AID TB resistance line probe assay for rapid detection of resistant Mycobacterium tuberculosis in clinical samples

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KEYWORDS
Tuberculosis; Molecular diagnostic testing; Multi-drug resistance; Extensively drug-resistant tuberculosis

Summary
Objectives: To determine the sensitivity and specificity of AID TB Resistance line probe assay (AID Diagnostika, Germany) to detect Mycobacterium tuberculosis and its resistance to first- and second-line drugs in clinical samples using BACTEC 460TB as the reference standard.

Methods: The test consists on three strips to detect resistance to isoniazid/rifampicin, fluoroquinolones/ethambutol, and kanamycin/amikacin/capreomycin/streptomycin, respectively. This test was performed on 65 retrospectively selected clinical samples corresponding to 32 patients.

Results: A valid result was obtained for 92.3% (60/65), 90.8% (59/65) and 78.5% (51/65) of the samples tested, considering the three strips, respectively. Global concordance rates between AID and BACTEC for detecting resistance to isoniazid, rifampicin, fluoroquinolones, ethambutol, kanamycin/capreomycin and streptomycin were 98.3% (59/60), 100% (60/60), 91.5%

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(54/59), 72.9% (43/59), 100% (51/51) and 98.0% (50/51), respectively. Regarding the discordant results obtained between AID and BACTEC, the alternative molecular methods performed (GenoType MTBDRplus, GenoType MTBDRsl [Hain Lifescience, Germany] and/or pyrosequencing) confirmed the genotypic result in 90.9% (20/22) of the cases.

Conclusions: AID line probe assay is a useful tool for the rapid detection of drug resistance in clinical samples enabling an initial therapeutic approach. Nevertheless, for a correct management of drug resistant tuberculosis patients, molecular results should be confirmed by a phenotypic method.

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Introduction

In 2012, World Health Organization (WHO) reported 8.6 million new tuberculosis (TB) cases and 1.3 million deaths.1 According to the resistance pattern, multidrug-resistant (MDR) TB is defined as Mycobacterium tuberculosis resistant to isoniazid (INH) and rifampicin (RIF), and extensively drug-resistant (XDR) TB strains are those additionally resistant to fluoroquinolones (FQ) and at least one of the second-line injectable drugs kanamycin (KAN), amikacin (AMK) or capreomycin (CM).2,3 The long time to achieve a diagnosis and the incorrect treatment regimens have led to the emergence and spread of drug-resistant TB.4 In 2012, WHO reported 84,000 confirmed MDR-TB cases worldwide, and 9.6% of these cases were XDR-TB.1

Drug resistance in M. tuberculosis emerges by the stepwise acquisition of genetic mutations in genes coding for drug targets or drug-converting enzymes.5,6 Regarding first-line drug resistance, INH resistant strains may harbor mutations in katG codon 315 and positions –8, –15 and –16 of the inhA promoter.7 Nevertheless, there is a significant percentage of strains that may carry mutations in other still unknown genomic regions. As for RIF, mutations in the 81-bp core region of rpoB have been detected in 95–99% of resistant isolates.7,8 The most common mutations are located in codons 531, 526 and 516.9,10,11 Considering ethambutol (EMB), mutations in embCAB have been detected in resistant strains, being the embB306 the codon most commonly affected.12,13 Finally, streptomycin (STR) resistant strains usually harbor mutations in rpsL codons 43 and 88 or in rrs gene regions 530 and 915.7,14,15

With regard to second-line drugs, most of the FQ-resistant strains harbor mutations in gyrA and gyrB. The most common mutations are located in codons 90, 91 and 94, in the quinolone resistance determining region (QRDR) of gyrA.16,17 Concerning injectable drugs, cross-resistance between KAN, AMK and CM has been reported.18 Mutations in the rrs gene at positions 1401 and 1484 have been associated with resistance to the three drugs, while mutations at 1402 are associated with resistance to KAN and CM.7,19 In addition, resistance to KAN has also been associated with mutations at positions –10, –14 and –37 in the promoter region of eis.20

