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Use of the Genotype MTBDR Assay for Rapid Detection of Rifampin and Isoniazid Resistance in *Mycobacterium tuberculosis* Complex Isolates

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A commercially available DNA strip assay (Genotype MTBDR; Hain Lifescience, Nehren, Germany) was evaluated for its ability to detect mutations conferring resistance to rifampin (RMP) and isoniazid (INH) in clinical *Mycobacterium tuberculosis* complex isolates. A total of 103 multidrug-resistant (MDR; i.e., at least resistant to RMP and INH) and 40 fully susceptible strains isolated in Germany in 2001 in which resistance mutations have been previously defined by DNA sequencing and real-time PCR analysis were investigated. The Genotype MTBDR assay identified 102 of the 103 MDR strains with mutations in the *rpoB* gene (99%) and 91 strains (88.4%) with mutations in codon 315 of *katG*. All 40 susceptible strains showed a wild-type MTBDR hybridization pattern. The concordance between the MTBDR assay and the DNA sequencing results was 100%. Compared to conventional drug susceptibility testing, the sensitivity and specificity were 99 and 100% for RMP resistance and 88.4 and 100% for INH resistance, respectively. In conclusion, the MTBDR assay is a rapid and easy-to-perform test for the detection of the most common mutations found in MDR *M. tuberculosis* strains that can readily be included in a routine laboratory work flow.

The rapid determination of drug resistance in clinical isolates of *Mycobacterium tuberculosis* is the prerequisite for the initiation of effective chemotherapy ensuring successful treatment of the patient and preventing further spread of drug-resistant isolates (17). The development of drug resistance in *M. tuberculosis* isolates is the result of random genetic mutations in particular genes conferring resistance (30). Based on this knowledge, molecular assays have been established that allow the prediction of drug resistance in clinical *M. tuberculosis* isolates within one working day and potentially are the most rapid methods for the detection of drug resistance (16). The utility of such assays depends on different aspects, such as the technical requirements of each method and the ability of different tests to detect the most common drug resistance mutations in a given area.

The mutations that predominate in rifampin (RMP)-resistant *M. tuberculosis* isolates are located in an 81-bp "core region" of the *rpoB* gene (ca. 95% of all RMP-resistant strains (1, 27). In contrast, the mutations causing isoniazid (INH) resistance are located in several genes and regions (24). Approximately 50 to 95% of INH-resistant strains have been found to contain mutations in codon 315 of the *katG* gene (14, 15, 26), 20 to 35% contain mutations in the *inhA* regulatory region (15, 18, 26), and an additional 10 to 15% have mutations in the *ahpC-oxyR* intergenic region (8, 18, 26), often in conjunction with *katG* mutations outside of codon 315 (25).

In order to allow the detection of the majority of mutations causing RMP resistance and also a high proportion of mutations causing INH resistance in *M. tuberculosis* isolates, a DNA strip assay was developed (Genotype MTBDR; Hain Lifescience, Nehren, Germany). It is based on a multiplex PCR in combination with reverse hybridization to identify either wild-type sequences or specific mutations.

The Genotype MTBDR assay was evaluated by investigating 103 MDR and 40 susceptible strains isolated in Germany in 2001. The results of the MTBDR assay were compared to data obtained by DNA sequencing and real-time PCR analysis of the respective chromosomal regions.

MATERIALS AND METHODS

Strains analyzed. A set of 103 MDR and 40 randomly chosen fully susceptible *M. tuberculosis* complex strains obtained from patients living in Germany in 2001 was analyzed. The study population represents more than 90% of all MDR cases isolated in Germany in 2001 (9). Drug susceptibility testing (DST) for INH and RMP was performed with the modified proportion method in the BACTEC 460TB system (Becton Dickinson Microbiology Systems, Cockeysville, Md.) according to the manufacturer's instructions.

