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Molecular Characterization of Rifampin- and Isoniazid-Resistant *Mycobacterium tuberculosis* Strains Isolated in Poland

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A total of 105 rifampin (RMP)- and/or isoniazid (INH)-resistant strains of *Mycobacterium tuberculosis* isolated from different parts of Poland in 2000 were screened for mutations associated with resistance to these drugs by two molecular methods, namely sequence analysis and real-time PCR technology. Three loci associated with drug resistance were selected for characterization: they were *rpoB* (RMP), *katG*, and the regulatory region of *inhA* (INH). Nineteen different mutations were identified in 64 RMP-resistant strains, and five new alleles were described. The most common point mutations were in codons 531 (41%), 516 (16%), and 526 (9%) of the *rpoB* gene. Mutations were not found in two (3%) of the isolates. In the case of resistance to INH, six different mutations in the *katG* gene of 83 resistant strains were detected. Fifty-seven (69%) isolates exhibited nucleotide substitutions at codon 315. One strain harbored a mutation affecting codon 279 (Gly279Thr). Twelve of 26 INH-resistant strains with the wild-type codon 315 (14.5% of all strains tested) had the mutation −15C→T in the regulatory region of *inhA*. A full correlation between the DNA sequence analysis and real-time PCR data was obtained. We conclude that the real-time PCR method is fast and reliable for the detection of RMP and INH resistance-associated mutations in *M. tuberculosis* clinical isolates.

Mycobacterium tuberculosis is one of the most harmful human pathogens worldwide, causing about 8 million new tuberculosis (TB) cases and between 2 million and 3 million deaths yearly (29). The upsurge of TB has been accompanied by a rise in the frequency of *M. tuberculosis* strains that are resistant to one or more primary anti-TB drugs. In Poland, 10,049 new and 1,428 previously treated TB cases were reported in 2000 (notification rate, 29.7 of 100,000). Primary drug resistance has been monitored since 1960, with decreasing frequencies occurring up to 1997. In 2000, a twofold increase in the primary resistance rate was observed in comparison with that in 1997 (6.1 versus 3.6%) (1). At the same time, an increased level of primary multidrug resistance (MDR) (resistance to at least isoniazid [INH] and rifampin [RMP]) was also observed (1.2 versus 0.6%) (1).

Early diagnosis of the disease and the rapid identification of resistance to primary anti-TB agents are essential for the efficient treatment and control of MDR strains. It is known that resistance to INH and RMP is a key factor in determining the effectiveness of the currently recommended standard treatment regimens. The elucidation of the mechanism of action of these drugs, which was accomplished only recently, has led to the development of new rapid diagnostic methods (4, 7, 14, 19, 22).

The rapid detection of RMP resistance is of particular importance, since it also represents a valuable surrogate marker for MDR resistance, which is a tremendous obstacle to TB therapy (10). Collectively, DNA sequencing studies have demonstrated that >95% of RMP-resistant (Rmp^r) *M. tuberculosis* strains have a mutation within the 81-bp hot-spot region (codons 507 to 533) of the RNA polymerase β subunit (*rpoB*) gene (23). The prevalence of the mutations determined so far varies for *M. tuberculosis* strains obtained from different countries. Thus, it is important to determine the distribution of resistance mutations at the level of each country prior to molecular tests being introduced for routine diagnostics (4).

Unlike RMP resistance, INH resistance is apparently controlled by a more complex genetic system that involves several genes (21). However, extensive studies have demonstrated that INH resistance is most frequently associated with a specific mutation in *katG* (codon 315), a gene that encodes the catalase-peroxidase enzyme in *M. tuberculosis* (18). Also, InhA (enoyl-ACP-reductase), an enzyme involved in mycolic acid biosynthesis, was identified as a main target of INH, and mutations in the regulatory region of the *inhA* gene have been linked to INH resistance (12).

The aim of the present study was to determine the drug resistance profiles of 105 drug-resistant *M. tuberculosis* strains isolated in Poland and to detect mutations present in the *rpoB* and *katG* genes as well as in the regulatory region of the *inhA* gene. Two molecular methods were used. In the first one, mutations that have been associated with RMP and/or INH resistance were determined by automated DNA sequence analysis. The results were compared with those obtained by real-time PCR technology.

