Journal of Clinical Microbiology

Detection of First- and Second-Line Drug Resistance in Mycobacterium tuberculosis Clinical Isolates by Pyrosequencing

Anna Engström, Nora Morcillo, Belen Imperiale, Sven E. Hoffner and Pontus Juréen

J. Clin. Microbiol. **2012, 50(6):2026. DOI: 10.1128/JCM.06664-11.**
Published Ahead of Print 29 March 2012.

JCM
Journals.ASM.org

Detection of First- and Second-Line Drug Resistance in *Mycobacterium tuberculosis* Clinical Isolates by Pyrosequencing

Anna Engström,[a,b] Nora Morcillo,[c] Belen Imperiale,[c] Sven E. Hoffner,[a,b] and Pontus Juréen[a,b]

Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden[a]; Department of Preparedness, Swedish Institute for Communicable Disease Control, Solna, Sweden[b]; and Reference Laboratory of Tuberculosis Control Program of Buenos Aires Province, Dr. Cetrángolo Hospital, Buenos Aires, Argentina[c]

Conventional phenotypic drug susceptibility testing (DST) methods for *Mycobacterium tuberculosis* are laborious and very time-consuming. Early detection of drug-resistant tuberculosis (TB) is essential for prevention and control of TB transmission. We have developed a pyrosequencing method for simultaneous detection of mutations associated with resistance to rifampin, isoniazid, ethambutol, amikacin, kanamycin, capreomycin, and ofloxacin. Seven pyrosequencing assays were optimized for following loci: *rpoB*, *katG*, *embB*, *rrs*, *gyrA*, and the promoter regions of *inhA* and *eis*. The molecular method was evaluated on a panel of 290 clinical isolates of *M. tuberculosis*. In comparison to phenotypic DST, the pyrosequencing method demonstrated high specificity (100%) and sensitivity (94.6%) for detection of multidrug-resistant *M. tuberculosis* as well as high specificity (99.3%) and sensitivity (86.9%) for detection of extensively drug-resistant *M. tuberculosis*. The short turnaround time combined with multilocus sequencing of several isolates in parallel makes pyrosequencing an attractive method for drug resistance screening in *M. tuberculosis*.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, persists as a global public health problem. Drug-resistant bacteria, especially multidrug-resistant (MDR) *M. tuberculosis* and extensively drug-resistant (XDR) *M. tuberculosis*, pose challenges for the prevention and control of this deadly disease (64). MDR *M. tuberculosis* is defined as an isolate resistant to at least the two main first-line anti-TB drugs, rifampin (RIF) and isoniazid (INH), whereas XDR *M. tuberculosis* is defined as an MDR isolate that is also resistant to a fluoroquinolone (FQ) and at least one of the following second-line injectable agents: amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) (8). TB control and prevention programs are based on early diagnosis followed by rapid identification of drug resistance (62). Generally, detection of drug-resistant *M. tuberculosis* is performed by culture and drug susceptibility testing (DST) in liquid or on solid media. However, these procedures are laborious and take several weeks to months to complete. The development of rapid molecular methods, which can be performed within 1 or 2 days, is important for the timely identification of appropriate TB treatment. In fact, the World Health Organization (WHO) endorses the use of genotypic methods that target specific molecular mutations associated with resistance to individual drugs (63). Two examples of commercially available methods are Xpert MTB/RIF and GenoType MTBDR (16–18), which are predominately based on a lack of probe hybridization to wild-type loci, indirectly indicating the presence of mutations. The Xpert MTB/RIF system is also limited in that it detects only RIF resistance.

Spontaneous chromosomal mutations are the genetic basis for drug resistance in *M. tuberculosis* (40, 68), and a limited number of mutations account for the majority of phenotypic resistance to anti-TB drugs. RIF interacts with the ß-subunit (encoded by *rpoB*) of the RNA polymerase and inhibits the early steps of transcription (5). Resistance to RIF is almost entirely coupled to mutations within an 81-bp region of the *rpoB* gene, called the RIF resistance-determining region (RRDR), comprising codons 507 to 533 (14, 40, 46). In accordance with standard practice, these codon numbers correspond to those found in the *Escherichia coli rpoB* sequence, as designated on the basis of alignment of the translated sequence, and do not represent the actual codon positions in *M. tuberculosis rpoB* (30, 32, 58). The mechanisms of resistance to isoniazid (INH), though extensively investigated, remain incompletely understood (60). INH is a prodrug that is activated by the catalase-peroxidase enzyme KatG, encoded by the *katG* gene (22). The primary target of INH in *M. tuberculosis* is believed to be the enoyl-acyl carrier protein reductase InhA, which is involved in mycolic acid synthesis (3, 39, 43). Mutations in *katG*, specifically at codon 315, lead to high-level INH resistance (40, 67). Low-level resistance to INH is attributed to mutations in the promoter region of the *mabA-inhA* operon (here denoted *inhA*) leading to overexpression of InhA (26, 60). Ethambutol (EMB) inhibits arabinosyl transferases, encoded by the *embCAB* operon, thereby interfering with biosynthesis of the cell wall component arabinogalactan (55, 59). Mutations associated with resistance to EMB are primarily found in the *embB* gene (40). Amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) inhibit protein synthesis by binding to the ribosome (21, 31, 38). Resistance to all three drugs is associated with mutations in the 16S rRNA gene *rrs*, specifically at nucleotide positions 1401, 1402, and 1484 (1, 11, 28, 54, 57). A mutation at nucleotide 1401 or 1484 is associated with resistance to all these agents, whereas a mutation at nucleotide 1402 is associated with CAP resistance and low-level KAN resistance (11, 28). Overexpression of *eis* (encoding the aminoglycoside acetyltransferase Eis), caused by mutations in the promoter region, confers low-level resistance to KAN (66). Fluoroquinolo-

