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Use of DNA Extracts from Ziehl-Neelsen-Stained Slides for Molecular Detection of Rifampin Resistance and Spoligotyping of *Mycobacterium tuberculosis*

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Multidrug resistance among new cases of tuberculosis (TB) is increasingly becoming a significant problem in countries with a high prevalence of TB and with inadequate therapies for TB. Rifampin resistance is widely used as a marker for multidrug-resistant (MDR) TB; therefore, a new approach to the retrospective measurement of rifampin resistance without the need of a viable culture has been introduced. In many developing countries culture is unavailable and diagnosis relies on clinical manifestations and the results of Ziehl-Neelsen staining of sputum smears. We determined rifampin resistance directly with DNA extracts from Ziehl-Neelsen-stained slides by identification of mutations in the *rpoB* gene using reverse line blot hybridization and DNA sequencing. Analysis of the *rpoB* gene revealed that samples containing rifampin-resistant *Mycobacterium tuberculosis* carried altered codons representing amino acid positions 516, 526, and 531 of the RNA polymerase. Although the sensitivities of both methods were equal (84%), sequencing of the *rpoB* gene was more accurate in identifying mutations in the core region of the *rpoB* gene. Sequence analysis of the *rpoB* gene in extracts from Ziehl-Neelsen-stained slides may be used to quantify more precisely the magnitude of MDR TB and, more importantly, provide information on trends in the development of resistance on a global scale. The nature of rifampin resistance and the genotype can be determined by analysis of Ziehl-Neelsen-stained slides in a laboratory equipped for sequencing and spoligotyping without the need to ship biohazardous materials.

An estimated 1.7 billion people, representing one-third of the world's population, are latently infected with *Mycobacterium tuberculosis*. With approximately 8.8 million new cases each year and 2.9 million people dying from tuberculosis (TB) yearly, with 99% of the deaths occurring in developing countries (49), this infectious disease constitutes an impressive health burden. Sputum smear microscopy is the most cost-effective diagnostic technique used to diagnose TB and is used in most developing countries, where culturing is usually impossible. Detection of smear-positive patients is crucial in the fight against TB because they are responsible for spreading TB in the community (49). Early diagnosis, effective treatment, and successful cessation of transmission are major strategies in the control of TB. The advent of molecular detection and differentiation of strains of members of the genetically closely related *Mycobacterium tuberculosis* complex have proved useful in providing an understanding of the various aspects of the epidemiology and transmission of TB. In addition, molecular methods can be used to determine the genetic changes that lead to antibiotic resistance. However, the use of such methods is restricted to well-equipped laboratories.

Multidrug resistance (MDR) has been defined by the World Health Organization as resistance to at least isoniazid and rifampin (50). Surveillance for MDR TB is important to study its spread within and across borders and to determine the risk factors for transmission of MDR TB. Such data and data on monodrug resistance and MDR also enable evaluation of the effectiveness of treatment programs for MDR TB like the Directly Observed Treatment Short-Course Plus program and of measures to curtail the transmission of MDR TB.

Several groups have reported on the epidemiology, treatment, and control of MDR TB in countries like the Czech Republic (20), Germany (26), Portugal (31), Italy (36), and Azerbaijan and Siberia (30). While data were available from at least 11 of the 22 countries that account for 80% of the new cases of TB worldwide, the global project of the World Health Organization should be expanded in order to define more precisely the magnitude of drug-resistant TB and to quantify this problem and its trends (49–51).

In bacteria resistance to rifampin almost invariably involves alterations of RNA polymerase, which is encoded by the RNA polymerase subunit β gene (*rpoB*). Missense (single or double) mutations, deletions, or insertions within the 81-bp core region of the *rpoB* gene were found to be responsible for rifampin resistance in more than 95% of rifampin-resistant *M. tuberculosis* strains (Table 1). Some mutations are known to be associated with high-level rifamycin (rifampin, rifabutin, rifapentine, and KRM-1648) cross-resistance (47), but some studies have had conflicting results (18, 25). Because the molecular mechanisms of resistance to other anti-TB drugs are much less restricted to particular genomic loci and are far more complex,

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TABLE 1. Overview of all reported mutations occurring in codons 479 through 580 of the *M. tuberculosis rpoB* gene[a]

