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Pseudo-Outbreak of Pre-Extensively Drug-Resistant (Pre-XDR) Tuberculosis in Kinshasa: Collateral Damage Caused by False Detection of Fluoroquinolone Resistance by GenoType MTBDR*sl*

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Fluoroquinolones are the core drugs for the management of multidrug-resistant tuberculosis (MDR-TB). Molecular drug susceptibility testing methods provide considerable advantages for scaling up programmatic management and surveillance of drug-resistant TB. We describe here the misidentification of fluoroquinolone resistance by the GenoType MTBDR*sl* (MTBDR*sl*) (Hain Lifescience GmbH, Nehren, Germany) line probe assay (LPA) encountered during a feasibility and validation study for the introduction of this rapid drug susceptibility test in Kinshasa, Democratic Republic of Congo. The double *gyrA* mutation 80Ala and 90Gly represented 57% of all fluoroquinolone mutations identified from MDR-TB patient sputum samples, as confirmed by DNA sequencing. This double mutation was previously found to be associated with susceptibility to fluoroquinolones, yet it leads to absent hybridization of a wild-type band in the MTBDR*sl* and is thus falsely scored as resistance. Our findings suggest that MTBDR*sl* results must be interpreted with caution when the interpretation is based solely on the absence of a wild-type band without confirmation by visualization of a mutant band. Performance of the MTBDR*sl* LPA might be improved by replacing the *gyrA* wild-type probes by additional probes specific for well-documented *gyrA* mutations that confer clinically relevant resistance.

Fluoroquinolones (FQs) are essential drugs for the management of multidrug-resistant tuberculosis (MDR-TB) (1). Resistance to FQs is associated with poor treatment outcome in MDR-TB and is also one of the defining conditions of extensively drug-resistant tuberculosis (XDR-TB). XDR-TB is defined as MDR-TB with additional resistance to any FQ and a second-line injectable drug such as kanamycin, amikacin, or capreomycin, and pre-XDR-TB is defined as MDR-TB associated with resistance to FQ or a second-line injectable, but not both (2–5). In areas with high rates of TB and MDR-TB, it is extremely important to monitor resistance to these drugs, especially where FQs are widely used for treatment of other bacterial infections. Molecular drug susceptibility testing (DST) methods have considerable advantages for scaling up programmatic management and surveillance of drug-resistant TB. Currently, compared to conventional DST, these methods offer improvements in speed of diagnosis, standardized testing, potential for high-burden settings, and lower levels for laboratory biosafety (6). In 2008, the World Health Organization (WHO) endorsed molecular line probe assay (LPA) technology for rapid detection of MDR-TB that brings results within 2 days—even from clinical specimens (7). The GenoType MTBDR*plus* assay (MTBDR*plus*) (Hain Lifescience GmbH, Nehren, Germany) was one of the first commercially available LPAs. Because of its accuracy and rapidity, genotypic detection of rifampin (RMP) and isoniazid resistance with the MTBDR*plus* has emerged as an essential tool for the diagnosis of MDR-TB (8, 9). It has also been suggested as an alternative approach for conducting drug resistance surveys (DRS) in settings with limited capacity to perform phenotypic DST (10). In 2009, the company Hain Lifescience introduced a new format of the LPA, the GenoType MTBDR*sl* test (MTBDR*sl*), for the rapid determination of genetic mutations associated with resistance to FQs, second-line injectable drugs, and ethambutol. The main mechanism of resistance to FQs in *Mycobacterium tuberculosis* is caused by mutations affecting DNA gyrase, which consists of the GyrA and GyrB subunits, encoded by the *gyrA* and *gyrB* genes, respectively (11). Most mutations conferring bacterial resistance to FQs occur in a short segment termed the quinolone resistance-determining region (QRDR) in the *gyrA* gene (12, 13). Analysis of the QRDR alone by genotypic tests has been suggested as sufficient for rapid identification of the vast majority of FQ-resistant *M. tuberculosis* strains, with an estimated sensitivity around 85% for FQ resistance (14, 15). The identification of resistance to FQs by the MTBDR*sl* is based on this principle. The format of the MTBDR*sl* is similar to that of the MTBDR*plus* and it also has a turnaround time of 48 h. Compared to phenotypic DST, the sensitivity of the MTBDR*plus* for detection of FQ resistance by identification of the best known *gyrA* mutations (but, for instance, not the *gyrB* mutations) has been generally evaluated as suboptimal. For this reason, the MTBDR*plus* has not yet been endorsed by the WHO, but its specificity is considered to be very high (15, 16). Here we report that, in addition, the specificity of the MTBDR*sl*

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LPA was inadequate for the detection of FQ resistance during a validation study of the assay in Kinshasa.

