

RAPID, SIMPLE, AND CULTURE-INDEPENDENT DETECTION OF *rpsL* CODON 43 MUTATIONS THAT ARE HIGHLY PREDICTIVE OF STREPTOMYCIN RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

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Abstract. The substitution of codon 43 in the gene *rpsL* is the single most common mutation found in streptomycin-resistant *Mycobacterium tuberculosis*. The characterization of this mutation has been hampered by the need for prior cultivation of the mycobacteria, the need for DNA sequencing, or both. In this report we describe a simple and culture-independent technique to detect this mutation directly from sputum samples, requiring little more than a polymerase chain reaction (PCR) machine and a simple agarose minigel. There is no need for labeled probes or DNA sequencing. In a preliminary test of feasibility, interpretable results were obtained from all of 16 smear-positive and 1 of 4 smear-negative, culture-positive samples. Two of two samples containing *M. tuberculosis* with *rpsL* codon 43 mutations were correctly identified.

INTRODUCTION

Streptomycin (SM) is a first-line antituberculosis drug but it must be administered parenterally. In high income countries, it is often substituted by ethambutol if a fourth drug is desired in addition to rifampicin, isoniazid (INH), and pyrazinamide during the initial phase of therapy. In low income countries, however, even smear-positive tuberculosis patients may have to be treated with a combination of the inexpensive drugs thioacetazone and INH, and SM is recommended as a third drug during the initial 2 months of chemotherapy.¹ In these situations, the detection of drug resistance to SM in addition to INH would be highly desirable not only for the individual patient but also for the timely detection of a changing resistance situation in a given population. Due to limitations in financial resources, it may not be possible to determine drug resistances or even to culture mycobacteria from many of these patients. It has further been argued that simple methods to detect drug resistances would be useful for monitoring the efficacy of tuberculosis control strategies and that paradoxically, non-culture approaches may be easier to implement than the laborious and costly techniques required for conventional *M. tuberculosis* culture and susceptibility testing.² Thus, in principle, the characterization of genomic mutations that are highly predictive for drug resistances offers the possibility for a culture-independent resistance detection. In this report, we describe a simple PCR approach to detect the most frequently found mutation in SM resistant *Mycobacterium tuberculosis*, a substitution in codon 43 of the gene *rpsL*, directly from sputum samples. We describe the results of a preliminary study and compare them to those obtained by the proportion method and by DNA sequencing.

MATERIALS AND METHODS

Sputum samples and DNA isolation. Twenty sputum samples were collected from 20 patients from Germany (no. 1–6 and 17–20), Italy (no. 7), France (no. 8–12), and Spain (no. 13–16), known or suspected to be infected with SM

resistant *M. tuberculosis* (Table 1). The samples were processed as for routine culture by shaking for 20 minutes at room temperature with an equal volume of decontaminating solution (2% NaOH, 1.5% sodium citrate, 5 g/l *N*-acetyl-L-cysteine). After adjusting the volume to 50 ml with phosphate buffered saline (PBS, pH 7.4) the suspension was centrifuged at $3,500 \times g$ for 25 minutes and the pellet was resuspended in 1 ml PBS. Approximately half of this material was used for microscopy and cultures. Resistance was determined by the 1% proportion method (DIN [German Institute for Standardization] standard no. 58943, part 8). All SM resistant isolates were resistant at a level of at least 4 $\mu\text{g/ml}$. The other half of the decontaminated mycobacterial suspension was heated to 80°C or higher for at least 10 minutes before mailing it to the PCR laboratory. There, the suspension was pelleted in a microcentrifuge for 2 minutes, resuspended in 100 μl 1 M NaOH, 2% Triton X-100, boiled for 5 minutes and neutralized with 100 μl unbuffered 1 M Tris-HCl.³ After pelleting debris in a microcentrifuge for 3 minutes the DNA in the aqueous phase was adsorbed to 5 μl of a silica gel suspension (GeneClean; BIO 101, La Jolla, CA) and eluted into 100 μl 10 mM Tris-HCl (pH 8.3).