Affected codon	Amino acid change	Base change(s)	Reference	Affected codon	Amino acid change	Base change(s)	Reference
479	Met→Leu	ATG→TTG	41	521	Leu→Met	CTG→ATG	48
504	Glu→Ala	GAG→GCG	38	521	Leu→Leu[b]	CTG→TTG	40
505	Phe→Leu	TCG→TTG	9	522	Ser→Leu	TCG→TTG	3
507	Gly→Asp	GGC→GAC	19	522	Ser→Gln	TCG→CAG	40
Del	510–513	GCTGAGCCA	11	522	Ser→Ser	TCG→TCC	38
509	Ser→Thr	AGC→ACC	41	Del	522–525	CTGGGGTTGACC	2
509	Ser→Arg	ACG→CGG	48	523	Gly→Trp	GGG→TGG	32
510	Gln→His	CAG→CAT	40	524	Leu→Ser	TTG→TCC	41
511	Leu→Pro	CTG→CCG	10	524	Leu→Trp	TTG→TGG	44
511	Leu→Asp	CTG→GAC	18	525	Thr→Pro	ACC→CCC	44
511	Leu→Val	CTG→GTC	40	525	Thr→Ile	ACC→ATC	32
511	Leu→Arg	GTG→CGG	16	525	Thr→Asn	ACC→AAC	9
511	Leu→Leu[b]	CTG→CTA	40	526	His→Tyr	CAC→TAC	3
512	Ser→Thr	AGC→ACC	16	526	His→Asp	CAC→GAC	3
513	Gln→Leu	CAA→CTA	11	526	His→Arg	CAC→CGC	12
513	Gln→Lys	CAA→AAA	3	526	His→Leu	CAC→CTC	7
513	Gln→Pro	CAA→CCA	3	526	His→Pro	CAC→CCC	8
513	Gln→His	CAA→CTA	3	526	His→Glu	CAC→CAA	16
513	Gln→Glu	CAA→GAA	21	526	His→Asn	CAC→AAC	1
513	Gln→Arg	CAA→CGA	10	526	His→Gln	CAC→CAG	44
Ins	513	TTC	16	526	His→Cys	CAC→TGC	10
Ins	513–514	TTCATG	16	526	His→Gly	CAC→GGC	34
Del	513–514	CAATTC	25	526	His→Leu	CAC→CTC	52
Del	513–515	AATTCATGG	16	526	His→Leu	CAC→CTG	44
514	Phe→Leu	TTC→TTG	42	526	His→Thr	CAC→ACC	6
514	Phe→Phe[b]	TTC→TTT	25	527	Lys→Gln	AAG→CAG	12
514	Phe→Val	TTC→GTC	44	527	Lys→Asn	AAG→AAC	40
Ins	514	TTC	33	Del	527	AAG	44
515	Met→Ile	ATG→ATA	44	528	Arg→Arg[b]	CGC→CGT	25
515	Met→Val	ATG→GTG	52	528	Arg→Pro	CGC→CCC	40
Del	514–516	TTCATGGAC	44	528	Arg→His	CGC→CAC	40
Del	515–518	ATGGACCAGAAC	44	529	Arg→Gln	CGA→CAA	18
Del	515–517	ATGGACCAG	52	529	Arg→Lys	CGA→AAA	52
516	Asp→Val	GAC→GTC	3	531	Ser→Leu	TCG→TTG	3
516	Asp→Tyr	GAC→TAC	16	531	Ser→Trp	TCG→TGG	3
516	Asp→Glu	GAC→GAG	12	531	Ser→Cys	TCG→TGT	16
516	Asp→Gly	GAC→GGC	10	531	Ser→Tyr	TCG→TAT	6
516	Asp→Asn	GAC→AAC	33	531	Ser→Pro	TCG→CCG	3
516	Asp→Asn	GAC→AAC	38	531	Ser→Gln	TCG→CCG	33
516	Asp→Phe	GAC→TTC	7	531	Ser→Ala	TCG→GCG	3
Del	516–517	TGG	10	531	Ser→Phe	TCG→TTC	3
Del	516–517	GACCAG	10	531	Ser→Phe[b]	531→TTT	52
Del	517	CAG	48	533	Leu→Pro	CTG→CCG	3
Del	517–518	CAGAAC	16	541	Glu→Gly	GAG→GAT	32
518	Asn→Ser	AAC→TCC	11	553	Ser→Ala	TCG→GCG	32
518	Asn→His	AAC→CAC	19	562	Glu→Ala	GAA→GCA	37
518	Asn→Thr	AAC→ACC	40	564	Pro→Leu	CCT→CTT	23
Del	518	AAC	38	565	Glu→Ser	GAG→AGT	41
519	Asn→Lys	GAC→AAA	48	Del	566	GGG	41
521	Leu→Pro	CTG→CCG	39	572	Ile→Phe	ATC→TTC	3
521	Leu→Pro	CTG→CTT	39	580	Arg→Gln	CGC→CAG	41

[a] The data represent a compilation of the mutations described in the literature. The references given are representatives of studies describing these mutations. Mutations occurring in *M. tuberculosis* isolates with one or more point mutations, deletions, (Del), and insertions (Ins), are listed as a single event in this table.
[b] Silent mutation.

molecular detection of resistance will remain too difficult for some time. However, resistance to rifampin alone seems to be a rare event, making it an excellent marker for detection of MDR TB.

We introduce a new approach to the retrospective measurement of rifampin resistance without the need for a viable culture. In this study we evaluated the use of rifampin oligonucleotide typing (rifoligotyping) and sequencing of the *rpoB* gene in DNA extracts from acid-fast bacillus-positive Ziehl-Neelsen (ZN)-stained slides of sputum obtained from TB patients. A representative collection of ZN-stained slides from sputum smears can easily be organized both in countries with a low incidence of MDR TB and in countries with a high incidence of MDR TB. Data on the alterations in the *rpoB* genes of *M. tuberculosis* bacteria leading to rifampin resistance can be obtained from these ZN-stained slides, thus facilitating surveillance for resistance to this major component of treatment without expensive culturing procedures. The DNA ex-

