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 MTBDRplus (MTBDRsl) (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 MTBDRsl 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 MTBDRsl and is thus falsely scored as resistance. Our findings suggest that MTBDRsl 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 MTBDRsl 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.
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 MTBDRplus and MTBDRsl 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the city of Kinshasa is notifying more than 80% of all MDR-TB suspects in the country (DRC National TB Program, unpublished data).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 were included as part of routine care in 10 TB clinics which were purposefully selected as sentinel sites in Kinshasa. The data were completely delinked from patients occurring for this study. The data were completely delinked from any personal identifiers before analysis.

**RESULTS**

**MTBDRsl 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 MTBDRplus. All 239 extracts with RMP resistance were also tested by MTBDRsl. 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.

The distribution of the 14 gene mutations found in the 12 FQ-resistant samples with a MUT band on the MTBDRsl strip 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 MTBDRsl as FQ resistant. DNA sequencing confirmed mutations in the gyrA QRDR for 23 (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 MTBDRsl 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 94Arg mutation. Mutations in this group were detected directly with the INRB laboratory, following FQ susceptibility testing by hybridization with the gyrA probes in MUT3C or MUT3A and were missing corresponding WT bands, except for the 94Gly, 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 94Gly) showed only a gyrA MUT3A band on the MTBDRsl 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 MTBDRsl. 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-
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 MTBDRs results, the proportion of FQ resistance among MDR-TB (i.e., RMP-resistant) samples was alarming, at 15%. Of these FQ-resistant samples, 63% were indirectly detected by MTBDRs through lack of hybridization with WT2 in MTBDRs without a WT 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.

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 treatment outcomes (30Ala and 90Gly represent 57% of all 23 confirmed gyrA 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 QDRD 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 MTBDRs 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.

<table>
<thead>
<tr>
<th>Type of patienta</th>
<th>gyrA MTBDRs patternb</th>
<th>gyrA sequencing data, codons, and amino acid (nucleotide) substitutions</th>
<th>No. (%) detected</th>
</tr>
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<tbody>
<tr>
<td>Cat. 4</td>
<td>ΔWT2 plus MUT3C</td>
<td>94Gly (GGC)</td>
<td>(8)</td>
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<tr>
<td></td>
<td>plus ΔWT3</td>
<td>90Gly (GGG)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>plus MUT3C</td>
<td>94Gly (GGC)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90Ala (GCC)</td>
<td>(4)</td>
</tr>
<tr>
<td>Cat. 2</td>
<td>MUT3A</td>
<td>94Tyr (TAC)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>94Ala (GCC)</td>
<td>90Gly (GGG)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>94Ala (GCC)</td>
<td>80Ala (GCC), 90Gly (GGG),</td>
<td>(36)</td>
</tr>
<tr>
<td></td>
<td>ΔWT2</td>
<td>90Gly (GGG)</td>
<td>(4)</td>
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</tr>
<tr>
<td></td>
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<td>WT</td>
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<tr>
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<tr>
<td></td>
<td>WT</td>
<td>WT</td>
<td>(4)</td>
</tr>
</tbody>
</table>

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.
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 MTBDRsl 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 QDRD, 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 were also not observed among category 4 recurrences. Their high prevalence may then 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.

Our results warrant caution in the interpretation of the MTBDRsl 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 MTBDRsl 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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