Culture-Independent Prediction of Isoniazid Resistance in *Mycobacterium tuberculosis* by *katG* Gene Analysis Directly From Sputum Samples

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Background: The molecular prediction of isoniazid (INH) resistance in *Mycobacterium tuberculosis* is hampered by the need for specialized equipment, expertise, high costs, a limited range of detectable mutations, or several of these factors. The rationale for the study was to find a practical alternative and to demonstrate generally valid problems.

Methods and Results: DNA extracted from decontaminated sputum pellets was used to amplify a 0.26 kb target sequence within the *katG* gene. Mutations of codon 315, frequently found in isoniazid-resistant isolates, could be discriminated in a simple agarose minigel format following an *Aci*I digest of the nested polymerase chain reaction (PCR) product. Within a panel of 22 sputum samples, INH resistance could be predicted in 5 of 10 samples containing isoniazid-resistant *M. tuberculosis*. The protocol is robust, requires little expertise and no specialized equipment, and provides the test results within 2 days.

Conclusion: The results show the feasibility to rapidly and easily detect mutations highly predictive of isoniazid resistance. Nevertheless, this, like any other molecular resistance prediction test, is affected by often neglected factors, including mutation prevalences, the phenomenon of heteroresistance, and a possible bias toward one's own method.

Key words: PCR-RFLP, heteroresistance, genotyping.

Many isoniazid (INH) resistances are linked to mutations at a single genetic locus, codon 315 of the catalase-peroxidase gene (*katG*), which leads to a high level of resistance at 1 μg/mL INH or higher [1,2]. INH-resistant isolates without *katG* codon 315 substitutions may have mutations elsewhere in this gene, mostly at codon 463 [3–5], but in contrast to codon 315 mutations these can also be found in INH sensitive isolates [2,4,6,7]. Few INH-resistant isolates have deletions of the entire *katG* gene [8–10], mutations of the gene *inhA* [2,3,5,10], or mutations in other genes, for example *ahpC* [11,12].

At least in principle, the characterization of suitable mutations directly from sputum samples suggests itself for the rapid prediction of resistances in

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Mycobacterium tuberculosis. Several methods have been suggested to achieve this goal, including DNA sequencing and single strand conformation polymorphism (SSCP) analysis. Unfortunately, these methods require specialized equipment (in addition to a thermocycler) and have not yet found a wide application outside research laboratories. As a more practical alternative, restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) products has been suggested in which a *Msp*I restriction site is created by a particular *katG* codon 315 mutation [13]. However, in a subsequent comparison [14] of this method with other genotyping techniques, including SSCP, RFLP was less sensitive because it could detect only one particular mutation out of five already known to occur in INH-resistant *M. tuberculosis* isolates [2,15].

In this report we propose a different PCR-RFLP technique to predict INH resistances directly from sputum samples involving the restriction enzyme *Aci*I. This enzyme recognizes the wild-type genotype and its recognition sequence is lost not only by one but several codon 315 mutations associated with INH resistance. This approach not only increases the spectrum of detectable *katG* codon 315 mutations, but also allows the use of a simple agarose gel for product analysis.

Material and Methods

Sputum Samples and DNA Isolation

Twenty-two Ziehl-Neelsen positive sputum samples were collected from 22 patients from Italy (no. 1–6), Germany (no. 7–18), and France (no. 19–22) known or suspected to be infected with INH-resistant *M. tuberculosis*. The samples were processed as for routine culture preparations by shaking 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 4,170*g* for 25 minutes, and the pellet was washed with 1 mL PBS. Approximately half of this material was used for microscopy and cultures. Resistance was determined by the 1% proportion method (DIN 58943, part 8). All INH-resistant isolates were resistant at a level of at least 0.25 μg/mL INH. The other half of the decontaminated mycobacterial suspension was pelleted in a microcentrifuge for 12 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 [16]. After pelleting debris in a microcentrifuge for 3 minutes, the DNA in the aqueous phase was then adsorbed to 5 μL of a silica gel suspension (Geneclean; BIO 101, La Jolla, CA) and eluted into 30 μL TE buffer (10 mM Tris-HCl, [pH 8.3], 1 mM EDTA).