Genotypic characterization. Strains used for DNA preparation were grown on Löwenstein-Jensen slants. Extraction of genomic DNA from mycobacterial strains were performed in accordance with a standardized protocol described elsewhere (29). All strains were analyzed by real-time PCR and DNA sequencing of the key regions involved in the development of resistance (*rpoB*, *katG*, *inhA*, and *ahpC* [7]; for primers and probes, see reference 23). Direct sequencing of selected PCR products was carried out with an ABI Prism 3100 capillary sequencer (Applied Biosystems) and the ABI Prism BigDye Terminator kit v.1.1 according to the manufacturer's instructions.

Genotype MTBDR assay. The Genotype MTBDR assay (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer. Briefly, for amplification 35 μl of primer-nucleotide mix (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U of Hot Start *Taq* polymerase (QIAGEN, Hilden, Germany), and 5 μl of a 1:100 dilution preparation of chromosomal DNA (29) in a final volume of 50 μl were used. The use of a 1:100 dilution of chromosomal DNA, however, is not mandatory. We achieved identical results if 3 μl of crude cell lysates (one loopful of cells was suspended in 400 μl of distilled water, boiled for 20 min, and sonicated for 15 min) was used for PCR amplification (data not shown). The amplification protocol consisted of 15 min of denaturing at 95°C; followed by 10 cycles of 30 s at 95°C and 120 s at

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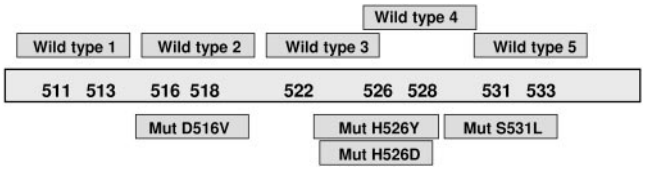

FIG. 1. Locations of probes within the 81-bp hot spot cluster of the *rpoB* gene.

58°C; followed by 20 additional cycles of 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C; with a final extension at 70°C for 8 min. Hybridization and detection were performed in an automated washing and shaking device (Profiblot; Tekan, Maennedorf, Switzerland). Steps taken to avoid amplicon contamination are the manual pipetting of the amplicon, the usage of separate wells and tubes for each stripe, and extensive rinsing after each use. The program was started after 20 μl of the amplification products was mixed with 20 μl of denaturing reagent (provided with the kit) for 5 min in separate troughs of a plastic well. Automatically, 1 ml of prewarmed hybridization buffer was added, followed by a stop to put the membrane strips into each trough. The hybridization procedure is performed at 45°C for 0.5 h, followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin-conjugated with alkaline phosphatase and substrate buffer is added. After final washing, strips were air dried and fixed on paper. Each strip contains 17 probes, including five amplification and hybridization controls to verify the test procedures, five *rpoB* wild-type and four mutant probes, and one *katG* wild-type and two mutant probes (Fig. 1 and 2).

RESULTS AND DISCUSSION

For 103 MDR and 40 randomly chosen fully susceptible *Mycobacterium tuberculosis* strains, isolated in Germany in 2001, genotypic analysis of RMP and INH resistance was carried out with the MTBDR DNA strip assay. The test is based on a multiplex PCR in combination with reverse hybridization with membrane strips coated with target-specific oligonucleotides. The reverse hybridization is followed by biotin-streptavidin-mediated detection of hybridized PCR fragments. The presence of wild-type or mutated DNA sequences in the 81-bp hot spot region of *rpoB* (Fig. 1 and 2) and at codon 315 of *katG* (Fig. 2) is then shown by clear-cut hybridization signals on the membrane strips, which can easily be analyzed by using a template that is supplied with the kit. In addition, the multiplex PCR and the strip include an amplification control specific for *M. tuberculosis* complex isolates and an amplification control detecting all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content.

The organization of the membrane strip and examples of typical test results are shown in Fig. 1 and 2, respectively. The presence of mutations is indicated either by loss of hybridization to the oligonucleotide representing the wild-type sequence and hybridization to the corresponding oligonucleotide representing the mutated sequence or by loss of hybridization to the respective wild-type sequence only (Table 1 and Fig. 1 and 2).

