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Rifampin Drug Resistance Tests for Tuberculosis: Challenging the Gold Standard

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The rapid diagnosis of rifampin resistance is hampered by a reported insufficient specificity of molecular techniques for detection of *rpoB* mutations. Our objective for this study was to document the prevalence and prognostic value of *rpoB* mutations with unclear phenotypic resistance. The study design entailed sequencing directly from sputum of first failure or relapse patients without phenotypic selection and comparison of the standard retreatment regimen outcome, according to the mutation present. We found that among all *rpoB* mutations, the best-documented "disputed" rifampin resistance mutations (511Pro, 516Tyr, 526Asn, 526Leu, 533Pro, and 572Phe) made up 13.1% and 10.6% of all mutations in strains from Bangladesh and Kinshasa, respectively. Except for the 511Pro and 526Asn mutations, most of these strains with disputed mutations tested rifampin resistant in routine Löwenstein-Jensen medium proportion method drug susceptibility testing (DST; 78.7%), but significantly less than those with common, undisputed mutations (96.3%). With 63% of patients experiencing failure or relapse in both groups, there was no difference in outcome of first-line retreatment between patients carrying a strain with disputed versus common mutations. We conclude that rifampin resistance that is difficult to detect by the gold standard, phenotypic DST, is clinically and epidemiologically highly relevant. Sensitivity rather than specificity is imperfect with any rifampin DST method. Even at a low prevalence of rifampin resistance, a rifampin-resistant result issued by a competent laboratory may not warrant confirmation, although the absence of a necessity for confirmation needs to be confirmed for molecular results among new cases. However, a result of rifampin susceptibility should be questioned when suspicion is very high, and further DST using a different system (i.e., genotypic after phenotypic testing) would be fully justified.

Prompt diagnosis of multidrug-resistant tuberculosis (MDR-TB) has been the main obstacle to its correct management and control. This problem would seem to have been solved with the development of molecular techniques applicable also in high-prevalence, low-income settings, such as the Genotype MTBDR*plus* and Gene Xpert MTB/RIF assays. However, though very rapid and highly sensitive, these tests are not considered highly specific for the diagnosis of rifampin resistance (1). From calculations based on this experimentally imperfect specificity, the World Health Organization (WHO) has thus recommended that an Xpert result indicating resistance must be confirmed by another technique when rifampin resistance prevalence is below 15% (2). Obviously, this requirement reverses the gains made in the early and rapid diagnosis of MDR-TB in most settings.

Phenotypic TB drug susceptibility testing (DST) is the gold standard and has hitherto not been questioned, although we are well aware of important differences between the various techniques, particularly for some drugs, e.g., ethambutol (3). Traditionally considered to be highly accurate and reliable, rifampin DST was also found not to be that straightforward in the rounds of proficiency testing among the supranational TB reference laboratories (SRL) (4). Strains yielding highly discordant results in these high-profile laboratories carried specific *rpoB* mutations, and results were shown to depend on method and details of the technique used (5). The Bactec 460 radiometric and Mycobacteria Growth Indicator Tube (MGIT 960) automated DST methods systematically classified strains as susceptible that were usually resistant by the absolute concentration or proportion method on Löwenstein-Jensen (LJ) medium as well as agar medium. This has now been confirmed for a larger number and range of mutated strains (6).

What remains unclear is the importance of such discordant strains with "disputed" *rpoB* mutations in terms of the relative frequency and impact on outcome of rifampin-based standard therapy. They are commonly considered to be very rare (7), and their MICs can be below the conventional critical concentration (8). For this reason, they are often considered susceptible, despite reports on adverse treatment outcome (9). Moreover, their prevalence may be underestimated because very few large molecular surveys have been performed without phenotypic DST preselection. Their low MICs and in our own experience often also pronounced fitness loss make them difficult to grow and thus to detect in phenotypic DST. Moreover, the critical concentrations used in DST have not been established to detect each and every clinically resistant strain (10, 11).

