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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

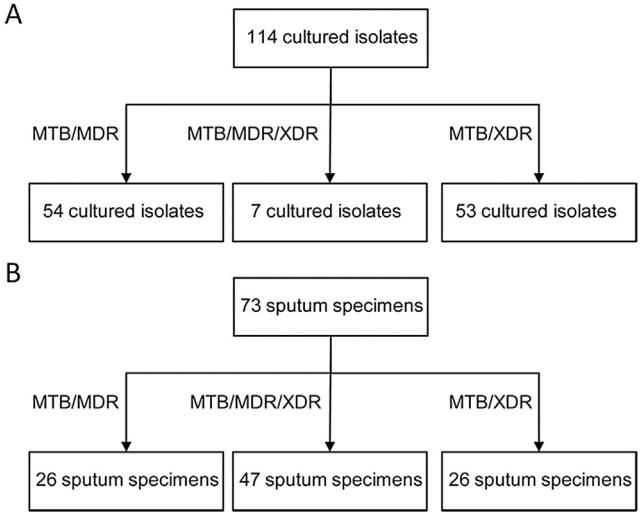

Figure 1 Number of cultured isolates and sputum specimens retrospectively selected for the study and analyzed by the MTB/MDR, MTB/XDR, or both MTB/MDR/XDR tests. A: a total of 61 cultured isolates were analyzed by the MTB/MDR test and 60 were analyzed by the MTB/XDR test. B: among the sputum specimens,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

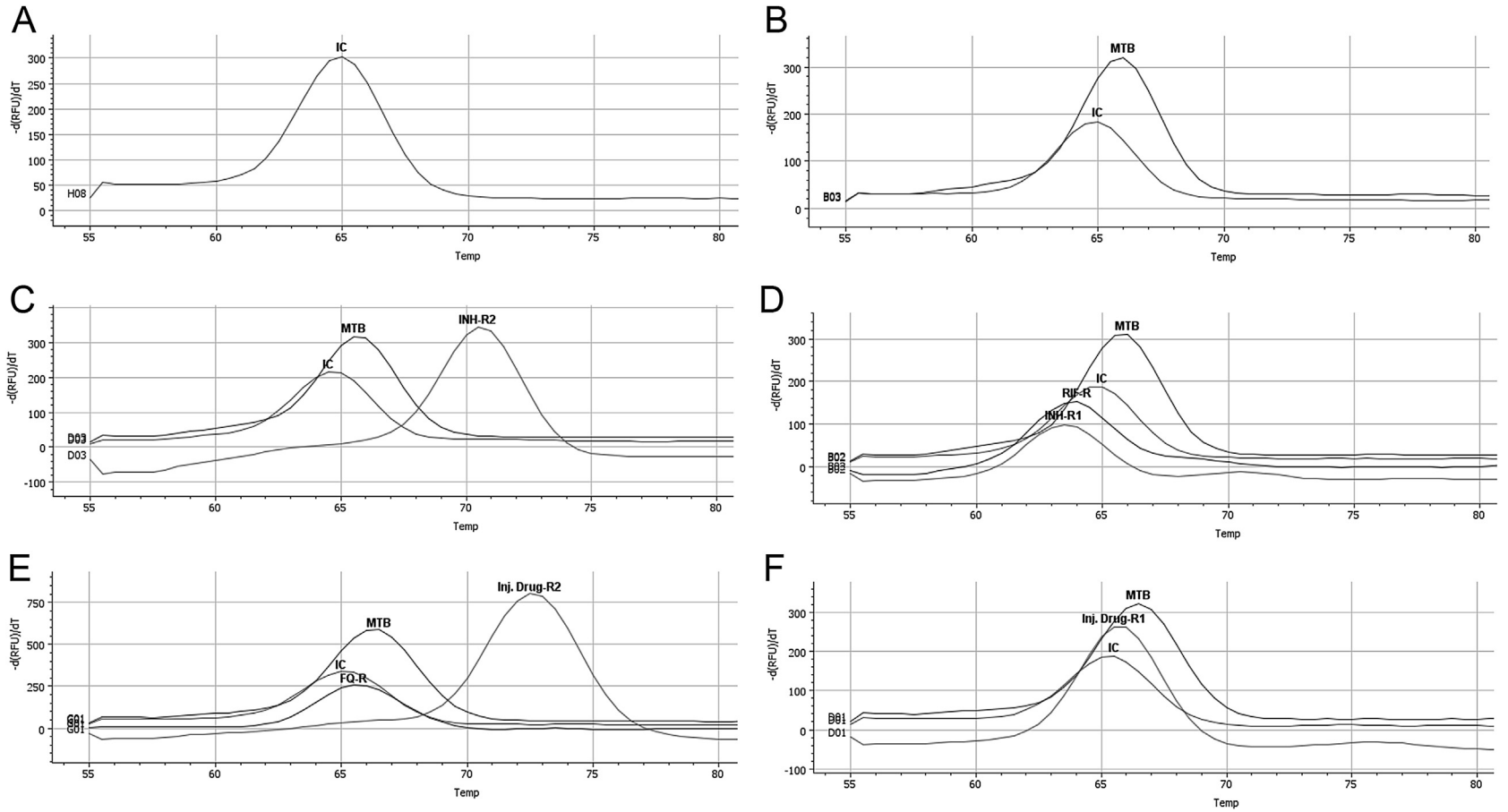

Figure 2 Examples of results obtained with Anyplex II MTB/MDR/XDR. Plot of the rate of change of the relative fluorescence units with time (-d(RFU)/dT) versus the temperature (Temp, °C). A: example of an excluded result due to absence of MTB melting curve. B: example of a INH^S and RIF^S isolate. C: 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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