Primer construction and nested PCR. The outer primers MYC-21 (5'-CGC TGC AGC AGC TGG T(A,C)C GCA A-3') and MYC-22 (5'-CGG AAT TCG GTT CTT (G,C)AC ACC CTG-3') have been described previously.⁴ The inner primers MYC-36 (5'-AAG GTC AAG ACC GCG GC-3') and MYC-37 (5'-TGA TCT TGT AGC GCA CAC-3') were constructed to asymmetrically encompass codon 43 (AAG) with its overlapping *Mbo*II restriction enzyme site (GAA-GA).⁵ For the first PCR, 1 μl each of 50 μM solutions of the outer primers MYC-21 and MYC-22, and for the second (nested) PCR equal amounts and concentrations of the inner primers MYC-36 and MYC-37 were used. The PCR reactions were done employing a hot start technique in which 3 μl of the DNA eluate and 34 μl water for the first PCR, or 2 μl from the first PCR and 38.3 μl water for the second PCR, were heated to 96°C for 2 minutes after the addition of 1 μl each of 50 mM solutions of the primers, 2.5 μl (first PCR) or 1.7 μl (nested PCR) of 50 mM MgCl_2 , 2.5 μl di-

TABLE 1
Characterization of sputum specimens*

No.	Country of origin	Ziehl-Neelsen microscopy [†]	Culture media				SM susceptibility (by culture)	Other resistances [‡] (by culture)	<i>rpsL</i> genotype by PCR-RFLP	<i>rpsL</i> genotype by sequencing
			LJ	S	B	C				
1	Germany	+++	pos.	pos.	pos.	n.d.	res.	I	w.t.	w.t.
2	Germany	++	pos.	pos.	pos.	n.d.	sens.	–	w.t.	w.t.
3	Germany	+++	pos.	pos.	pos.	n.d.	sens.	–	w.t.	w.t.
4	Germany	0	neg.	neg.	pos.	n.d.	res.	–	n.p.	n.p.
5	Germany	+++	pos.	pos.	pos.	n.d.	sens.	–	w.t.	w.t.
6	Italy	+++	pos.	n.d.	pos.	n.d.	res.	I, R, E	w.t.	w.t.
7	France	+/-	pos.	n.d.	n.d.	pos.	sens.	–	w.t.	w.t.
8	France	++	pos.	n.d.	n.d.	pos.	sens.	–	w.t.	w.t.
9	France	+++	pos.	n.d.	n.d.	pos.	sens.	–	w.t.	w.t.
10	France	+++	pos.	n.d.	n.d.	pos.	res.	–	mut.	AGG
11	France	+/-	pos.	n.d.	n.d.	pos.	res.	I	w.t.	w.t.
12	France	++	pos.	n.d.	pos.	n.d.	res.	I, R, E	w.t.	w.t.
13	Spain	++	pos.	n.d.	pos.	n.d.	sens.	I, R	w.t.	w.t.
14	Spain	++	pos.	n.d.	pos.	n.d.	sens.	I, E	w.t.	w.t.
15	Spain	+	pos.	n.d.	pos.	n.d.	sens.	I, R	w.t.	w.t.
16	Spain	+++	pos.	n.d.	pos.	n.d.	sens.	I	w.t.	w.t.
17	Germany	0	pos.	pos.	pos.	n.d.	res.	I, R, E	n.p.	n.p.
18	Germany	0	pos.	pos.	pos.	n.d.	res.	I, R	n.p.	n.p.
19	Germany	++++	pos.	pos.	pos.	n.d.	res.	I, R, E	mut.	AGG
20	Germany	0	pos.	pos.	neg.	n.d.	res.	I, R, E	w.t.	w.t.

* LJ = Löwenstein-Jensen; S = Stonebrink; B = Bactec; C = Coletsos; pos. = positive; neg. = negative; res. = resistant; sens. = sensitive; w.t. = wild-type; mut. = mutation; n.d. = not done; n.p. = no PCR product.