In this report we describe the distribution of *rpoB* mutations found by DNA sequencing directly from sputum in systematic samples from two populations of first retreatment cases. We also analyzed standardized first-line drug retreatment outcome by mutation, comparing the clinical prognostic value of pheno-

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typic and genotypic DST. Based on our results, we challenge consideration of phenotypic methods as the gold standard for rifampin DST.

MATERIALS AND METHODS

Patients and specimens. Since the mid-1990s, strains from Bangladesh, or sputa transported in cetylpyridinium chloride (CPC) from Kinshasa (Democratic Republic of Congo), were tested by DST on solid medium at the Antwerp SRL for drug resistance surveillance through systematic sampling of smear-defined recurrences (failure and relapse/reinfection) after primary treatment (category 1) (12). For a number of years, a portion of the sputum samples were sent as ethanol-preserved samples for *rpoB* sequencing, in the context of retrospective studies on acquired rifampin resistance (13). These systematic sample data were used to determine the mutation prevalence rates reported here.

For the second part of the study, analysis regarding the impact of particular mutations on treatment outcome, all Bangladesh first-line retreatment episodes (category 2) were included for which an *rpoB* sequence at the start was recorded in the laboratory database, so that not only those included in the acquired resistance studies above were part of the analysis.

Reference laboratory tests. Primary culture and first-line DST on LJ with final reading at 6 weeks were performed using standard methods, as previously described (14). Rapid, direct DST in liquid medium on microscopy slides was performed at the local laboratories, with rifampin critical concentrations of 0.5 and 1.0 μg/ml and reading at 10 to 14 days (15).

Sequencing was performed later and independently of phenotypic DST. DNA extracts from clinical specimens were prepared using the automated Boom extraction method (16, 17). Primers for amplification of the *rpoB* gene covered codons 176 to 672, including all areas from which rifampin resistance mutations have been described and not only the rifampin resistance-determining region (RRDR; codons 507 to 533) (18). All RRDR mutations, plus others previously reported for the *rpoB* gene (7, 19), were considered potentially relevant for rifampin resistance and kept for analysis.

Data and analysis of treatment outcome impact. Individual Bangladesh patient data were captured using Epi-Info 6.04d (Centers for Disease Control and Prevention, Atlanta, GA). Epidata (www.epidata.dk) was used for the reference laboratory database and for data analysis. For multiple isolates, a single rifampin sequence and/or phenotypic DST result was kept per treatment episode, with a mutation or resistance result taking precedence in case of discordance. Unique treatment episode identifiers allowed linkage of both databases to assess the impact of initial *rpoB* mutations on treatment outcome, after a visual identity check using other variables.

Mutations were identified by *rpoB* codon number (*Escherichia coli* numbering) and amino acid substitution. Based on published data regarding the MIC (ratio to the critical DST concentration, <1 to 8) and frequent discordant results in studies, as well as DST proficiency testing rounds among the SRLs (4–8, 20–26), the following mutations were classified as conferring "disputed" resistance: 511Pro, 516Tyr, 526Asn, 526Leu, 526Ser, 533Pro, and 572Phe. The most common, high resistance mutations were grouped as "undisputed" resistance: 526Arg, 526Asp, 526Tyr, and 531Leu. Several other mutations shown below in the tables might belong to one of these groups as well but had been described too rarely to be classified beyond doubt. Multiple mutations are presented as such, with each mutation shown in alphabetical order. For 14 cases of heteroresistance with mutant as well as wild-type DNA, only the mutation is shown.

The usual TB control program definitions, based on smear microscopy, were applied (27). Posttreatment follow-up was passive, with continuous update of individual electronic treatment records. Recurrence-free cure was defined as cure or treatment completion without a smear-positive recurrence registered at any time.

Ethical considerations. This study retrospectively used specimens and data collected in the course of routine patient care and resistance surveillance, performed without ethics review or informed consent. On some stored specimens we conducted retrospective laboratory tests. Analysis was done on deidentified results.