MATERIALS AND METHODS

Patients and specimens. From March 2011 to June 2013, a feasibility and validation study with both the MTBDR*plus* and MTBDR*sl* LPAs was carried out at the National Public Health Laboratory of the Democratic Republic of Congo (DRC)—the Institut National de Recherche Biomédicale (INRB)—in Kinshasa. Kinshasa is the capital city of DRC, with an estimated 10 million inhabitants. With a total of 137 TB clinics, the city of Kinshasa is notifying more than 80% of all MDR-TB suspects in the country (DRC National TB Program, unpublished data). We prospectively collected sputum specimens of 587 MDR-TB suspects in 50 out of the 137 TB clinics which were purposefully selected as sentinel sites in Kinshasa for rapid detection of drug-resistant TB. Consenting MDR-TB suspects defined according to the WHO categories of treatment (17) have been consecutively included in the study, in addition to smear-positive contacts of known MDR-TB cases.

MTBDR*sl*. Sputum specimens without any additives were transported from the TB clinics. Patient information related to their demographic data and clinical TB history was collected. Sputum specimens were processed according to standard methods previously described, which included decontamination and processing with NaOH according to a modified Petroff technique, with a final concentration of 2% (18). The sediment obtained was tested by the MTBDR*plus* according to the manufacturer's instructions (19). All specimens showing resistance to RMP and isoniazid or to RMP alone by the MTBDR*plus* were concurrently tested by the MTBDR*sl* from the same DNA extract. Strips were interpreted according to the manufacturer's instructions (20). For each gene, the test evaluates the presence of wild-type (WT) and/or mutant (MUT) sequences, thus covering all high-confidence resistance mutations. For *gyrA* these are 90Val, 91Pro, and the codon 94 mutations Ala, Asn, Gly, His, and Tyr. When all the WT probes of a specific gene appear as bands on the strip, there is no detectable mutation within the region examined and the strain is considered sensitive to the corresponding drugs. In case of a mutation, the amplicon cannot bind to the corresponding WT probe, but it may bind to one of the MUT probes provided this specific mutation is represented on the strip. The absence of a WT band or appearance of a MUT band at least as strong as the amplification control band must be interpreted as resistance to the respective drugs.

The INRB laboratory follows a strict unidirectional workflow for all molecular testing. For quality control, each test batch included a known pan-susceptible TB strain (H37Rv). Negative controls (water) were included during all steps of the procedure. The strips were interpreted on a regular schedule by two different readers (M.K.K. and M.A.) who were blinded to the results of genetic sequencing. Discrepancies between both readers were uncommon (<10%) and were resolved by consensus.

Genetic sequencing. Sequencing of the *gyrA* was performed at the Supra National Reference Laboratory (SRL) in Antwerp, Belgium, on all available DNA extracts from Kinshasa harboring FQ-resistant patterns on LPA. The methodology for PCR amplification and sequencing of genes encoding gyrase A and B has been published elsewhere (21).

Ethical considerations. The study was approved by the Ethics Committee of the University of Antwerp, Belgium, and the National Tuberculosis Program DRC. All data and sputum specimens were collected in the context of routine care and no additional data collection or contact with patients occurred for this study. The data were completely delinked from any personal identifiers before analysis.

RESULTS

MTBDR*sl* test. Of 587 consecutive individual MDR-TB suspects who submitted their sputum specimens at TB clinics during the study period, a total of 211 MDR-TB and 28 RMP monoresistant results were obtained from DNA extracts tested with MTBDR*plus*. All 239 extracts with RMP resistance were also tested by MTBDR*sl*. Of those, 87% (209/239) yielded an interpretable test for FQ *gyrA*, with the remainder invalid due to absence of the *gyrA* control band. As shown in Table 1, of 209 samples with an interpretable result, 177 (85%) were identified as FQ susceptible and 32 (15%) as FQ resistant. Out of 32 FQ-resistant samples, 20 (63%) were identified as resistant based only on the lack of hybridization with WT probe number 2 (WT2), while for the other 12 samples, one or two specific mutation bands appeared.