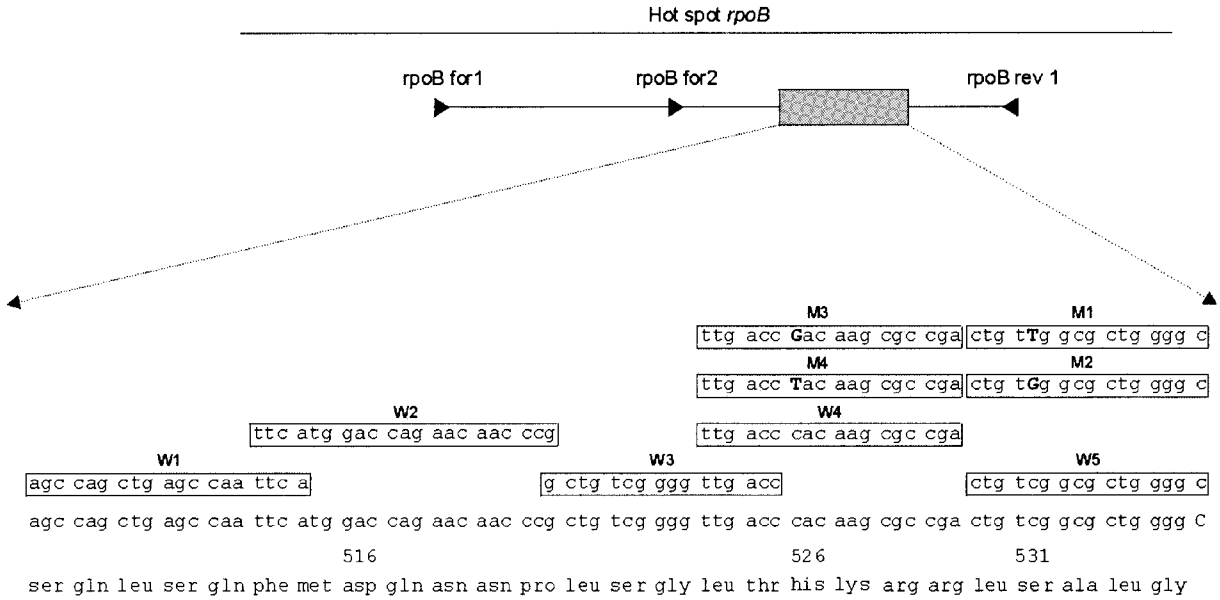

FIG. 1. Schematic representation of the *rpoB* gene, primers, and probes used for rifoligotyping and sequencing. The arrowheads indicate the positions of primers rpoB-for1, rpoB-for2, and rpoB-rev1. The gray box corresponds to the area covered by the mutant (M) and wild-type (W) probes used for rifoligotyping (boxed sequences). The codon numbering is based on that used for *Escherichia coli*, as described by Telenti et al. (43), and are not the positions of the actual *M. tuberculosis rpoB* codons (24).

tracts of ZN-stained slides can also be used for the spoligotyping method, described earlier (45), which enables data on molecular epidemiology to be linked to resistance data.

MATERIALS AND METHODS

Strains and ZN-stained sputum smears. Twenty-six clinical isolates of *M. tuberculosis* and 37 ZN-stained sputum smears were included in this study. Smears and corresponding isolates were retrieved from sputum samples from patients originating from various parts of the world. There were 15 smears and 15 strains from Moldova, 8 smears and 4 strains from The Netherlands, and 14 smears and 7 strains from Kazakhstan (from a prison). The drug susceptibilities of all isolates were determined by the conventional proportion method with Löwenstein-Jensen medium described by Canetti et al. (5). The isolates in 21 samples were resistant to rifampin (>4 μg/ml), and those in 16 samples were sensitive. Three ZN-stained slides (slides R19 to R21) contained sputum from patients who had undergone directly observed short-course treatment.

Extraction of DNA from mycobacterial cultures. DNA extracts were prepared by suspending approximately 10 mg of wet bacterial cells into 250 μl of sterile distilled water (Sigma) and heating the mixture at 100°C for 30 min to kill the cells and to induce cell lysis (53). DNA was extracted by using a Capture Column DNA extraction kit according to the instructions of the manufacturer (Gentra Systems). The samples were stored at −20°C until use for DNA amplification.

DNA extraction from ZN-stained microscopic preparations. Prior to DNA extraction all ZN-stained preparations were examined for acid-fast bacilli and were rated on the Bronkhorst scale (4). After microscopic examination the mineral oil was removed with xylene. ZN-stained material was scraped off from the microscopic slides after the addition of 25 μl of distilled sterile water. Seventy-five microliters of Chelex suspension (45) was added, and after thorough mixing of the samples, the samples were incubated for 30 min at 97°C. The samples were centrifuged at 13,000 × *g* for 10 min. The supernatant was transferred to a fresh microcentrifuge tube and used directly for PCR. The individuals who performed the laboratory work were blinded to the rifampin resistance status of the isolates in the various experimental tests.