One of the most evaluated methods aimed to detect resistance to first- and second-line drugs are the line probe assays (LPA).21 This method is based on PCR amplification of specific gene regions associated with drug resistance and the subsequent detection of genetic mutations by hybridization of the PCR product to specific probes immobilized to a nitrocellulose membrane. LPA can be performed not only in clinical isolates, but also directly in clinical samples. In 2008 GenoType MTBDRplus, aimed to detect resistance to INH and RIF, was endorsed by the WHO for use in smear positive samples.22 Later on, GenoType MTBDRsl was developed for detecting resistance to FQ, KAN/AMK/CM and EMB. During the last years, these tests have been the only commercially available LPA for the molecular detection of first- and second-line drug resistance in M. Tuberculosis. Recently, a new commercial LPA named AID TB Resistance (AID Diagnostika, Germany) has been developed.

The present study was undertaken to determine the diagnostic accuracy of AID TB Resistance for the detection of M. tuberculosis and its resistance to INH, RIF, FQ, EMB, KAN, CM and STR directly in clinical samples comparing the results with those obtained by the reference phenotypic method BACTEC 460TB.

Materials and methods

Clinical samples

A total of 65 respiratory clinical samples (62 sputum samples, 1 bronchoalveolar lavage sample, 1 bronchial aspirate sample, 1 gastric fluid sample) from 32 patients were retrospectively selected. The study was approved by the institutional ethics committee. From 10 patients, more than one sample were obtained. All samples had been collected at the time of diagnosis or during a maximum period of 2 months; they were not obtained by split, and therefore, there was no repeat testing of a single sample. Samples were processed as follows. First, they were digested and decontaminated using Kubica’s N-acetyl-L-cysteine NaOH method.23,24 After decontamination, auramine-rhodamine acid-fast staining was performed from the concentrated sediment. Specimens that were positive by fluorochrome staining were confirmed with Ziehl-Neelsen staining. The auramine-rhodamine smears were graded on a scale from 0 to 3+. Three samples were smear negative and 62 were smear positive. A total of 10 samples had an acid fast bacillus count of one to ten per 100 fields (smear 1+), 10 samples had one to nine bacilli per field (smear 2+), and 42 samples had more than nine bacilli per field (smear 3+). The concentrated sediment was suspended in 2 ml sterile phosphate buffer (pH 7.0) and an aliquot was cultured on Lowenstein-Jensen solid and
Drug susceptibility

First- and second-line phenotypic DST was performed at the time of diagnosis with the radiometric method BACTEC 460TB. Critical concentrations for INH, RIF, EMB, STR, moxifloxacin (MOX), KAN and CM were 0.1 μg/ml, 2 μg/ml, 7.5 μg/ml, 6 μg/ml, 0.5 μg/ml, 5 μg/ml and 1.25 μg/ml, respectively. In this study, BACTEC 460TB was considered the gold standard method.

