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GenoType MTBDR*sl* performance on clinical samples with diverse genetic background

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ABSTRACT: We evaluate the performance of the GenoType® MTBDR*sl* (Hain Lifescience Nehren, Germany) for the detection of second-line resistant tuberculosis and we correlate the frequency of mutations to different *Mycobacterium tuberculosis* genotypes.

We tested 175 strains and 59 clinical specimens interpreting the results according to the Standards for Reporting of Diagnostic Accuracy recommendations. All the strains were also investigated by spoligotyping and Mycobacterial Interspersed Repetitive Units–Variable Number of Tandem Repeats typing.

The performances of the MTBDR*sl* in detecting resistance to fluoroquinolones (FQ), second-line injectable drugs (SLID), and ethambutol (EMB) on clinical isolates were similar (specificity ~99%, sensitivity ~70%, and positive predictive value (PPV) ~99%).

Of the 59 respiratory specimens, three samples were classified as ''indeterminate''. The specificity in detecting resistances was similar for FQs and EMB 100% (95% CI 92.7–100%) and 100% (95% CI 83.9–100%), respectively with a PPV of 100% (95% CI 64.6–100%) and 100% (95% CI 87.9–100%), respectively. Detection of SLID showed a specificity of 89.1% (95% CI 77.0–95.3%) and a PPV of 58.3% (95% CI 32.0–80.7%). Sensitivity for FQ-resistance detection was 100% (95% CI 64.6–100%), whereas for SLID and EMB it was 89.1% (95% CI 77.0-95.3%) and 86.1% (95% CI 71.3-93.9%), respectively. We detected a significant association between mutations in the *rrs* gene and Beijing lineage.

The MTBDR*sl* can be used to ''rule in'' extensively drug-resistant strains of tuberculosis in a high risk group; the low sensitivity and negative predicted value (NPV) make confirmation by conventional drug susceptibility testing mandatory when mutations are not identified. NPV for SLID is higher in Beijing strains, showing that the predictive values of the molecular tests are related to the genetic background.

KEYWORDS: Extensively drug-resistant tuberculosis, GenoType® MTBDR*sl*, molecular drug-susceptibility testing, multidrug-resistance tuberculosis, rapid diagnosis, tuberculosis

Drug-resistant tuberculosis (TB) remains a major public health concern worldwide [1]. The efficacy of short-course chemotherapy is compromised by the emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains, *i.e.* strains resistant to at least rifampicin and isoniazid, and of extensively drug-resistant (XDR) strains, namely MDR strains with additional resistance to any fluoroquinolone (FQ) and at least one of three second-line injectable drugs (SLIDs): capreomycin, kanamycin, amikacin [2, 3]. Up to 28.3% and 61.6% of MDR-TB cases were reported among new and previously treated TB cases, respectively, and by 2010 at least one case of XDR-TB was reported in 68 countries [1].

Rapid detection of MDR-TB and XDR-TB is necessary in order to refer patients to specialist centres, thereby ensuring correct treatment under adequate infection control conditions [4, 5].

Currently, the direct detection of gene mutations associated with drug resistance is the only rapid method for prediction of resistance. The line probe assays (LiPA), strip-based DNA-probe tests relying

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on multiplex nucleic acid amplification and reverse hybridisation, are commercially available for the detection of multiple antibiotic resistances.

In particular, the GenoType MTBDR and MTBDR*plus* assays (both Hain Lifescience, Nehren, Germany) showed to be highly sensitive and specific assays for the prediction of rifampicin and, at a lower extent, of isoniazid resistances on sputum smear-positive samples [6].

In 2008, the World Health Organization issued (WHO) a policy statement for rapid screening of patients at risk of MDR-TB, providing recommendations on the use of molecular LiPA in TB national programmes [6, 7]. Their use greatly reduced the time in which MDR-TB cases were detected in high-risk populations, thereby preventing the development of resistance due to patient mismanagement.