Concerning RMP resistance, a mutation in the *rpoB* gene was detected in 102 of 103 MDR isolates, whereas none of the 40 susceptible strains carried a mutation in this region. Altogether, 13 different MTBDR hybridization patterns representing a minimum of 10 different mutations in the *rpoB* hot spot region were found (Table 1). Codon 531 was most frequently affected in 78 of the 103 strains (75.7%), with 73 strains showing the specific nucleotide exchange TCG to TTG, resulting in the amino acid exchange of serine to threonine. The five other strains had a mutation located in the region from codons 531

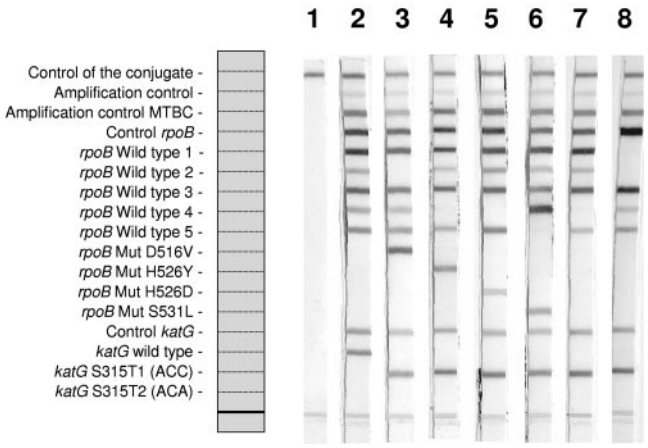

FIG. 2. Representative results for patterns obtained with the Genotype MTBDR assay. The positions of the oligonucleotides and the marker line are given on the left side. The specificity and targeted genes of the lines are shown from top to bottom as follows: conjugate control; amplification control (23S rRNA); *M. tuberculosis* complex-specific control (23S rRNA); control of *rpoB* amplification 5 to 9; *rpoB* wild-type probes located in the 81-bp hot spot region (the localization is shown in Fig. 1); *rpoB* mutant (Mut) probes with mutations in codons 516, 526, and 531; control of *katG* amplification; *katG* codon 315 wild-type probe; and *katG* codon 315 mutation probes (sequences in parenthesis). Along the bottom is a band for orientation of the strip. Patterns of the strips for MDR and susceptible *M. tuberculosis* strains are shown on the right. Lane 1, negative control; lane 2, H37Rv (wild type); lane 3, *rpoB* D516V, *katG* S315T1; lane 4, *rpoB* H526Y, *katG* S315T1; lane 5, *rpoB* H526D, *katG* S315T1; lane 6, *rpoB* S531L, *katG* S315T1; lane 7, *rpoB* H526N, *katG* S315T1; lane 8, *rpoB* 514-to-516 deletion, *katG* S315T1.

to 535. However, these mutations could not be further specified by the strip assay since the corresponding mutations were not targeted by an oligonucleotide on the strip.

Other mutations were detected in *rpoB* codon 526 in 14 (13.6%), and *rpoB* codon 516 in three strains (2.9%). In 5 of these strains the particular mutations could be clearly identified directly by hybridization to a specific oligonucleotide targeting the mutated sequence, and in 12 strains they could be identified indirectly by omission of a specific wild-type signals (Table 1).

In two strains hybridization patterns showing the wild-type pattern together with signals at the position of a mutant probe were observed, indicating the presence of a mix of wild-type and mutated DNA sequences. In these strains the presence of wild-type and mutated sequence was confirmed by the sequencing analysis. Overall, the Genotype MTBDR assay detected 102 of 103 possible mutations conferring RMP resistance compared to DNA sequencing and real-time PCR data (7) (Table 1). Only one strain that is phenotypically resistant to RMP was classified as susceptible by Genotype MTBDR due to the presence of a hybridization pattern typical for the wild-type sequences in the *rpoB* hot spot region. This strain carried a mutation in codon 176 of *rpoB*, which, because of its low frequency, was not included in the test (6). Based on our results, the Genotype MTBDR assay revealed a high sensitivity and specificity (99 and 100%, respectively) for the detection of RMP resistance compared to the conventional DST.