RESULTS

Of the 1,018 Bangladesh sputum samples from retreatment patients that were tested, 108 failed to amplify, 28 contained DNA from mycobacteria other than *M. tuberculosis* (nontuberculosis mycobacteria), and 882 (86.6%) yielded a TB-specific *rpoB* amplicon. For 1,390 Kinshasa samples tested, these figures were, respectively, 102, 15, and 1,273 (91.6%). Core region and other previously described resistance-conferring mutations were not detected from 707 (80.2%) Bangladesh and 1,019 (80.1%) Kinshasa sequences.

Table 1 shows the mutations detected, their frequencies, and growth on culture. There were 35 different alleles among 175 mutations from Bangladesh, versus 30 alleles from 254 mutations from Kinshasa, and only 17 alleles occurred in both populations. The 531Leu substitution made up half or more of both series, followed by 7 to 10% each for 526Asp and 526Tyr for Bangladesh and 516Val for Kinshasa. Together, these four mutations represented two-thirds of either population. The disputed resistance group accounted for 13.1% in Bangladesh and 10.6% in Kinshasa. Among the remaining were 16 (5.7%/2.4%, Bangladesh/Kinshasa) double mutations and 16 (4.6%/3.1%) highly unusual mutations. Some of the latter had not been described previously (508Asn, 516Gln, 518Ser, 523Glu, and INS511) or were reported only as part of a double mutation (535Ser, 536Ser).

Overall recovery in culture was very low from Kinshasa samples (177/251; 70.5%), which is at least partly explained by often very long transport delays. For Bangladesh samples, culture positivity did not seem to be less frequent in the disputed resistance group (139/167; 91%), but lower sensitivity of cultures appeared to be associated with specific mutations (516Val and 533Pro; 60 to 70% [$P = 0.29$]). All but 1 of 16 double mutation samples grew, but only 4/16 with unusual mutations grew, which was significantly lower than for the other groups ($P < 0.001$). From each study site, there was also one sample with a triple mutation, but none of these yielded culture growth.

Table 2 shows *M. tuberculosis* isolates that tested resistant to rifampin on LJ at the standard 40-μg/ml critical concentration, sorted by *rpoB* mutation found in the sputum, for both populations together. Altogether, 295/315 (93.3%) of the strains tested phenotypically resistant. Also, disputed mutations were usually found resistant (32/38 [84.2%], 95% confidence interval [CI], 68.1% to 93.4%), except 511Pro and 526Asn (1/3 and 2/4 only). The difference was just significant with the undisputed group (220/230; 95.5% CI, 91.6% to 97.7%; $P = 0.02$).

Table 3 shows an analysis of phenotypic DST results by *rpoB* mutation, for the LJ proportion method as well as local rapid slide DST. This series was expanded to include all Bangladesh isolates in our database with a mutation, and we assumed that the same mutation was present in subsequent isolates from the same patient treatment episode. Of 894 tests, 91.9% (95% CI, 89.9% to 93.6%) was resistant on LJ, versus 96.4% (95% CI, 90.5% to 98.8%) of 111 on slide DST (statistically nonsignificant, $P = 0.055$). Of the disputed resistance mutation strains, only 78.7% and 84.2% were found resistant on LJ and slide DST, mainly caused by half the 511Pro and an occasional 516Tyr or 526Asn strain testing susceptible. On LJ, the difference was significantly different from the

TABLE 1 *rpoB* mutations found in first-recurrence sputum samples from Bangladesh and Kinshasa: relative frequency and growth on culture