Received 17 December 2011 **Returned for modification** 17 January 2012
Accepted 21 March 2012
Published ahead of print 29 March 2012
Address correspondence to Anna Engström, anna.engstrom@ki.se.
Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.06664-11

nes (FQs), e.g., ofloxacin (OFL), which bind to DNA gyrase, inhibit proper regulation of supercoiling and cause chromosomal double-strand breaks (65). DNA gyrase is a heterotetramer consisting of two A and two B subunits, encoded by the genes *gyrA* and *gyrB* (34, 61). FQ resistance-associated mutations are predominantly found in a short region, codons 88 to 94, of the *gyrA* gene (53, 56, 65).

Pyrosequencing is a semiautomated sequencing method based on real-time monitoring of DNA synthesis, optimized to analyze short DNA sequences. It is based on the quantitative detection of released pyrophosphate during DNA synthesis. In a cascade of enzymatic reactions, light is generated at intensities proportional to the numbers of incorporated nucleotides (33, 42). Pyrosequencing has previously been described as a method for detection of drug resistance in *M. tuberculosis* (2, 4, 23).

The objective of this study was to develop and evaluate a molecular method for detection of drug resistance-associated mutations in clinical isolates of *M. tuberculosis* by pyrosequencing technology. Apart from further optimization of the current *rpoB* assay (23), we have designed pyrosequencing assays for *katG*, *embB*, *rrs*, *gyrA*, and the promoter regions of *inhA* and *eis*. These assays facilitate rapid molecular detection of resistance to RIF, INH, EMB, AMK, KAN, CAP, and OFL in clinical isolates. Phenotypic and genotypic DST results were compared for determination of the sensitivity and specificity of the molecular assays for each drug.

MATERIALS AND METHODS

Bacterial strains and phenotypic drug susceptibility testing. The pansusceptible reference strain *M. tuberculosis* H37Rv (ATCC 25618) and 290 clinical isolates of *M. tuberculosis* from Europe, Asia, Africa, and Latin America were selected from the culture collection at the WHO Supranational TB Reference Laboratory at the Swedish Institute for Communicable Disease Control in Solna, Sweden, and included in this study. The classification of strains as susceptible or resistant was based on DST performed earlier using WHO-recommended standardized and quality assured methods for *M. tuberculosis* and, in some cases, complemented by MIC determinations (11).

Gene amplification. Genomic DNA was isolated as described elsewhere (23), or by the method of Sandegren and colleagues (45), but using only one chloroform extraction step. Parts of genes *rpoB*, *katG*, *embB*, *rrs*, and *gyrA* and of the promoter regions of *inhA* and *eis* were amplified using PCR. Primers and amplicon sizes are presented in Table S1 in the supplemental material. Amplifications were performed in a final volume of 50 μl with 1× PCR buffer, 2 mM $MgCl_2$ for all loci (except for *rpoB*, for which 1 mM $MgCl_2$ was used), 100 μM each dNTP (Applied Biosystems, Warrington, United Kingdom) (for *rpoB*, *inhA*, *eis* and *gyrA*) or 200 μM each dNTP (for *embB*, *rrs* and *katG*), 200 nM each primer, 1 U of AmpliTaq Gold (Applied Biosystems, Branchburg, NJ), and 10 ng of DNA. Thermocycling conditions are specified in Table S1 in the supplemental material.

Pyrosequencing. Seven pyrosequencing assays were optimized for the following loci: *rpoB*, *katG*, *embB*, *rrs*, *gyrA*, and the promoter regions of *inhA* and *eis*. A previously reported *rpoB* assay was further optimized by using only two sequencing primers, F1 and F9 (see Table S1 in the supplemental material) and a directed dispensation order instead of cyclic dispensation (23), thus reducing the number of sequencing reactions needed per sample. The directed dispensation order for *rpoB* was designed to detect all mutations in the RRDR reported by Ramaswamy and Musser (40). All four nucleotides are repeatedly dispensed in cyclic dispensation, whereas the nucleotide dispensation order is preprogrammed according to a known sequence in directed dispensation. Cyclic dispensation order was used for *katG*, *embB*, *gyrA*, and the promoter region of *inhA*. Apart from the *rpoB* locus, directed dispensation order was also used for *rrs* and the promoter region of *eis*.

Preparation of templates and sequencing reactions were performed according to the instructions of the manufacturers (Biotage AB, Uppsala, Sweden, and Qiagen GmbH, Hilden, Germany). Briefly, each locus was amplified by PCR with a biotinylated reverse primer for immobilization onto streptavidin-coated Sepharose beads (GE Healthcare, Uppsala, Sweden). The PCR product was converted into a single-stranded DNA template and purified using a vacuum preparation tool. A sequencing primer (0.4 μM) was subsequently annealed to the single-stranded DNA (see Table S1 in the supplemental material). Pyrosequencing was carried out in a PSQ 96MA instrument (Pyrosequencing AB, Uppsala, Sweden) using a PSQ Gold 96 SQA reagent kit (Biotage AB, Uppsala, Sweden, and Qiagen GmbH, Hilden, Germany) and a nucleotide dispensation order as specified in Table S1 in the supplemental material. Pyrograms were interpreted and mutations were detected using both peak height and sequence output. Sequences were analyzed in BioEdit version 7.0.4.1 (13) and Geneious Pro version 4.8.4 (Biomatters Ltd., Auckland, New Zealand) by alignment to the *M. tuberculosis* H37Rv loci (GenBank accession no. NC_000962; NCBI bank) (7) and the sequence results of *M. tuberculosis* H37Rv (ATCC 25618).

