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DETECTION OF DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* STRAINS ISOLATED IN SERBIA BY THE GENOTYPE MTBDR*sl* ASSAY

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Abstract - The new GenoType MTBDR*sl* assay (Hain Lifescience) detects mutations most frequently associated with resistance to fluoroquinolones (FLQ), aminoglycosides/cyclic peptides (AG/CP), and ethambutol (EMB) and thus, enables rapid identification of extensively drug-resistant (XDR) *Mycobacterium tuberculosis* strains. A set of 19 multidrug-resistant (MDR) strains isolated in Serbia in 2011 was tested by the MTBDR*sl* assay and by conventional drug susceptibility testing (DST). The sensitivity and specificity of the MTBDR*sl* assay were as follows: 100% for both for FLQ; 100% for both for AG/CP; and 58.3% and 85.7%, respectively, for EMB. The sensitivity for detection of XDR strains was 100%. Mutations in the *gyrA*, *rrs*, and *embB* genes established in local *M. tuberculosis* strains resistant to the respective drugs have been reported as the most prevalent in other studies as well. We recommend the MTBDR*sl* assay as a screening test for the preliminary detection of XDR-TB cases in Serbia, but not as a replacement of the conventional second-line DST.

Key words: *Mycobacterium tuberculosis*, multidrug-resistant tuberculosis, extensively drug-resistant tuberculosis, GenoType MTBDR*sl*

INTRODUCTION

The emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant tuberculosis (XDR-TB) present a major health problem of global significance. MDR *Mycobacterium tuberculosis* strains are resistant to at least rifampin and isoniazid, while XDR are defined as MDR strains displaying additional resistance to a fluoroquinolone (FLQ), and one of the three injectable second-line drugs, namely amikacin (AM), kanamycin (KM) or capreomycin (CM) (Sotgiu et al., 2009; Caminero, 2010). In 2010, there were an estimated 650,000 prevalent cases of MDR at a global level (WHO, 2011a), while the proportion of MDR-TB among new TB cases ranged from 0% to 28.3%. As far as XDR-TB is concerned, it has been estimated that 40,000 cases occur annually worldwide (WHO, 2008). The global spread of XDR *M. tuberculosis* strains is obvious, since 69 countries had reported at least one case of XDR-TB by March 2011 (WHO, 2011b). Patients with MDR-TB and, in particular, XDR-TB, have a significantly poorer prognosis than patients with TB caused by drug-susceptible *M. tuberculosis* strains. They have a higher probability of death, treatment failure, longer hospitalization and treatment duration (Johnston et al., 2009; Sotgiu et al., 2009). Rapid detection of MDR-TB and XDR-TB and, thereby rapid drug susceptibility testing (DST) to first- and second-line drugs, are critically

important to the successful control of TB (WHO, 2011b).

DST for XDR strains may be performed by using the conventional approach that requires the cultivation of *M. tuberculosis* and has a slow turnaround time. The time needed for testing with the use of solid media is four weeks or more, beginning from the time that a positive culture is available. Even with the use of rapid commercial liquid-based culture systems, such as Bactec MGIT 960, the turnaround time is approximately one week (Woods et al., 2007). Molecular methods provide new possibilities for the rapid detection of first- and second-line drug resistance in *M. tuberculosis* strains. These tests enable the identification of a resistant strain within one to two days, and can be performed in both cultures and clinical samples (WHO, 2009; Parsons et al., 2011). The GenoType *Mycobacterium tuberculosis* drug resistant second-line assay (MTBDR*sl*) (Hain Lifescience, Nehren, Germany) is a recently developed line-probe assay for molecular detection of resistance to FLQ, aminoglycosides/cyclic peptides (AG/CP), and ethambutol (EMB). The assay uses multiplex PCR DNA amplification followed by reverse hybridization to detect the presence of *M. tuberculosis* DNA and the mutations most frequently associated with resistance to FLQ, AG/CP, and EMB.