Recently, a second generation LiPA MTBDR*sl* was developed to detect the most frequent mutations responsible for resistance to the XDR-TB defining drugs, FQ and SLIDs, plus ethambutol (EMB). Few studies compared the MTBDR*sl* to genetic sequencing of clinical isolates and assessed its performance by comparison with the gold standard, the phenotypic drug-susceptibility testing (DST) [8–12]; furthermore, only one such study evaluated it directly on clinical specimens [8]. More data are, therefore, needed to better understand the usefulness of this assay in the clinical practice.

So far, no study reported genotyping information concerning strains analysed. Association between genotypes and mutation frequency in genes involved in resistance was only accomplished for first-line drug (FLD) resistance and no data are available for genes involved in second-line drug (SLD) resistance [13, 14]. Here, we evaluated the performance of the MTBDR*sl* by comparison with DST and sequencing on 175 clinical isolates and 59 clinical specimens. Furthermore, for the first time, we reported associations between mutations involved in resistances to SLIDs and different *M. tuberculosis* genotypes. We provided the proof-of-principle on how the performances of a molecular test can be altered in different geographical settings by considering the phylogeographical distribution of *M. tuberculosis*.

MATERIALS AND METHODS

Clinical isolates

175 *M. tuberculosis* cultures, with known phenotypic resistant patterns, were studied by the MTBDR*sl* assay (table 1).

TABLE 1 Phenotypic drug-susceptibility testing of the 175 clinical isolates and 59 clinical specimens included in the study

Drug	Clinical isolates		Clinical specimens	
	R	S	R	S
FQs	57	118	7	52
SLIDs	85	90	10	49
EMB	84	91	17	29

FQs: fluoroquinolones; SLIDs: second-line injectable drugs; EMB: ethambutol; R: resistant; S: susceptible.

Clinical specimens

DNA obtained from 59 clinical specimens from MDR cases that had been decontaminated, according to international guidelines using *N*-acetyl-cysteine/NaOH procedure, were included in the study. All samples were acid-fast bacilli-positive, according to sputum smear microscopy. The susceptibility phenotypes of the corresponding strains had been determined.

DST

Susceptibility to FLD and SLD was performed by the BACTEC™ MGIT™ 960 system (MGIT) (BD Bioscience, Erebodegem, Belgium) [15].

DNA extraction

Genomic DNA was extracted from strains and clinical specimens as described elsewhere [16].

GenoType® MTBDRsl assay

Amplification of DNA extracted from isolates was performed accordingly to the manufacturer's instruction. For the sputum specimens, a modified amplification protocol was applied: 40 cycles instead of 20 and 2 U HotStarTaq DNA polymerase were used.

Sequencing

Genomic regions harbouring drug-resistance mutations were amplified and sequenced using primers specific for each genomic region (table S1).

Spoligotyping

The amplified direct repeat locus of clinical isolates was investigated using the spoligotyping kit (Ocimum Biosolutions, Hyderabad, India) following the manufacturer's instructions. Spoligotyping results were entered, in binary format, in MIRU-VNTR*plus* web database to identify spoligotype international types (SITs) and strain main lineages [17]. Best-matching lineages were identified by similarity search (distance setting: <0.1).

Microplate Alamar blue assay

The assay was performed according to standard procedure with minor modifications [18]. EMB concentrations ranged from 256 $\mu g \cdot mL^{-1}$ to 1 $\mu g \cdot mL^{-1}$. *M. tuberculosis* H37Rv was used as a drug-susceptible reference strain. Strains showing a minimum inhibitory concentration (MIC) $\geqslant 4$ $\mu g \cdot mL^{-1}$ were considered EMB resistant.

Statistical analysis

To evaluate the MTBDR*sl*, we compared the LiPA performance to DST performed by MGIT and to direct sequencing as reference standards following the Standards for Reporting of Diagnostic Accuracy (STARD) recommendations [19]. Mid-p exact test was applied to assess the significance of different mutations frequencies among different genotypes. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratios and diagnostic accuracy values were calculated according to the Wilson score. All statistical analyses were carried out using the Open Source Epidemiologic Statistics for Public Health version 2.3.1 [20].