PCR amplification. Five microliters of the extracted DNA from the cultured *M. tuberculosis* complex bacteria and 10 or 2.5 μl of the DNA extracts from the ZN-stained slides were used in the PCR. PCR for spoligotyping was performed as described by van der Zanden et al. (45). PCR for rifoligotyping was carried out in 55 μl of a reaction mixture containing 50 mM KCl, 2.9 mM $MgCl_2$, 0.23 mM (each) deoxynucleoside triphosphate, 0.22 μg of TaqStart, 1 U of *Taq* polymerase, 1 U of uracil DNA glycosylase, 14.5 mM Tris (pH 9.0), and 50 pmol (each) of primer rpoB-for1 (5′-TGGTCCGCTTGCACGAGGGTCAGA-3′) and primer rpoB-rev1 (5′-biotin label-CTCAGGGGTTTCGATCGGGCACAT-3′), which yielded a 437-bp PCR-amplified fragment of the *rpoB* gene (Fig. 1). Nonspecific annealing was avoided by incubating the *Taq* polymerase with the TaqStart monoclonal antibody for 15 min at room temperature before addition to the reaction mixture. The mixture was overlaid with 1 drop of PCR-grade mineral oil (Sigma) and was incubated for 3 min at 37°C for uracil DNA glycosylase incubation and at 10 min at 96°C for uracil DNA glycosylase inactivation (22) and DNA denaturation. Touchdown cycles comprising denaturation at 96°C for 1 min, primer annealing at 72°C −69°C for 1 min (two cycles at each 1°C decrement), and primer extension at 72°C for 1 min were conducted. This was followed by 40 cycles of amplification, each of which consisted of 96°C for 1 min, 69°C for 1 min, and 72°C for 1 min. The PCR products were kept at −20°C until further analysis.

Rifoligotyping. The PCR products were analyzed by hybridization by the reverse line blotting technique (17). Five wild-type oligonucleotide probes (probes W1 to W5) whose sequences are complementary to the wild-type sequence encoding amino acid codons 509 to 534 of the *rpoB* gene and whose sequences partially overlap were used (Fig. 1). The sequences of the probes span the hot-spot region for the mutation associated with rifampin resistance, as indicated in Fig. 1. If a mutation is present in this genomic region, and hence in the PCR product, no hybridization with the corresponding wild-type probe occurs under the stringent conditions applied. Consequently, the absence of a hybridization signal with one of the wild-type probes is indicative of the presence of a mutation and implies a resistance genotype. In addition, four mutant probes which specifically hybridize with PCR products carrying particular mutations were used. Probes M1, M2, M3, and M4 hybridized with the PCR products carrying the most frequently occurring mutations: Ser531Leu, Ser531Trp, His526Asp, and His 526Tyr.

Synthetic oligonucleotide probes with a 5′-terminal amino group covalently linked to a carboxyl group of a negatively charged Biodyne C membrane were used for reverse line blotting. The concentrations of the oligonucleotide probes ranged from 12.5 to 20 pmol/150 μl, and the probes had melting temperatures between 58.8 and 64.6°C. After hybridization for 45 min at 50°C and washing for 20 min at 57°C, the hybridized DNA was detected by a 30-min incubation with peroxidase-labeled streptavidin, essentially as described by Kamerbeek et al. (15). Optimization of the hybridization conditions and probe concentrations was performed with DNA from strains whose *rpoB* genes had been sequenced.

Sequencing of *rpoB* gene. To obtain sufficient DNA for sequencing, the 437-bp PCR fragment used for rifoligotyping was reamplified by a seminested PCR with primer rpoB-rev1 and primer rpoB-for2 (5′-CGATCACACCGCAGACG-3′),

which resulted in 178-bp amplicons (Fig. 1). The reaction mixtures contained, in a final volume of 55 μl, 10 μl of Q buffer (Qiagen GmbH, Hilden, Germany), 5.5 μl of *Taq* buffer (10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCl), 50 pmol each of primers rpoB-rev1 and rpoB-for2, 0.23 mM (each) deoxynucleotide triphosphate, 1 U of SuperTaq polymerase, 0.22 μg of TaqStart monoclonal antibody, and 437-bp amplicons diluted 1:100. The cycling parameters included an initial denaturation at 96°C for 1 min, followed by 1 min at 93°C, primer annealing at 63°C −60°C for 1 min (two cycles at each 1°C decrement), and primer extension at 68°C for 1 min. This was followed by 20 subsequent cycles of amplification: 93°C for 1 min, 60°C for 1 min, and 68°C for 1 min, followed by an additional cycle at 68°C for 5 min to complete the elongation of the intermediate PCR products.

PCR products were checked for integrity on ethidium bromide-stained agarose gels and purified with the Qiaquick PCR purification kit (Qiagen). For the DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used according to the protocol of the manufacturer (Applied Biosystems, Foster City, Calif.). Unincorporated dye terminators were removed with a Multiscreen assay system (Millipore, Molsheim, France) according to the protocol of the manufacturer, and the reaction products were separated and detected with an ABI Prism 3700 automatic DNA sequencer (PE Biosystems). Both strands of the polymorphic region of the *rpoB* gene were sequenced. Sequence assembly and editing were performed with the Seqman module of the Lasergene (version 5.0) software package (DNAstar Inc., Madison, Wis.), and the sequence was compared with the *rpoB* gene sequence obtained from the genome sequence of *M. tuberculosis* laboratory strain H37Rv (NC000962).