The major mechanism of resistance to FLQ in *M. tuberculosis* is a change of the DNA gyrase that consists of gyrA and gyrB subunits encoded by the *gyrA* and *gyrB* genes, respectively. Most mutations occur in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene (codons 90, 91, and 94) (Antonova et al., 2008; Mokrousov et al., 2008; Zhang and Yew, 2009). The percentage of FLQ-resistant clinical *M. tuberculosis* isolates with evident *gyrA* mutations was found to range from 70% to 90% (Antonova et al., 2008; Mokrousov et al., 2008). Resistance to AM, KM, and CM in over 90% of clinical *M. tuberculosis* isolates is caused by mutations in the *rrs* gene, particularly at positions 1401, 1402, and 1484 (Maus et al., 2005; Jugheli et al., 2009; Zhang and Yew, 2009). Maus et al. (2005) reported the following expression patterns of resistance to the three injectable drugs: the *rrs* A1401G mutants show low-level resistance to CM and high-level resistance to AM and KM; the *rrs* C1402T mutants are susceptible to AM, but display low-level resistance to KM and high-level resistance to CM; and the *rrs* G1484T mutants have high-level resistance to all three drugs. Resistance to EMB is most frequently associated with mutations in the *embCAB* operon and particularly with mutations in the *embB* codon. It has been reported that up to 68% of EMB-resistant *M. tuberculosis* strains carry a mutation in *embB*306 (Plinke et al., 2006; Starks et al., 2009; Zhang and Yew, 2009).

In 2011, the number of prevalent MDR-TB cases in Serbia was 56. As of September 2009, all patients diagnosed with MDR-TB have been enrolled in treatment for MDR-TB. DST to second-line antituberculosis drugs for MDR *M. tuberculosis* strains isolated in Serbia is performed in the Supranational Reference Laboratory (SRL), National Reference Center for Mycobacteria, Forschungszentrum, Borstel, Germany. The first case of XDR-TB in Serbia was discovered when the first collection of MDR strains was sent to the SRL in 2008. The total number of patients with XDR-TB revealed so far is seven.

The aim of the present study was to use the GenoType MTBDR*sl* assay as a new diagnostic tool for the detection of XDR *M. tuberculosis* among MDR strains isolated in Serbia during 2011 and thereby to assess the performance of the assay in local settings.

MATERIALS AND METHODS

Mycobacterium tuberculosis isolates

In total, 20 MDR *M. tuberculosis* strains were isolated in Serbia during 2011. The strains were identified as *M. tuberculosis* by the GenoType MTBC assay (Hain Lifescience), while their susceptibility to first-line drugs was established by the proportion method on Löwenstein-Jensen medium and the GenoType MTBDR*plus* assay (Hain Lifescience). These strains were sent to the SRL for first- and second-line DST that was performed by the proportion method on Löwenstein-Jensen medium and the BACTEC MGIT 960

method (MGIT 960; Becton Dickinson Diagnostic Systems, Sparks, MD). The testing included examination of susceptibility to EMB, ofloxacin (OFL) as a representative of FLQ, AM, and CM.

GenoType MTBDRsl assay

The number of MDR *M. tuberculosis* strains tested by the MTBDR*sl* assay was 19, since one culture failed to grow at the time of the testing. The assay was performed as recommended by the manufacturer. PCR reactions comprised 5 μl of the mycobacterial DNA, 35 μl of a primer-nucleotide mixture (provided with the kit), an amplification buffer containing 2.5 mM $MgCl_2$, and 1.25 U *Taq* DNA polymerase (Fermentas UAB, Lithuania) in 50 μl. The amplification parameters were 95°C for 15 min; than 10 cycles comprising 30 s at 95°C and 120 s at 58°C; additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C; followed by an extension at 70°C for 8 min prior to a 6°C hold. Hybridization and detection were performed in a shaking water bath TwinCubator (Hain Lifescience). The first step was mixing 20 μl of the amplicons with 20 μl of denaturing reagent (provided with the kit) for 5 min in a plastic well. After that, 1 ml of prewarmed hybridization buffer was added per well, and the membrane strips were placed into each well. The hybridization was performed at 45°C for 30 min, followed by two washing steps. Streptavidin conjugated with alkaline phosphatase and a corresponding substrate was used for colorimetric detection of hybridized amplicons.

The MTBDR*sl* strip contains 22 probes: one amplification and one hybridization control, *M. tuberculosis* specific probe, and PCR control bands for all targeted regions (*gyrA*, *rrs*, and *embB*). For the detection of resistance to FLQ, three *gyrA* wild-type probes (WT1, WT2, and WT3) encompassed the region of the gene encoding amino acids 85 to 97, while six probes (*gyrA* MUT1 A90V, *gyrA* MUT2 S91P, *gyrA* MUT3A D94A, *gyrA* MUT3B D94N/Y, *gyrA* MUT3C D94G, *gyrA* MUT3D D94H) specifically targetted the most common mutations in the *gyrA* gene. For the detection of AG/CP resistance, two probes covered the wild-type region of *rrs*, while the *rrs* MUT1 and MUT2 are probes designed to assess nucleotide exchanges A1401G and G1484T. Detection of resistance to EMB is based upon *embB* wild-type probe WT1 that targets the *embB* codon 306, and the *embB* MUT1A and MUT1B probes that bind to nucleotide exchanges ATG/ATA (M306I) and ATG/GTG (M306V). If all of the wild-type probes produced positive staining and the mutant probes showed no staining, the isolate was considered susceptible. In contrast, the omission of at least one wild-type probe and/or the staining of any mutant probe indicated strain resistance to the respective drug.

