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Detection of *embB* codon 306 mutations in ethambutol resistant *Mycobacterium tuberculosis* directly from sputum samples: a low-cost, rapid approach

H. Rinder,[1]* K. T. Mieskes,[1] E. Tortoli,[2] E. Richter,[3] M. Casal,[4] M. Vaquero,[4] E. Cambau,[5] K. Feldmann[6] and T. Löscher[1]

[1] *Department of Infectious Diseases and Tropical Medicine, Klinikum Innenstadt, University of Munich, Germany,* [2] *Careggi Hospital, Firenze, Italy,* [3] *National Reference Centre for Mycobacteria, Borstel, Germany,* [4] *Mycobacteria Reference Center, University of Córdoba, Spain,* [5] *Pitié-Salpêtrière School of Medicine, University of Paris, France,* [6] *Institute of Laboratory Medicine, Asklepios Clinics, Gauting, Germany*

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Substitutions of codon 306 in the gene *embB* are the most common mutations found in ethambutol resistant *Mycobacterium tuberculosis*. The characterization of these mutations has been hampered by the need for prior cultivation of the mycobacteria, or the need for DNA sequencing, or both. Here, we describe a simple and culture-independent technique to detect *embB* codon 306 mutations directly from sputum samples, requiring little more than a PCR machine and a simple agarose minigel. There is no need for labelled probes or DNA sequencing. In a preliminary test of feasibility, interpretable results were obtained from 21 of 24 selected sputum samples, 12 of which were determined to contain ethambutol resistant *M. tuberculosis* after culture. All of six samples with *embB* codon 306 mutations were correctly identified. Although an exact validation of this technique is beyond the scope of this technical report, we conclude from well-known *embB* codon 306 mutation prevalence figures that approximately one half of EMB resistant cases could already be predicted within 2 working days, with little equipment or hands-on time needed, instead of weeks required for conventional resistance testing.

KEYWORDS: *Mycobacterium tuberculosis*, ethambutol, resistance, *embB*.

INTRODUCTION

Ethambutol (EMB) is a first-line drug in the therapy of tuberculosis. Combined with isoniazid (INH), rifampicin, and pyrazinamide, it is recommended as an alternative to streptomycin as a fourth drug during the initial phase of therapy until susceptibilities are known, unless the patient has no individual risk factors for drug resistance and lives in an area where primary INH resistance is below 4%.

In the majority of cases, resistance to EMB is associated to resistances to other anti-tuberculosis drugs.[1] Therefore, an early detection of EMB resistance would not only abolish risks of adverse reactions, particularly optic neuritis, of an actually ineffective EMB treatment, but it would also give a strong indication for the need for a modification of the therapy regimen.

It is unfortunate that the median times (and average times must be expected to be even longer) needed

* Author to whom correspondence should be addressed at: Department of Infectious Diseases and Tropical Medicine, University of Munich, Leopoldstr. 5, 80802 Munich, Germany. Tel: +49 89 2180 3618; Fax: +49 89 336 112; E-mail: rinder@lrz.uni-muenchen.de

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for cultivation and susceptibility testing has been reported to be between 21 and 60 days, depending on the methods used.[2] If the initial therapeutic regimen is proven ineffective, that time not only increases the time the patient remains infective with a drug resistant strain, but it is also lost for the patient. The early start of an effective therapy with at least two drugs to which the strain is susceptible is a key requirement for a positive treatment outcome in multidrug-resistant tuberculosis.[3,4]

The application of molecular methods for the detection of genomic mutations indicative of drug resistance have been suggested to accelerate reporting times, but they are not widely used because they either require specialized equipment, or are expensive, or both. In this report, we describe a low-cost, low-technology approach to detect, directly from sputum samples, the most frequently found mutation in EMB resistant *Mycobacterium tuberculosis*, a substitution in codon 306 of the gene *embB*.