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TABLE 1. Primers and probes used for PCR amplification and detection of drug-resistant *M. tuberculosis* strains

Target	Primer or probe	Sequence (5′-3′)[h]	Nucleotides within target[a]	Product size (bp)	Annealing temp (°C)	T_m (°C)
rpoB	TR9	TCGCCGCGATCAAGGAGT[b]	2335–2352	158	62	61.0
	TR8	GTGCACGTCGCGGACCTCCA[b]	2492–2473			68.4
	rpo sensor[c]	640-ACCCACAAGCGCCGACTGCTGG-P[d]	2412–2432			70.4
	rpo anchor[c]	TTCATGGACCAGAACAACCCGCTGTCGGT-F	2379–2407			73.8
	RPO1 sensor[e]	CAGCTGAGCCAATTCATGGACC-F	2367–2388			65.2
	RPO1 sensor[e]	640-AACAACCCGCTGTCGGGG	2390–2407			65.2
katG	TB86	GAAACAGCGGCGCTGATCGT[b]	2759–2778	209	67	64.3
	TB87	GTTGTCCCATTTCGTCGGGG[b]	2968–2948			62.8
	TB sensor[c]	640-TCACCAGCGGCATCGAGGTCGT-P[d]	2916–1937			69.4
	TB anchor[c]	CGTATGGCACCGGAACCGGTAAGGACGC-F	2886–2913			75.3
	TaqMan MGB probes	FAM-ACCAGCGGCATCG-Q	2918–2930			66.3
		VIC-TCACCACAGGCATCG-Q	2916–2930			65.1
		VIC-ACCACCGGCATCG-Q	2918–2930			64.9
inhA	TB92	CCTCGCTGCCCAGAAAGGGA[f]	56–75	248	64	65.2
	TB93	ATCCCCCGGTTTCCTCCGGT[f]	303–284			66.5
	TB ATT[g]	640-CCCGACAACCTATCATCTCGCC-P[d]	223–202			63.1
	TB anchor[g]	CCCCTTCAGTGGCTGTGGCAGTC-F	248–226			68.1

[a] The positions of the primers and probes correspond to GenBank accession numbers L27989, X68081, and U66801 for the *rpoB* gene, the *katG* gene, and the *inhA* locus, respectively.
[b] Primer sequence described by Telenti et al. (23).
[c] Probe sequence described by Torres et al. (25).
[d] P, phosphorylation of the 3′ end of the probe to prevent extension during PCR.
[e] Probe sequence described by Garcia de Viedma et al. (7).
[f] Primer sequence described by Gonzalez et al. (8).
[g] Probe sequence described by Torres et al. (26).
[h] Fluorophores were as follows: 640, Red 640; F, fluorescein; FAM, 6-carboxy-fluorescein; Q, quencher (TAMRA [6-carboxy-*N,N,N′,N′*-tetramethylrhodamine]); VIC, fluorescent reporter. For TaqMan MGB probes, designed to detect wt codon 315 of *katG* (AGC) and two mutations (ACA and ACC), these sequences are underlined.

MATERIALS AND METHODS

Bacterial strains. The *M. tuberculosis* strains examined for this study were isolated from TB patients in Poland in 2000 during the second national survey of drug resistance. Strains obtained in different regions of the country were provided by the National Tuberculosis Reference Laboratory in Warsaw. In this survey, 221 INH-resistant (Inh^r) and 104 Rmp^r strains were identified [both monoresistant and multiply resistant to another drug(s)] (1). Of these, 105 drug-resistant *M. tuberculosis* strains were examined, comprising 83 Inh^r (37.6%) and 64 Rmp^r (61.5%) strains (numbers include 59 MDR strains). As controls, *M. tuberculosis* strain H37Rv (susceptible) and four INH-sensitive and three RMP-sensitive clinical isolates were included for the analysis.

Primary isolation, differentiation, and drug susceptibility testing were performed with Löwenstein-Jensen medium and the BACTEC 460-TB system (Becton-Dickinson, Sparks, Md.), as reported earlier (1).