Sensitivity and specificity. Phenotypic DST was considered the gold standard for comparison with the results obtained by pyrosequencing. Sensitivity was calculated as the number of true-positive specimens (phenotypically resistant and pyrosequencing resistant) divided by the number of true-positive specimens plus the number of false-negative specimens (phenotypically resistant and pyrosequencing susceptible). Specificity was calculated as the number of true-negative specimens (phenotypically susceptible and pyrosequencing susceptible) divided by the number of true-negative specimens plus the number of false-positive specimens (phenotypically susceptible and pyrosequencing resistant).

RESULTS

We have developed a pyrosequencing method for simultaneous detection of mutations associated with resistance to RIF, INH, EMB, AMK, KAN, CAP, and OFL in *M. tuberculosis* clinical isolates. In comparison to phenotypic DST, the molecular method demonstrated an overall specificity of 97.2% and a sensitivity of 83.2%. For detection of MDR *M. tuberculosis* isolates, a specificity of 100% and a sensitivity of 94.6% were noted. The corresponding figures for detection of XDR *M. tuberculosis* isolates were 99.3% and 86.9%, respectively.

For RIF resistance detection, the sensitivity was 94.7% and the specificity was 100% (Table 1). Of 209 RIF-resistant isolates, 198 had a mutation in the RRDR, and the vast majority (189 of 198) had a mutation in codon 516, 526, or 531. The *rpoB* locus of all but five isolates could be sequenced using directed dispensation order and sequencing primers F1 and F9. Four of these had insertions or deletions not designed for the directed dispensation order; instead, these were sequenced using four primers (F1, F7, F9, and F13) (23) and cyclic dispensation order. One isolate could not be sequenced with primer F1, and as a control, the RRDR was sequenced by Sanger sequencing (24). This isolate harbored an L511P mutation but also a mutation in codon 505 (F→V), which is where the F1 primer anneals. The vast majority of isolates had a missense mutation in the RRDR (Table 2). In one case, the silent mutation G536G occurred together with the S531L mutation. Eleven of the RIF-resistant clinical isolates had a wild-type *rpoB* locus.

The sensitivity and specificity for INH were 94.4% and 100%, respectively (Table 1). In total, 215 isolates were resistant to INH, and 203 of those had a mutation in *katG* (the majority had an S315T mutation), in the promoter region of *inhA* (the majority had a C-15T mutation), or in both loci (Table 2). Twelve clinical

TABLE 1 Performance of pyrosequencing as a molecular drug susceptibility test method

Pyrosequencing result[c]	No. of isolates with the indicated result by phenotypic DST or % sensitivity or specificity[a]													
	RIF		INH		EMB		AMK		KAN[b]		CAP		OFL	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S
R	198	0	203	0	82	21	69	0	82	2	62	4	54	0
S	11	71	12	74	52	117	15	155	16	126	16	139	8	184
Sensitivity[d] (%)	94.7		94.4		61.2		82.1		83.7		79.5		87.1	
Specificity[d] (%)	100.0		100.0		84.8		100.0		98.4		97.2		100.0	

[a] R, resistant; S, susceptible.
[b] Isolates harboring *eis* promoter mutations at positions −10 and −12 were considered pyrosequencing susceptible.
[c] R, isolates with a mutation in *rpoB* (RIF), *katG* or *inhA* (INH), *embB* (EMB), *rrs* (AMK and CAP), *rrs* and *eis* (KAN), or *gyrA* (OFL); S, isolates with a wild-type locus.
[d] Data were determined on the basis of phenotypic drug susceptibility testing as the gold standard.

isolates were phenotypically determined to be resistant to INH but had wild-type *katG* and *inhA* loci.

The sensitivity for EMB was 61.2%, and the specificity was 84.8% (Table 1). Only 82 of the 134 phenotypically resistant isolates harbored a mutation in the *embB* locus. Also, among the EMB-susceptible isolates, 21 had an *embB* 306 mutation. Mutations in *embB* codons 313 and 315 were detected in EMB-resistant isolates (Table 2).

The sensitivity and specificity for AMK were 81.2% and 100%, respectively. For KAN, the sensitivity was 83.7% and the specificity was 98.4% (Table 1). The majority the AMK- and KAN-resistant isolates harbored a mutation in the *rrs* locus (Table 3). All but one isolate with −14 or −37 *eis* mutations were KAN resistant, and the majority of −10 and −12 mutations were found among susceptible isolates. A C-15G mutation was detected in a KAN-susceptible isolate. The sensitivity and specificity for CAP were 79.5% and 97.2%, respectively (Table 1). Most CAP-resistant isolates had an *rrs* mutation at position 1401; however, this mutation was also detected in four susceptible isolates. Few possible mutations in *rrs* and a directed dispensation order made it possible to achieve a read length of 95 bp, which covered nucleotide positions 1401, 1402, and 1484 in one sequence run.

For OFL, the sensitivity and specificity were 87.1% and 100%, respectively (Table 1). Missense mutations in *gyrA*, specifically in codon 94, were detected in the vast majority of OFL-resistant isolates but were absent in all OFL-susceptible isolates.

By investigation of the pyrosequencing pyrograms, five heteroresistant isolates, i.e., a mixed template consisting of both wild-type and mutated sequences, were identified (Tables 2 and 3). The total turnaround time from inactivation of bacteria to interpreted sequencing results was approximately 8 h.