Genotypic drug resistance characterization

Clinical specimens were incubated at 95 °C during 30 min for M. tuberculosis inactivation before DNA extraction. DNA extraction was performed using Maxwell™ 16 Viral Total Nucleic Acid Purification Kit (Promega, USA). Immediately after DNA extraction, AID TB Resistance assay was performed following manufacturer’s instructions. Hybridization and detection were performed with AutoLipa (Innogenetics, Belgium), an automated washing and shaking device. For those cases with unspecific background bands after the hybridization step, DNA was diluted 1:10 and the assay was repeated from the PCR step. This test is a LPA consisting of three modules termed INH/RIF, FQ/EMB and AG. This last module detects mutations related to resistance to the following aminoglycosides (AG): KAN, AMK and STR; and to the cyclic peptide CM. Four control probes (Conjugate control, Amplification control, Mycobacterium genus control and M. tuberculosis complex control) are present in each strip to verify the test procedures. In order to consider a result valid, all four control bands should be present; otherwise, the result is considered invalid. INH/RIF module consists of 14 reaction zones and detects mutations in inhA positions –16, –15 and –8, katG codon 315 and rpoB codons S16, S26 and S31. FQ/EMB module consists of 17 reaction zones and detects mutations in gyrA codons A90V, S91P, D94A, D94N, D94Y and D94G, and embB codons M306V and M306I. AG module consists of 19 reaction zones and detects mutations in rrs positions A1401G, C1402T and G1484 C/T, rrs positions C513T, A514C, G515C and C517T and rpsL codons A43G, A88G and A88C. The presence of all wild-type hybridization bands in combination with the absence of mutation bands indicates that M. tuberculosis is susceptible to the drug considered. The absence of at least one wild-type hybridization band and/or the presence of any mutation bands indicates resistance to the considered drug. In combination with a mutation band in a target gene indicates heteroresistance, a combination of both susceptible and resistant M. tuberculosis. Researchers who read and recorded AID results were blind to the BACTEC 460TB result. Discordant results between AID and BACTEC 460TB were analyzed by GenoType MTBDRsl, GenoType MTBDRplus, GenoType MTBDRsa (Hain Lifescience GmbH, Germany) and/or pyrosequencing. Both commercial assays were performed following the manufacturer’s instructions and pyrosequencing was performed as previously described.²⁶–²⁸ Briefly, the pyrosequencing method consists of a PCR amplification followed by the pyrosequencing reaction. PCR and pyrosequencing primers for rpoB, katG, inhA, gyrA, rrs, and embB were previously described. Mutations detected by pyrosequencing are located in codons 516, 526 and 531 of rpoB, codon 315 of katG, positions –5, –8, –15 and –16 of inhA, codons 80–81 and 88 to 95 of gyrA, positions 1401, 1402, and 1484 of 16S rrs, and codon 306 of embB. Pyrosequencing reaction and data analysis were performed as recommended by the PSQ96MA and SQA software manufacturer (Qiagen GmbH, Hilden, Germany).

Statistical analysis

AID values of sensitivity and specificity, with their corresponding 95% confidence intervals (CI), agreement values and kappa coefficients were calculated considering as reference method BACTEC 460TB. Kappa (k) values below 0.40 indicate weak correlation, values between 0.41–0.60 indicate good agreement and values above 0.60 indicate strong agreement. The commercial statistical software package used was SPSS 15.0 (SPSS Inc, USA).

Results and discussion

Sixty-five clinical samples were tested by the three AID modules. Phenotypic resistance profiles to INH, RIF, FQ, EMB, KAN, CM and STR according to BACTEC are presented in Table 1. Forty-five samples corresponding to 13 patients were MDR, and there were no XDR samples. A valid result was obtained for 60 out of 65 (92.3%), 59 out of 65 (90.8%) and 51 out of 65 (78.5%) samples, considering the three separate modules (INH/RIF, FQ/EMB and AG), respectively. The 25 invalid results corresponded to strips in which conjugate, amplification and Mycobacterium genus controls were present, but M. tuberculosis complex control and bands referring to resistance were missing or extremely faint. As shown in Table 2, the smear result seems to affect the valid result rate, specially for the AG module, since the number of valid results tends to increase when samples are

<table>
<thead>
<tr>
<th>Drug</th>
<th>INH</th>
<th>RIF</th>
<th>EMB</th>
<th>STR</th>
<th>FQ</th>
<th>KAN</th>
<th>CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant (%)</td>
<td>49 (75.4)</td>
<td>45 (69.2)</td>
<td>37 (56.9)</td>
<td>31 (47.7)</td>
<td>7 (10.8)</td>
<td>20 (30.8)</td>
<td>20 (30.8)</td>
</tr>
<tr>
<td>Susceptible (%)</td>
<td>16 (24.6)</td>
<td>20 (30.8)</td>
<td>28 (43.1)</td>
<td>34 (52.3)</td>
<td>58 (86.2)</td>
<td>45 (69.2)</td>
<td>45 (69.2)</td>
</tr>
</tbody>
</table>