The relatedness of all resistant strains was investigated by studying restriction fragment length polymorphisms (RFLPs) of the IS*6110* element (28). The spoligotyping (spacer oligotyping) method (11) was additionally used to confirm the relationships of the strains.

DNA preparation. A rapid DNA extraction procedure for the direct testing of *M. tuberculosis* on Löwenstein-Jensen slants was performed. A loopful of organisms was suspended in 1 ml of sterile water and the bacteria were lysed by boiling for 20 min. The cells were centrifuged (12,000 × *g* for 5 min) and then frozen overnight at −20°C. The supernatant was used as a template for amplification.

Standard PCR. The DNA extract was amplified with the primers listed in Table 1. Primer pairs TR8-TR9 and TB86-TB87 were used to amplify the regions within the *rpoB* and *katG* genes, respectively (22). Primers TB92 and TB93 were applied for the amplification of a fragment containing the regulatory region of the *inhA* locus (8). Prior to PCR, a preincubation step (95°C for 15 min) was performed to activate the HotStart *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany). The PCR was carried out for 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C (for *rpoB*), 67°C (for *katG*), or 64°C (for *inhA*) for 30 s, and extension at 72°C for 45 s in an MJ Research thermocycler. PCR products were examined by gel electrophoresis and purified by use of a QIAquick PCR purification kit (Qiagen).

DNA sequence analysis. The PCR primers were also used for the direct sequencing of both strands of the amplification products with an automated ABI Prism 377 DNA sequencer and corresponding kits from the same manufacturer (Applied Biosystems, Foster City, Calif.). The Blast 2 Sequences computer program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov/BLAST/).

Probes for detection of mutations by real-time PCR. The sequences of the probes used for the present study are listed in Table 1. For the detection of mutations in the *rpoB* gene, two pairs of fluorescence resonance energy transfer (FRET) probes, the *rpo* (25) and RPO1 (7) probes, were used. Both pairs of probes were labeled with fluorescein (at the 3′ ends) and Red 640 (at the 5′ ends) and were homologous to the wild-type (wt) sequence. Thus, mutations in the DNA region covered by the probes led to different melting temperatures (T_ms) for the probes when they were hybridized to the target.

For *katG*, a set of standard FRET probes and three TaqMan minor groove binder (MGB) probes were applied. The FRET probes covered the region containing codon 315, which is the most frequent target for mutations related to INH resistance. This pair of probes was labeled with fluorescein (TB anchor) and Red 640 (TB sensor) (25). Additionally, three *katG*-specific TaqMan MGB probes were designed to be homologous to wt codon 315 (AGC) or to one of the most frequent substitutions, ACC or ACA. These oligonucleotides contained a reporting fluorescent dye (FAM or VIC) at the 5′ end and a quencher dye (TAMRA) at the 3′ end. During PCR, the 5′→3′ exonuclease activity of *Taq* DNA polymerase cleaves the downstream probe as it extends. As the probe is degraded, the reporter dye is separated from the quencher. This results in increased fluorescence as amplification proceeds. Because the cleavage occurs only if the probe hybridizes to the target, the fluorescence detected originates from specific amplification.

For the detection of mutations in the *inhA* locus, a pair of conventional FRET probes was used (anchor-sensor design) (26). The 5′-Red 640-labeled sensor (TB ATT) covered the segment containing the regulatory region (Table 1).

All primers and FRET probes were synthesized by TIB MOLBIOL (DNA Synthesis Service, Berlin, Germany), and TaqMan MGB probes were synthesized by Applied Biosystems (Weiterstadt, Germany).

Real-time PCR assay. The PCR mixtures, in a final volume of 20 μl, contained 2.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, a 1 μM concentration of each primer, a 0.2 μM concentration of each probe (except for the RPO1 and TaqMan MGB probes, which were added to final concentrations of 0.04 and 0.1 μM, respectively), 1 U of HotStart *Taq* DNA polymerase (Qiagen), and 2 μl of template DNA. Additionally, the reaction

TABLE 2. DNA sequencing and real-time PCR data for *rpoB* mutations in Rmp^r *M. tuberculosis* strains from Poland