DISCUSSION

Bacteriological methods are highly sensitive and specific for detection of resistance to most anti-TB drugs; however, they are labor-intensive and very time-consuming. Consequently, molecular diagnostics can be used for rapid detection of drug-resistant *M. tuberculosis*. We have developed seven pyrosequencing assays for detection of mutations associated with resistance to first- and second-line anti-TB drugs and evaluated the method on a panel of 290 clinical isolates of *M. tuberculosis*.

Pyrosequencing is a robust technique that is easy to perform in a high-throughput manner. In the method developed here, 12 strains could simultaneously be sequenced in a 96-well plate. Some of the most frequent resistance-associated mutations in *M. tuberculosis* are located in very short regions of genes or promoter regions, whereas others are more dispersed. By using directed pyrosequencing dispensation order for *rpoB*, *rrs*, and the promoter region of *eis*, the entire resistance-associated locus could be covered in only one or two sequencing reactions. The successful sequencing of 95 bp of the *rrs* gene shows not only that it is possible to optimize longer reads for pyrosequencing but also that it is possible to do so using a GC-rich template (65%) (7). Cyclic dispensation was used for *katG*, the promoter region of *inhA*, *embB*, and *gyrA*, where mutations are confined to a short stretch of the locus. Cyclic dispensation increases tolerance for variations and eliminates the risk of failing to detect as-yet-unidentified mutations but typically renders shorter sequence reads. An advantage of sequencing compared to line-probe assays is the discriminatory power on the genotypic level. For instance, sequencing can distinguish between missense and silent mutations and discriminate against mutations not associated with drug resistance. This is in contrast to the commercially available GenoType MTBDR assays, which partly define resistance by the absence of wild-type sequence (17, 18). Pyrosequencing also requires less hands-on time than GenoType MTBDR and is more flexible, as the number of assays and the choice of included loci easily can be adjusted. Furthermore, the pyrosequencing method offers sensitivity and specificity comparable to those of Sanger sequencing (6) but is easier to handle and thus more suitable for investigation of a large number of samples. On the other hand, a drawback with pyrosequencing, as well as with other commercially available molecular methods (16–18), is that they cannot cover whole genes. Thus, *pncA* gene mutations, associated with resistance to the first-line drug pyrazinamide (48), cannot be sufficiently covered by these methods. Pyrogram analysis revealed that five phenotypically resistant isolates were heteroresistant. Heteroresistance is due either to segregation of a single strain into resistant and susceptible organisms or to superinfection with two different strains (one susceptible and one resistant) (19). The former scenario describes the evolution of resistance that arises from suboptimal treatment.

Introduction of directed dispensation order for the *rpoB* locus reduced the number of sequencing reactions needed for the RRDR to only two without compromising the detection of missense mutations. However, four isolates had in-frame deletions and insertions; thus, cyclic dispensation and four sequencing primers (F1, F7, F9, and F13) had to be employed in those cases. The *rpoB* assay is suboptimal in cases where missense mutations, insertions, or deletions have occurred specifically at the site where the sequencing primer anneals. This is,

TABLE 2 Mutations identified within loci associated with resistance to the first-line drugs rifampin, isoniazid, and ethambutol in clinical isolates of *M. tuberculosis*[a]

Drug	Locus(i)	Amino acid change(s)	Codon change(s)	Nucleotide change	No. of isolates with indicated phenotypic DST result	
					Resistant	Susceptible
RIF	*rpoB*	del TS 508-509	del nt 1279–1284 (ACCAGC)		1	
		del SQL 509-511	del nt 1282–1290 (AGCCAGCTG)		1[b]	
		S509T + D516Y	AGC/ACC + GAC/TAC		1	
		Q510H + D516Y	CAG/CAT + GAC/TAC		1	
		L511P	CTG/CCG		1	
		L511P + D516Y	CTG/CCG + GAC/TAC		2	
		L511P + D516G	CTG/CCG + GAC/GGC		1	
		L511R + D516Y	CTG/CGG + GAC/TAC		1	
		Q513P	CAA/CCA		2	
		D516G + L533P	GAC/GGC + CTG/CCG		2	
		D516V	GAC/GTC		11	
		D516Y	GAC/TAC		6	
		D516Y + H526Y + P535L	GAC/TAC + CAC/TAC + CCC/CTC		1	
		del N 519	del nt 1312–1314 (AAC)		1	
		H526D	CAC/GAC		3	
		H526L	CAC/GAC		3	
		H526N	CAC/AAC		2	
		H526R	CAC/CGC		1	
		H526Y	CAC/TAC		11	
		H526S + K527Q	ins nt 1331 (CGT) + del nt 1335–1337 (CAA)		1	
		H526Y + P535R	CAC/TAC + CCC/CGC		1	
		S531L	TCG/TTG		137	
		S531L + G536G	TCG/TTG + GGC/GGT		1	
		S531W	TCG/TGG		3	
		L533P	CTG/CCG		3	
INH	*katG*[c]	S315T	AGC/ACC		156	
		S315T	AGC/ACG		1	
		S315N	AGC/AAC		3	
		S315R	AGC/AGA		1	
	katG + *inhA*	S315T	AGC/ACC	T-8C	3	
		S315T	AGC/ACC	T-8G	1	
		S315T	AGC/ACC	C-15T	27	
		S315N	AGC/AAC	C-15T	1	
		S315T	AGC/ACC	G-17T	1	
	inhA			C-15T	9	
EMB	*embB*	M306V	ATG/GTG		58	5
		M306V	ATG/GTA		5	
		M306I	ATG/ATA		8	6
		M306I	ATG/ATT		7	8
		M306I	ATG/ATC		1	
		M306L	ATG/CTG		1	2
		A313V	GCC/GTC		1	
		Y315C	TAC/TGC		1	