TABLE 2 Fluoroquinolone resistance results obtained by GenoType® MTBDR*sl* assay and sequencing on 175 clinical isolates and 59 clinical specimens

M. tuberculosis	Phenotypic DST	MTBDR*sl*	Sequencing	n %
Clinical isolate				
	R	A90V	A90V	15 (8.6)
	R	D94G	D94G	11 (6.3)
	R	S91P	S91P	4 (2.3)
	R	D94A	D94A	2 (1.1)
	R	D94N/D94Y	D94N	2 (1.1)
	R	no WT	D94Y	2 (1.1)
	R	WT+D94A	WT+D94A	1 (0.6)
	R	WT+D94A	WT	1 (0.6)
	R	WT+D94G	WT	2 (1.1)
	R	WT+D94G	D94G	1 (0.6)
	R	D94A+D94G	D94A	1 (0.6)
	R	WT	WT	15 (8.6)
	S	WT	WT	116 (66.3)
	S	No WT	WT	1 (0.6)
	S	Indeterminate	WT	1 (0.6)
Clinical specimen				
	R	A90V		1 (1.7)
	R	D94G		2 (3.4)
	R	D94N/D94Y		3 (5.1)
	R	No WT		1 (1.7)
	S	WT		49 (83.1)
	S	Indeterminate		3 (5.1)

DST: drug-susceptibility testing; R: resistant; WT: wild type; S: susceptible.

RESULTS

Performance of MTBDRsl on clinical isolates

The performance of the MTBDR*sl* assay was evaluated on 175 clinical isolates. An indeterminate result was obtained in one isolate due to lack of hybridisation.

FQs

The panel strains included 57 phenotypically resistant isolates and 117 FQ-susceptible strains (table 2). FQ-resistant strains were correctly identified by MTBDR*sl* in 73.7% of cases. In six cases both wild-type (WT) and mutated DNA sequences were detected, a finding suggestive of a mixed infection. In two cases a mutation in the *gyrA* gene was inferred by the lack of hybridisation of the wild-type probe WT3; however, the aminoacid substitution D94Y was not detected by the specific probe. The codons 94 and 90 were the most frequently affected by mutations (26.3% and 19.3% of resistant strains, respectively). The mutation S91P was detected in 7.0% cases. 26.3% of strains, phenotypically resistant to FQ, were classified as susceptible because of hybridisation of all the WT *gyrA* probes (data confirmed by sequencing).

All but one sample of the phenotypically susceptible strains were correctly recognised by the test. Diagnostic performances are summarised in table 3. Comparison between GenoType® MTBDR*sl* and sequencing data analysis are reported in the online supplementary data (table S2).

Genotype analysis of clinical isolates with known FQ susceptibility was investigated (table S3). Among them, 76.9% of the FQ-resistant strains were affected by *gyrA* mutations (tables 4, S4). Within non-Beijing lineages, the proportion of *gyrA* mutated and non-mutated FQ-resistant strains was grossly overlapping; no significant differences were observed between Beijing and non-Beijing lineages ($p>0.05$).

SLIDs

Out of the 174 strains evaluated, 84 were resistant to at least one SLID and 90 were susceptible to all drugs (table 1). 60 resistant strains (71.4%) were detected by the MTBDR*sl* assay (table 5), among which five showed a double pattern. In one, a mutation in a subregion of the *rrs* gene was inferred by lack of hybridisation with the probe WT1 (nucleotides 1401–1402). One strain showing hybridisation with the probe MUT2 (specific for the mutation g1484t) turned out to be WT by sequencing. The substitution a1401g was the most frequent affecting 57 (67.9%) out of the 84 resistant strains (table S5). The substitution c1402t was observed only once (1.2%). 24 strains, which were phenotypically resistant to SLID, were classified as susceptible; the WT sequence in *rrs* was confirmed by sequencing.

All strains phenotypically susceptible to the three SLIDs were correctly recognised by the test, diagnostic performances are summarised in table 3. Comparison of MTBDR*sl* and sequencing data analysis are reported online in the supplementary data (table S2).

Among the strains with known phenotypic results to all three SLIDs, the a1401g substitution correlated with phenotypic resistance to at least two drugs among kanamycin, capreomycin, amikacin (57 (87.7%) out of 65 cases).

The Beijing spoligotype was the most prevalent (table S3; mutation details are given in table S6). Among SLID-resistant strains the *rrs* mutations were present in 78.3% of the Beijing lineage and in only 59.0% of the non-Beijing lineage (table 4). The difference was statistically significant ($p<0.01$).