Affected codon	Nucleotide change(s)	Amino acid change	No. (%) of strains with mutation	Deviation of T_m (°C) from that for wt[a]	
				RPO1 probe (65.4°C)	*rpo* probe (63.2°C)
531	TCG→TTG	Ser→Leu	24 (37.5)		+1.92 to +2.62
	TCG→TGG	Ser→Trp	2 (3.1)		ND
526	CAC→ACC	His→Thr	2 (3.1)		−1.78
	CAC→GAC	His→Asp	2 (3.1)		−3.08 to −3.38
	CAC→TGC	His→Cys	1 (1.6)		ND
	CAC→TAC	His→Tyr	1 (1.6)		−1.78
522	TCG→TTG	Ser→Leu	1 (1.6)	ND	
517	CAA→CTA[b]	Gln→Leu	1 (1.6)	−3.83	
516	GAC→TAC	Asp→Tyr	6 (9.4)	−2.33 to −2.83	
	GAC→GTC	Asp→Val	3 (4.7)	−2.53	
	GAC→GTG[b]	Asp→Val	1 (1.6)	−2.83	
513	CAA→CTA	Gln→Leu	2 (3.1)	−3.83	
	CAA→AAA	Gln→Lys	2 (3.1)	−6.33	
514	TTC	Phe (Ins)[c]	1 (1.6)	−5.33	
516 and 529	GAC→GGC and CGA→CAA	Asp→Gly and Arg→Gln	1 (1.6)	ND	
515 and 516	ATG→ATT[b] and GAC→TAC	Met→Ile and Asp→Tyr	8 (12.5)	−6.33 to −6.63	
513 and 526	CAA→CTA and CAC→AAC	Gln→Leu and His→Asn	1 (1.6)	ND	
512 and 516	AGC→ATC[b] and GAC→GGC	Ser→Ile and Asp→Gly	1 (1.6)	−7.33	
510 and 516	CAG→CAC[b] and GAC→TAC	Gln→His and Asp→Tyr	2 (3.1)	−6.33	
None	None	None (wt)	2 (3.1)	−0.13 to −0.17	

[a] Deviations in the T_ms for the RPO1 (7) and *rpo* (25) probes in comparison with the value for the reference wt sequence (given in parentheses); ND, not determined.
[b] New allele.
[c] Ins, insertion.

mixture for *rpoB* amplification included 8 μl of Q solution (Qiagen). For preparation of the components for the *katG* gene PCR with TaqMan MGB probes, 10 μl of a commercial ready-to-use reaction mix (universal PCR master mix; Roche) was used.

The cycling parameters used for experiments with FRET probes were as follows: 95°C for 10 min and 35 to 40 amplification cycles at 94°C for 15 s; 62, 67, or 64°C (annealing temperatures for *rpoB*, *katG*, and *inhA*, respectively) for 20 s; and 72°C for 30 s. PCR cycling was followed by a melting-curve analysis from 50 to 85°C, with fluorescence readings taken every 1°C. The T_m of each of the FRET probes for the wt sequence was calculated empirically as the average value of the T_ms obtained in at least three independent experiments. When a probe binds to a mismatched sequence, the T_m deviates with respect to that of a perfectly matched wt sequence. The standard deviations (SD) of the T_ms for probes *rpo*, RPO1, TB (*katG*), and TB (*inhA*) were 0.10, 0.33, 1.20, and 0.36°C. In all cases in which the deviations in the T_m were more than two times the SD, a mutation was suspected.

For PCRs with TaqMan MGB probes, the conditions for cycling were 94°C for 10 min followed by 40 to 45 cycles of 92°C for 15 s and 60°C for 1 min. All reactions were performed in a Rotor-Gene 2000 instrument (Corbett Research, Mortlake, Australia).

