented 28 nucleotide peaks.

The result for each isolate consisted of a single pyrogram combining the simultaneous pyrosequencing of the 5' flanking regions of all the IS*6110* copies present in the genome (Figure 2). Therefore, the pyrogram corresponds to an artificial sequence obtained from the merged sequences of all the 5' flanking regions. PyroTyping profiles from isolates from each case study were compared by the naked eye. When the same PyroTyping profiles were obtained, isolates were considered to be clustered, whereas when PyroTyping profiles were different, isolates were considered to be discriminated. Clustering or discrimination results obtained by PyroTyping were compared with those obtained by RFLP and spoligotyping. Spoligotyping and RFLP results were blinded to the researchers who interpreted PyroTyping results.

To assess the reproducibility of PyroTyping, three independent reactions were performed using DNA extracted from the *M. tuberculosis* reference strain H37Rv. In addition, to assess the limit of detection of the assay, six additional reactions were performed using ten-fold serial dilutions of DNA extracted from H37Rv (100ng to 0.001ng).

AUTHOR CONTRIBUTIONS STATEMENT

Conception and design of the study: CA; CP; JD
Acquisition of data: BM; NG; SB, LH, SS; JR
Analysis and interpretation of data: BM; AL; SS; CP; JD
Drafting the article: BM; AL, CP; JD
Revising it critically for important intellectual content and final approval of the version to be published: all authors.

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SUPPLEMENTARY MATERIAL. Spoligotyping results for the 94 isolates included in the study. Case and isolate number, spoligotypes, SIT, and clade are shown. SIT and clades were identified according to SITVITWEB.