TABLE 1 Genotypic pattern obtained by MTBDR*sl* line probe assay on 209 sputum DNA extracts from documented multidrug-resistant tuberculosis in Kinshasa

MTBDR*sl* result	*gyrA* pattern[a]	No. (%) of strains
Resistant (n = 32)	MUT3A	4 (12.5)
	MUT3C	3 (9.3)
	MUT1	2 (6.2)
	MUT3A plus MUT3C	2 (6.2)
	MUT2	1 (3.1)
	ΔWT2	20 (62.5)
Sensitive (n = 177)	WT1 plus WT2 plus WT3	177 (84.7)

[a] MUT1, MUT2, MUT3A, and MUT3C, mutation bands appearing; WT1, WT2, and WT3, wild-type DNA bands appearing; ΔWT2, wild-type 2 band disappearing.

The distribution of the 14 gene mutations found in the 12 FQ-resistant samples with a MUT band on the MTBDR*sl* strips is shown in Table 1. The predominant mutations identified as conferring FQ resistance were *gyrA* MUT3A (94Ala) (6/14 [43%]), followed by MUT3C (94Gly) (5/14 [36%]), MUT1 (90Val) (2/14 [14%]), and MUT2 (91Pro) (1/14 [7%]), with Arg94Ala and Arg94Gly found twice as a double mutation. No MUT3B (94Asn or Tyr) or MUT3D (94His) mutations were found in our study.

DNA sequencing results. *gyrA* sequencing was performed on 25 (25/32 [78%]) DNA extracts identified by MTBDR*sl* as FQ resistant. DNA sequencing confirmed mutations in the *gyrA* QRDR for 23/25 (92%), but two contained only WT DNA. Table 2 shows the types of mutations detected by DNA sequencing and their frequencies, stratified by WHO patient category and compared to MTBDR*sl* results. There were 4 patients in WHO category 4 (recurrence after second-line TB treatment) versus 12 in category 2 (recurrence after re-treatment with first-line drugs) and 9 in category 1 (recurrence after primary treatment with first-line drugs). The 94Gly substitution was detected in three of the four DNA extracts from category 4 patients, once as a triple mutation (80Ala, 90Gly, and 94Gly), while the fourth showed a 94Ala mutation. Mutations in this group were detected directly with MTBDR*sl* by hybridization with the *gyrA* probes in MUT3C or MUT3A and were missing corresponding WT bands, except for the 94Ala, which showed as MUT3A and MUT3C bands without loss of WT3. Eleven samples from WHO category 2 patients were confirmed to contain multiple mutations, but one showed only wild-type DNA on sequencing. The two multiple mutations (94Tyr and 94Ala and 80Ala, 90Gly, and 94Ala) showed only a *gyrA* MUT3A band on the MTBDR*sl* strip. The expected MUT3B was missing for the first, and for both all of the wild-type bands were still present. The nine remaining, with an 80Ala and 90Gly mutation on sequencing, showed only as an absent WT2 band in the MTBDR*sl*. From WHO category 1 patient samples, two single and six double mutations were identified by DNA sequencing, but only wild-type DNA was found in one sample. Both single muta-

TABLE 2 MTBDR*sl* patterns versus *gyrA* mutations detected by DNA sequencing among 25 of the 32 DNA extracts, by WHO patient category