RESULTS

Detection of mutations in the *rpoB* gene. Sixty-four Rmp^r *M. tuberculosis* clinical isolates (including 59 MDR strains) were subjected to DNA sequencing analysis of the hypervariable (hot-spot) *rpoB* region. Nineteen different types of mutations were identified (Table 2). Most of them were single-nucleotide mutations (69%) involving six codons. No silent substitutions in the 158-bp *rpoB* gene fragment examined were observed for any of the *M. tuberculosis* strains analyzed in this study. The codons most frequently affected by point mutations were codons 531, 516, and 526, with frequencies of 41, 16, and 9%, respectively. Eighteen strains (28%) exhibited more complex mutations. One strain, identified as a member of the Beijing family, had an insertion of Phe between codons 514 and 515. Thirteen Rmp^r strains of *M. tuberculosis* (20%) contained point mutations in two separate codons, specifically in codons 513 and 526 (1.6%), 516 and 529 (1.6%), 512 and 516 (1.6%), 510 and 516 (3.1%), and 515 and 516 (12.5%). The last three double mutations have not been described previously. The most frequent of them (Met515Ile plus Asp516Tyr) was found in eight strains. Seven of these isolates had identical IS*6110* RFLP patterns, indicating their epidemiologic relationship. However, direct epidemiologic links could be established for only two patients of this cluster. Additionally, two strains had novel mutations in single codons. One of these two strains contained a GAC→GTG (Asp-to-Val) mutation in codon 516, and in the other strain codon 517 had the mutation CAA→CTA (Gln to Leu). In total, five new alleles were identified in the present study, with all involving changes in one or two codons. Two Rmp^r strains (3.1%) contained no mutations in the sequenced region, although these strains were phenotypically resistant to RMP. None of the three RMP-sensitive control isolates had any mutation.

Fifty-eight Rmp^r strains (including 55 MDR isolates) that were previously characterized by DNA sequencing and three RMP-susceptible clinical isolates were available for analysis by the faster technology of real-time PCR. The three susceptible strains showed T_ms within the range obtained for the wt reference strain *M. tuberculosis* H37Rv. All strains with a changed *rpoB* core region were correctly identified in the real-time PCR assay. With the *rpo* sensor probe (25), a change from the wt sequence to a sequence with a mutation at codon 531 (TCG→TTG) was detected in 24 strains as an increase in the T_m of the probe (from 1.92 to 2.62°C; Table 2), whereas for the 5 strains with three different mutations at codon 526, the T_m of the probe was 1.78 to 3.38°C lower (depending on the mutation) than that of the wt strain (Table 2). With the RPO1 probes (7), mutations in the 5′ half of the core in 29 strains were also detected by reductions in the T_ms of the probes

TABLE 3. DNA sequencing and real-time PCR data for mutations in the *katG* gene and the *inhA* regulatory region in Inh^r *M. tuberculosis* strains from Poland

DNA target	No. of strains tested	Nucleotide change(s)	Amino acid change	No. (%) of strains with mutation	Deviation of T_m (°C) from that for wt for FRET probes[a]	Result with TaqMan MGB probe[b]		
						ACC	ACA	AGC (wt)
katG codon 315	83	AGC→ACC	Ser→Thr	50 (60.2)	−2.41 to −4.41	+	−	−
		AGC→ACT	Ser→Thr	4 (4.8)	−8.51	−	−	−
		AGC→ACA	Ser→Thr	1 (1.2)	−8.41	−	+	−
		AGC→AAC	Ser→Asn	1 (1.2)	−4.71	−	−	−
		AGC→ATC	Ser→Ile	1 (1.2)	−3.21	−	−	−
katG codon 279		GGC→ACC	Gly→Thr	1 (1.2)	+0.29	−	−	+
		None	None (wt)	25 (30.1)	−1.71 to +1.09	−	−	+
inhA	26[c]	−15C→T		12 (14.5)	+4.85 to +5.25			
		None		14 (16.5)	−0.45 to +0.55			
katG and *inhA*	83[d]	None	None (wt)	13 (15.7)				

[a] Deviations in the T_ms for the *katG* (25)- and *inhA* (26)-specific probes in comparison with the value for the reference wt sequence (74.8 and 62.1°C, respectively).
[b] Three different probes were designed to detect mutations ACC and ACA and the AGC wt sequence in *katG* codon 315. +, positive result; −, negative result.
[c] The strains had a wt codon 315 in the *katG* gene.
[d] All Inh^r strains were included.

(from −2.33 to −7.33°C; Table 2). The real-time PCR assay results were in full accordance with the nucleotide sequencing data.