RESULTS

In total, 19 MDR *M. tuberculosis* strains isolated in Serbia during 2011 were tested by the GenoType MTBDR*sl* assay. Seven strains (36.8%) were susceptible to all drugs, i.e. FLQ, AG/CP, and EMB (Table 1). Three strains (15.8%) were identified as XDR *M. tuberculosis* strains since they displayed simultaneous resistance to FLQ and AG/CP. All three strains were susceptible to EMB. Out of seven strains (36.8%) exhibiting monoresistance, six (31.5%) were resistant to EMB, and one (5.3%) to FLQ. Combined resistance was noted in two strains (10.6%). These results were not fully concordant with the results of the conventional DST in liquid and solid media performed for the same strains in the SRL. The distinct MTBDR*sl* patterns of 19 strains tested by the assay compared to the results of the conventional DST are presented in Table 2. Out of seven fully susceptible strains identified by the MTBDR*sl* assay, two were falsely susceptible to EMB. The same three strains identified as XDR by the MTBDR*sl* assay were also recognized by the conventional DST, but two of these strains were resistant to EMB as well. Out of six strains established as monoresistant to EMB by the MTBDR*sl*, five were truly resistant to this drug. The strain that was monoresistant to FLQ by the MTBDR*sl* was detected as resistant to both FLQ and EMB with conventional DST. In summary, the results of DST obtained by the MTBDR*sl* and those obtained by conventional DST were fully consistent for five pansusceptible strains, five strains monoresistant to EMB, and two strains

displaying combined resistance. As far as the XDR strains are concerned, the results of MTBDR*sl* and phenotypic DST were concordant in terms of resistance to FLQ and AG/CP.

All five isolates that were resistant to FLQ had D94G (GAC-GGC) exchange in the *gyrA* gene, indicated by the omission of the WT3 band together with the appearance of the MUT3C band. All these strains were found to be FLQ-resistant by conventional DST. Four strains that showed resistance to AG/CP had the A1401G exchange in *rrs* gene, which indicates high-level resistance to AM and KM associated with low-level resistance to CM. Resistance to both AM and CM was established phenotypically in all of them. Among the eight strains recognized as resistant to EMB by the MTBDR*sl* assay, seven had M306V (ATG-GTG) exchange in the *embB* gene. This finding was confirmed by the conventional DST in six strains. In the remaining strain displaying resistance to EMB, the omission of the wild-type band was noted and this result was consistent with the results of phenotypic testing. The pattern indicating heteroresistance, i.e. the simultaneous appearance of wild type and mutant bands, to any of the drugs included in the MTBDR*sl* assay was not noted among *M. tuberculosis* strains tested in our study.

Sensitivity and specificity of the GenoType MTBDRsl assay

The sensitivity and specificity of the MTBDR*sl* test were calculated by comparing the results from the line probe assay to the results of the conventional DST in liquid and solid media taken as a reference. The sensitivity values of the MTBDR*sl* assay to detect resistance to the drugs tested were as follows: 100% for FLQ, 100% for AG/CP, and 58.3% for EMB. The specificity values were 100% for FLQ, 100% for AG/CP, and 85.7% for EMB.

DISCUSSION

The MTBDR*sl* assay detects the most prevalent mutations in the *gyrA*, *rrs*, and *embB* genes linked to resistance to FLQ, AG/CP, and EMB, respectively. Therefore, the assay is specifically designed to rapidly detect XDR- and EMB-resistant *M. tuberculosis* strains among MDR strains. As noted above, the conventional DST to second-line anti-tuberculosis drugs is not performed in our country. MDR strains are sent to the SRL annually, which significantly delays the detection of possible cases of XDR-TB. The MTBDR*sl* assay may be an appropriate solution for this situation for two obvious reasons. First, the previous studies that evaluated the assay reported that it reliably detects the most common mutations involved in resistance to FLQ and AG/CP (Hillemann et al., 2009; Brossier et al., 2010; Huang et al., 2011). Second, other line probe assays provided by the same manufacturer are already part of the diagnostic algorithm in our NRL and thus, implementation of the MTBDR*sl* assay is attainable.