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smear 2+ or 3+. Nevertheless, the number of smear negative samples included in this study is low. Previous studies evaluating the performance of GenoType line probe assays directly in clinical samples reported overall rates of valid results that ranged from 78.5% to 100%, including smear positive and smear negative samples. Considering only smear negative samples, this rate ranged from 46.2% to 100%, respectively (Table 4). These data are in concordance with the previously reported in other studies evaluating the performance of GenoType MTBDRplus directly in clinical samples. In this study, none of the molecular methods detected one INH-resistant sample by exploring the most common mutations at katG and inhA. However, mutations in the ahpC-oxyR intergenic region or in kasA have been found in INH resistant isolates. Finally, our sample may harbor a mutation or in other yet unknown genomic regions.

As for RIF, the results between the test and BACTEC 460TB were in complete agreement for all the 43 RIF\textsuperscript{a} and the 17 RIF\textsuperscript{b} samples. Hence, sensitivity and specificity of AID were 100% (Table 4). In a recent meta-analysis Bwanga et al. reported values of 99% of sensitivity and specificity for the detection of resistance to RIF by GenoType MTBDRplus considering both clinical isolates and samples. This high level of agreement between molecular and phenotypic DST is due to the fact that mutations associated with RIF resistance are mainly located in the 81bp-core region of \textit{rpoB}, and mutations outside this region are uncommon.\textsuperscript{38}

### FQ and EMB resistance detection

Concerning FQ, 2 out of 6 phenotypically resistant samples were correctly identified by AID. The remaining 4 samples identified as FQ sensitive by the assay were also identified as FQ\textsuperscript{c} by both GenoType MTBDR\textsuperscript{sl} and pyrosequencing. Fifty-two out of the 53 samples identified as FQS by BACTEC 460TB were identified as sensitive by AID. The remaining 4 samples identified as sensitive by the assay, this result was in agreement with the result obtained by both GenoType MTBDR\textsuperscript{sl} and pyrosequencing. All 14 samples identified as INH\textsuperscript{d} by BACTEC 460TB were correctly identified as sensitive by AID. Sensitivity and specificity values were 97.8% and 100%, respectively (Table 4). These data are in concordance with the previously reported in other studies evaluating the performance of GenoType MTBDRplus directly in clinical samples. In this study, none of the molecular methods detected one INH-resistant sample by exploring the most common mutations at katG and inhA. However, mutations in the ahpC-oxyR intergenic region or in kasA have been found in INH resistant isolates. Finally, our sample may harbor a mutation or in other yet unknown genomic regions.

Concerning EMB, 22 out of 28 phenotypically resistant samples were correctly identified by AID. The remaining samples identified as EMB sensitive by the assay were also identified as EMB\textsuperscript{e} by both GenoType MTBDR\textsuperscript{sl} and pyrosequencing. All 14 samples identified as EMB\textsuperscript{f} by BACTEC 460TB were identified as sensitive by AID. The remaining 4 samples identified as sensitive by the assay, this result was in agreement with the result obtained by both GenoType MTBDR\textsuperscript{sl} and pyrosequencing. All 14 samples identified as INH\textsuperscript{d} by BACTEC 460TB were correctly identified as sensitive by AID. Sensitivity and specificity values were 97.8% and 100%, respectively (Table 4). These data are in concordance with the previously reported in other studies evaluating the performance of GenoType MTBDRplus directly in clinical samples. In this study, none of the molecular methods detected one INH-resistant sample by exploring the most common mutations at katG and inhA. However, mutations in the ahpC-oxyR intergenic region or in kasA have been found in INH resistant isolates. Finally, our sample may harbor a mutation or in other yet unknown genomic regions.