Detection of mutations in the *katG* gene. Eighty-three *M. tuberculosis* strains that were resistant to INH [alone or in combination with another drug(s)] were investigated by DNA sequencing of a 209-bp *katG* fragment including codon 315, the codon at which mutations are most frequently associated with INH resistance. Fifty-seven (68.7%) Inh^r isolates had mutations at codon 315 (Table 3). The wt codon, AGC (Ser), was altered to ACC (Thr) in 50 strains, ACT (Thr) in 4 strains, ACA (Thr) in 1 strain, AAC (Asn) in 1 strain, and ATC (Ile) in 1 strain. One strain harbored a double mutation affecting codon 279 (GGC→ACC [Gly→Thr]). In 25 (30.1%) cases, no mutation was found in the analyzed fragment. Four INH-susceptible control isolates possessed the wt sequence.

All 83 Inh^r strains and 4 INH-sensitive controls were subjected to real-time PCR analysis, and the results were compared to those obtained by DNA sequencing. Two different types of probes (FRET and TaqMan MGB) were used to detect the most prevalent mutations associated with INH resistance. The analysis (Table 3) indicated the presence of deviations in the T_ms of the FRET probes specific for *katG* (25), which suggests the presence of mutations, for all 57 strains (T_m reductions, −2.41 to −8.51°C). For the four INH-sensitive isolates, the T_ms of the probes were within the range of the SD for the wt reference strain. A mutation at codon 279 in one strain could not be detected because it was outside the region spanned by the probe used. A similar result for this strain was obtained with three different TaqMan MGB probes designed to detect wt codon 315 (AGC) and its two most frequent mutations, ACC and ACA. All ACC and ACA mutations in codon 315 as well as the wt AGC sequence were correctly identified by the TaqMan MGB probes (Table 3). Only three other substitutions at codon 315 (ACT, AAC, and ATC) were not detected by these particular probes. Overall, except for four explainable cases, there was full agreement between the results of real-time PCR and those of sequencing, both according to the presence or absence of mutations (FRET and TaqMan MGB probes) and according to the presence of two particular mutations for Inh^r (TaqMan MGB assay).

Detection of mutations in the *inhA* regulatory region. Twenty-six Inh^r *M. tuberculosis* strains that had no mutations at *katG* codon 315 (including one strain carrying a mutation at codon 279) were investigated in a real-time PCR test. A set of standard FRET probes was used to detect changes in a 22-bp fragment upstream of the start site of *inhA* (26). For 4 INH-susceptible control isolates and 14 Inh^r strains (including one strain with a mutation at codon 279 of *katG*), the T_ms were within the range obtained for the reference strain, and therefore they were not considered mutants (Table 3). For the remaining 12 (14.5%) Inh^r strains, deviations in the T_ms of the probes suggested mismatches, as there were increases in the T_ms of the probes (from 4.85 to 5.25°C; Table 3) compared to those for the wt reference strain.

To confirm the alterations in the *inhA* regulatory region in the 12 Inh^r strains, we determined the DNA sequence of the 248-bp fragment of this locus. We found that all of the strains carried a single point mutation, C→T, at position 15 upstream of the start site of the *inhA* gene (Table 3). This mutation has previously been reported by other groups. The present understanding of this mutation is that it probably confers resistance by a drug titration effect. Overall, 58 (69.9%) Inh^r *M. tuberculosis* strains investigated in this study harbored mutations in the *katG* gene, 12 (14.5%) strains carried a mutation in the regulatory region of the *inhA* gene, and the remaining 13 (15.7%) isolates had no detectable changes in the two targets analyzed. In all cases, full concordance was obtained between the real-time PCR results and DNA sequencing data.

DNA fingerprinting analysis. Two standardized DNA fingerprinting techniques, spoligotyping (11) and IS*6110* RFLP typing (28), were used to differentiate the strains in order to assess possible genetic similarities between them. The numbers of IS*6110* copies per isolate varied from 5 to 20 (A. Sajduda, A. Brzostek, and J. Dziadek, unpublished results). A total of 73 different IS*6110* fingerprint patterns were identified for the 105 analyzed strains. Fifty-nine strains showed unique fingerprints, which indicates their epidemiologic independence. However,