MATERIALS AND METHODS

Sputum samples and DNA isolation

A total of 24 sputum samples were collected from 24 patients from Germany (no. 1–4 and 21–22), Italy (no. 5–11 and 23–24), France (no. 12–18), and Spain (no. 19–20), known or suspected to be infected with EMB resistant *M. tuberculosis* (Table 1). The samples were processed as for routine culture preparations by shaking 20 min at room temperature with an equal volume of decontaminating solution (2% (w/v) NaOH, 1·5% (w/v) sodium citrate, 5 g/l *N*-acetyl-L-cysteine). After adjusting the volume to 50 ml with phosphate buffer (35·6 mM KH_2PO_4, 31·1 mM $Na_2HPO_4 \cdot 2H_2O$, pH 6·8) the suspension was centrifuged at 3500 ***g*** for 20 min and the pellet was resuspended in 1 ml phosphate buffer. Approximately half of this material was used for microscopy and cultures. After growth, susceptibility testing was performed by the 1% proportion method (DIN 58943, part 8). All EMB resistant isolates were resistant at a level of at least 1 µg/ml EMB. The other half of the decontaminated mycobacterial suspension was heated to 80°C or higher for at least 10 min before mailing it to the PCR laboratory. There, the suspension was pelleted in a microcentrifuge for 2 min, resuspended in 100 µl 1 M NaOH, 2% (v/v) Triton X-100, boiled for 5 min and neutralized with 100 µl unbuffered 1 M Tris-HCl.[5] After pelleting debris in a microcentrifuge for 3 min the DNA in the aqueous phase was adsorbed to 5 µl of a silica gel suspension ('Geneclean'; BIO 101, La Jolla, CA, USA) and eluted into 100 µl Tris-buffer (10 mM Tris-HCl, pH 8·3).

Primer construction and nested PCR

The outer primers MYC-44 (5'-CGT CTA GAT CGC GTT GTG GCG CCT-3') and MYC-45 (5'-CGC TGC AGT CCA CAG ACT GGC GTC GC-3') and the inner primers MYC-46 (5'-CTG CTC TGG CAT GTC AT-3') and MYC-47 (5'-AGC GGA AAT AGT TGG AC-3') were constructed to asymmetrically encompass codon 306 (ATG) which overlaps with an *Nla*III restriction enzyme site (CATG). The inner primers were targeted close to the next neighbouring *Nla*III sites, located 44 bp upstream and 30 bp downstream, in order to avoid additional restriction products that might interfere with the subsequent restriction fragment length polymorphism (RFLP) analysis. The PCR reactions were done employing a 'hot start' technique in which 3 µl of the DNA eluate and 38·2 µl water (or 2 µl of the first PCR and 39·4 µl water for the second, nested PCR) were heated to 96°C for 2 min after the addition of 1 µl each of 50 mM solutions of the primers, 0·8 µl (first PCR) or 0·6 µl (nested PCR) of 50 mM $MgCl_2$, 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. For both the first and the nested PCR, the most sensitive signal-to-background relation was observed after 50 cycles each of denaturation at 92°C for 60 s, annealing at 48°C (first PCR) or 46°C (nested PCR) for 60 s, and extension at 72°C for 60 s.

Restriction endonuclease digests

Four microlitres of the nested PCR reaction, 4 µl water, 1 µl of digestion buffer (Nr. 4; New England Biolabs), and 1 µl (10 u/µl) of *Nla*III were incubated at 37°C for 90 min. Alternatively, very faint PCR products could still be analysed after silica gel adsorption of the remaining PCR product either directly from the PCR product, or from the 103-bp band following electrophoresis and excision from a 2·5% (w/v) agarose gel containing 0·2 µg/ml ethidium bromide, and elution into 8 µl of TE buffer. The latter would also be an option if excessive primer-dimer formation was observed which could later interfere with the RFLP pattern. Restriction products were visualized in a 3% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate (pH 8·5); 1 mM EDTA) containing 0·2 µg/ml ethidium bromide. The agarose is best dissolved on a hot plate