Mutation(s) in *rpoB* sequence	Bangladesh		Kinshasa	
	Frequency [*n* (%)]	Positive cultures [no. positive/no. tested[a] (%)]	Frequency [*n* (%)]	Positive cultures [no. positive/no. tested[a] (%)]
508Asn			1 (0.4)	0/1
508Ile	1 (0.6)	0/1		
509Thr&526Leu	1 (0.6)	1/1		
511Arg&516Gly			1 (0.4)	1/1
511Pro	3 (1.7)	3/3		
511Pro&512Cys	1 (0.6)	1/1		
511Val			1 (0.4)	1/1
513Glu&516Phe			2 (0.8)	2/2
513Glu&516Val			1 (0.4)	1/1
513Lys	1 (0.6)	1/1	1 (0.4)	1/1
513Lys&526Asp	2 (1.1)	2/2		
513Pro			2 (0.8)	2/2
514Leu&516Val&531Leu	1 (0.6)	0/1		
515Ile			1 (0.4)	0/1
515Ile&533Pro			1 (0.4)	1/1
515Thr&516Gly	1 (0.6)	1/1		
516Asn	1 (0.6)	0/1		
516Gln			1 (0.4)	0/1
516Lys&526Asn	1 (0.6)	1/1		
516Phe	2 (1.1)	2/2	2 (0.8)	1/2
516Phe&531Leu	1 (0.6)	1/1		
516Tyr	4 (2.3)	4/4	5 (2.0)	4/5
516Tyr&533Pro	1 (0.6%)	1/1		
516Val	6 (3.4)	3/5	18 (7.1)	11/18 (61)
518Ser	1 (0.6)	0/1		
522Gln			8 (3.1)	8/8
522Leu	2 (1.1)	1/2	1 (0.4)	0/1
523Glu	1 (0.6)	0/1	1 (0.4)	0/1
524Trp&525Pro&DEL526_527			1 (0.4)	0/1
526Arg	5 (2.9)	3/4	2 (0.8)	2/2
526Arg&531Glu	1 (0.6)	1/1		
526Asn	2 (1.1)	2/2	4 (1.6)	2/4
526Asn&533Pro			1 (0.4)	0/1
526Asn&572Leu	1 (0.6)	1/1		
526Asp	12 (6.9)	11/12 (86)	5 (2.0)	4/5
526Leu	5 (2.9)	4/4	6 (2.4)	4/6
526Tyr	17 (9.7)	16/17 (94)	6 (2.4)	5/6
531Gln	1 (0.6)	0/1		
531Leu	82 (46.9)	66/77 (86)	160 (63.0)	113/157 (72)
531Phe	1 (0.6)	0/1		
531Trp	2 (1.1)	2/2	1 (0.4)	1/1
533Pro	7 (4.0)	5/7	7 (2.8)	5/7
535Ser	1 (0.6)	0/1	4 (1.6)	3/4
536Ser	1 (0.6)	0/1		
572Phe	2 (1.1)	2/2	5 (2.0)	3/5
DEL509_511			3 (1.2)	1/3
DEL513_515	3 (1.7)	3/3	2 (0.8)	0/1
INS511	1 (0.6)	1/1		
Total no. of isolates with a mutation(s)	175 (100.0)	139/167 (83.2)	254 (100.0)	177/251 (70.5)
No. with disputed resistance	23 (13.1)	20/22 (90.9)	27 (10.6)	18/27 (66.7)
No. with undisputed resistance	116 (66.3)	96/110 (87.3)	173 (68.1)	124/170 (72.9)
No. of isolates with double mutations	10 (5.7)	10/10 (100.0)	6 (2.4)	5/6
No. of isolates with unusual mutation(s)	8 (4.6)	1/8	8 (3.1)	3/8

[a] Percentages are shown only if the denominator was at least 10.

undisputed group (96.3% resistant; CI, 94.2% to 97.7%). Double mutation strains always tested resistant in both systems.