[a] ins, insertion; del, deletion; nt, nucleotide.
[b] Heteroresistant isolate, i.e., mixed wild-type and mutated sequences.
[c] The *katG* locus could not be amplified for one of the phenotypically resistant isolates.

however, not a concern for the downstream sequences of F1, as the read length is generally sufficient to cover two sequencing primers, and mutations at the F1 primer site are rarely found among clinical isolates. In fact, sequencing primer F1 anneals upstream of the RRDR. Still, repeatedly failed sequencing reactions provide an indication that the sequencing primer cannot anneal to the specific annealing site, and we consider that the method failed to detect drug resistance in the case where an isolate had mutations in codons 505 and 511. Eleven phenotypically RIF-resistant isolates in this study did not harbor a mutation in the RRDR and were thus misclassified as susceptible by the molecular method. This may reflect the inherent limitations of phenotypic DST but could also be due to *rpoB* mutations outside the RRDR (14, 49) or elsewhere in the genome.

The INH assay (*katG* and *inhA* promoter region) was 100% specific and demonstrated high sensitivity. The link between INH

TABLE 3 Mutations identified within loci associated with resistance to the second-line drugs amikacin, kanamycin, capreomycin, and ofloxacin in clinical isolates of *M. tuberculosis*

Drug	Locus(i)	Amino acid change(s)	Codon change(s)	Nucleotide change(s)	No. of isolates with indicated phenotypic DST result	
					Resistant	Susceptible
AMK	*rrs*			A1401G	69[a]	
KAN	*rrs*			A1401G	65[b]	
	rrs + *eis*			A1401G + G-6T	1	
				A1401G + C-12T	1	
				A1401G + G-37T	1[c]	
	eis			G-10A	14	38
				C-12T	1	5
				C-14T	11	1
				C-15G		1
				G-37T	3	
CAP	*rrs*			A1401G	62[b]	4[b]
OFL	*gyrA*	D89N	GAC/AAC		1	
		A90V	GCG/GTG		23	
		A90V + S91P	GCG/GTC + TCG/CCG		1	
		A90V + D94N	GCG/GTG + GAC/AAC		1	
		S91P	TCG/CCG		4[b]	
		D94G	GAC/GGC		13	
		D94G	GAC/GGT		1	
		D94A	GAC/GCC		6	
		D94N	GAC/AAC		3	
		D94H	GAC/CAC		1	
		D94Y	GAC/TAC		1	

[a] Two isolates were heteroresistant, i.e., mixed wild-type and mutated sequences.
[b] One isolate was heteroresistant.
[c] Heteroresistant isolate.

and catalase peroxidase (KatG) originally came from the observation that some highly resistant clinical isolates of *M. tuberculosis*, with *katG* deleted, were catalase negative (67). We suspect that one of the INH-resistant isolates in this study had this genotype, as we were consistently unable to amplify the *katG* gene of this isolate, and this result was confirmed by the failure of another independent *katG* PCR assay. As the pyrosequencing assay is designed to detect mutations, we consider that the method failed to detect drug resistance in this case. It is possible that the 12 phenotypically resistant isolates lacking a mutation in either locus have a mutation elsewhere in the genome. Mutations in the open reading frame of *inhA* confer INH resistance (3); however, these mutations are rarely found in clinical isolates and usually occur together with mutations in *katG* and the promoter region of *inhA* (60). Consequently, we chose not to include the opening reading frame of *inhA* in the molecular method, as well as other genes, such as *aphC* and *kasA*, as there are limited data on their role in INH resistance (25, 60).

The *embB* pyrosequencing assay detected mutations in both phenotypically EMB-resistant and EMB-susceptible isolates (Table 2), suggesting that these mutations are a poor indicator of EMB resistance. The sensitivity (61.2%) and specificity (84.8%) of detection of EMB resistance were indeed lower than were seen with the other drugs; however, this does not necessarily mean that the assay has a low predictive value for clinically relevant EMB resistance. Conventional phenotypic EMB DST for *M. tuberculosis* is notoriously problematic (12, 27). Several studies, including allelic exchange experiments, have demonstrated a strong association between *embB* mutations, specifically at codon 306, and EMB resistance (35–37, 44, 51, 52). It has also been shown that there is a narrow range between the MICs of EMB-susceptible and EMB-resistant isolates of *M. tuberculosis* (15, 47). Some mutations, e.g., the M306I substitution, give rise to a modest increase in the MIC (37, 44, 52), which may explain why this particular mutation was found in so many EMB-susceptible isolates in this study. It is likely that the EMB MIC increases only moderately for the *embB* A313V substitution, as we detected it in a phenotypically resistant isolate, and others have found it in EMB-susceptible isolates (6). A possible clinical implication is that all *embB* 306 mutants should be treated as isolates with altered EMB susceptibility, even if they appear to be EMB susceptible by conventional DST. Mutations in codon 497 in the *embB* gene have been reported in EMB-resistant clinical isolates (20) and could possibly be included to increase the sensitivity of the method. Furthermore, mutations in *embC* and *embA* may also be involved (41).