EMB

83 phenotypically EMB-resistant and 91 EMB-susceptible strains were tested; table 6 shows the comparison between LiPA and sequencing.

Among 83 phenotypically resistant strains, 55.4% were correctly identified by MTBDR*sl*. In three cases the mutation inferred by the lack of hybridisation with the WT probe was confirmed by genetic sequencing that detected substitutions corresponding to M306I (atg/atc) and M306L respectively. The most frequent mutations were M306V and M306I observed in 42.2%, and 12.0% of phenotypically resistant strains, respectively. 37 (44.6%) strains phenotypically resistant to EMB were classified as susceptible (hybridisation with the WT probe, confirmed by sequencing). Table S7 shows *emb*B differences.

Phenotypical susceptibility to EMB was correctly recognised in 54.9% of strains. In 28.6% of the 41 discrepant cases both MTBDR*sl* and direct sequencing identified a specific mutation at codon 306. 12 additional strains identified by MTBDR*sl* as resistant, by the lack of WT hybridisation, harboured the

TABLE 3 Statistical parameters of the performances of the GenoType® MTBDR*sl* assay on 175 clinical isolates and 59 clinical specimens

Drug	Clinical isolates		Clinical specimens	
	MTBDR*sl*	DST	MTBDR*sl*	DST
Fluoroquinolones				
Sensitivity	99.4 (96.8–99.9)	73.7 (61.0–83.4)	94.9 (86.1–98.3)	100 (64.6–100)
Specificity		99.2 (95.3–99.9)		100 (92.7–100)
PPV		97.7 (87.9–99.6)		100 (64.6–100)
NPV		88.6 (82.0–92.9)		100 (92.7–100)
Likelihood ratio pos.		86.2 (11.9–62.2)		Undefined
Likelihood ratio neg.		0.3 (0.2–0.3)		0
Diagnostic accuracy		90.8 (85.6–94.3)		100 (93.6–100)
Second-line injectable drugs				
Sensitivity	99.4 (96.8–99.9)	71.4 (61.2–80.0)	91.5 (81.7–96.3)	87.5 (52.9–97.8)
Specificity		100 (95.9–100)		89.1 (77.0–95.3)
PPV		100 (94.0–100)		58.3 (32.0–80.7)
NPV		79.0 (70.6–85.4)		97.6 (87.7–99.6)
Likelihood ratio pos.		Undefined		8.1 (5.2–12.4)
Likelihood ratio neg.		0.3 (0.3–0.3)		0.1 (0.02–1.0)
Diagnostic accuracy		86.2 (80.3–90.6)		88.8 (77.8–94.8)
Ethambutol[#]				
Sensitivity	99.4 (96.8–99.9)	69.7 (61.0–77.1)	94.9 (86.1–98.3)	86.1 (71.3–93.9)
Specificity		96.2 (87.0–98.9)		100 (83.9–100)
PPV		97.7 (92.0–99.4)		100 (87.9–100)
NPV		57.5 (47.0–67.3)		80.0 (60.9–91.1)
Likelihood ratio pos.		18.1 (6.7–47.8)		Undefined
Likelihood ratio neg.		0.3 (0.3–0.3)		0.1 (0.09–0.2)
Diagnostic accuracy		77.6 (70.8–83.2)		91.1 (80.7–96.1)

Data are presented as % (95% CI %). DST: drug-susceptibility testing; interval; PPV: positive predictive value; NPV: negative predictive value; pos: positive; neg: negative; inject: injectable. [#]: the phenotypic DST result is adjusted according to the minimum inhibitory concentration results.

TABLE 4 Genotype analysis of clinical *Mycobacterium tuberculosis* isolates with known drug-susceptibility testing and mid-p exact test on the distribution of mutations among lineages

Drug	*M. tuberculosis* strain		p-value
	Beijing	Other[#]	
Fluoroquinolones			
gyrA			
Wild-type	23.1	29.0	0.166
Mutated	76.9	71.0	
Second-line injectable drugs			
rrs			
Wild-type	21.7	41.0	0.002[¶]
Mutated	78.3	59.0	
Ethambutol			
embB			
Wild-type	29.5	30.0	0.470
Mutated	70.5	70.0	

Data presented as % unless otherwise stated. [#]: all other lineages excluding Beijing.[¶]: statistically significant.