The outcomes for category 2 standardized retreatment under field conditions are shown by *rpoB* mutation in Table 4. For the 345 treatment episodes, average success without recorded relapse was 21% (CI, 17.3% to 26.2%), versus 63% (CI, 58.1% to 68.5%) failure or relapse recurrence, with a 4.2 ratio of failures to relapses. These proportions barely differed between the disputed and un-

TABLE 2 Phenotypic rifampin resistance of *M. tuberculosis* isolates with particular *rpoB* mutations (Bangladesh and Kinshasa series combined) detected using the LJ proportion method at a critical concentration of 40 μg/ml

rpoB sequence mutation(s)	No. of isolates:		% resistant (95% CI)[a]
	Tested	Resistant	
509Thr&526Leu	1	1	
511Arg&516Gly	1	1	
511Pro	3	1	
511Pro&512Cys	1	1	
511Val	1	0	
513Glu&516Phe	2	2	
513Glu&516Val	1	1	
513Lys	2	2	
513Lys&526Asp	2	2	
513Pro	2	2	
515Ile&533Pro	1	1	
515Thr&516Gly	1	1	
516Lys&526Asn	1	1	
516Phe	3	3	
516Phe&531Leu	1	1	
516Tyr	8	7	
516Tyr&533Pro	1	1	
516Val	14	14	100 (73.2–100.0)
522Gln	8	8	
522Leu	1	1	
526Arg	5	5	
526Arg&531Glu	1	1	
526Asn	4	2	
526Asn&572Leu	1	1	
526Asp	15	15	100 (74.7–100.0)
526Leu	8	8	
526Tyr	21	20	95 (74.1–99.8)
531Leu	179	170	95 (90.4–97.5)
531Trp	3	3	
533Pro	10	9	90 (54.1–99.5)
535Ser	3	0	
572Phe	5	5	
DEL509_511	1	1	
DEL513_515	3	3	
INS511	1	0	
Wildtype	944	56	6 (4.5–7.7)
Any mutation	315	294	93.3 (89.8–95.7)
Disputed resistance	38	32	84.2 (68.1–93.4)
Undisputed resistance	230	220	95.5 (91.6–97.7)
Double mutations	15	15	100 (74.7–100.0)
Unusual mutations	4	0	

[a] Percentages are shown only if the denominator (total number of isolates) was at least 10.

disputed groups, with exactly the same percentage of recurrence and hardly more relapse-free registered cures (27% of 70 versus 20% of 214 episodes; nonsignificant). There might have been more relapses relative to failures in the disputed group (ratio, 2.4), particularly with the 511Pro and 533Pro mutations. All five unusual mutations within the RRDR with treatment outcome available were recorded as relapse-free cures.

DISCUSSION

Our results show that *rpoB* mutations that result in rifampin resistance that is regularly or even systematically missed by standard, WHO-endorsed DST methods are not uncommon. Selecting only the more commonly described of these disputed mutations (511Pro, 516Tyr, 526Asn, 526Leu, 533Pro, and 572Phe), we found that they made up over 10% of all *rpoB* mutations among failure and relapse cases from Bangladesh as well as Kinshasa. A systematic series from Hong Kong with over 3,000 isolates screened by molecular technique without phenotypic DST preselection found 21% prevalence among 89 RRDR mutated strains, counting only 511Pro, 526Leu, and 533Pro (28). However, this series must have consisted largely of new cases. We are not aware of other large series screened without conventional DST preselection, which leads to systematic underestimation of their prevalence. However, just 533Pro, the most easily detected disputed mutation, occurs at a rather constant frequency of 3 to 6%, according to many reports (29). There are reasons to believe that 511Pro and 516Tyr might be detected at similar frequencies, if they were not so easily missed in phenotypic DST, while 572Phe also would be reported more frequently if molecular DST did not target only the RRDR (codons 507 to 533). Moreover, there is a whole range of rarely reported and thus ill-known mutations that might very well also belong to the disputed group.

Although our SRL is known to declare such strains most often resistant in the phenotypic DST proficiency testing rounds (4), it also missed about 8% of all, or 20% of disputed, rifampin resistance in routine work. Feuerriegel et al. reported that in a systematic series of Sierra Leone retreatment case isolates retested by DNA sequencing, 5/21 (24%) *rpoB* mutations were found among strains classified as rifampin susceptible by phenotypic DST. All but one strain originally classified as resistant carried the undisputed 531Leu or 526Arg or Tyr mutations, while among the "susceptible" isolates there were three 516Tyr and one each of the 511Pro and 533Pro mutations. Those authors confirmed these disputed strains to be susceptible and concluded there was 94% specificity with DNA sequencing because repeat testing with the MGIT 960 system confirmed their MICs were below the conventional 1.0-μg/ml breakpoint (10).