46 strains (44%) clustered into 14 groups consisting of two to seven isolates with identical IS*6110* RFLP patterns, representing possible cases of recent transmission. Direct epidemiologic links could not be established for most of the cases in clusters. However, the transmission of the same drug-resistant *M. tuberculosis* strain among the patients of one cluster or their infection by contact with one or more index patients is further supported by the observation that in most clusters all of the strains exhibited the same polymorphism in the Rmp^r and/or Inh^r-associated genes and identical or very similar drug resistance profiles (data not shown). Also, only five of the clusters had isolates with different Rmp^r and/or Inh^r mutations, indicating an independent acquirement of resistance to these drugs. Although the epidemiologic data obtained in the present study are preliminary, they show that the transmission of drug-resistant *M. tuberculosis* strains may have occurred in Poland and seems to have contributed to the emergence of drug-resistant TB in this country.

DISCUSSION

In this study, a total of 105 Inh^r and/or Rmp^r *M. tuberculosis* strains, isolated in Poland in 2000 during the second national drug resistance survey, were investigated by DNA sequencing and a real-time PCR assay to evaluate drug resistance mechanisms.

Our findings of mutations in the *rpoB* and *katG* genes were comparable to those reported for strains from other parts of the world, especially the common mutations, which reflect a global pattern (16). The *rpoB* codons 531, 526, and 516 are the most frequently mutated codons worldwide, although variations in the relative frequencies of mutations in these codons have been described for *M. tuberculosis* isolates from different geographic locations (reviewed in reference 4). Also, in our study most of the strains with the Rmp^r phenotype (69%) contained missense mutations which led to amino acid substitutions at the Ser531 (41%), Asp516 (16%), and His526 (9%) residues. A comparison of these results with the results of other studies showed that the mutations at codon 531 also predominate in Poland (4, 23, 27). In contrast with previously published data, the frequency of mutations at codon 516 was found to be high in Poland (4, 23). A high frequency of changes at this codon was also described recently for MDR *M. tuberculosis* isolates from East Hungary (3), Latvia (27), North India (20), and East Asia (15). A characteristic finding was the high frequency of double mutations occurring in two separate codons (20%). Altogether, we found five novel alleles, which broadens the range of known mutations in the *rpoB* gene. The high incidence of these less common changes in Polish strains can be explained by either geographic variations in the frequencies of particular *rpoB* mutations or a sample bias. Sequence analysis identified no mutation in two (3.1%) of the strains tested, although they were resistant to RMP as determined by the proportion method. A recent study revealed that a mutation associated with RMP resistance can also be located outside the 81-bp *rpoB* core region, although this does not occur frequently (9). Other possibilities are that in these resistant strains, other rare *rpoB* mutations, heteroresistance (a mixture of susceptible and resistant subpopulations), or less likely, another mechanism of resistance may be involved (3, 10).

In Inh^r strains, neither insertions nor deletions (complete or partial) of *katG* were found, which is evidence of the rare occurrence of these mutations in clinical isolates, although they were reported previously by other authors (20, 24). We observed that almost 70% of the strains studied carried the Ser315Thr substitution, which is in concordance with the bulk of the published data (16). However, the frequency of this mutation was lower in Polish strains than the 93.6% of strains in northwestern Russia (13), 91% of strains in Latvia (26), and 85.7% of strains in Lithuania (2). In accordance with previous reports, the most frequent mutation was AGC to ACC at *katG* codon 315 (60%). A novel double mutation, AGC to ACT, which also leads to an amino acid change from Ser to Thr, was found in four strains. However, three of these isolates seemed to be epidemiologically related, according to their identical patterns in the IS*6110* RFLP analysis. We also identified one strain with a double mutation at codon 279 (GGC→ACC) that has not been described previously. This strain carried wt *katG* codon 315 and showed no mutation in the regulatory region of *inhA* (see below). We assume that this mutation is involved in the INH resistance of this strain.