M306I mutation (codon substitution occurred in atg306atc (n=8) and atg306att (n=4)). In two cases the MTBDR*sl* showed a double pattern; sequencing resulted WT in the first case and detected only the M306I mutation in the second case. Finally, in the last discrepant case, a faint hybridisation signal lead to misinterpretation but, after re-checking, the presence of M306I substitution was acknowledged.

The MICs of 33 drug-susceptible isolates harbouring *emb*B mutations (table 7) confirmed a resistant phenotype in 31 cases. Diagnostic performances are summarised in table 3. Comparison between MTBDR*sl* and sequencing data analysis are reported in table S2.

The spoligotype of 175 strains in which EMB resistance was investigated is reported in table S3 (phenotypic DST result is adjusted according to the MIC results). The proportion of mutated and non-mutated EMB-resistant strains within Beijing and non-Beijing lineages (table 4 and table S8) was similar ($p>0.05$).

Overall performances of the MTBDR*sl* on different strain lineages are reported in table S9.

Performance of MTBDRsl on clinical specimens

Of the 59 respiratory specimens tested with MTBDR*sl* 53 showed valid results. Three samples (5.1%) did not provide

TABLE 5 Second-line drug resistance and susceptibility: a comparison of results obtained by GenoType® MTBDR*sl* assay and sequencing for *Mycobacterium tuberculosis* resistance and susceptibility to second-line injectable drugs in 175 clinical isolates and 59 clinical specimens

M. tuberculosis	Phenotypic DST	MTBDR*sl*	Sequencing	n (%)
Clinical isolate				
	R	a1401g	a1401g	53(30.3)
	R	no WT	c1402t	1 (0.6)
	R	WT+a1401g	WT+a1401g	1 (0.6)
	R	WT	WT	24 (13.7)
	R	a1401g+g1484t	a1401g	3 (1.7)
	R	WT+a1401g	WT	1 (0.6)
	R	g1484t	WT	1 (0.6)
	R	Indeterminate	WT	1 (0.6)
	S	WT	WT	90 (51.4)
Clinical specimen				
	R	a1401g		4 (6.8)
	R	WT+a1401g		3 (5.1)
	R	WT		1 (1.7)
	R	Indeterminate		2 (3.4)
	S	WT		41 (69.5)
	S	WT+g1484t		4 (6.8)
	S	No WT (c1402t)		1 (1.7)
	S	Indeterminate		3 (5.1)

DST: drug-susceptibility testing; R: resistant; WT: wild type; S: susceptible.

valid hybridisation for any of the three genomic regions and were classified as ''indeterminate''. The overall diagnostic performances are summarised in table 3.

FQ

Table 2 shows the comparison between LiPA results and the DST performed on the corresponding strain. The MTBDR*sl* identified seven resistant strains, all confirmed by phenotypic DST; the remaining 49 samples, characterised by a WT pattern, were FQ-susceptible by DST. Overall concordance was 94.9%.

SLIDs

Table 5 shows the comparison between LiPA results and the DST performed on the corresponding strain. Twelve samples were classified resistant by MTBDR*sl*; in four a mutated pattern (a1401g) was detected, in one hybridisation of the WT probe was lacking and in seven a double pattern was present. Seven samples showing positive hybridisation with the a1401 g probe were confirmed to be resistant by phenotypic DST. On the contrary specimens lacking hybridisation with the WT probe or hybridising with the a1484t probe were phenotypically susceptible to all three SLIDs. From the 42 samples showing a WT hybridisation, 41 were phenotypically susceptible while one was amikacin-resistant. Two samples, phenotypically resistant to kanamycin, failed hybridisation with the *rrs* probe and were considered indeterminate. The concordance between the MTBDR*sl* assay and phenotypic DST was 81.4%.

EMB

The MTBDR*sl* identified EMB resistance in 31 samples by detection of substitutions and lack of hybridisation with the WT probe (table 6). Only 12 cases were confirmed resistant by phenotypic DST. From the 25 specimens showing a WT hybridisation pattern, 20 were confirmed as susceptible by DST whereas five were found to be resistant. Overall concordance was 55.9%.