In our view, the conventional gold standard, i.e., phenotypic DST, fails for these strains. We have previously shown that rapid phenotypic DST, specifically automated MGIT, classifies many *rpoB* mutated strains as susceptible (or fails to yield a valid result), while such strains usually test resistant by other methods in various proficient laboratories (5), and most have clearly raised MICs (6). They may be so hard to detect because of fitness loss and slower growth in the presence of the drug compared to the drug-free controls, particularly if the resistance level is relatively low (30). Indirect laboratory evidence that the disputed and other very rare, little-known mutations (i.e., 516Gly, 515Thr) do confer some degree of resistance can be inferred from their overrepresentation in double and triple mutations. These occurred at levels of several percent in our series, far too frequent to have come up simultaneously. The fitness cost of each mutation must thus have been less determinant than the continued selective pressure for further increased resistance levels, implying that resistance due to the first mutation was indeed borderline. At the same time, the first mutation conferred sufficient resistance to allow the bacilli to replicate during rifampin treatment. This explains why no double mutation was composed of two undisputed mutations, but at most one, which we hypothesize to have arisen last. We would even postulate that the clinical significance of very rare single mutations can be deducted from their occurrence in multiple mutations, such as the 535Ser or 536Ser mutations in our series, even

TABLE 3 Rifampin resistance observed with routine phenotypic DST, by *rpoB* mutation

rpoB mutation(s)	DST on LJ			Slide DST		
	No. tested	No. rifampin resistant	% resistant (95% CI)[a]	No. tested	No. rifampin resistant	% (95% CI)[a]
509Arg&526Gln	1	1				
509Thr&526Leu	3	3		1	1	
511Arg&531Leu	2	2				
511Pro	30	14	47	4	2	
511Pro&515Leu	3	3				
511Pro&515Val	1	1		1	1	
511Pro&516Val	1	1				
511Pro&526Arg	2	2				
511Pro&526Leu	1	1				
511Pro&531Leu	7	7				
512Arg&516Gly	2	2				
513Glu	2	2				
513Lys	14	14	100	2	2	
513Lys&526Asp	2	2				
513Pro	4	3				
515Arg&516Val	1	1				
515Ile&516Tyr	4	4				
515Thr&516Gly	7	7		1	1	
516Lys&526Asn	3	3		1	1	
516Phe	16	15	94			
516Phe&531Leu	1	1				
516Tyr	34	30	88	5	5	
516Val	57	56	98	4	4	
522Gln	1	1				
522Leu	13	11	85			
526Arg	13	13	100	1	1	
526Arg&531Glu	1	1				
526Asn	5	3				
526Asp	31	31	100	3	3	
526Cys	4	3				
526Gly	1	1				
526Leu	27	26	96	1	1	
526Pro	3	2				
526Tyr	83	79	95	16	16	
531Gly	2	1		1	1	
531Leu	392	377	96	56	55	
531Phe	1	1				
531Trp	16	14	88			
533Pro	67	55	82	8	7	
572Leu&526Asn	3	3		1	1	
572Phe	15	12	80	1	1	
DEL513_515	6	5		3	3	
DEL517	2	2				
INS511	1	0				
INS512_513	2	1				
INS513_514	5	5		1	1	
INS514	2	0				
Any mutation	894	822	91.9 (89.9–93.6)	111	107	96.4 (90.5–98.8)
Disputed resistance	112	81	78.7 (71.8–84.3)	16	19	84.2 (59.5–95.8)
Undisputed resistance	558	535	96.3 (94.2–97.7)	78	77	98.7 (91.9–99.9)
Double mutations	45	45	100.0 (90.2–100)	5	5	

[a] Percentages are shown only if the denominator was at least 10.

though this could not be concluded from their MICs or the clinical outcomes.