With regard to INH resistance, a mutation in *katG* codon

TABLE 1. MTBDR test results for the detection of mutations conferring RMP resistance compared to DNA sequencing data of 103 MDR *M. tuberculosis* strains isolated in Germany

MDR strain group (*n*)	MTBDR pattern		MTBDR result (location of *rpoB* mutation)	Sequencing data		No. (%) of strains
	Positive with wild-type probes	Positive with mutant probes[a]		Affected codon(s) in *rpoB*[b]	Nucleotide/amino acid change(s)	
Strains with unique pattern (101)	1,2,3,4	Mut S531L	S531L			
				531	TCG→TTG/Ser→Leu	73 (70.9)
	1,2,3,4		531–535	531	TCG→TGG/Ser→Trp	3 (2.9)
	1,2,3,4		531–535	531	TCG→TTT/Ser→Phe	2 (1.9)
	1,2,3,5		525–529	526	CAC→AAC/His→Asn	4 (3.9)
	1,2,3,5		525–529	526	CAC→CTC/His→Leu	3 (2.9)
	1,2,3,5		525–529	526	CAC→CGC/His→Arg	3 (2.9)
	1,2,3,5	Mut H526Y	H526Y	526	CAC→TAC/His→Tyr	1 (1.0)
	1,2,3,5	Mut H526D	H526D	526	CAC→GAC/His→Asp	1 (1.0)
	1,2,3,5		525–529	526	CAC→TGC/His→Cys	1 (1.0)
	1,3,4,5	Mut D516V	515–520	516	GAC→GTC/Asp→Val	2 (1.9)
	1,3,4,5		515–520	516	GAC→TAC/Asp→Tyr	1 (1.0)
	1,2,4,5		521–525	522	TCG→CAG/Ser→Gln	1 (1.0)
	1,2,4,5		521–525	522	TCG→TTG/Ser→Leu	1 (1.0)
	1,3,4,5		515–520	518	AAC→ATC/Asn→Ile	1 (1.0)
	2,3,4,5		510–514	513	CAA→CCA/Gln→Pro	1 (1.0)
	1,3,4,5		515–520	517	Del[c]	1 (1.0)
	3,4,5		510–520	514–516	Del	1 (1.0)
	1,2,3,4,5		*rpoB* wild type	176	GTC→TTC/Val→Phe	1 (1.0)
Strains with multiple patterns (2)	1,2,3,(4),5	Mut H526Y	H526H/Y	526	CAC→TAC/His→Tyr	1 (1.0)
	1,(2),3,4,5	Mut S531L	S531L	519/522/531	Del; TCG→TTG/Ser→Leu and TCG→TTG/Ser→Leu	1 (1.0)

[a] Mut, mutation.
[b] According to Telenti et al. (26). GenBank accession number L27989 for the *rpoB* gene.
[c] Del, deletion.

315 was detected in 91 of the 103 MDR strains (88.4%) (Table 2) but in none of the 40 susceptible strains. In the majority of MDR strains distinct nucleotide exchanges in *katG* codon 315 could be verified by hybridization to the oligonucleotide probe targeting the particular mutations: 87 strains carried the ACC (S315T1), and two carried the ACA (S315T2) mutation. In two further strains (3.9%) mutations in codon 315 were indicated by the absence of the wild-type hybridization signal only. Twelve of the MDR strains showed a wild-type hybridization pattern in the MTBDR assay. Sequencing analysis revealed that three of these (2.9%) had mutations in the ribosome-binding site region of *inhA*, and two (1.9%) had a nucleotide exchange in the regulatory region of the *ahpC* gene (7). Presumably, the remaining seven strains carry a mutation in other genomic regions not analyzed in this investigation, such as *kasA*, or *ndh* (10, 13, 23, 25). In conclusion, Genotype MTBDR detected all mutations in *katG* 315. Compared to conventional DST, the sensitivity and specificity of the MTBDR assay for the detection of INH resistance were 88.4 and 100%, respectively.