### Table 2

<table>
<thead>
<tr>
<th>Module tested</th>
<th>Smear result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smear 1+ (%)</td>
<td>Smear 2+ (%)</td>
</tr>
<tr>
<td>INH/RIF</td>
<td>8/10 (80.0)</td>
<td>9/10 (90.0)</td>
</tr>
<tr>
<td></td>
<td>1/3 (33.3)</td>
<td>6/10 (60.0)</td>
</tr>
<tr>
<td>FQ/EMB</td>
<td>10/10 (100)</td>
<td>40/42 (95.2)</td>
</tr>
<tr>
<td>AG</td>
<td>6/10 (60.0)</td>
<td>6/10 (60.0)</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Drug and BACTEC result (no. of clinical samples)</th>
<th>AID result</th>
<th>Resistant</th>
<th>Sensitive</th>
<th>Mix R/S</th>
<th>Invalid</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>45 (91.8)</td>
<td>1 (2.1)</td>
<td>3 (6.1)</td>
<td>2 (12.5)</td>
<td>5 (7.7)</td>
</tr>
<tr>
<td>Sensitive</td>
<td>0 (0)</td>
<td>14 (87.5)</td>
<td>2 (12.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45 (69.2)</td>
<td>15 (23.1)</td>
<td>5 (7.7)</td>
<td>2 (12.5)</td>
<td>5 (7.7)</td>
</tr>
<tr>
<td>RIF</td>
<td>43 (95.6)</td>
<td>0 (0)</td>
<td>4 (8.8)</td>
<td>1 (2.1)</td>
<td>5 (7.7)</td>
</tr>
<tr>
<td>Sensitive</td>
<td>0 (0)</td>
<td>17 (85.0)</td>
<td>3 (15.0)</td>
<td>5 (8.6)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>Total</td>
<td>43 (66.2)</td>
<td>17 (26.1)</td>
<td>5 (7.7)</td>
<td>5 (8.6)</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>FQ</td>
<td>2 (28.6)</td>
<td>4 (57.1)</td>
<td>5 (6.4)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>0 (0)</td>
<td>52 (89.7)</td>
<td>1 (1.7)</td>
<td>5 (8.6)</td>
<td>5 (8.6)</td>
</tr>
<tr>
<td>Total</td>
<td>2 (3.1)</td>
<td>56 (86.2)</td>
<td>1 (1.5)</td>
<td>6 (9.2)</td>
<td>5 (8.6)</td>
</tr>
<tr>
<td>EMB</td>
<td>21 (56.8)</td>
<td>14 (37.8)</td>
<td>2 (5.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>2 (7.1)</td>
<td>22 (78.6)</td>
<td>4 (14.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23 (35.4)</td>
<td>36 (55.4)</td>
<td>6 (9.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KAN/CM</td>
<td>17 (85.0)</td>
<td>0 (0)</td>
<td>3 (15.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>0 (0)</td>
<td>34 (75.6)</td>
<td>11 (24.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17 (26.2)</td>
<td>34 (52.3)</td>
<td>14 (21.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* Samples identified as heteroresistant.

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this same test, Feng et al. reported a sensitivity of 87% and a specificity of 97% considering strains and specimens altogether.42 FQR samples/strains not detected by any molecular assays exploring the QRDR of \textit{gyrA} may harbor a mutation in other \textit{gyrA} gene regions or in \textit{gyrB}.16 In addition, although there is cross-resistance within fluoroquinolone drugs, MOX minimum inhibitory concentrations are usually lower than those of other FQ.16 Thus, if a genotypic test indicates FQ resistance whereas the phenotypic DST reveals susceptibility, this discordance might be due to the fluoroquinolone considered and the critical concentration recommended for the DST.