RESULTS

Spoligotyping with extracts from ZN-stained sputum smears. As shown previously (45), the spoligotyping technique is sensitive enough to genotype small numbers of mycobacteria in extracts from ZN-stained slides. In the study presented here, spoligotyping of the isolates on all except 1 (Table 2, slide R6) of the 37 ZN-stained slides with a score of +1 or more on the Bronkhorst scale resulted in complete spoligotyping patterns. One ZN-stained slide (Table 2, slide R6) with a score of +5 on the Bronkhorst scale yielded an incomplete spoligotyping pattern. For five culture-positive samples (slides R6, R10, and R11 [Table 2] and slides S11 and S16 [Table 3]), microscopy of the ZN-stained slides did not reveal acid-fast bacilli, yet the extracts from three of these slides (slide R6 [Table 2] and slides S11 and S16 [Table 3]) yielded complete spoligotyping patterns. Spoligotyping revealed patterns specific for the Beijing family of *M. tuberculosis* in 14 of the 21 samples with rifampin-resistant isolates: 4 of the 9 isolates in the samples from Moldova, 2 of the 4 isolates in the samples from The Netherlands, and all 8 isolates in the samples from Kazakhstan (Table 2). Six of the 16 rifampin-sensitive isolates were of the Beijing type: 2 of the 6 isolates in the samples from Moldova, none of the 4 isolates in the samples from The Netherlands, and 4 of the 6 isolates in the samples from Kazakhstan (Table 3). The Beijing genotype has previously been found to be often associated with MDR and seems to represent an important genogroup in the worldwide TB epidemic. In this study, 14 of the 20 (70%) isolates with the Beijing genotype were rifampin resistant. In contrast, only 7 of the 17 (41%) isolates that were not of the Beijing genotype were resistant. Even the analysis of the small number of isolates in this study shows the high prevalence of rifampin resistance among isolates of the Beijing genotype.

Detection of mutations in the *rpoB* gene by rifoligotyping. The DNA extracts obtained from the cultured *M. tuberculosis* strains were used to compare the performance of rifoligotyping with that of sequencing of the polymorphic region of the *rpoB* gene from isolates recovered on ZN-stained slides. Furthermore, the nature of the mutations observed in the *rpoB* gene was used to determine the association between the genotype and the phenotypic resistance pattern. The use of DNA extracts from slides with a score of +2 or more on the Bronkhorst scale invariably yielded rifoligotyping patterns. No rifoligotyping pattern was obtained for two of the nine ZN-stained slides with a score of +1 on the Bronkhorst scale. Four ZN-stained slides revealed no acid-fast bacilli, and none of them yielded rifoligotyping patterns.

Characterization of mutations in the *rpoB* gene by DNA sequencing. Initially, DNA sequencing was attempted for the PCR products created for rifoligotyping. However, in most cases this PCR did not yield sufficient amounts of DNA products for DNA sequencing when the PCR was applied to extracts from ZN-stained slides. This problem was circumvented by reamplifying the *rpoB* fragment by a seminested PCR. Sequence analysis revealed that all rifampin-resistant isolates with the exception of strain R5 had a single base-pair mutation; strain R5 had a double base-pair mutation in a single codon (GAC516TTC) in the core region of *rpoB* (Table 2). For virtually all isolates sequence analysis confirmed the presence of the mutations identified by rifoligotyping; the exceptions were the isolates from samples R5 and R14. The results of rifoligotyping and *rpoB* sequencing of the extract from the ZN-stained slide were in complete accordance for sample R5. This was also true for the analysis of the DNA from the strain cultured from the corresponding sputum sample. However, the results for the isolates from the slide and the culture were completely different. The most likely explanation for this observation is that the strain or the slide numbers were mixed up in one of the laboratories. The isolate from sample R14 gave a positive, yet weak hybridization with wild-type probe W2, suggesting that the sample contained a rifampin-sensitive *M. tuberculosis* strain. However, sequence analysis revealed that this was a false-positive hybridization, as codon 516 of the *rpoB* gene of the isolate from this sample was altered. Four of the rifampin-resistant strains carried an altered codon 516 (GAC→GTC [Asp→Val; 3 strains] and GAC→TTC [Asp→Phe; 1 strain]); 1 strain carried an altered codon 526 (CAC→TAC [His→Tyr]), and 15 strains carried an altered codon 531 (TCG→TTG [Ser→Leu; 12 strains] and TCG→TGG [Ser→Trp; 3 strains]).

DISCUSSION

We previously showed that DNA extracts from stained microscopic preparations can be used as a target for amplification of *M. tuberculosis* DNA by spoligotyping (45). In the study presented here, we showed that this technique can also be exploited to monitor rifampin resistance by reverse line hybridization (rifoligotyping) and DNA sequencing without the need for culture.