DISCUSSION

The concordance between MTBDR*sl* and sequencing was >95% for all genomic regions analysed. Discrepancies were mainly due to double patterns (WT+mutation) whose problematic detection by sequencing is known. Among clinical isolates, WT+mutation patterns were rarely found (six cases for *gyrA* gene, five for *rrs* gene and two for *embB* gene) and exclusively in phenotypically resistant isolates. Only in one case the MTBDR*sl* reported a mutation (a1484t, *rrs* gene) that could not be detected by sequencing. The high agreement between MTBDR*sl* and sequencing is in line with previous observations [8–12].

While on clinical isolates the molecular assay was repeated in three cases to obtain a valid result (1.7%), on clinical specimens the molecular assay was repeated in nine cases (16.1%). The majority of anomalies were related to the *rrs* amplification (*e.g.* faint band staining, no amplification). In some cases sample freezing and thawing may have decreased the PCR performance. The wide range of confidence intervals of the performances on clinical specimens reflects the low number of resistant clinical specimens available for this study and the high frequency of double pattern (especially for SLIDs-resistance). However, prevalence of 11.9% for FQ-resistance and of 15.3% for SLID-resistance was observed. Despite the lack of clear data, prevalence for FQ-resistance or SLID-resistance among TB cases within low-burden countries is within the range of 10%–15%.

TABLE 6 Ethambutol resistance: comparison of results obtained by GenoType® MTBDR*sl* assay and by sequencing on 175 clinical isolates and 59 clinical specimens

M. tuberculosis	Phenotypic DST	MTBDR*sl*	Sequencing	n (%)
Clinical isolates				
	R	M306V	M306V	35 (20.0)
	R	M306I	M306I	8 (4.6)
	R	No WT	M306I[#]	2 (1.1)
	R		M306L	1 (0.6)
	R	WT	WT	37 (21.1)
	R	Indeterminate	M306V	1 (0.6)
	S	M306V	M306V	16 (9.1)
	S	M306I	M306I	10 (5.7)
	S		WT	1 (0.6)
	S	No WT	M306I[#]	8 (4.6)
	S		M306I[¶]	4 (2.3)
	S	WT+M306V	WT	1 (0.6)
	S	WT	WT	50 (28.6)
	S	M306I+M306V	M306I	1 (0.6)
Clinical specimens				
	R	M306V		11 (18.6)
	R	M306I		2 (3.4)
	R	WT		5 (8.5)
	R	Indeterminate		2 (3.4)
	S	WT		20 (33.9)
	S	M306V		7 (11.9)
	S	M306I		9 (15.3)
	S	WT+M306V		1 (1.7)
	S	No WT		1 (1.7)
	S	Indeterminate		1 (1.7)

DST: drug-susceptibility testing; R: resistant; WT: wild type; S: susceptible; [#]: atg→atc; [¶]: atg→att.

Thus, our sample size provides the evaluation of the diagnostic performances of the MTBDR*sl* in a real setting.

FQs

As to clinical samples and isolates, the MTBDR*sl* was 99% specific in detecting FQ resistance, showing a PPVs of ~98%. In contrast, sensitivity was significantly lower (73.7%), probably because of mutations conferring FQ-resistance in genomic regions not targeted by MTBDR*sl* [21, 22]. The NPV (88.6%) makes confirmation by conventional DST mandatory whenever a susceptible MTBDR*sl* pattern is obtained.

SLID

The performances of the MTBDR*sl* on SLIDs were similar to those of FQ (99.4% specificity, 71.4% sensitivity and 100% PPV). Indeed, the assay targets only one region involved in the development of resistant phenotypes [22, 23]. Due to the low NPV value (79.0%), confirmation by conventional DST is required for every strain presenting susceptible MTBDR*sl* pattern.

Five false SLIDs resistances significantly affected the MTBDR*sl* performance in clinical specimens. Four were characterised by double pattern compatible with a mixed infection (susceptible and a resistant strain) while the fifth, identified as resistant by lack of hybridisation with the WT probe, presented anomalies in other molecular assays, suggesting presence of inhibitors or DNA degradation. However, a point of note is that the patient was still sputum smear-positive after 12 months, despite being treated for MDR-TB according to the WHO guidelines [4].