Primer Construction and PCR

Sequence information of the *M. tuberculosis katG* gene deposited at GenBank (accession number X68081) [17] was used to construct nested PCR primers. The internal primers MYC-32 (5′-TGG AGC AGA TGG GCT TGG-3′) and MYC-33 (5′-CAG TGG CCA GCA TCG TCG-3′) were constructed to asymmetrically encompass codon 315 (AGC) with its overlapping *Aci*I restriction enzyme site (GCGG). Further *Aci*I sites are located 117 bp and 162 bp downstream (Fig. 1). These sites serve the purpose of internal controls for the subsequent *Aci*I digestion because they yield invariant digestion products of 45 bp and 34 bp for both wild-type and mutant genotypes. In addition to these, the wild type sequence will give two more products of 68 bp and 117 bp, whereas the INH resistant genotype with a codon 315 substitution affecting the first *Aci*I site will result in an undigested product of 185 bp. The outer primers MYC-30 (5′-CGA ACC CGA GGC TGC TCC-3′) and MYC-31 (5′-CAC CCG CAG CGA GAG GTC-3′) were constructed adjacent to the internal primers. For the first PCR, 1 μL each of 50 μM solutions of the outer primers MYC-30 and MYC-31, and for the second (nested) PCR equal amounts and concentrations of the inner primers MYC-32 and MYC-33 were used. The PCR reactions were done by us-

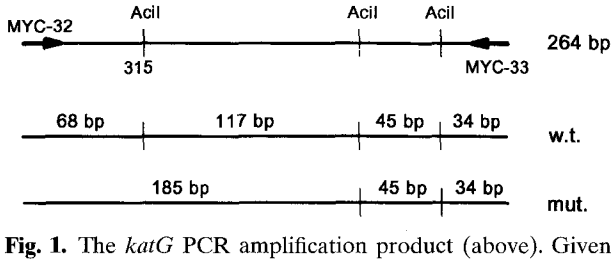

Fig. 1. The *katG* PCR amplification product (above). Given below are the expected *Aci*I restriction fragment sizes from wild-type (w.t.) products and those with codon 315 mutations (mut.) affecting the overlapping *Aci*I restriction site.

ing a "hot start" technique in which 25 μL of the DNA eluate with 14.5 μL water (or 2 μL of the first PCR and 37.5 μL water for the second PCR) were denatured at 96°C for 2 minutes after the addition of 1 μL each of 50 mM solutions of the primers, 1.5 μL of 50 mM $MgCl_2$, 1 μL dimethyl sulfoxide 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 45 cycles of denaturation at 92°C for 60 seconds, annealing at 57°C for 60 seconds and extension at 72°C for 60 seconds for both PCR reactions.

Restriction Endonuclease Digestion

In all, 4 μL of the nested PCR reaction, 4 μL water, 1 μL of digestion buffer (Nr. 3; New England Biolabs, Beverly, MA) and 1 μL (5 u/μL) of *Aci*I (New England Biolabs) were digested for 90 minutes at 37°C. Alternatively, very faint PCR products could still be analyzed after silica gel adsorption of the remaining 47 μL of PCR product either directly or from the 264 bp band after electrophoresis and excision from an agarose gel and elution into 8 μL of 10 mM Tris-HCl [pH 8.3], 1 mM EDTA (TE) buffer. Restriction products were visualized in a 3% agarose gel containing 0.2 μg/mL ethidium bromide. The best diagnostic aid is the absence of a 117 bp band in mutated sequences, which is independent of possible partial digestion products at 185 bp and which separates well from smaller digestion products, even in an agarose gel. The completeness of the digestion could be controlled easily by the absence of the intact 264 bp amplification product.