Regarding EMB, 21 out of the 35 phenotypically resistant samples were identified as resistant by the line probe assay. For the remaining 14 samples identified as sensitive, this result was in complete agreement with the results obtained by GenoType MTBDR \textit{sl} and pyrosequencing. Twenty-two out of the 24 samples identified as sensitive by BACTEC 460TB were correctly identified as sensitive by AID. The remaining 2 samples, identified as resistant by the molecular assay, were also detected as resistant by GenoType MTBDR\textit{sl} and pyrosequencing. These two samples harbored the mutation \textit{embB} M306I (codon ATA), that was confirmed by pyrosequencing. In this study, AID assay was 60.0% sensitive and 91.7% specific for the detection of EMB resistance (Table 4). These results are in concordance with those obtained in other studies assessing the yield of GenoType MTBDR\textit{sl} in clinical samples, which reported sensitivities that ranged from 33.3% to 72.2% and specificities that oscillated from 52.6% to 100%.27,32,33,40 GenoType MTBDR\textit{sl} sensitivity and specificity values estimated in a meta-analysis considering strains and specimens together were 68.0% and 80.0%, respectively.42 These low sensitivity values may be due to the presence of mutations in codons other than \textit{embB} 306, that are neither explored by AID nor GenoType MTBDR\textit{sl}. These mutations have been located in other genes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>AID TB resistance</th>
<th>Agreement between AID TB resistance and BACTEC</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity (%) (95% CI)</td>
<td>Specificity (%) (95% CI)</td>
<td>Agreement (%)</td>
</tr>
<tr>
<td>INH</td>
<td>45/46 (97.8) (87.0–99.9)</td>
<td>14/14 (100) (73.2–100)</td>
<td>59/60 (98.3)</td>
</tr>
<tr>
<td>RIF</td>
<td>43/43 (100) (89.8–100)</td>
<td>17/17 (100) (77.1–100)</td>
<td>60/60 (100)</td>
</tr>
<tr>
<td>FQ</td>
<td>2.6 (33.3) (6.0–75.9)</td>
<td>52/53 (98.1) (88.6–99.9)</td>
<td>54/59 (91.5)</td>
</tr>
<tr>
<td>EMB</td>
<td>21/35 (60.0) (42.2–75.6)</td>
<td>22/24 (91.7) (71.5–98.5)</td>
<td>33/59 (72.9)</td>
</tr>
<tr>
<td>KAN/CM</td>
<td>17/17 (100) (77.1–100)</td>
<td>34/34 (100) (87.4–100)</td>
<td>51/51 (100)</td>
</tr>
<tr>
<td>STR</td>
<td>22/22 (100) (81.5–100)</td>
<td>28/29 (96.6) (80.4–99.8)</td>
<td>50/51 (98.0)</td>
</tr>
</tbody>
</table>

CI, confidence interval. SE, standard error.

\(^a\) Invalid results obtained were excluded for these calculations.

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in *embB* codons 319 and 497, and also in *embC* and *embA* genes. On the other hand, several studies reported different specificity values, that have been attributed to the complexity of the phenotypic methods to determine resistance to EMB.32,40

**KAN/CM and STR resistance detection**

All the samples included in our study were resistant to both KAN and CM. The results between AID and BACTEC 460TB were in complete agreement for all the 17 resistant and the 34 sensitive samples. Therefore, AID sensitivity and specificity values to detect both injectable drugs were 100% (Table 4). Feng et al. estimated the sensitivity (44% and 82% for KAN and CM, respectively) and specificity (99% and 97% for KAN and CM, respectively) of GenoType MTBDRsl considering together strains and specimens.42 Sensitivity and specificity values reported differ considerably between studies.27,40,41 This may be due to the presence of cross-resistance between KAN, AMK and CM.18 The presence of A1401G mutation in **rrs** has been detected in isolates resistant to the three injectable drugs, but also in isolates resistant to both KAN and AMK but susceptible to CM.40 That may decrease the specificity of the molecular test used to detect resistance to CM. On the other hand, isolates that are resistant to KAN only, or resistant to both KAN and AMK, may harbor a mutation in **eis** promoter region.40 In these cases, a molecular test that does not explore this region would present a decreased sensitivity for detecting resistance to these drugs.