To our knowledge, this is the first report of hetero-resistance related to SLIDs. DST could not confirm a mixed, resistant and susceptible, population. However, it is possible that a predominant proportion of WT bacteria conceal the resistance in the phenotypic test as observed by HILLEMANN *et al.* [8].

Several groups correlated *rrs* mutations with cross-resistance to amikacin, kanamycin and capreomycin [23, 24]. We observed that the a1401g substitution correlated with phenotypic resistance to at least two SLID (87.5% of cases). Once confirmed, detection of such mutation could provide a useful hint in a patient's management. Like HILLEMANN *et al.* [8] we identified a strain resistant to all three SLIDs harbouring a c1402t substitution in *rrs*, which is in contrast with a report correlating this mutation to phenotypic resistance to capreomycin and kanamycin only [8, 24]. Thus, a relationship between *rrs* mutations and SLID cross-resistances is still unknown.

EMB

MTBDR*sl* provided discrepant results on EMB resistance. In comparison with phenotypic DST, the MTBDR*sl* showed low specificity and sensitivity (55%), mainly due to *embB* mutations

TABLE 7 Correlation of mutations, detected by sequencing of the ethambutol (EMB)-resistance determining region, to the minimum inhibitory concentrations (MICs) of 33 clinical isolates phenotypically susceptible[#] to EMB

ERDR sequence	Conventional DST	MIC MABA		n
		$\mu g \cdot mL^{-1}$	Result	
M306V	S	32	R	1
	S	16	R	5
	S	8	R	4
	S	4	R	1
M306I	S	8	R	5
	S	4	R	4
M306I[¶]	S	16	R	2
	S	8	R	2
	S	4	R	2
	S	2	S	1
M306I[+]	S	8	R	1
	S	2	S	1
S297A	S	16	R	1
	S	4	R	1
S296H	S	8	R	1
S347I	S	8	R	1

ERDR: EMB-resistance determining region; DST: drug-susceptibility testing; MABA: microplate Alamar blue assay; S: susceptible; R: resistant; [#]: BACTEC™ MGIT™ 960 system was used for detection; [¶]: atg→atc; [+]: atg→att.

at codon 306 in 41 phenotypically susceptible strains. Similar results were obtained in clinical specimens.

MIC values resolved the discrepancies between LiPA and DST but raised concerns on phenotypic EMB DST by MGIT. Our results support the findings of PLINKE *et al.* [25] reporting that strains, phenotypically susceptible to EMB and carrying mutations at codon 306 of the *embB* gene, were found resistant after retesting. These findings raise concerns regarding the standardisation of phenotypic DST for EMB. In fact, recent results on the proficiency testing of DST in supranational TB reference laboratories reported by VAN DEUN *et al.* [26] highlighted a lack of consistency in DST results for EMB. In particular, sensitivity was reported to be ~93%, the worst performance among all the first line drugs tested (rifampin, isoniazid and streptomycin). EMB testing was also the DST showing the higher number of rounds with sensitivity <80% and the lower with sensitivity >100%. In addition, also the reproducibility was found to be poor [26]. Despite the lack of clear experimental evidences, we could hypothesise a role in resistance determination for the medium used for the test; thus the cut-off value selected to perform DST for EMB by MGIT (5 $\mu g \cdot mL^{-1}$) might underestimate resistance. Indeed, SCARPARO *et al.* [27] showed that the MGIT system provided very major errors (false-susceptible results) only for EMB using as a cut-off value 5 $\mu g \cdot mL^{-1}$. Thus, when MIC results were used for assessment of EMB tests, MTBDR*sl* specificity and sensitivity were raised up to 96.2% and 69.7%, respectively, and the PPV increased from 56.2% to 97.7% in clinical isolates.

Low NPV values (57.5%) are likely to be caused by the presence on the MTBDR*sl* of only the codon 306 of *embB*.