[†] 0 = no acid fast rods in entire smear; +/- = 1–3 acid fast rods in entire smear; + = 4–10 acid fast rods per 100 fields of vision; ++ = 10–100 acid fast rods per 100 fields of vision; +++ = 100–1,000 acid fast rods per 100 fields of vision; ++++ = more than 1,000 acid fast rods per 100 fields of vision (1000× magnification).

[‡] Resistances to isoniazid (I), rifampicin (R), and ethambutol (E) were tested.

methyl sulfoxide (first PCR only) and two drops of mineral oil. After cooling to 85°C, 6 µl of a freshly prepared mixture of 5 µl buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 0.5 µl of dNTP mix (25 mM each) and 0.5 µl (5 u/µl) of *Taq* DNA polymerase was added. The most sensitive signal-to-background relation was observed after 50 cycles for the first, and 30 cycles for the nested PCR of denaturation at 92°C for 60 seconds, annealing at 55°C (first PCR) or 65°C (nested PCR) for 60 seconds, and extension at 72°C for 60 seconds.

Restriction endonuclease digests. 4 µl of the nested PCR reaction, 4 µl water, 1 µl of digestion buffer (Nr. 4; New England Biolabs), 0.5 µl (12 u/µl) of MboII, and 0.5 µl (15 u/µl) of EcoO109I were mixed and incubated at 37°C for 90 minutes. Alternatively, very faint PCR products could still be analyzed after silica gel adsorption of the remaining PCR product, or, alternatively, after excision and silica gel adsorption of the 241 bp band following electrophoresis in a 2.5% agarose gel containing 0.2 µg/ml ethidium bromide. The elution medium in both cases was 8 µl TE buffer. Restriction products were visualized in a 2.5% agarose gel containing 0.2 µg/ml ethidium bromide.

DNA cloning and sequencing. For direct sequencing, the PCR products were excised from the agarose gel. The DNA was then purified by silica gel adsorption, eluted with 8 µl of TE buffer, and 7 µl of this solution were heated with 1 µl (5 µM) of the primer MYC-36 in a boiling water bath for 5 min. After heating, the solution was snap-frozen in dry ice/ethanol, and the regular sequencing protocol was followed thereafter using a Sequenase 2.0 kit (United States Biochemical Corporation, Cleveland, OH). When there was not enough DNA for direct sequencing or when direct sequencing gave ambiguous results, the PCR products were ligated into pBluescript II SK⁻ vectors (Stratagene, La Jolla, CA), and the constructs were then used to transform XL1-

Blue cells (Stratagene) in order to generate plasmid templates for sequencing.

RESULTS

Nested PCR reactions on DNA with and without *rpsL* codon 43 substitutions gave PCR products of the expected, identical sizes of 0.24 kb (Figure 1). Three of four smear-negative but culture-positive sputum samples did not yield PCR products (Table 1). After MboII digestion, DNA from all of 10 sputum samples subsequently determined to contain SM sensitive *M. tuberculosis* and DNA from five samples with SM resistant *M. tuberculosis* showed bands at 0.13 kb and 0.09 kb, while two sputum samples with SM resistant *M. tuberculosis* lacked these bands (no. 10 and no. 19, Figure 1). In some samples, there were byproducts of 0.16 kb or the undigested PCR product at 0.24 kb, but these did not affect the interpretation of the digestion pattern.

An internal control of the authenticity of the PCR product and completeness of the digestion is the invariant 27 bp product from both mutated and wild type sequences (Figure 1). It is produced by restriction at the EcoO109I site starting 12 nucleotides upstream of the MYC-37 binding site (Figure 2). Even in the absence of an MboII site there is a visible size reduction of the PCR product, even if the 27 bp fragment cannot be seen (Figure 1, samples no. 10 and no. 19). In addition to this 27 bp product, the wild type sequence will give two more products of 86 bp and 128 bp, while the SM resistant genotype with a codon 43 substitution will result in an undigested product of 214 bp (Figure 2). The best diagnostic aid is the absence of digestion products between 0.1 kb and 0.2 kb (Figure 1, samples no. 10 and no. 19).