With the exception of one sample, the results of rifoligotyping and sequence analysis were in complete agreement. The isolate in the only sample with discrepant results was determined to be rifampin sensitive by both rifoligotyping of DNA extracted from the slide and sequencing of the corresponding strain from culture. However, DNA sequencing showed that the strain carried a mutation in the *rpoB* gene that led to resistance, and this was confirmed by phenotypic characteriza-

TABLE 2. Spoligotyping and rifoligotyping patterns and sequence analysis for DNA obtained from rifampin-resistant *M. tuberculosis* isolates and the corresponding ZN-stained slides[a]

Sample no.	Country	Spoligotyping pattern: Strain	Spoligotyping pattern: ZN	Rifoligotyping pattern: Strain	Rifoligotyping pattern: ZN	*rpoB* mutation: Strain	*rpoB* mutation: ZN	Score for ZN-stained smear on Bronkhorst scale
R1	Moldova	■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+2
R2	Moldova	■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	■□■■■ □□□□	■□■■■ □□□□	516-GTC	516-GTC	+2
R3	Moldova	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■□ □■□□	■■■■□ □■□□	531-TGG	531-TGG	+5
R4	Moldova	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+5
R5	Moldova	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■□■■■ □□□□	■■■■□ ■□□□	516-TTC	531-TTG	+5
R6	Moldova	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■□■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+5
R7	Moldova	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■□ □■□□	■■■■□ □■□□	531-TGG	531-TGG	+2
R8	Moldova	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■□ ■□□□	Neg	531-TTG	Neg	Neg
R9	Moldova	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+2
R10	The Netherlands	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	Neg	■□■■■ □□□□	Neg	516-GTC	Neg	Neg
R11	The Netherlands	■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	Neg	■■■■□ □■□□	Neg	531-TGG	Neg	Neg
R12	The Netherlands	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+2
R13	The Netherlands	No strain available	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	ND	■■■□■ □□□■	ND	526-TAC	+1
R14	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■■ □□□□	■■■■■ □□□□	516-GTC	516-GTC	+2
R15	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+3
R16	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+2
R17	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+2
R18	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■□ ■□□□	■■■■□ ■□□□	531-TTG	531-TTG	+2
R19	Kazakhstan	No strain available	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	ND	■■■■□ ■□□□	ND	531-TTG	+1
R20	Kazakhstan	No strain available	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	ND	■■■■□ ■□□□	ND	531-TTG	+1
R21	Kazakhstan	No strain available	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	ND	Neg	ND	Neg	+1

[a] Characteristics of the rifampin-resistant strains that were isolated (strain) and extracts from ZN-stained slides (ZN) for different patients from Moldova, The Netherlands, and Kazakhstan. Spoligotyping patterns are read from left to right and indicate spacers 1 to 43 from the direct repeat region, as described by Kamerbeek et al. (15). A fragment of the *rpoB* gene was amplified from bacterial cultures and ZN-stained slides and tested for mutations by hybridization to membrane-bound oligonucleotides giving rifoligotyping patterns corresponding to alleles for sensitivity (squares 1 to 5; probes W1 to W5, respectively) and resistance alleles (squares 6 to 9; probes M1 to M4, respectively). ■, hybridization; □, no hybridization; Neg, negative; ND, not determined.

tion of the strain. All other samples containing resistant *M. tuberculosis* strains and the strains in those samples with sensitive mycobacteria were correctly identified by both methods. Three mutations (531TTG, 531TGG and 526TAC) could be identified by rifoligotyping in DNA extracts from 15 of the 21 slides. The fourth mutation (516GTC), which was present in three strains, was not identified, but the lack of a hybridization signal with the wild-type probe covering this region indicated that the *rpoB* gene was altered. In contrast, DNA sequencing disclosed all mutations leading to resistance in the strains analyzed. Patnaik et al. (29) also performed sequencing of PCR products and sequencing of DNA extracted from slides. They used sputa that were spread onto slides and dried on a hot plate (75°C). In contrast, our approach used previously examined ZN-stained slides. ZN staining is a very harsh treatment which kills all mycobacteria. Heating of the samples, as done by Patnaik et al. (29), does not result in complete inactivation of *M. tuberculosis* and may present a danger of infection of laboratory staff during processing. Furthermore, samples evaluated in real time are stained with ZN, and to reduce the numbers of samples that must be analyzed, only ZN staining-positive samples are subjected to analysis for mutations leading to resistance.

The rifoligotyping and DNA sequencing results from this study corroborate the results from previous reports that more than 70% of the *rpoB* mutations occur in codons 531 and 526 (14). An earlier report suggested that there is no significant difference in the distribution of mutations among rifampin-resistant *M. tuberculosis* isolates from different countries (10). A review of the literature revealed 59 reports describing *rpoB* mutations in approximately 3,000 strains from 44 different countries. Table 4 summarizes the information from those reports and shows there may be differences in the geographic distributions of *rpoB* mutations; e.g., mutation 522TTG, which has been found in Asia, Australia, Russia, and the Americas, has not been reported in Europe. Similarly, mutation 533CCG was found in most parts of the world but has not been reported in the Middle East. Two other mutations, 513CCA and 516GTC, were more often seen in Asia, Australia, and Russia than in other parts of the world. However, it is difficult to compare the findings presented in the 59 studies because the reports are not always clear regarding the origins of the strains, the methodologies used, and the epidemiological relatedness of the strains. Furthermore, there are large differences in the numbers of strains used in the various studies. Consequently, no solid conclusions can be drawn concerning the distribution of mutations in the *rpoB* gene. Therefore, a study with a better design is needed to determine whether there are differences in the geographic distributions of mutations in the *rpoB* genes of rifampin-resistant *M. tuberculosis* strains. Such a study should use the same methodology, the number of samples should depend on the prevalence TB and resistance, the samples must be collected during the same time period, and the geographic origin should be well defined and balanced.