DST should predict what clinicians can expect as action from the drug, whatever the method used, or MIC or mutation type found. Based on almost two-thirds of bacteriologically adverse outcomes for standard first-line retreatment in an excellent TB control project, our data showed that these disputed mutations have exactly the same poor clinical prognosis as the most frequent undisputed mutations. A few reports had already suggested that such strains have clinical relevance. Williamson

TABLE 4 Outcome of category 2 cases (first-line retreatment regimen), by *rpoB* mutation detected at time of prime treatment failure or relapse[a]

Mutations(s) in *rpoB* sequence	No. of episodes	Relapse-free cure		FL and RL[b]		
		n	% (95% CI)[a]	No. reported	% (95% CI)[a]	FL/RL ratio
508Ile	1	1		0		
509Thr&526Leu	1	1		0		
511Arg&531Leu	1	0		1		Only FL
511Pro	21	3	14	15	71	1.1
511Pro&512Cys	1	0		1		Only FL
511Pro&515Leu	1	0		1		Only FL
511Pro&516Val	1	0		1		Only FL
513Glu	1	0		1		Only FL
513Lys	3	1		1		Only FL
513Lys&526Asp	1	0		0		
513Pro	1	0		1		Only FL
515Thr&516Gly	2	1		1		Only FL
516Phe	5	1		4		1.0
516Phe&531Leu	1	0		0		
516Tyr	7	2		4		Only FL
516Val	15	1	7	12	80	5.0
516Val&531Leu	1	0		0		
518Ser	1	1		0		
522Leu	4	1		3		Only FL
523Glu	1	1		0		
526Arg	7	2		6		Only FL
526Arg&531Glu	1	0		1		Only FL
526Asn	5	3		2		Only FL
526Asp	17	4	24	8	47	1.0
526Cys	2	0		2		Only FL
526Leu	10	2	20	7	70	2.5
526Tyr	31	9	29	19	61	18.0
531Leu	159	27	17	101	64	6.0
531Trp	6	1		5		4.0
533Pro	20	7	35	12	60	1.4
535Ser	1	1		0		
536Ser	1	1		0		
572Phe	5	2		2		Only FL
DEL513_515	3	0		3		2.0
INS512_513	3	0		3		Only RL
INS513_514	3	1		2		1.0
Any mutation	345	74	21 (17.3–26.2)	219	63 (58.1–68.5)	4.2
Disputed resistance	70	19	27 (17.5–39.3)	44	63 (50.4–73.9)	2.4
Undisputed resistance	214	42	20 (14.7–25.7)	134	63 (55.7–69.0)	5.8
Double mutations	11	2	18 (3.2–52.2)	6	55 (24.6–81.9)	Only FL
Unusual mutations	5	5	0			

[a] Percentages are shown only if the denominator was at least 10.
[b] FL, failure; RL, relapse.

reported four cases from New Zealand, retrospectively detected by using Xpert, among MGIT-DST susceptible cases, among whom three had failed treatment while one was found postmortem. All cases represented disputed mutations, single or combined (511Pro&515Ile, 526Asn&532Val, 516Tyr, 526Leu) (9). van Ingen reported a small outbreak with a 516Tyr strain from Holland (31). That these strains can be highly meaningful on the population level was suggested also by the report of Ioerger et al. Their genome analysis of MDR and extremely drug-resistant strains from the KwaZulu-Natal outbreak showed the high transmissibility of a 516Tyr as well as a 516Gly&533Pro mutant strain (32). It is possible that the very high HIV prevalence in this population facilitated transmission. However, it is also conceivable that patients with such disputed resistant strains have a prolonged period of infectiousness resulting from delayed diagnosis of MDR-TB because of susceptible DST results, followed by prolonged, treatment with low effectiveness and repeated relapse. In our own experience, patients afterwards documented to have had strains with these mutations were started on curative MDR treatment only after several first-line retreatment relapses, or they were erroneously switched back from effective MDR to first-line treatment upon receipt of a susceptible DST result, with an adverse outcome.

