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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