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-32 in a boiling water bath for 5 minutes. 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 the unambiguous sequence determination of individual PCR product molecules was desired, 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) to generate plasmid templates for sequencing.

Results

Nested PCR reactions on DNA with and without a *katG* codon 315 substitution gave PCR products of the expected, identical sizes of 0.26 kb. Sputum samples with *M. xenopi*, *M. malmoense*, or *M. avium* complex did not yield PCR products. The same was true for three samples with INH-resistant *M. tuberculosis* from Italy (Table 1). After *Aci*I digestion, DNA from the nine sputum samples subsequently determined to contain INH-sensitive *M. tuberculosis* showed bands at 0.12 kb, whereas 5 of 10 sputum samples with INH-resistant *M. tuberculosis* lacked this band (Fig. 2).

The results of the *Aci*I digestions were confirmed by sequencing in all cases (Table 1). Two of the sample (nos. 13 and 21) gave direct sequencing products that were too faint to be readable on an autoradiography film and had to be cloned before sequencing. All 8 clones from sample 21 and 9 of 11 clones from sample 13 possessed the wild-type sequence (AGC) for *katG* codon 315, the remaining 2 clones from sample 13 displayed the silent mutation AGT.

When six clones from sample 16, representing an isolate found to be INH sensitive after cultivation, with only a weak 0.12 kb PCR-RFLP product derived from sputum DNA, were sequenced, three were wild type and one each had an AGC→AAC substitution in codon 315, an GGC→AGC substitution in codon 316 resulting in a loss of the *Aci*I restriction site, and an ACC→AC deletion in codon 314. When 13 clones from sample 18, representing an INH resistant isolate with a 0.12 kb PCR-RFLP product, were sequenced, 11 were wild type, 1 had an AGC→AAC substitution in codon 315, and 1 showed a GGC→GAC substitution in codon 316 resulting in a loss of the *Aci*I restriction site.

Discussion

How Frequent are *katG* Codon 315 Mutations?

Even in recent literature, grossly divergent figures can be found to answer the seemingly trivial

Table 1. Characterization of Sputum Specimens

No.	Species by Culture	INH Susceptibility (by Culture)	Other Resistances* (by Culture)	*katG* Genotype by PCR-RFLP	*katG* Genotype by Sequencing
1	*M. tuberculosis*	res.	R, E	mut.	ACC
2	*M. tuberculosis*	res.	R, E	n.p.	n.p.
3	*M. tuberculosis*	res.	R, S, E	mut.	ACA
4	*M. tuberculosis*	res.	R, E	n.p.	n.p.
5	*M. tuberculosis*	res.	R, E	n.p.	n.p.
6	*M. tuberculosis*	res	—	w.t.	w.t.
7	*M. tuberculosis*	res.	S	mut.	ACC
8	*M. tuberculosis*	sens.	—	w.t.	w.t.
9	*M. xenopi*	res.	R, E	n.p.	n.p.
10	*M. tuberculosis*	res.	—	mut.	ACC
11	*M. malmoense*	res.	—	n.p.	n.p.
12	*M. tuberculosis*	sens.	—	w.t.	w.t.
13	*M. tuberculosis*	sens.	—	w.t.	w.t.
14	*M. tuberculosis*	sens.	S	w.t.	w.t.
15	*M. avium* complex	res.	R, S, E	n.p.	n.p.
16	*M. tuberculosis*	sens.	—	w.t.	w.t.
17	*M. tuberculosis*	sens.	—	w.t.	w.t.
18	*M. tuberculosis*	res.	R, S, E	w.t.	w.t.
19	*M. tuberculosis*	res.	R	mut.	ACC
20	*M. tuberculosis*	sens.	—	w.t.	w.t.
21	*M. tuberculosis*	sens.	—	w.t.	w.t.
22	*M. tuberculosis*	sens.	—	w.t.	w.t.