Because of this failure of the phenotypic gold standard, molecular rifampin DST techniques thus perform with higher specificity than generally believed. The original publication introducing GeneXpert for TB concluded a 98.1% specificity for rifampin resistance, although sequencing had shown RRDR mutations in all nine discordant phenotypically susceptible strains (33). Six be-

longed to the disputed mutations (four 511Pro, one 516Tyr, and one 533Pro), one mutation belonged to the undisputed (526Tyr), one to the unusual mutations (DEL518; MDR in our laboratory), and one represented a silent mutation (514PheTTT [C. Boehme and S. Ruesch-Gerdes, personal communication]). These sequence results were not accepted for resolution of discrepancies because of doubts regarding the significance of the mutations (34), but our data indicate that the Xpert rifampin specificity in that study was in fact 99.8% if we kept only the silent mutation as a false resistance result. Other reports on Xpert false rifampin resistance results have mentioned mutations found by sequencing (35). True-false resistance without any mutation detected by sequencing has been documented occasionally for earlier versions of the MTB/RIF cartridge, and possibly more with extrapulmonary tuberculosis (36, 37).

Based on testing of mainly retreatment cases, silent RRDR mutations occur at below 0.5% frequency in our laboratory. Also, highly unusual mutations that (almost) failed to grow on culture, never showed rifampin resistance on DST, and for which no adverse treatment outcome could be documented, occurred in the failure/relapse series presented here. Their significance remains truly doubtful. However, our data suggest that these silent and doubtful mutations represent only a small percentage of all RRDR mutations targeted by commercial molecular DST techniques. The predictive value of a rifampin resistant result may thus be estimated at over 95%, probably independent of total rifampin resistance prevalence. The current WHO guidelines (34) can then be simplified: except when rapid DNA sequencing is possible, an (Xpert) molecular rifampin-resistant result does not need to—and should not be—confirmed when there is a low prevalence of resistance. Even confirmation by line probe assay (LPA) may not work, because it sometimes misses the 533Pro mutation (38, 39). Unfortunately, the confusion brought in recent years by parallel LPA and (MGIT) phenotypic DST, regularly causing discordant results, has led to modifications, so that the LPA MTBDR*Plus* version 2 became totally unable to detect 533Pro. Fortunately, this seems to have been rectified already.

We are not implying that molecular rifampin DST should now become the gold standard, since it is well known to also miss some rifampin resistance, particularly if only the RRDR is covered. But the evaluation of diagnostic DST methods should include discrepant resolution using the reference technique of the other DST type (sequencing covering all known mutations in case of phenotypic DST, and the proportion method on solid medium in case of genotypic DST). For individual diagnosis, the best approach seems to be to accept any rifampin-resistant result as true, yet to be suspicious of missed resistance in clinically highly probable cases.

The main limitation of our study is that it concerned first-recurrence patients only. It cannot be excluded that silent and unusual mutations might be proportionally more frequent among new cases not suspected of rifampin resistance, reducing the predictive value of molecular DST. Ultimate proof of its universally adequate specificity will have to be determined via resistance surveys among new cases, using parallel phenotypic and genotypic DST complemented by sequencing of discordant strains and long-term follow-up of standardized treatment outcomes. Further research is also needed to determine the clinical significance of unusual RRDR mutations.

To conclude, rifampin resistance that is difficult to detect by the gold standard, phenotypic DST, is clinically and epidemiologically highly relevant and occurs too frequently to continue to be ignored. The underlying *rpoB* mutations are readily detected by gene sequencing, which should be used to correct the phenotypic gold standard when evaluating the performance characteristics of rifampin resistance diagnostic tests. The problem of any rifampin DST method is not imperfect specificity but suboptimal sensitivity. The predictive value of an Xpert or LPA resistance result may be very high also when there is low prevalence, but this requires further study that includes new cases. A susceptible result should be questioned when suspicion is very high, and further DST using a different system (i.e., genotypic after phenotypic) would be fully justified.

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