Since 31% of the Inh^r strains in this study had no mutation at *katG* codon 315, it is likely that mutations in other genes, such as the *inhA* locus, significantly contribute to resistance. Previous studies have shown that mutations in the upstream region of the *inhA* locus result in increased levels of InhA (NADH-dependent enoyl-acyl carrier protein reductase) expression, thereby elevating the drug target levels and producing INH resistance via a titration mechanism (16). We searched for mutations in the *inhA* regulatory region of 26 Inh^r *M. tuberculosis* strains with a wt codon 315 in *katG* and found a substitution at position 15 upstream of the start codon in 12 (14.5%) isolates. This frequency was similar to the 13.5% frequency in strains from Lithuania (2) but higher than the 4.3% frequency in strains from Seville, Spain (24). Since a polymorphism in the *inhA* regulatory region accounted for almost 15% of all Inh^r strains examined in this study, we suggest screening for such a polymorphism in all isolates from Poland that have no detectable changes at codon 315 of the *katG* gene. Fourteen (16.5%) strains showed a lack of mutations in both the *katG* gene and the *inhA* locus fragments analyzed here. According to previous studies, there are some 18 additional genes that have been implicated in INH resistance, including *kasA*, *ndh*, and the *oxyR-ahpC* intergenic region (17). These genes either are involved in mycolic acid biosynthesis or are overexpressed as a response to the buildup or cellular toxicity of INH. Therefore, the alterations responsible for INH resistance in these 14 strains may be associated with any of these loci.

The prevalence of Rmp^r mutations within the *rpoB* core region (97%) as well as Inh^r mutations at *katG* codon 315 and the *inhA* locus (69 and 14.5%, respectively) in *M. tuberculosis* strains from Poland indicated the potential for a rapid diagnostic test for the detection of drug-resistant TB. Different genotypic assays have been proposed for the detection of mutations involved in drug resistance in *M. tuberculosis*, including a recent real-time PCR assay coupled to fluorescence detection. Formats that utilize molecular beacons (6) and biprobes (5) for the detection of RMP resistance have been reported.

However, as many as five different beacons are required to encompass the entire *rpoB* core region, and three biprobes described by Edwards et al. (5) detected mutations in only four codons of the *rpoB* gene. We evaluated a standard FRET probe set that was previously described by Torres et al. (25) for the detection of Rmp^r mutations at codons 526 and 531 of the *rpoB* gene and the RPO1 dual-sensor FRET probes that were recently described by García de Viedma et al. (7) to detect changes in the 5′ region of the *rpoB* core. Using both probe sets, we were able to detect all 17 different mutations in nine different codons. The majority of these alterations were not represented in previous reports (7, 25), thus further proving the efficiency of the method.

Also, the INH probe (TB sensor) utilized by Torres et al. (25) proved to be efficient for detecting mutations at *katG* codon 315. Using this probe, we were able to detect not only the previously described substitutions (ACC and AAC) but also three additional changes (ACT, ACA, and ATC) that were not present in the authors' collection. Analysis of the melting profiles of the three probes allowed the determination of the codon positions of mutations or even the distinction of different nucleotide substitutions at the same codon. As an alternative to FRET probes, we propose a set of three TaqMan MGB probes designed to detect the wt sequence AGC and the two most frequent substitutions, ACC and ACA. These short oligonucleotides are perfectly suited to search for mutations at a particular position, as in the case of *katG* codon 315. Compared to FRET probes, the TaqMan MGB probes offer the advantage of detecting a particular mutation that may have further implications, since different mutations may be associated with different levels of resistance.

Since almost 15% of the strains studied had a C→T nucleotide substitution at position 15 upstream of the start site of the *inhA* gene, we applied a pair of standard FRET probes to search for mutations in the regulatory region of *inhA* (26). Mutations in this region cause the overexpression of this gene. The *inhA*-specific probe sequence was designed to bind to a mutant sequence at position −15 in the putative promoter site (A instead of C). In all cases, the T_ms for the mutants were ca. 5°C higher than the T_m for the wt reference strain, proving this test efficient for detecting mutations in the regulatory region of *inhA*. We emphasize that in all cases studied, the results obtained by real-time PCR amplification corresponded to the nucleotide sequence data.

In the present study, we determined the occurrence and frequencies of different kinds of mutations at various target loci in drug-resistant clinical isolates of *M. tuberculosis* from Poland. We developed a basis for the detection of mutations underlying RMP and INH resistance in *M. tuberculosis* in Poland by the application of real-time PCR technology. This molecular method is fast and reliable and could be directly applied for examinations of clinical material.

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