Despite the relatively long distance between the mutations in *rrs*, the pyrosequencing assay for this locus could be optimized for only one sequencing reaction without compromising mutation detection. The specificities were high for AMK, KAN, and CAP (100%, 98.4%, and 97.2%, respectively), whereas the sensitivities were lower (82.1%, 83.7%, and 79.5%). The A1401G mutation in *rrs* was observed in isolates resistant to all the three agents. One isolate, which was found to be phenotypically resistant to AMK, KAN, and CAP, was identified as susceptible by the molecular

method. It is possible that this isolate was heteroresistant but that the proportion of resistant organisms was not large enough to be detected by the pyrosequencing assay. Nevertheless, alternative drug resistance mechanisms cannot be ruled out. Four CAP-susceptible isolates were identified as resistant by the pyrosequencing assay (*rrs* 1401 mutation); however, the MIC (4 mg/liter) for these strains was close to the critical concentration for CAP (11). Overexpression of *eis* confers low-level KAN resistance (66). Mutations at certain positions, such as −14 and −37, give rise to a higher overexpression of *eis* than other *eis* promoter mutations (66) and can thus be considered more sensitive genetic markers for KAN resistance. All but one of the isolates with −14 or −37 mutations in this study were classified as phenotypically resistant. Mutations at position −10 and, in particular, at position −12 seem to give rise to a MIC close to the critical concentration, as isolates harboring these mutations have been reported as both KAN susceptible and KAN resistant (6, 11, 66). Because of their poor predictive value, we chose not to take mutations at these positions into account when calculating sensitivity and specificity for KAN. The previously unreported C-15T *eis* promoter mutation was detected in a KAN-susceptible isolate; however, further studies must be undertaken in order to clarify its role in *eis* expression. It should also be noted that all but one of the AMK-resistant clinical isolates lacking an *rrs* mutation did have a mutation (G-10A, C-14T, or G-37T) in the promoter region of *eis*. Overexpression of the Eis protein also leads to inactivation of AMK, but the inactivation is 3-fold less than that seen for KAN (66). As with EMB, this suggests that isolates with *eis* promoter mutations should be treated as isolates with altered KAN, and plausibly AMK, susceptibility, regardless of the phenotypic DST results. CAP resistance is also known to be conferred by mutations in *tlyA* (29); however, we chose not to include this gene in the molecular method, since *tlyA* mutations are rare (6, 11) and some have also been found also in CAP-susceptible isolates (11). Furthermore, in order to include *tlyA* as a genetic marker for CAP, the entire open reading frame must be analyzed, and this is not practically feasible by pyrosequencing.

The specificity of the OFL assay was 100%, although the sensitivity was lower. Eight of the 62 phenotypically OFL-resistant isolates lacked a mutation in the *gyrA* locus. In these cases, it is not certain that a mutation accounting for this phenotype would be found elsewhere in *gyrA*, or in *gyrB*, as other drug resistance mechanisms, such as efflux pumps, have been suggested to contribute to FQ resistance in *M. tuberculosis* (10). The *gyrB* locus was not included in this assay, as mutations in this gene occur at a much lower frequency and usually in association with *gyrA* mutations (9, 68). There were equal distributions of the *gyrA* S95T mutation among both susceptible and resistant clinical isolates; in fact, it was detected in 88% of the cases in both groups. This mutation is considered to be a polymorphism not associated with resistance to FQs (50).

In general, the noted sensitivity rates for detection of resistance (Table 1) reflect the incomplete understanding of resistance mechanisms associated with all drugs investigated in this study. Phenotypically resistant isolates lacking mutations in known resistance-associated loci present a problem for all molecular tests designed to detect mutations associated with resistance. This emphasizes the need for intensifying research that aims at identifying novel resistance mechanisms in *M. tuberculosis*.

We have developed a high-throughput molecular method for simultaneous detection of resistance to RIF, INH, EMB, AMK, KAN, CAP, and OFL in *M. tuberculosis*. A rapid and specific diagnosis is highly important not only for the individual patient but also from the general public perspective, since rapid modification to an effective drug therapy would reduce the spread of TB in the society. The short turnaround time in combination with a multilocus sequencing of several isolates in parallel makes pyrosequencing an attractive method for drug resistance screening in *M. tuberculosis*. As the method is PCR based, it has a great potential to be further developed for application directly to clinical samples, e.g., sputum samples.

ACKNOWLEDGMENTS

This work was supported by EC project FP7-HEALTH-2007-A-201690 (FAST-XDR-DETECT) and the National Council of Science and Technology (CONICET), Argentina.

We gratefully acknowledge Juan-Carlos Palomino, Anandi Martin, and Fabienne Paasch for assistance with the study design and providing clinical isolates, Jim Werngren and Juan Carlos Toro for technical assistance, Ramona Groenheit, Solomon Ghebremichael, and Alexandra Pennhag for kindly providing DNA samples, and Diarmaid Hughes, Margareta Krabbe, and David Herthnek for valuable comments on the manuscript.