Regional differences in *rpoB* mutations may be associated with the types of treatment and the rifamycin analogs used (33). Particular mutations in either codon 531 or codon 526 lead to high-level resistance to all rifamycins. In contrast, mutations in codons 511, 516, 519, and 522 result in high-level resistance to rifampin and rifapentine but susceptibility to both

TABLE 3. Spoligotyping and rifoligotyping patterns and sequence analysis for DNA obtained from rifampin-sensitive *M. tuberculosis* isolates and corresponding ZN-stained slides

Sample no.	Country	Spoligotyping pattern		Rifoligotyping pattern		*rpoB* mutation		Score for ZN-stained smear on Bronkhorst scale
		Strain	ZN	Strain	ZN	Strain	ZN	
S1	Moldova	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■■■■■	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+5
S2	Moldova	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+2
S3	Moldova	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+1
S4	Moldova	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+1
S5	Moldova	■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■□□□□■■■□■■■	■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■□□□□■■■□■■■	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+5
S6	Moldova	■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■□□□□■■■□■■■	■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■□□□□■■■□■■■	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+5
S7	The Netherlands	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□	□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□	■■■■■ □□□□	■■■■■ □□□□	Wt	Wt	+1
S8	The Netherlands	No strain available	□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	ND	■■■■■ □□□□	ND	Wt	+1
S9	The Netherlands	No strain available	■■■■■■■■■■■■■■■□■■■■■■■□■■■■■■■■□□□□■■■■■■■	ND	■■■■■ □□□□	ND	Wt	+2
S10	The Netherlands	No strain available	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□	ND	□□□□□ □□□□	ND	Wt	+3
S11	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■■ □□□□	Neg	Wt	Neg	+1
S12	Kazakhstan	No strain available	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	ND	■■■■■ □□□□	ND	Wt	+2
S13	Kazakhstan	No strain availabel	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	ND	■■■■■ □□□□	ND	Wt	+4
S14	Kazakhstan	No strain available	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	ND	■■■■■ □□□□	ND	Wt	+2
S15	Kazakhstan	No strain available	■■■■■■■□□■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■■■■■	ND	■■■■■ □□□□	ND	Wt	+2
S16	Kazakhstan	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	■■■■■ □□□□	Neg	Wt	Neg	Neg

[a] Characteristics of the rifampin-sensitive strains at were isolated (strain) and extracts from ZN-stained slides (ZN) for different patients from Moldova, The Netherlands, and Kazakhstan. Spoligotyping patterns are read from left to right and indicate spacers 1 to 43 from the direct repeat region, as described by Kamerbeek et al. (15). A fragment of the *rpoB* gene was amplified from bacterial cultures and ZN-stained slides and tested for mutations by hybridization to membrane-bound oligonucleotides giving rifoligotyping patterns corresponding to alleles for sensitivity (squares 1 to 5; probes W1 to W5, respectively) and resistance (squares 6 to 9; probes M1 to M4, respectively). ■, hybridization; □; no hybridization; Neg, negative; ND, not determined; Wt, wild type.

TABLE 4. Summary of geographic distributions of the mutations in the *rpoB* gene of *M. tuberculosis* most frequently reported in the literature[a]

Codon and amino acid change	Base change	Occurrence (no. of countries)[b]				
		Asia, Australia, Russia (16)	Europe (13)	Africa (5)	Middle Eastern (3)	The Americas (7)
511 Leu→Pro	CTG→CCG	2	6	2	1	2
513 Gln→Pro	CAA→CCA	6	1		1	3
513 Gln→His	CAA→CTA	2	3	1		3
516 Asp→Val	GAC→GTC	13	11	3	2	6
516 Asp→Tyr	GAC→TAC	7	3	3		4
516 Asp→Glu	GAC→GAG			1		1
516 Asp→Gly	GAC→GGC	2	4	2		1
516 Asp→Ala	GAC→GTC	4	1	1		1
516 Asp→Asn	GAC→AAC		1		1	
516 Asp→Phe	GAC→TTC	1	2			
522 Ser→Leu	TCG→TTG	6		1	1	6
526 His→Tyr	CAC→TAC	12	7	4	2	7
526 His→Asp	CAC→GAC	8	10	3	1	7
526 His→Arg	CAC→CGC	5	6	4		4
526 His→Leu	CAC→CTC	5	5	4		3
531 Ser→Leu	TCG→TTG	12	10	4	3	6
531 Ser→Trp	TCG→TGG	4	7	2	1	4
533 Leu→Pro	CTG→CCG	6	7	3		6
Total		15	20	9	5	10

[a] The *rpoB* mutations detected and the corresponding region are summarized for 59 countries.

[b] The numbers indicate how many times the *rpoB* mutation was reported in literature for each region. With the exception of codon 516, only the most frequently observed *rpoB* mutations are displayed. All mutations were determined by DNA sequencing.

rifabutin and KRM-1648 (47). Yet, strains susceptible to both rifabutin and KRM-1648 may develop resistance to KRM-1648 through secondary mutations in the *rpoB* gene (28). Furthermore, some mutations, e.g., 533CCG, have been described to cause both high-level resistance (25) and low-level resistance (18, 27). These differences reflect the complex interaction between the drug and its target at the molecular level, in which the affected amino acid of the RNA polymerase seems to be crucial (3).