The high prevalence of mutations within the *rpoB* core region (99%), as well as of mutations at *katG* codon 315 (88.4%) in the MDR *M. tuberculosis* strains isolated in Germany, indicated the potential of a rapid diagnostic test for detection of drug-resistant *M. tuberculosis*. As stated here, the detection of mutations in the *rpoB* gene is a useful strategy for diagnosis of

TABLE 2. MTBDR test results for the detection of mutations conferring INH resistance in comparison with DNA sequencing data of MDR *M. tuberculosis* strains (n = 103) isolated in Germany

MTBDR pattern	MTBDR result	Sequencing data		No. (%) of strains
		Mutation in *katG*, *inhA*, or *ahpC*[a]	Nucleotide/amino acid change(s)	
katG T1	S315T	*katG* 315	AGC→ACC/Ser→Thr	87 (84.5)
katG T2	S315T	*katG* 315	AGC→ACA/Ser→Thr	2 (1.9)
	S315X	*katG* 315	AGC→AAC/Ser→Asn	2 (1.9)
Wild type	*katG* wild type	*inhA*209	C→T	3 (2.9)
Wild type	*katG* wild type	*ahpC*	C(-52)T	1 (1.0)
Wild type	*katG* wild type	*ahpC*	G(-48)A	1 (1.0)
Wild type	*katG* wild type	No mutation		7 (6.8)

[a] According to Telenti et al. (26). GenBank accession numbers X68081 for the *katG* gene, U66801 for the *inhA* gene, and U16243 for the *ahpC-oxyR* intergenic region.

drug resistance to RMP in *M. tuberculosis* complex since the mutations in the hot spot region are predominant (average, >95%) (19). Additional analysis of codon 315 of *katG* facilitates the detection of the most-frequent mutations associated with INH resistance (30). These mutations are present in ca. 50% of all INH-resistant isolates worldwide and are even more predominant in strains originating from Russia and the states of the former Soviet Union (7, 12, 14).

Based on the knowledge of the molecular mechanisms of drug resistance in *M. tuberculosis*, different genotypic assays have been proposed for the detection of mutations, including recent real-time PCR formats that utilize molecular beacons (5), biprobes (4), or TaqMan probes (23, 28). However, as many as five different beacons were required to encompass the entire *rpoB* core region, and three biprobes described by Edwards et al. (4) detected mutations in only four codons of the *rpoB* gene. Above all, for the measurement of fluorescence, a real-time PCR instrument is beneficial, and indeed essential, if a melting-curve analysis is required for post-PCR analysis. Recently, a molecular biology technique based on the PCR and a reverse hybridization procedure (INNO-LiPA Rif TB assay; Innogenetics, Ghent, Belgium) has been evaluated for the detection of *rpoB* mutations (2, 3, 22), but the assay is restricted to the detection of RMP resistance.

In contrast, the Genotype MTBDR assay allows the rapid and specific detection of the most-frequent mutations leading to IHN and RMP resistance in clinical *M. tuberculosis* isolates by applying an easy-to-perform test format. Both the PCR technology and the reverse hybridization technique used for the test have been proven to be robust and reproducible, and the results are easy to interpret without the extensive expert knowledge required for the interpretation of real-time PCR data or DNA sequencing data. Furthermore, it can easily be implemented in routine work flows, especially when other strip assays are already established, such as in differentiation of several mycobacterial species (11) and differentiation within the *M. tuberculosis* complex (20, 21). All tests can be run by using the same platform technology. On the other hand, one must be aware that the Genotype MTBDR assay has the same limitations as other molecular tests for the detection of antibiotic resistance, and therefore, it cannot totally replace traditional culture-based methods for DST. This is basically due to the fact that none of the molecular tests established targets all possible genes or mechanisms (some are not identified yet) involved in resistance, and thus, a variable proportion of resistant strains will not be detected. The second inherent limitation is the detection limit of ca. 10% mutant DNA in a mixture of wild-type and mutant DNA. If the proportion of resistant cells in an isolate is less than that amount, it can hardly be detected by molecular methods, whereas classical susceptibility testing might give a more sensitive test result in these cases. Nevertheless, the MTBDR culture assay appears to be a valuable tool that allows the detection of resistant *M. tuberculosis* isolates within one working day and can easily be included in routine workflow. Considering the high rates of resistant and MDR isolates in several parts of the world, especially in Eastern Europe, such a test has the potential to complement and accelerate the variety of different measures in laboratory diagnostics that are necessary for improved tuberculosis control in the future.

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