Table 1. Characterization of Sputum Specimens

No.	Country of origin	Ziehl-Neelsen microscopy[a]	Culture media				EMB susceptibility (by culture)	Other resistances[b] (by culture)	*embB* genotype by PCR-RFLP	*embB* genotype by sequencing
			LJ	S	B	C				
1	Germany	+ + + +	pos	pos	pos	n.d.	sens	—	wt	wt
2	Germany	+ + +	pos	pos	pos	n.d.	sens	—	wt	wt
3	Germany	+ + +	pos	pos	pos	n.d.	sens	—	wt	wt
4	Germany	+ + +	pos	pos	pos	n.d.	sens	—	wt	wt
5	Italy	+ +	pos	n.d.	n.d.	n.d.	res	I, R	mut.	GTG
6	Italy	+ +	pos	n.d.	n.d.	n.d.	res	I, R	wt	wt
7	Italy	+ + +	pos	n.d.	n.d.	n.d.	res	I, R	mut.	GTG
8	Italy	+ + +	pos	n.d.	pos	n.d.	res	I, R, S	mut.	GTG
9	Italy	+ +	pos	n.d.	n.d.	n.d.	res	I, R	n.p.	n.p.
10	Italy	+ + +	pos	n.d.	n.d.	n.d.	res	I, R	wt	wt
11	Italy	+ +	pos	n.d.	pos	n.d.	sens	I	wt	wt
12	France	+ + +	pos	n.d.	n.d.	pos	sens	—	wt	wt
13	France	0	pos	n.d.	n.d.	pos	sens	—	wt	wt
14	France	+ + +	pos	n.d.	n.d.	pos	sens	—	n.p.	n.p.
15	France	+ +	pos	n.d.	n.d.	pos	sens	—	wt	wt
16	France	+ + + +	pos	n.d.	n.d.	pos	sens	—	wt	wt
17	France	+ +	pos	n.d.	n.d.	pos	res	I, R, S	mut.	ATC
18	France	0	pos	n.d.	n.d.	pos	sens	I	wt	wt
19	Spain	+ +	pos	n.d.	pos	n.d.	res	I	wt	wt
20	Spain	+ + +	pos	n.d.	pos	n.d.	sens	I	wt	wt
21	Germany	+ + + +	pos	pos	pos	n.d.	res	I, R, S	mut.	GTG
22	Germany	0	pos	pos	neg	n.d.	res	I, R, S	n.p.	n.p.
23	Italy	+ +	pos	n.d.	pos	n.d.	res	I, R	wt	wt
24	Italy	+	pos	n.d.	n.d.	n.d.	res	I, R	mut.	ATA

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

[a] 0, no 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; + + +, 100–1000 acid fast rods per 100 fields of vision; + + + +, more than 1000 acid fast rods per 100 fields of vision (1000 × magnification).

[b] Resistances to isoniazid (I), rifampicin (R), and streptomcin (S) were tested.

with an integrated magnetic stirrer in an Erlenmeyer flask with a smaller, inverted flask on top to reduce evaporation. As the solution becomes more viscous, the stirrer speed should be increased as much as possible, but without squirting. The hot plate should be turned off early enough to allow only a short final boiling of the then clear solution. The ethidium bromide is added to the cooled solution just before pouring the gel.

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-46 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, USA). When there was not enough DNA for direct sequencing or when direct sequencing gave ambiguous results, the second, nested PCR was repeated with primers MYC-46A (5′-CGT CTA GAC TGC TCT GGC ATG TCA T-3′) and MYC-47A (5′-CGC TGC AGC GGA AAT AGT TGG AC-3′), containing *Xba*I and *Pst*I linkers, respectively. The products were ligated into pBluescript II SK$^-$ vectors (Stratagene, La Jolla, CA, USA), 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 *embB* codon 306 substitutions gave PCR products of the expected, identical sizes of 0·1 kb (Fig. 1). Two of 21 smear-positive, and 1 of 3 smear-negative samples did not yield PCR products (Table 1). After *Nla*III digestion, DNA from all of 11 sputum samples