REFERENCES

1. **Alangaden GJ, et al.** 1998. Mechanism of resistance to amikacin and kanamycin in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **42**:1295–1297.
2. **Arnold C, et al.** 2005. Single-nucleotide polymorphism-based differentiation and drug resistance detection in Mycobacterium tuberculosis from isolates or directly from sputum. Clin. Microbiol. Infect. **11**:122–130.
3. **Banerjee A, et al.** 1994. inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science **263**:227–230.
4. **Bravo LT, et al.** 2009. Pyrosequencing for rapid detection of Mycobacterium tuberculosis resistance to rifampin, isoniazid, and fluoroquinolones. J. Clin. Microbiol. **47**:3985–3990.
5. **Campbell EA, et al.** 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. Cell **104**:901–912.
6. **Campbell PJ, et al.** 2011. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **55**:2032–2041.
7. **Camus JC, Pryor MJ, Medigue C, Cole ST.** 2002. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. Microbiology **148**:2967–2973.
8. **Centers for Disease Control.** 2006. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs—worldwide, 2000–2004. MMWR Morb. Mortal. Wkly. Rep. **55**:301–305.
9. **Chakravorty S, et al.** 2011. Rapid detection of fluoroquinolone-resistant and heteroresistant Mycobacterium tuberculosis by use of sloppy molecular beacons and dual melting-temperature codes in a real-time PCR assay. J. Clin. Microbiol. **49**:932–940.
10. **da Silva PE, Von Groll A, Martin A, Palomino JC.** 2011. Efflux as a mechanism for drug resistance in Mycobacterium tuberculosis. FEMS Immunol. Med. Microbiol. **63**:1–9.
11. **Engström A, Perskvist N, Werngren J, Hoffner SE, Jureen P.** 2011. Comparison of clinical isolates and in vitro selected mutants reveals that tlyA is not a sensitive genetic marker for capreomycin resistance in Mycobacterium tuberculosis. J. Antimicrob. Chemother. **66**:1247–1254.
12. **Griffith ME, Bodily HL.** 1992. Stability of antimycobacterial drugs in susceptibility testing. Antimicrob. Agents Chemother. **36**:2398–2402.
13. **Hall TA.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41**:95–98.
14. **Heep M, et al.** 2001. Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical Mycobacterium tuberculosis isolates. J. Clin. Microbiol. **39**:107–110.
15. **Heifets LB, Iseman MD, Lindholm-Levy PJ.** 1986. Ethambutol MICs and