* Resistances to rifampicin (R), streptomycin (S), and ethambutol (E) were tested.
Abbreviations: res., resistant; sens., sensitive; mut., mutation; w.t., wild type; n.p., no PCR product.

question of how frequent *katG* codon 315 mutations are in INH-resistant isolates. An extensive international survey detected *katG* codon 315 mutation in 55% of INH-resistant isolates [2]. Similar proportions have been found in isolates from the United States (52%) [18], South Africa (68%) [15], and Germany and Sierra Leone (54%) [19]. Interestingly, although all isolates were sequenced in the previously mentioned studies, much smaller prevalences (0 to 14%) have been reported from investigations where SSCP was used to screen for mutations [1,3–5,11]. However, SSCP analysis cannot be regarded as infallible and when one of these studies was double-checked by sequencing the same isolates, the original report [4] had to be retracted as an SSCP artifact, and the initially proposed figure of 0% codon 315 mutations had to be corrected to 52% [18].

Can Molecular Resistance Prediction Substitute for Culture?

Especially in, but not limited to INH resistance, there are drug-resistant isolates that do not possess "specific" mutations, that is, mutations not found in drug-sensitive isolates. Therefore, the performance of genotype-based methods for the prediction of resistances in general and that to INH in particular is dependent not only on the specificity and sensitivity of the particular nucleic acid based detection method itself, but also on the specificity (degree of absence in sensitive isolates) and sensitivity (prevalence in resistant isolates) of the investigated resistance mutation (or mutations) characterized by that method. Even if optimistic claims are accurate that PCR based methods may reach or even surpass culture techniques in sensitivity for the detection of mycobacteria [20], it can never be ruled out that some PCR-negative samples may still be detected by culture, including drug-resistant isolates. Therefore, it is true for the proposed method of INH resistance prediction and probably for molecular resistance predictions in general that molecular techniques are beneficial only in addition to, but not instead of, cultivation. In a possible practical application of the described INH resistance prediction method, a negative result (wild-type sequence) will have no effect on clinical decision making, whereas a positive test (mutation) may result in a significant advantage for each patient when INH resistance can be predicted the next day after the sputum sample is produced in-

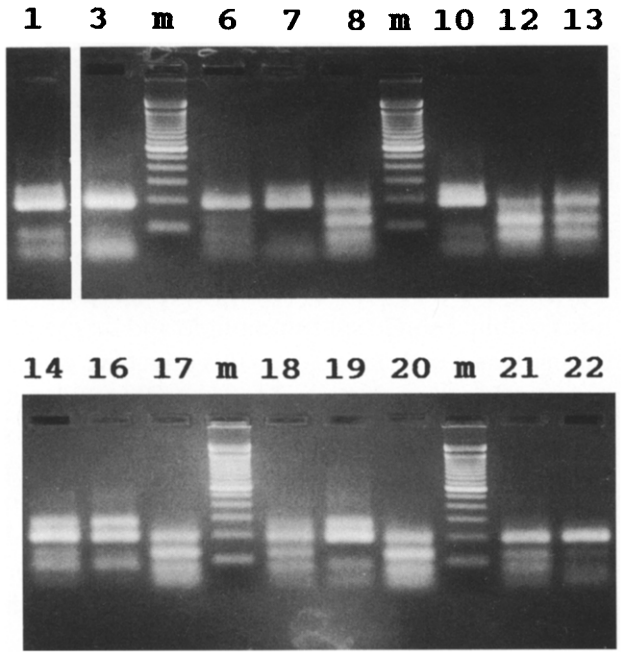

Fig. 2. *Aci*I restriction products of *katG* PCR products of INH-sensitive and INH-resistant *M. tuberculosis* from sputum samples. The absence of a 0.12 kb digestion product is indicative of INH resistance (see discussion). m: DNA size marker with bands at 100 bp intervals starting at 100 bp.

stead of after weeks when the resistance test by culture is available.