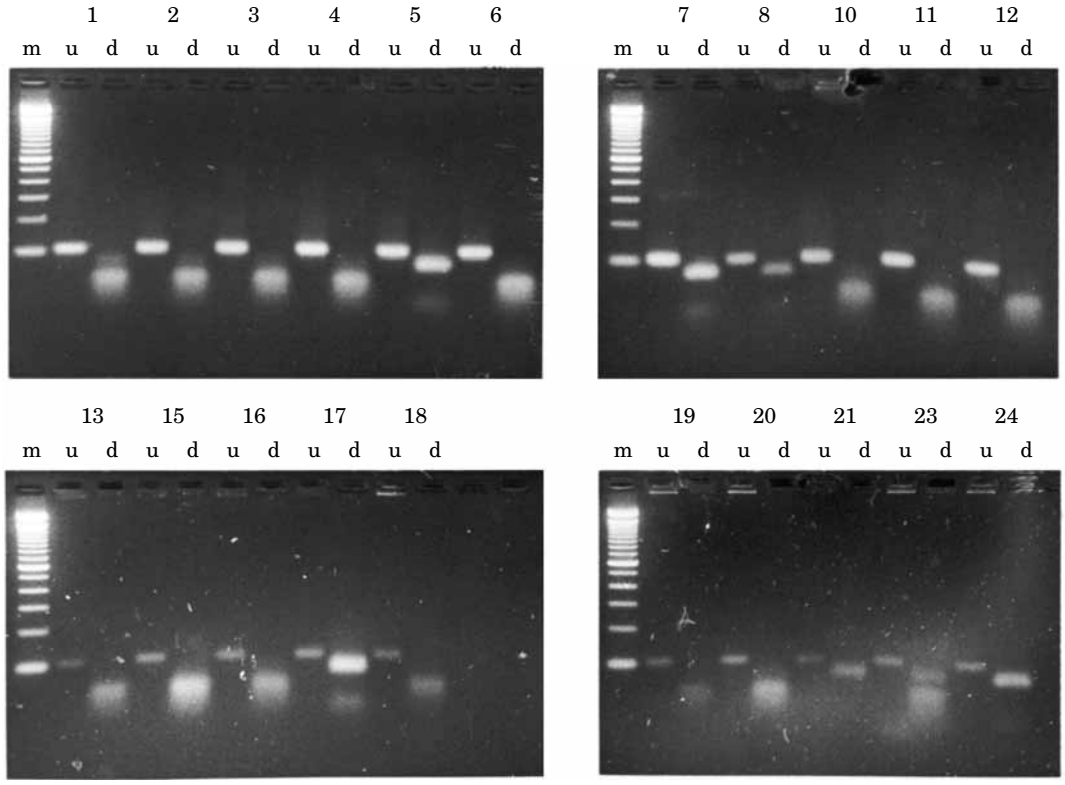

Fig. 1. *Nla*III restriction products of *embB* PCR products of EMB-sensitive and EMB-resistant *M. tuberculosis* from sputum samples. The absence of digestion products in the range of 0·03–0·04 kb is indicative of EMB resistance. m, DNA size marker with bands at 100-bp intervals starting at 100 bp; u, undigested PCR product; d, *Nla*III-digested PCR product.

subsequently determined to contain EMB sensitive *M. tuberculosis* which also yielded a PCR product, and DNA from four samples with EMB resistant *M. tuberculosis* showed products in the range of 0·03 kb to 0·04 kb, while 6 sputum samples, all of them determined to contain EMB resistant *M. tuberculosis* after culture, lacked these bands (no. 5, 7, 8, 17, 21 and 24, Fig. 1).

An internal control of the authenticity of the PCR product and of the completeness of the digestion are the invariant 11-bp and 18-bp products from both mutated and wild-type sequences (Fig. 2). Therefore, even in samples with the mutated genotype which have lost the *Nla*III site overlapping codon 306, there is a visible reduction in the size of the PCR product compared to that of the original product, which can be seen even if the 11-bp and 18-bp products themselves are too faint to be visible (Fig. 1). The wild-type sequence will give two additional products of 30 bp and 44 bp (Fig. 2). The best diagnostic aid to predict EMB resistance is the absence of products in the range of 0·03–0·04 kb.

The results of the restriction enzyme digestions were confirmed by sequencing in all cases (Table 1). The 6 samples lacking digestion products in the range of 0·03 kb to 0·04 kb (no. 5, 7, 8, 17, 21 and 24) contained substitutions of the wild-type codon 306 ATG (Met) to GTG (Val) in four isolates, and to ATC or to ATA (both Ile) in the remaining two samples.

DISCUSSION

Only recently, the *emb* locus was discovered as the drug target for EMB, first in *M. avium*.[6] Then, substitutions of codon 306 in the *M. tuberculosis* gene *embB* were shown to be the most frequent and most predictive mutations for EMB resistance.[7] In the two largest studies to date, this codon was found to be mutated in 39 of 67 (58%) and in 13 of 28 (46%) EMB resistant isolates, but not in 28 and 44 EMB sensitive isolates.[7,8] The described PCR-RFLP technique takes advantage of the fact that the recognition site of the endonuclease *Nla*III (CATG) overlaps completely with the wild-type *embB* codon 306 DNA sequence (ATG). Therefore, any mutation of this codon will lead to a loss of the *Nla*III site.