MBCs for Mycobacterium avium complex and Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **30**:927–932.
16. **Helb D, et al.** 2010. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J. Clin. Microbiol. **48**:229–237.
17. **Hillemann D, Rusch-Gerdes S, Richter E.** 2007. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of Mycobacterium tuberculosis strains and clinical specimens. J. Clin. Microbiol. **45**:2635–2640.
18. **Hillemann D, Rusch-Gerdes S, Richter E.** 2009. Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of Mycobacterium tuberculosis strains and clinical specimens. J. Clin. Microbiol. **47**:1767–1772.
19. **Hofmann-Thiel S, et al.** 2009. Mechanisms of heteroresistance to isoniazid and rifampin of Mycobacterium tuberculosis in Tashkent, Uzbekistan. Eur. Respir. J. **33**:368–374.
20. **Huang WL, Chi TL, Wu MH, Jou R.** 2011. Performance assessment of the GenoType MTBDRsl test and DNA sequencing for detection of second-line and ethambutol drug resistance among patients infected with multidrug-resistant Mycobacterium tuberculosis. J. Clin. Microbiol. **49**: 2502–2508.
21. **Johansen SK, Maus CE, Plikaytis BB, Douthwaite S.** 2006. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2′-O-methylations in 16S and 23S rRNAs. Mol. Cell **23**:173–182.
22. **Johnsson K, Schultz PG.** 1994. Mechanistic studies of the oxidation of isoniazid by the catalase peroxidase from Mycobacterium tuberculosis. J. Am. Chem. Soc. **116**:7425–7426.
23. **Jureen P, et al.** 2006. Rapid detection of rifampin resistance in Mycobacterium tuberculosis by pyrosequencing technology. J. Clin. Microbiol. **44**: 1925–1929.
24. **Jureen P, Werngren J, Hoffner SE.** 2004. Evaluation of the line probe assay (LiPA) for rapid detection of rifampicin resistance in Mycobacterium tuberculosis. Tuberculosis (Edinb.) **84**:311–316.
25. **Kiepiela P, Bishop KS, Smith AN, Roux L, York DF.** 2000. Genomic mutations in the katG, inhA and aphC genes are useful for the prediction of isoniazid resistance in Mycobacterium tuberculosis isolates from Kwazulu Natal, South Africa. Tuber. Lung Dis. **80**:47–56.
26. **Larsen MH, et al.** 2002. Overexpression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in Mycobacterium smegmatis, M. bovis BCG and M. tuberculosis. Mol. Microbiol. **46**:453–466.
27. **Madison B, et al.** 2002. Multicenter evaluation of ethambutol susceptibility testing of mycobacterium tuberculosis by agar proportion and radiometric methods. J. Clin. Microbiol. **40**:3976–3979.
28. **Maus CE, Plikaytis BB, Shinnick TM.** 2005. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **49**:3192–3197.
29. **Maus CE, Plikaytis BB, Shinnick TM.** 2005. Mutation of tlyA confers capreomycin resistance in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **49**:571–577.
30. **Miller LP, Crawford JT, Shinnick TM.** 1994. The rpoB gene of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **38**:805–811.
31. **Moazed D, Noller HF.** 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature **327**:389–394.
32. **Musser JM.** 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin. Microbiol. Rev. **8**:496–514.
33. **Nyrén P.** 2007. The history of pyrosequencing. Methods Mol. Biol. **373**: 1–14.
34. **Piton J, et al.** 2010. Structural insights into the quinolone resistance mechanism of Mycobacterium tuberculosis DNA gyrase. PLoS One **5**:e12245.
35. **Plinke C, et al.** 2009. Tuberculosis ethambutol resistance: concordance between phenotypic and genotypic test results. Tuberculosis (Edinb.) **89**: 448–452.
36. **Plinke C, Rusch-Gerdes S, Niemann S.** 2006. Significance of mutations in embB codon 306 for prediction of ethambutol resistance in clinical Mycobacterium tuberculosis isolates. Antimicrob. Agents Chemother. **50**: 1900–1902.
37. **Plinke C, Walter K, Aly S, Ehlers S, Niemann S.** 2011. Mycobacterium tuberculosis embB codon 306 mutations confer moderately increased resistance to ethambutol in vitro and in vivo. Antimicrob. Agents Chemother. **55**:2891–2896.
38. **Prammananan T, et al.** 1998. A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in Mycobacterium abscessus and Mycobacterium chelonae. J. Infect. Dis. **177**:1573–1581.
39. **Quémard A, et al.** 1995. Enzymatic characterization of the target for isoniazid in Mycobacterium tuberculosis. Biochemistry **34**:8235–8241.
40. **Ramaswamy S, Musser JM.** 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tuber. Lung Dis. **79**:3–29.
41. **Ramaswamy SV, et al.** 2000. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **44**:326–336.
42. **Ronaghi M, Uhlen M, Nyren P.** 1998. A sequencing method based on real-time pyrophosphate. Science **281**:363, 365.
43. **Rozwarski DA, Grant GA, Barton DH, Jacobs WR, Jr, Sacchettini JC.** 1998. Modification of the NADH of the isoniazid target (InhA) from Mycobacterium tuberculosis. Science **279**:98–102.
44. **Safi H, Sayers B, Hazbon MH, Alland D.** 2008. Transfer of embB codon 306 mutations into clinical Mycobacterium tuberculosis strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob. Agents Chemother. **52**:2027–2034.
45. **Sandegren L, et al.** 2011. Genomic stability over 9 years of an isoniazid resistant Mycobacterium tuberculosis outbreak strain in Sweden. PLoS One **6**:e16647.
46. **Sandgren A, et al.** 2009. Tuberculosis drug resistance mutation database. PLoS Med. **6**:e2.
47. **Schön T, et al.** 2009. Evaluation of wild-type MIC distributions as a tool for determination of clinical breakpoints for Mycobacterium tuberculosis. J. Antimicrob. Chemother. **64**:786–793.
48. **Scorpio A, Zhang Y.** 1996. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat. Med. **2**:662–667.
49. **Siu GK, et al.** 2011. Mutations outside the rifampicin resistance-determining region associated with rifampicin resistance in Mycobacterium tuberculosis. J. Antimicrob. Chemother. **66**:730–733.
50. **Sreevatsan S, et al.** 1997. Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. U. S. A. **94**:9869–9874.
51. **Sreevatsan S, et al.** 1997. Ethambutol resistance in Mycobacterium tuberculosis: critical role of embB mutations. Antimicrob. Agents Chemother. **41**:1677–1681.
52. **Starks AM, Gumusboga A, Plikaytis BB, Shinnick TM, Posey JE.** 2009. Mutations at embB codon 306 are an important molecular indicator of ethambutol resistance in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **53**:1061–1066.
53. **Sun Z, et al.** 2008. Comparison of gyrA gene mutations between laboratory-selected ofloxacin-resistant Mycobacterium tuberculosis strains and clinical isolates. Int. J. Antimicrob. Agents **31**:115–121.
54. **Suzuki Y, et al.** 1998. Detection of kanamycin-resistant Mycobacterium tuberculosis by identifying mutations in the 16S rRNA gene. J. Clin. Microbiol. **36**:1220–1225.
55. **Takayama K, Kilburn JO.** 1989. Inhibition of synthesis of arabinogalactan by ethambutol in Mycobacterium smegmatis. Antimicrob. Agents Chemother. **33**:1493–1499.
56. **Takiff HE, et al.** 1994. Cloning and nucleotide sequence of Mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone resistance mutations. Antimicrob. Agents Chemother. **38**:773–780.
57. **Taniguchi H, et al.** 1997. Molecular analysis of kanamycin and viomycin resistance in Mycobacterium smegmatis by use of the conjugation system. J. Bacteriol. **179**:4795–4801.
58. **Telenti A, et al.** 1993. Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis. Lancet **341**:647–650.
59. **Telenti A, et al.** 1997. The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. Nat. Med. **3**:567–570.
60. **Vilchèze C, and Jacobs WR, Jr.** 2007. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu. Rev. Microbiol. **61**:35–50.
61. **Wang JC.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665–697.
62. **World Health Organization.** 2009. Guidelines for surveillance of drug resistance in tuberculosis. WHO/HTM/TB/2009.422. World Health Organization, Geneva, Switzerland.
63. **World Health Organization and Stop Partnership TB.** 2010. The global plan to stop TB 2011-2015: transforming the fight towards elimination of tuberculosis. World Health Organization, Geneva, Switzerland.

64. **World Health Organization.** 2010. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response, WHO/HTM/TB/2010, 3rd ed. World Health Organization, Geneva, Switzerland.
65. **Xu C, Kreiswirth BN, Sreevatsan S, Musser JM, Drlica K.** 1996. Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis. J. Infect. Dis. **174**:1127–1130.
66. **Zaunbrecher MA, Sikes RD, Jr, Metchock B, Shinnick TM, Posey JE.** 2009. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U. S. A. **106**:20004–20009.
67. **Zhang Y, Heym B, Allen B, Young D, Cole S.** 1992. The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature **358**:591–593.
68. **Zhang Y, Yew WW.** 2009. Mechanisms of drug resistance in Mycobacterium tuberculosis. Int. J. Tuberc. Lung Dis. **13**:1320–1330.