The results of the restriction enzyme digestions were confirmed by sequencing in all cases (Table 1). The 2 samples lacking digestion products between 0.1 and 0.2 kb (no. 10

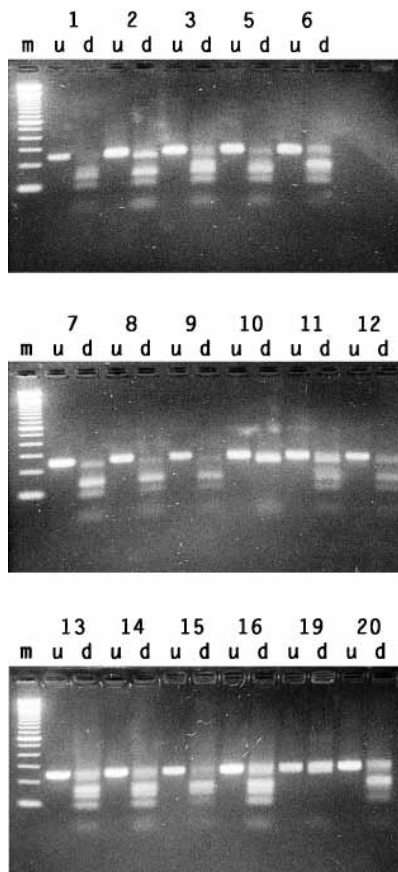


FIGURE 1. *Mbo*II and *Eco*O109I restriction products of *rpsL* PCR products of SM-sensitive and SM-resistant *M. tuberculosis* from sputum samples. The absence of digestion products between 0.1 and 0.2 kb is indicative of SM resistance. u = undigested PCR product; d = *Mbo*II- and *Eco*O109I-digested PCR product; m = DNA size marker with bands at 100 bp intervals starting at 100 bp.

and no. 19) contained an AAG (Lys) to AGG (Arg) substitution of codon 43.

DISCUSSION

We describe a rapid, culture independent, low technology approach to detect *rpsL* codon 43 substitutions that are highly predictive of SM resistance. As with any other molecular resistance test, the detection rate is dependent on the prevalence of the particular mutations that are characterized. The prevalence of *rpsL* codon 43 substitutions in SM resistant *M. tuberculosis* can be estimated from eight previous reports, in each of which 25 or more SM resistant isolates (cultures) were examined.^{4,6-12} In total, the mutation was found in 146 (42%) of 345 SM resistant cultures. It was not found in any of the 228 SM sensitive isolates investigated. Among the individual reports there was a considerable variation in the prevalence figures, ranging from 28%–56%. It has been noted earlier that there is a geographic variation in the prevalences of mutations associated with SM resistance.⁴ Keeping this in mind, it appears safe to say that, at least on average, approximately one in three SM resistant isolates can be expected to have a *rpsL* codon 43 substitution.

No prediction for SM resistance can be made for samples

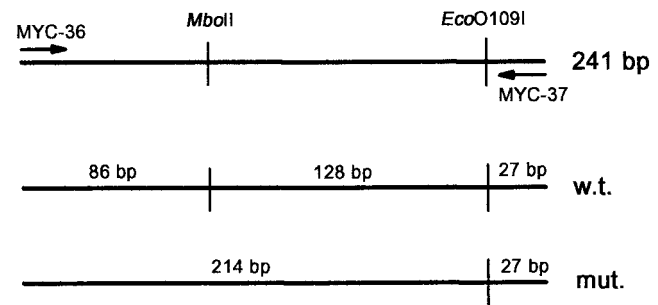


FIGURE 2. Amplification product of the *rpsL* gene with primer and restriction sites (above). Given below are the expected *Mbo*II and *Eco*O109I restriction fragment sizes from the wild-type (w.t.) products and those with codon 43 mutations (mut.) affecting the overlapping *Mbo*II restriction site.