A previous study indicated that the nature and frequency of occurrence of mutations in the *rpoB* gene were independent of their IS*6110*-based restriction fragment length polymorphism analysis and spoligotyping patterns (31, 33). In our study we found that 70% of the strains of the *M. tuberculosis* Beijing genotype were rifampin resistant. Thirty-eight of the samples were from a prison in Kazakhstan; 86% of these strains were of the Beijing genotype, and 50% of these strains were MDR. The frequent appearance of the Beijing genotype in outbreaks and its prevalence worldwide in association with drug-resistant TB suggest that it may have the potential to spread, although evidence for this is lacking.

Rifoligotyping is relatively easy to perform and requires inexpensive and simple equipment. The method is sensitive and fast, making it possible to perform PCR and hybridization in a single day. However, the interpretation of the results is not always easy. Differences in the intensities of the spots make it difficult to determine whether weak spots are truly reactive or not. Furthermore, we have noticed that mutations in the *rpoB* gene may alter the hybridization characteristics of neighboring wild-type probes. This may lead to extra strong or relatively

weak spots in assays with oligonucleotide probes that do not react with the region where the mutation resides. In addition, it is difficult to design an array of probes that detect single base-pair substitutions, as all probes must have the same hybridization characteristics. Also, the requirement to detect a single base-pair change demands extremely accurate posthybridization wash conditions, limiting the variation in the washing temperature that is allowed to 1 to 2°C. An example of these difficulties was the hybridization with all of the wild-type probes in sample R14, which led to an interpretation of rifampin sensitivity for the resistant *M. tuberculosis* strain in this sample. Like rifoligotyping, most other molecular techniques used, like PCR–enzyme-linked immunosorbent assay (10), PCR-based reverse hybridization line probe assay (Ino-LiPA; Innogenetics N.V., Ghent, Belgium) (2), and single-stranded conformational polymorphism analysis (3), fail to detect all mutations in the core region of the *rpoB* gene. Moreover, silent mutations result in the loss of a signal with wild-type probes and cannot be discriminated from mutations that lead to rifampin resistance without the use of proper probes for detection of the mutation. This may lead an incorrect classification of sensitive strains as rifampin resistant. These problems can be avoided by using DNA sequencing of the core region of the *rpoB* gene. Sequence analysis yields unambiguous results, enables the detection of new mutations, and makes it possible to discriminate silent from nonsilent mutations. In the method described in this study, DNA sequencing of the PCR products of DNA extracts from slides was possible only after reamplification by a seminested PCR. Reamplification was required because the amount of amplified DNA obtained in the first PCR was mostly insufficient for reliable sequencing. The sequencing can be carried out in any laboratory that has PCR and DNA sequencing facilities but takes 2 days to perform, which is twice as long as rifoligotyping. DNA sequencing requires expensive equipment, but the costs per analysis are not extremely high. Not counting the costs for DNA extraction, we calculated the costs of PCR-rifoligotyping to be approximately $4.50 per sample, whereas double PCR and sequencing cost $6.50 per sample.

The methods described in this report are intended to be used to study changes in the resistance patterns by using material only from ZN-stained slides. The study aimed at detecting trends in the development of resistance and not in the detection and identification of MDR in strains from individual patients. In many regions of the world, particularly in developing countries, cultures are not used to confirm a diagnosis of TB in an individual patient, and as a result, drug susceptibility testing results are not available for the strains from any given patient with TB. Often, these are the regions where the rates of TB are soaring and MDR *M. tuberculosis* is prevalent. Rapid detection of drug resistance is critical for achieving favorable clinical outcomes and in preventing the continued transmission of disease (35). For these regions of the world, centralized laboratories that process ZN-stained slides for the detection of resistance by molecular methods such as the relatively simple rifoligotyping method may contribute to the diagnosis and proper treatment of TB. However, among the set of 37 slides that we analyzed in our study, 6 (16%) did not yield a rifoligotyping pattern or an *rpoB* sequence. This means that the sensitivities of these methods may not be high enough for the diagnosis of TB in individual patients.

Analysis of the results of tests for anti-TB drug resistance and genotyping of *M. tuberculosis* isolates collected from different geographic region of the world may help in the study of the epidemiology of the disease and may help to improve the control of TB by optimizing treatment regimens. Surveillance for rifampin resistance is a good surrogate to measure the efficiencies of TB control programs (46), because the present emergence of MDR TB is a reflection of the difficulties encountered in treatment (13). To optimize a tailored regimen in situations of MDR TB, phenotypic testing for sensitivity to first- and second-line drugs remains necessary. Molecular methods like rifoligotyping and DNA sequencing of the *rpoB* gene in *M. tuberculosis* DNA extracted from ZN-stained slides could provide simple methods for the rapid retrieval of information on the global evolution of anti-TB drug resistance and may provide a better understanding of the occurrence of primary resistance and the international transmission of MDR TB. Due to its accuracy and flexibility, DNA sequencing seems to be the best method for this type of research.

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