This study reports the results of the first application of the assay in our laboratory. We wanted to determine whether the GenoType MTBDR*sl* might be used as a diagnostic tool for the rapid and reliable detection of XDR *M. tuberculosis* strains. The collection of 19 MDR *M. tuberculosis* strains isolated in Serbia during 2011 was used for the survey. The susceptibility profiles of these strains established by conventional DST in the SRL were taken as a reference. The GenoType MTBDR*sl* assay, in general, produced easily interpretable results. The only exceptions were amplification control and the *gyrA* wild-type probe 2 that often produced faint bands and therefore sometimes hindered interpretation of the results. The weak intensity of the amplification control was not specifically commented on by the other studies that evaluated the assay (Hillemann et al., 2009; Brossier et al., 2010; Kiet et al., 2010; Huang et al., 2011), but it is apparent in the figures presenting the hybridization patterns obtained with the MTBDR*sl* (Hillemann et al., 2009; Brossier et al., 2010). We did not encounter this problem with other line probe assays provided by the same manufacturer. Regardless of these minor technical difficulties, our study showed the high accuracy of the GenoType MTBDR*sl* assay in the detection of resistance to FLQ and AG/CP. Namely, the assay identified 100% of the FLQ-resistant isolates and 100% of the AG/CP-resistant

Table 1. Susceptibility patterns of 19 MDR *M. tuberculosis* strains tested with the GenoType MTBDR*sl*[a]

Number of strains (%)	FLQ	AG/CP	EMB
7 (36.8)	S	S	S
6 (31.6)	S	S	R
3 (15.8)	R	R	S
1 (5.3)	R	S	S
1 (5.3)	S	R	R
1 (5.3)	R	S	R

[a] R = resistant; S = susceptible.

Table 2. GenoType MTBDR*sl* test results for the detection of FLQ, AG/CP, and EMB resistance in 19 MDR *M. tuberculosis* strains[a]

MTBDR*sl* pattern (gyrA, rrs, embB)[b]	Amino acid change (nucleotide changes)	MTBDR*sl* result	DST result[c]	Number (%) of strains
FLQ				
gyrA WT	WT	FLQ^S	OFL^S	14 (73.7)
gyrA ΔWT3, MUT3C	D94G (GAC-GGC)	FLQ^R	OFL^R	5 (26.3)
AG/CP				
rrs WT	WT	$AG^S\,CP^S$	$AG^S\,CP^S$	15 (78.9)
rrs ΔWT1, MUT1	A1401G	$AG^R\,CP^R$	$AG^R\,CP^R$	4 (21.1)
EMB				
embB WT	WT	EMB^S	EMB^S	6 (31.6)
	WT	EMB^S	EMB^R	5 (26.3)
embB ΔWT1	/	EMB^R	EMB^R	1 (5.3)
embB ΔWT1, MUT1B	M306V (ATG-GTG)	EMB^R	EMB^R	6 (31.6)
	M306V (ATG-GTG)	EMB^R	EMB^s	1 (5.3)

[a] R = resistant; S = susceptible
[b] WT = wild type with all respective bands; ΔWT = omission of the respective band
[c] Drug resistance was determined by the conventional DST on LJ or MGIT.

isolates. The previous studies that evaluated the MTBDR*sl* assay did not report sensitivities of 100%, in particular for FLQ, but this is most probably related to the small number of isolates tested in our study. Hillemann et al. (2009) evaluated the ability of the MTBDR*sl* to detect resistance to FLQ and AM/CM in 106 clinical *M. tuberculosis* isolates and directly in 64 sputum specimens. The overall sensitivity of the assay was 90.2% for FLQ, 83.3% for AM, and 86.8% for CM. Therefore, this study showed that the MTBDR*sl* assay provides reliable detection of XDR *M. tuberculosis* strains when applied both to culture isolates and directly to smear-positive sputum specimens. Brossier et al. (2010) assessed the performance of the MTBDR*sl* by using a collection of 41 MDR and 8 XDR *M. tuberculosis* strains displaying a wide variety of molecular mechanisms of resistance to the respective drugs. The sensitivity of detection of resistance to FLQ, AM and CM was 87%, 100%, and 80%, respectively. In the study by Huang et al. (2011), the MTBDR*sl* assay accurately identified 85.1% of FLQ-resistant isolates, 84.2% of AM-resistant strains, and 71.4% of CM-resistant isolates among 234 MDR *M. tuberculosis* isolates. As far as detection of resistance to EMB is concerned, we established that sensitivity of the GenoType MTBDR*sl* assay was 58.3%. This