Type of patient[a]	*gyrA* MTBDR*sl* pattern[b]	*gyrA* sequencing data, codons, and amino acid (nucleotide) substitutions	No. (%) detected
Cat. 4	ΔWT3 plus MUT3C	94Gly (GGC)	2 (8)
	ΔWT2 plus ΔWT3 plus MUT3C	80Ala (GCC), 90Gly (GGG), 94Gly (GGC)	1 (4)
	MUT3A plus MUT3C	94Ala (GCC)	1 (4)
Cat. 2	MUT3A	94Tyr (TAC), 94Ala (GCC)	1 (4)
	MUT3A	80Ala (GCC), 90Gly (GGG), 94Ala (GCC)	1 (4)
	ΔWT2	80Ala (GCC), 90Gly (GGG)	9 (36)
	ΔWT2	WT[c]	1 (4)
Cat. 1	ΔWT2 plus MUT1	90Val (GTG)	1 (4)
	ΔWT2 plus MUT1	80Ala (GCC), 90Val (GTG)	1 (4)
	ΔWT2 plus MUT2	91Pro (CCG)	1 (4)
	ΔWT2	80Ala (GCC), 90Gly (GGG)	4 (16)
	ΔWT2	80Ala (GCC), 90Arg (AGG)	1 (4)
	ΔWT2	WT	1 (4)

[a] Cat. 1, 2, and 4, WHO patient categories of recurrences after first-line primary treatment, first-line re-treatment, and second-line treatment, respectively.
[b] ΔWT, omission of the respective wild-type band.
[c] WT, only wild-type DNA detected.

tions (90Val and 91Pro) and one of the six double mutations (80Ala and 90Val) were detected with the MTBDR*sl* by hybridization with the *gyrA* probe in MUT1 or MUT2 and absence of the WT2 band. The five remaining double mutations (80Ala and 90Gly and 80Ala and 90Arg) were all associated with lack of hybridization with WT2 in MTBDR*sl* without a MUT band appearing. Overall, results by sequencing showed that the double mutations 80Ala and 90Gly represent 57% of all 23 confirmed *gyrA* mutations among MDR-TB patients in Kinshasa, DRC.

DISCUSSION

This is the first assessment of the performance of LPA technology under routine diagnostic conditions in the capital city of DRC, one of the 22 TB high-burden countries. According to the MTBDR*sl* results, the proportion of FQ resistance among MDR-TB (i.e., RMP-resistant) samples was alarming, at 15%. Of these *gyrA* mutants, 63% were indirectly detected by MTBDR*sl* through lack of hybridization with the WT2 or with any mutant *gyrA* probes. However, besides a few cases without any mutation detected, DNA sequencing showed for more than half of those a combination of the mutations 80Ala and 90Gly, which has previously been demonstrated to confer FQ hypersusceptibility (22). Studies by the same group suggest that this hypersusceptibility may be caused by a stronger covalent bond and resulting enzyme blockage if the WT 90Ala is replaced by 90Gly because of its smaller side chain, while a bulkier side chain, as with the 90Val mutation, has the opposite effect and causes resistance (23). 80Ala is not detected by MTBDR*sl* LPA, since codon 80 is not covered by the test. The *gyrA* codons analyzed range from 89 to 93, including codon 90. Our sequence results revealed a mutation in 90Gly (GGG), while the only mutation probe included on the strip for position 90 is 90Val (GTG), explaining why only the absence of WT2 was found in these samples but no confirmation by appearance of a mutant probe. The MTBDR*sl*, which contains mutant DNA probes only for the most frequent *gyrA* QRDR mutations observed (12, 24) at codons 90, 91, and 94, has previously been assessed as highly specific in several countries worldwide (25–31). In our series in Kinshasa, the majority of the QRDR mutations observed were not associated with true FQ resistance. Mutation prevalence might differ by geographical areas and by preselection of patients (26). Negative controls, included in DNA extraction, PCR amplification, and hybridization, never showed evidence of contamination. Moreover, 14/20 profiles with a lacking WT2 were single occurrences found in as many runs, and the remaining six were found per two in three runs. For these reasons we believe that contamination or cross-contamination of the tests is highly unlikely to explain the high frequency of this unusual pattern.