Case number.Isolate number	Type of investigation	Spoligotype	Spoligotype43	SIT	Clade	Family
1.1	Contact tracing - family members	[illegible]	000000007760771	4	Orphan	Orphan
1.2	Contact tracing - family members	[illegible]	000000007760771	4	Orphan	Orphan
1.3	Contact tracing - family members	[illegible]	000000007760771	4	Orphan	Orphan
2.1	Cross-contamination	[illegible]	577777777760771	334	T1	T
2.2	Cross-contamination	[illegible]	577777777760771	334	T1	T
2.3	Cross-contamination	[illegible]	577777777760771	334	T1	T
3.1	Cross-contamination	[illegible]	776527242000000	Orphan	Orphan	Orphan
3.2	Cross-contamination	[illegible]	777777477400001	160	Orphan	Orphan
3.3	Cross-contamination	[illegible]	777777477400001	160	Orphan	Orphan
4.1	Cross-contamination	[illegible]	637777777720771	849	H3	Haarlem
4.2	Cross-contamination	[illegible]	776377003760771	Orphan	Orphan	Orphan
4.3	Cross-contamination	[illegible]	777717607760771	216	LAM5	LAM
4.4	Cross-contamination	[illegible]	777717607760771	216	LAM5	LAM
4.5	Cross-contamination	[illegible]	777717607760771	216	LAM5	LAM
4.6	Cross-contamination	[illegible]	777717607760771	216	LAM5	LAM
5.1	Cross-contamination	[illegible]	777767477400001	Orphan	Orphan	Orphan
5.2	Cross-contamination	[illegible]	777767477400001	Orphan	Orphan	Orphan
6.1	Contact tracing - family members	[illegible]	677737607760771	17	LAM2	LAM
6.2	Contact tracing - family members	[illegible]	677737607760771	17	LAM2	LAM
7.1	Contact tracing - spatio-temporal proximity	[illegible]	770777775777671	Orphan	Orphan	Orphan
7.2	Contact tracing - spatio-temporal proximity	[illegible]	770777775777671	Orphan	Orphan	Orphan
7.3	Contact tracing - spatio-temporal proximity	[illegible]	777777607760771	42	LAM9	LAM
7.4	Contact tracing - spatio-temporal proximity	[illegible]	777737777420771	35	H3	Haarlem
7.5	Contact tracing - spatio-temporal proximity	[illegible]	770777775777671	Orphan	Orphan	Orphan
7.6	Contact tracing - spatio-temporal proximity	[illegible]	703777340003171	Orphan	Orphan	Orphan
7.7	Contact tracing - spatio-temporal proximity	[illegible]	770777775777671	Orphan	Orphan	Orphan
7.8	Contact tracing - spatio-temporal proximity	[illegible]	777737777420771	35	H3	Haarlem
7.9	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
7.10	Contact tracing - spatio-temporal proximity	[illegible]	777777607760771	42	LAM9	LAM
7.11	Contact tracing - spatio-temporal proximity	[illegible]	777777677760771	291	T1	T
7.12	Contact tracing - spatio-temporal proximity	[illegible]	777777677760771	291	T1	T
7.13	Contact tracing - spatio-temporal proximity	[illegible]	777777607760771	42	LAM9	LAM
7.14	Contact tracing - spatio-temporal proximity	[illegible]	777777557760771	58	T5-Madrid2	T
7.15	Contact tracing - spatio-temporal proximity	[illegible]	770777707777671	326	AFRI_1	AFRI
8.1	Cross-contamination	[illegible]	677777600760771	Orphan	Orphan	Orphan
8.2	Cross-contamination	[illegible]	436177607760771	Orphan	Orphan	Orphan
8.3	Cross-contamination	[illegible]	677777600760771	Orphan	Orphan	Orphan
9.1	Contact tracing - family members	[illegible]	760000007760771	1574	LAM3	LAM
9.2	Contact tracing - family members	[illegible]	760000007760771	1574	LAM3	LAM
9.3	Contact tracing - family members	[illegible]	760000007760771	1574	LAM3	LAM
10.1	Contact tracing - roommate	[illegible]	777777777720771	50	H3	Haarlem
10.2	Contact tracing - roommate	[illegible]	777777777720771	50	H3	Haarlem
10.3	Contact tracing - roommate	[illegible]	771737777760771	Orphan	Orphan	Orphan
11.1	Cross-contamination	[illegible]	777777607760731	60	LAM4	LAM
11.2	Cross-contamination	[illegible]	777777607760731	60	LAM4	LAM
11.3	Cross-contamination	[illegible]	777777607760731	60	LAM4	LAM
12.1	Contact tracing - family members	[illegible]	760000007060771	Orphan	Orphan	Orphan
12.3	Contact tracing - family members	[illegible]	760000007060771	Orphan	Orphan	Orphan
13.1	Cross-contamination	[illegible]	774242777777771	Orphan	Orphan	Orphan
13.2	Cross-contamination	[illegible]	774242777777771	Orphan	Orphan	Orphan
14.1	Contact tracing - friends	[illegible]	777777607760771	42	LAM9	LAM
14.2	Contact tracing - friends	[illegible]	777777477400001	160	Orphan	Orphan
15.1	Contact tracing - family members	[illegible]	777777777520771	746	H3	Haarlem
15.2	Contact tracing - family members	[illegible]	777777777520771	746	H3	Haarlem
16.1	Contact tracing - roommate	[illegible]	777777777000371	775	Orphan	Orphan
16.2	Contact tracing - roommate	[illegible]	777777777000371	775	Orphan	Orphan
17.1	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
17.2	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
17.3	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
17.4	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
17.5	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
18.1	Contact tracing - spatio-temporal proximity	[illegible]	777737774020771	218	H1	Haarlem
18.2	Contact tracing - spatio-temporal proximity	[illegible]	777777607760761	1074	LAM9	LAM
19.1	Contact tracing - colleagues at work	[illegible]	777777557760771	58	T5-Madrid2	T
19.2	Contact tracing - colleagues at work	[illegible]	776177607760771	33	LAM3	LAM
20.1	Contact tracing - roommate	[illegible]	777777777760771	53	T1	T
20.2	Contact tracing - roommate	[illegible]	777777607760771	42	LAM9	LAM
21.1	Contact tracing - nursery school	[illegible]	774137600020771	1538	H3	Haarlem
21.2	Contact tracing - nursery school	[illegible]	774137600020771	1538	H3	Haarlem
22.1	Contact tracing - family members	[illegible]	777241777560771	Orphan	Orphan	Orphan
22.2	Contact tracing - family members	[illegible]	777241777560771	Orphan	Orphan	Orphan
22.3	Contact tracing - family members	[illegible]	777241777560771	Orphan	Orphan	Orphan
22.4	Contact tracing - family members	[illegible]	777241777560771	Orphan	Orphan	Orphan
23.1	Contact tracing - family members	[illegible]	777777557760771	58	T5-Madrid2	T
23.2	Contact tracing - family members	[illegible]	777777557760771	58	T5-Madrid2	T
23.3	Contact tracing - family members	[illegible]	777777557760771	58	T5-Madrid2	T
24.1	Contact tracing - colleagues at work	[illegible]	777777777720771	50	H3	Haarlem
24.2	Contact tracing - colleagues at work	[illegible]	776177607760771	33	LAM3	LAM
25.1	Contact tracing - family members	[illegible]	357777777760771	Orphan	Orphan	Orphan
25.2	Contact tracing - family members	[illegible]	357777777760771	Orphan	Orphan	Orphan
26.1	Contact tracing - colleagues at work	[illegible]	777777404760771	41	LAM7-TUR	LAM
26.2	Contact tracing - colleagues at work	[illegible]	777777404760771	41	LAM7-TUR	LAM
27.1	Contact tracing - family members	[illegible]	777737777760771	37	T3	T
27.2	Contact tracing - family members	[illegible]	777737777760771	37	T3	T
28.1	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.2	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.3	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.4	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.5	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.6	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.7	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
28.8	Contact tracing - spatio-temporal proximity	[illegible]	776140000000071	Orphan	Orphan	Orphan
29.1	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T
29.2	Contact tracing - spatio-temporal proximity	[illegible]	777777777760771	53	T1	T