finding is consistent with the results of the previous studies (Hillemann et al., 2009; Brossier et al., 2010; Kiet et al., 2010; Huang et al., 2011). They demonstrated the accuracy of the MTBDR*sl* test to be 56.2% to 69.2% for detecting EMB resistance. Summarized results of these studies indicate that the accuracy of the GenoType MTBDR*sl* assay in detecting resistance to FLQ, AG/CP, and, in particular, EMB is to some extent limited by mutations not included in the test and/or yet unidentified molecular mechanisms of resistance.

The molecular mechanisms of resistance to the respective drugs established in our study were predominantly homogeneous. This is presumably related to the small number of isolates tested by the MTBDR*sl* in our study, and the presence of other mutations and/or mechanisms conferring resistance to FLQ, AG/CP, and EMB in local *M. tuberculosis* strains cannot be excluded. As noted above, all FLQ-resistant isolates identified in our study had the same mutation, D94G (GAC-GGC) exchange in the *gyrA* gene. This mutation has been recognized as the most prevalent molecular mechanism of resistance to FLQ by other studies, but its frequency ranged from 33.3% to 47.7% (Mokrousov et al., 2008; Hillemann et al., 2009; Brossier et al., 2010; Kiet et al., 2010; Huang et al., 2011). All the isolates resistant to AG/CP recognized in our study harbored the A1401G mutation in the *rrs* gene, which was the most prevalent mutation in other studies as well (Hillemann et al., 2009; Jugheli et al., 2009; Brossier et al., 2010; Kiet et al., 2010; Huang et al., 2011). Although some of the previous studies reported CM-susceptible isolates with the A1401G mutation in the *rrs* gene (Alangaden et al., 1998; Hillemann et al., 2009; Jugheli et al., 2009; Huang et al., 2011), this was not the case in our study. The four *rrs* A1401G mutants were resistant both to AM and CM, according to the results of phenotypic testing. Out of seven strains truly resistant to EMB, i.e. phenotypically resistant strains, one was classified as EMB-resistant due to omission of the wild type probe, but without DNA sequencing the corresponding mutation could not have been recognized. The remaining six (85.7%) isolates had M306V (ATG-GTG) exchange in the *embB* gene. Predominance of this mutation among EMB-resistant isolates is also consistent with the previously reported results (Plinke et al., 2006: Hillemann et al., 2009; Starks et al., 2009; Brossier et al., 2010; Kiet et al., 2010; Huang et al., 2011). On the other hand, the conventional DST identified seven EMB-susceptible strains, but one of them had the same mutation. The presence of *embB* 306 mutations in EMB-susceptible strains has already been reported (Mokrousov et al., 2002; Brossier et al., 2010), and therefore the precise role of these mutations in resistance to EMB is still somewhat controversial. It is noteworthy that heteroresistance was not observed among the *M. tuberculosis* strains tested in our study. This is an important finding, since heteroresistance is considered a preliminary stage of full resistance, and high rates of heteroresistance, in particular to FLQ, have already been reported from different settings (Mokrousov et al., 2008; Hillemann et al., 2009; Kiet et al., 2010).

The overall data, including the results of our study, reveal a satisfactory accuracy of the GenoType MTBDR*sl* assay for the detection of resistance to FLQ and AG/CP, while the detection rates of resistance to EMB were significantly lower. Although the assay correctly identified all FLQ-resistant, AG/CP-resistant, and XDR isolates among the *M. tuberculosis* strains tested in our study, certain limitations of the study should be considered. First, while the collection of *M. tuberculosis* isolates tested is a representative collection in that all MDR strains isolated over a one-year period were included, the number of the isolates is rather small. Second, the assay detects the most prevalent mutations linked to resistance to FLQ and AG/CP, but the molecular background of resistance to these drugs in local *M. tuberculosis* strains remains largely unknown, and the presence of other mutations conferring resistance cannot be excluded. Therefore, we recommend the MTBDR*sl* assay as a screening test for preliminary detection of XDR-TB cases in our country, but not as a replacement of the conventional DST to second-line antituberculosis drugs.

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