Which Technique Should Be Used for INH Resistance Prediction?

First, the genomic targets to be characterized must be carefully selected and only thereafter the method that best characterizes them. Even though a *katG* codon 315 substitution is not found in all INH-resistant isolates; it is important to realize that it is highly predictive of INH resistance and has never been found in INH-sensitive *M. tuberculosis* isolates. This situation is quite different from, for example, mutations in codon 463. Given the limited sensitivity of codon 315 mutations for INH resistance prediction, its advantage to be highly specific should not be compromised by an attempt to raise sensitivity by characterizing other polymorphic sites such as *katG* codon 463. This codon may be found in additional INH resistant isolates, as well as in INH-sensitive ones. Sreevatsan et al. [14] compared three techniques for the detection of *katG* codon 315 mutations, PCR-RFLP, SSCP, and a method involving a structure-specific endonuclease (CFLP). They showed that PCR-RFLP was the least sensitive because the restriction enzyme *Msp*I could only detect AGC→ACC substitutions in codon 315. On the other hand, whereas CFLP was the most complicated and labor intensive, a fourth method involving a solid phase reverse hybridization (LiPA) as well as DNA sequencing were less cost effective. It has already been shown, in conjunction with *katG* codon 315 mutation prevalences, that SSCP cannot be regarded as infallible [18], and the sensitivity of this method is affected by numerous factors [21]. In a study on the rifampin resistance gene *rpoB*, sequencing was recommended to verify positive SSCP results [22]. Taken together, PCR-RFLP compares favorably to other genotyping methods because it is the easiest and most inexpensive method; the previously described method using *Msp*I [13] lacks sensitivity.

Failure to Produce PCR Products

In-house validations of molecular resistance prediction methods, especially by those who developed them, are generally satisfactory to excellent. Even when tests are used directly on sputum samples, failure to produce PCR products is often not encountered or not reported [23–25]. In contrast, significant rates of false-positive and false-negative test results, although not yet reported for resistance prediction but for the mere detection of mycobacteria, have been found in blinded, multicenter evaluations of PCR protocols [26,27]. In the present study, three samples failed to give a PCR product. Possible explanations are deletions of the *katG* gene that cause INH resistance, a rare event [8–10,19], and decay of the samples during transport and storage, because all three samples originated from the same shipment, which consisted of samples no. 1 through 6. Another reason for failures to produce PCR products can be mycobacteria other than *M. tuberculosis* (MOTT). Sputum samples with *M. xenopi*, *M. malmoense*, or *M. avium* complex did not yield PCR products with our primers. The *katG* genes of these organisms are not yet available through the GenBank database, but a comparison with the *katG* DNA sequence of *M. smegmatis* (accession no. X98718) showed between four and seven mismatches for each of the four PCR primers used, including the second or third base of their 3′ ends, thus not ful-

filling the minimum homology requirements for PCR primers [28]. The apparent inability to amplify DNA from several MOTT species may be seen as an advantage because *M. tuberculosis* may be rapidly detected in mixed infections, with a majority of organisms being MOTT.