Fundamentally, the problem is that interpretation of the MTBDR*sl* is based on absence of WT bands, which is equated with resistance (20). Errors are known to occur because of silent mutations, with a change of nucleotide resulting in a different code but for the same amino acid (26). The systematic error we report here was hitherto hardly known, i.e., a polymorphism not associated with resistance that appears to be more common in Kinshasa, DRC. In contrast to what was reported elsewhere (27–30), the predictive value for demonstration of FQ resistance of the assay in Kinshasa is therefore low, since the proportion of test results falsely indicating resistance to FQs was high. We erroneously alarmed the DRC National TB Program by reporting that FQ resistance had taken a big leap, based on these LPA results, before finding out through sequencing that the combinations of 80Ala (GCC) and 90Gly (GGG) mutations were in fact not associated with true FQ resistance. After this correction, the proportion of FQ resistance did drop to 5%. Considering sequencing as the gold standard, two samples had been wrongly classified as resistant because of a missing WT2 band. This may have been due to poor amplification compared to the control, leading to erroneous interpretation. Aberrant results were also seen for two samples with multiple mutations on DNA sequencing, but only a MUT3A band appearing on the LPA, and no wild-type band disappearing. Another sample with only a 94Ala mutation detected by sequencing showed two mutation bands on LPA, MUT3A, and MUT3C. These differences may have been caused by different proportions of the alleles present in the aliquots used for the different tests, possibly together with some remaining wild-type DNA. At a too low proportion, the alleles would not be reliably detected by either test. DNA sequencing performed in our study showed *gyrA* 80Ala (GCC) and 90Arg (AGG) in a double mutation, and to the best of our knowledge, this was never described previously.

In low-income countries with high TB burdens, resistance to FQ is not routinely tested because of the very limited laboratory infrastructure. New molecular techniques that do not have the same biosafety requirements as conventional techniques have the potential to overcome this problem and are an alternative to periodic or continuous surveillance of resistance against this important class of drugs. Local validation of a novel molecular assay will require assessing its accuracy compared to a reference, ideally composed of standard pheno- and genotypic techniques. For MDR-TB management, knowledge of FQ susceptibility is crucial, as FQs represent the core drugs in all the second-line drug regimens. Failure to detect mutations conferring resistance (i.e., poor sensitivity) or overdetection of false resistance (i.e., poor specificity) results in poor programmatic management of MDR-TB cases. FQ resistance in *M. tuberculosis* has a major impact on MDR-TB

patient outcome, and removal of FQ seriously jeopardizes the strength of the second-line regimen, so false-positive results should be avoided. Molecular differentiation of the *gyrA* mutations 80Ala and 90Gly has important clinical consequences, since these mutations are not associated with FQ resistance. In Kinshasa, managing MDR-TB cases based on results of the MTBDR*sl* LPA only would thus have been detrimental for patient prognosis.

In this study, a significant proportion of DNA extracts from MDR patients had mutations observed in the QRDR, suggesting a rapid increase or even an outbreak of pre-XDR-TB in Kinshasa. Although not confirmed as pre-XDR, an MDR-TB strain with clonal expansion in Kinshasa is suggested by the high frequency of the unusual combination of 80Ala and 90Gly mutations. These mutations do not confer any advantage due to FQ resistance and were also not observed among category 4 recurrences. Their high prevalence may thus point to continued MDR transmission due to delayed or absent detection and treatment of MDR-TB. Further studies using genotyping techniques with higher resolution should clarify the proportion of MDR-TB due to recent transmission.

This study has some limitations. First, we could not correlate the mutations with FQ resistance (level) in our population, since only a few strains were still available and local phenotypic DST appeared unreliable. In turn, this was caused by extremely high culture contamination rates in addition to numerous operational problems, illustrating the higher feasibility of genotypic than conventional DST, even at the capital. Second, DNA sequencing was not performed on susceptible RMP and on susceptible FQ to verify if this polymorphism is also frequent in Kinshasa in the absence of MDR-TB. Moreover, fingerprinting of these isolates was not done to identify whether the 80Ala (GCC) and 90Gly (GGG) mutations are characteristic of a single MTBc lineage that is common in Kinshasa. Further studies are necessary to characterize the biological significance and potential selective advantage of these mutations not conferring resistance.

Our results warrant caution in the interpretation of the MTBDR*sl* when the only sign of resistance is the absence of WT2 band hybridization, without the presence of confirmatory mutation bands. Such instances may lead to the false interpretation of the result as FQ resistance in settings with high prevalence of the 80Ala and 90Gly polymorphisms that do not confer resistance. Performance of the MTBDR*sl* LPA could thus be improved by omitting all *gyrA* WT probes and adding the few missing mutant probes well documented to confer FQ resistance, so that all clinically relevant mutations are confirmed by a mutant band.

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