when the wild type DNA sequence is present, indicated by *Mbo*II digestion products between 0.1 kb and 0.2 kb (Figure 1, samples no. 1–9, 11–18, and 20). This is because the wild type sequence could either come from SM sensitive *M. tuberculosis*, or from SM resistant ones without an *rpsL* codon 43 mutation. Further, a 214 bp product in the presence of 128 bp and 86 bp bands (for example in samples no. 2, 7, and 11) should not be used as an indicator for the mutant genotype for 2 reasons. First, this might be caused by an incomplete *Mbo*II restriction of what is actually the wild type genotype. Second, it could be caused by a thus far little studied phenomenon called heteroresistance.¹³ It describes a mixed population of resistant and sensitive *M. tuberculosis*, which would also result in the simultaneous generation of 214 bp, 128 bp, and 86 bp products. Therefore, a reliable statement can only be made for samples with a 214 bp digestion product, but without the 128 bp and 86 bp products. The best diagnostic aid is the absence of products between 0.1 kb and 0.2 kb. These samples can be expected to show the resistant phenotype after culture.

While it was not within the scope of this feasibility study to determine statistically significant figures on sensitivity and specificity, it is noteworthy that all of the 16 microscopically positive samples could be PCR-amplified, including samples with only one to three mycobacteria in the entire smear, and also one of the four microscopically negative, culture-positive samples. The sensitivity may therefore be expected to be comparable to the microscopic detection of mycobacteria. While in-house validations have reported better results for the detection of mycobacteria, blinded multicenter studies report sensitivities of the same order of magnitude.¹⁴⁻¹⁶ The observed detection limit is nevertheless appropriate for the most beneficial application scenario for this method, which is the detection of resistance in infectious patients rather than smear-negative ones.

We envisage two situations for the application of the described technique: First, in individual therapy in the absence of a DNA sequencing facility, especially when the time needed to determine resistances by culture can be expected to be more than the 35-day median determined in a national survey of 54 U.S. hospital mycobacteriology laboratories.¹⁷ During this time the patient might not receive an adequate therapy, remain infectious for a prolonged time, and needlessly take a potentially ototoxic drug. Even if only one in

three patients with SM resistant *M. tuberculosis* can be detected within 2 days instead of more than 5 weeks, this might still be of importance to that one individual.

Second, the described tool may prove useful in epidemiologic surveys when the objective is an estimate on SM resistance distribution, or in sentinel studies when the emphasis is on rapid detection of changes of the local resistance situation. In a global survey of drug resistance, the prevalence of streptomycin resistance in 6 surveyed African countries varied considerably from 1% in Zimbabwe to 29% in Sierra Leone.¹⁸ Such studies are difficult to undertake because the samples must not only be collected, stored and transported in a way that is suitable for the subsequent cultivation but there is also a need for facilities equipped to handle cultures of these potentially multidrug-resistant pathogens and perform resistance tests. In the protocol presented here, the mycobacteria may be killed by heat, transported as non-infectious samples and analyzed in a PCR laboratory without special safety precautions for infectious agents. There is no need for specialized equipment beyond a PCR machine and an agarose minigel chamber. No radioactive or non-radioactive labeled probes and no DNA sequencing is required. The entire procedure can be done in two working days. The preliminary feasibility study on 20 sputum samples has shown that all of 16 smear positive samples and one of four smear negative samples yielded PCR products that could be analyzed by restriction enzyme digestion. These 17 samples included 7 samples subsequently determined to be phenotypically SM resistant after culture, two of which possessed an *rpsL* codon 43 substitution. Both were correctly detected.

A comparably easy genotyping technique has recently been described for detection of isoniazid resistance.¹⁹ The areas of application described above would further benefit from a combination of the two techniques. This is especially true for low-income countries which depend on isoniazid, thioacetazone, and SM for the treatment of tuberculosis.¹ Even if the technique cannot be applied to identify individuals who would benefit the most from the limited resources of additional drugs, a more realistic application might be to detect a rise in resistance.

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