All PCR-RFLP Are Not Created Equal

The use of *Msp*I in PCR-RFLP resulted in unsatisfactory sensitivity [14] because the *katG* codon 315 wild-type sequence does not possess the *Mnl*I recognition site and only a G to C substitution of the second nucleotide of codon 315 will create it. This is only one of five known *katG* codon 315 substitutions found in INH-resistant strains of *M. tuberculosis* even though it appears to be the most common one (Table 2). Indeed, Nachamkin et al. [7] detected only 44% *katG* codon 315 mutations in a survey of 27 INH-resistant isolates using the *Msp*I PCR-RFLP. Another disadvantage is that invariant products of similar sizes require the use of a polyacrylamide gel for analysis [13]. The use of a different restriction enzyme, *Aci*I, which recognizes the wild-type genotype from cultured *M. tuberculosis*, has therefore been proposed [19]. Its recognition sequence is lost after four of the five recognized *katG* codon 315 substitution types associated with INH resistance. Only one substitution (AGC→CGC), reported once in 222 INH-resistant isolates (Table 2) would not give a RFLP pattern different from the wild type, whereas the PCR-RFLP with *Msp*I would have missed 10 more isolates. In contrast, the technique described here detects almost all known *katG* codon 315 substitution types. The PCR primers used also allow the prediction of INH resistances directly from sputum samples, and the restriction products can be analyzed in a simple agarose gel with preadded ethidium bromide, which is easier to prepare than acrylamide gels, does not require an extra staining step, and reduces handling of this mutagenic dye.

Are Cultures Representative of the Mycobacteria in Sputum?

A surprisingly little studied phenomenon is the possible occurrence of genotypically and phenotypically heterogeneous mycobacterial populations within a single sputum sample and its practical significance. References to this effect are either "personal communications," for example results of an SSCP analysis on artificially prepared mixtures of wild type and mutant strains [14], "unpublished data," for example that more than 50% of rifampin resistant isolates obtained in New York contained less than 80% rifampin-resistant organisms [29], or mere speculations about possible effects of such mixed populations on molecular genotyping techniques [30]. In the present study, in DNA amplified from sputum samples that were subsequently cultured and phenotypically classified as INH sensitive, we repeatedly observed the 185 bp band after *Aci*I digestion, which would be expected from INH-resistant isolates with a *katG* codon 315 mutation. An example of this phenomenon ("heteroresistance" [14]) with only little of the 117 bp product expected from the unmutated genotype, was sample 16. Direct sequencing of its PCR product confirmed the wild-type genotype, but when we cloned the DNA and sequenced six clones, we found the wild type in only three of them and, among other mutations, a clone with an AGC→AAC substitution that should produce the INH-resistant phenotype. Much can be said in favor of a pragmatic approach: INH resistance should be declared only if the 117 bp band is not visible at all (Fig. 2) (Table 1). If there is a 117 bp band, it may either come from INH-sensitive bacteria that may outgrow the resistant ones in culture, or from INH

Table 2. *katG* Codon 315 Substitutions in INH-Resistant *M. tuberculosis* Isolates

	Number of Isolates with *KatG* Codon 315 (AGC) Substitutions							
Study	→ACC	→AAC	→ACA	→ATC	→CGC	No Substitutions	No PCR Product	Total
Musser et al. [2]	44 (52%)	2 (2%)	1 (1%)	0	0	35 (41%)	3 (4%)	85
Haas et al. [15]	52 (60%)	5 (6%)	0	1 (1%)	1 (1%)	28 (32%)	0	87
Dobner et al. [19]	26 (52%)	0	1 (2%)	0	0	21 (42%)	2 (4%)	50
Total	122 (55%)	7 (3%)	2 (1%)	1 (0.5%)	1 (0.5%)	84 (38%)	5 (2%)	222

resistant bacteria without the *katG* codon mutation. Because the absence of this mutation does not allow for a resistance prediction anyway, and because almost half of all INH resistant *M. tuberculosis* do not carry this mutation, only the *katG* codon 315 mutation allows for a prediction, namely INH resistance—and, as we may add now, only in the absence of the wild type genotype. The RFLP method described here can detect a relatively small amount of wild-type sequence even within a majority of mutated genotypes in a heteroresistant mycobacterial population. The aim of a molecular resistance prediction test is not necessarily to accurately describe the situation in the sputum sample, but rather to predict the outcome of the resistance determination by culture. Therefore, the theoretically most satisfactory mutation characterization test for the majority of organisms in a sputum sample is not the best test, but the one that correctly predicts resistances most often and does this in a routine clinical laboratory or reference center.

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