Genetic diversity and frequency of mutations

Previous studies, demonstrated that the population genetics of *M. tuberculosis* is highly geographically structured [13, 28]: each of the main lineages is associated with particular geographical areas. In fact, a high correlation between genetic variability and geographical areas exists, and has been estimated taking into account human demographics and migration [28]. Thus, for each geographical setting it might be possible to identify a prevalent *M. tuberculosis* lineage and it has been hypothesised that phylogeographical diversity may influence the development of new diagnostics, drugs, and vaccines [13]. Some lineages show a higher frequency of mutations on ''canonical'' genes conferring antibiotic resistance. It seems likely that the performances of molecular assays may, therefore, be affected by the mycobacterial lineage transmitted within a particular geographical setting.

So far association studies between mycobacterial genotypes and incidence of mutations in drug-resistance genes are available only for rifampicin and isoniazid and are missing for SLID resistance genes [14]. The frequency of mutations within the major lineages identified by spoligotyping enables us to detect a statistically relevant association between *rrs* mutations and Beijing strains. In contrast, no association was found for EMB-related or FQ-related mutations.

To avoid inclusion of clonal strains in our analysis, we performed the Mycobacterial Interspersed Repetitive Units–Variable Number of Tandem Repeats (MIRU–VNTR) genotyping technique on all isolates belonging to the Beijing lineage. Once phenotypic DST, year and country of isolation, and single nucleotide polymorphisms within drug-resistance genes (*gyrA*, *rrs*, *embB*, *rpoB*, *inhA*, *katG*, *gidB*, *tlyA*, and *eis*) were analysed together with the MIRU–VNTR genotype (data not shown) the Beijing lineage showed a sufficient degree of diversity to exclude a clonal origin. Our data seemed, therefore, to confirm that Beijing strains may be characterised by higher mutation frequencies and that their cell-wall characteristics increased MDR and hyper-virulent phenotypes [14].

Clinical consequences

Determination of phenotypic DST to FLD and SLID requires 4–9 weeks [29, 30]. The use of rapid molecular assays reduces the time for diagnosing MDR-TB and XDR-TB to just 1–2 days. Can we speculate that the use of the MTBDR*sl* in clinical routine allows the identification of FQ-resistant and/or SLID-resistant cases in 1–2 days since the detection of MDR phenotype?

13 (46.4%) out of 28 XDR-TB strains of our panel were recognised by MTBDR*sl* to have both *gyrA* and *rrs* mutations. In seven cases (25%) the assay identified FQ resistance and in four cases (14.3%) to SLID resistance; four XDR-TB strains not harbouring mutations in the genes targeted by the MTBDR*sl* were misidentified as susceptible. Therefore, further investigations are needed to better understand the clinical add-on value of molecular assay in detecting XDR-TB cases correctly.

Due to the high PPV and specificity, the MTBDR*sl* allows detection of phenotypically resistant cases; anyway, low sensitivity and NPV make confirmation by conventional DST mandatory on all cases classified as susceptible by the molecular test. The study

results confirm that the field of application of MTBDR*sl* is the screening of selected MDR-TB patients at higher risk of XDR-TB (*e.g.* contacts of XDR-TB cases, treatment failures, screening of the index case contacts in specific outbreaks, *etc.*). Addition of further genomic targets involved in resistant phenotype to FQ and SLID could improve performances of the MTBDR*sl* in the next future.

Important advantages of the MTBDR*sl* test are the ability of identifying both of hetero-resistance and mutations conferring low-level resistance. Hetero-resistance is in fact the background for development of resistances and low-level resistance before high-level resistance [31].

To our current knowledge this study is the first to investigate the distribution of SLID-related mutations among *M. tuberculosis* lineages. It seems plausible that the MTBDR*sl* performs differently in different geographical settings. This finding is relevant in clinical practice, thus the contribution of molecular tests aiming at identifying drug resistances should be evaluated considering local epidemiological features.

In conclusion, our study strengthens previous findings concerning the performances of the MTBDR*sl* on a larger set of samples. In addition, we provided a correlation between strain lineages and frequency of mutations occurring in SLID-resistant strains, supporting the hypothesis that performances of molecular tests can be affected by the phylogeographical features.

STATEMENT OF INTEREST

A statement of interest for this study can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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