The technique also allows the detection of mixed

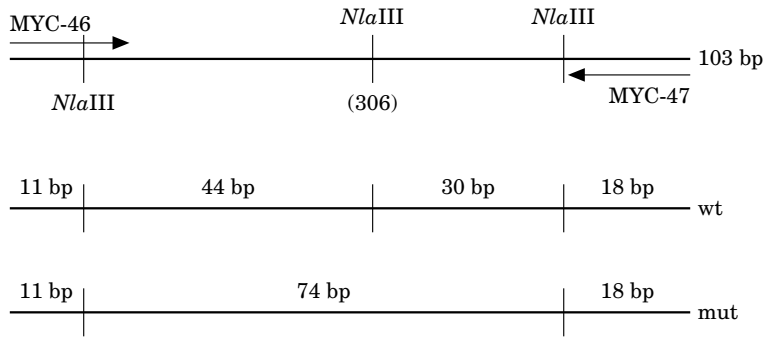

Fig. 2. Amplification product of the *embB* gene with primer and restriction sites (above). Given below are the expected restriction fragment sizes from the wild-type (wt) products and those with codon 306 mutations (mut) affecting the overlapping *Nla*III-restriction site.

populations of the resistant as well as the sensitive genotype within the same specimen. An example for this is sample no. 23 (Fig. 1). A partial digestion as an explanation can be ruled out, because the internal control, as explained in the results, does not show any undigested 103-bp PCR product. The finding is much rather compatible with a, thus far, little studied phenomenon called heteroresistance.[9] The term describes a mixed population of resistant and sensitive *M. tuberculosis*, which would also result in the simultaneous generation of 74-bp, 44-bp and 30-bp products. It is yet unclear how heteroresistance detected in the sputum sample affects the subsequent phenotypic susceptibility testing after cultivation. Therefore, the safest diagnostic criterion to predict EMB resistance is the absence of 30-bp and 44-bp digestion products in samples predicted to be EMB resistant.

As with any other genotype-based resistance prediction test, a limitation in characterizing *embB* codon 306 mutations is that no prediction can be made for samples containing the unmutated genotype, both in isolation and in mixed populations. The wild-type genotype could either come from EMB sensitive cells, or from those EMB resistant organisms that do not carry this mutation. The diagnostic categories are therefore not 'resistant' and 'sensitive', but resistant and 'no prediction possible'. For this reason, it is up to debate if or when a rapid resistance prediction test with high specificity but low sensitivity is useful. The answer may depend on the alternative, which is having to wait for weeks to detect ethambutol resistance by culture in all affected patients rather than detecting half of these cases within 2 days. The difference between the two options may be substantial not only for the individual patient with respect to potential adverse reactions to an unnecessarily given drug as well as the benefit of a timely start of a more effective therapy regimen, but also because of the possibility for rapid intervention strategies in an effort to limit transmissions. It is unfortunate that examples from other resistance genes show that despite its potential, genotypic analysis has not yet found a wide use in routine diagnostic laboratories. One reason for this is that some protocols are dependent on cultured mycobacteria for DNA preparations, thereby minimizing the actual amount of time saved because 2/3 to 3/4 of the time is needed for the initial cultivation. Others require specialized equipment and expertise, for example DNA sequencing and single-strand conformation polymorphism analysis. Yet another aspect is the basic cost per sample, which can be as high as US$ 35.00 for rifampicin resistance testing using a commercial kit (Innogenetics INNO-LiPA RifTB). The basic cost per sample for PCR plus sequencing has been estimated at US$ 10.00, while PCR and electrophoresis cost US$ 1.50 to 3.00.[10] It is therefore not surprising that an 'immediate need' has been ascertained to develop rapid, simple, and accurate assays to assess *M. tuberculosis* DNA polymorphisms.[11]

The presented PCR-RFLP method using the restriction enzyme *Nla*III is both simple and rapid. Results of a preliminary study on a test panel of 24 sputum samples, in comparison with phenotypic resistance determinations and genotypic analysis by DNA sequencing, indicated accuracy, too. All of six samples with phenotypically EMB resistant *M. tuberculosis* and mutations in *embB* codon 306, as determined by DNA sequencing, were correctly identified. We estimate the basic costs per sample at less than US$ 5.00, including 5 U *Taq* polymerase, 10 U *Nla*III, and other consumables. The test can be done within 2 working days with a minimum of actual hands-on time.

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