As for STR, all 22 samples identified as resistant by BACTEC 460TB were correctly identified as resistant by the test. Of the 29 phenotypically sensitive samples, AID identified 28 as sensitive. For the remaining sample, the mutation **rrs** C516T was detected, thus this sample was identified as resistant by the assay. STR resistance was not analyzed by GenoType MTBDRsl or pyrosequencing. Sensitivity and specificity values of AID were 100% and 96.6%, respectively (Table 4). Sensitivity of other molecular methods, such as sequencing, high resolution melting and array, to detect STR resistance in clinical isolates by exploring mutations in **rrs** and **rrs** genes varies between 36.6 and 87.5%, according to the different settings considered.36,45–47 In our study, the frequency of STR resistant isolates with identified mutations is higher than the previously reported in our geographical area.45,48 However, it is of note that in our study patients with STR resistant isolates were born in eastern Europe (5/6) or in India (1/6), where the frequency of STR resistant isolates with detected mutations is 85%.49 The specificity of AID for detecting STR resistance is in agreement with the values reported in other studies evaluating rapid tests.36,45–47

**Overall results**

Agreement values between AID and BACTEC 460TB results according to the drug considered are shown in Table 4. The highest kappa values were obtained in the detection of resistance to INH, RIF, KAN, CM and STR, which were above 0.95, while the lowest values were obtained for FQ and EMB, which were below 0.5.

Altogether, there were a total of 22 discordant results between AID and BACTEC 460TB, considering each drug individually: one for INH, 4 for FQ, 16 for EMB and 1 for STR. These discordant results corresponded to 22 samples obtained from 7 patients. Results obtained with the alternative molecular methods were in agreement with those obtained with AID in all cases, with two exceptions: the sample identified as FQ heteroresistant by the tested line probe assay and the STR-sensitive sample with the **rrs** C516T mutation detected, because STR resistance was not analyzed by GenoType MTBDRsl or pyrosequencing.

**Final considerations**

This study demonstrates the usefulness of AID assay to detect resistance to first- and second-line drugs in clinical specimens. This is a rapid molecular method that allows obtaining results in one working day, from the DNA extraction to the final report of the susceptibility pattern. The other tests used in this study to analyze the samples with discordant results between AID and BACTEC were GenoType MTBDRplus/MTBDRsl and pyrosequencing. These are also rapid methods with a similar turnaround time. In general terms, AID and GenoType are LPA that explore the same genes associated with drug resistance. Nonetheless, GenoType tests include more wild-type probes for **rpoB** and **gyrA** than AID, and the loss of hybridization signal for any of these additional wild-type probes theoretically increases the likelihood to detect drug resistance. On the contrary, AID explores mutations associated with STR resistance that are not included in GenoType tests. On the other hand, pyrosequencing is a more flexible method that allows the analysis of different targets of interest. Furthermore, is possible to investigate by this method other regions associated with drug resistance. Regarding the equipment required and the cost of the three tests presented, LPA reverse hybridization steps can be performed manually or with an automated washing and shaking device, whereas pyrosequencing requires more specific and expensive equipment. Similarly, the current WHO recommended method (Cepheid Xpert MTB/RIF System)50 utilizes expensive cartridge and equipment than AID test, but handling is easier than the multiple steps of LPA.

This is the second study reporting the performance of AID in clinical samples. Ritter et al. recently reported a good agreement between AID and the reference methods (DNA sequencing and/or phenotypical DST) although most of the samples were clinical isolates.23 Smear positive clinical specimens were also tested, with a rate of valid results higher than 95%, and the LPA showed complete agreement with DNA sequencing and phenotypic DST results. Nevertheless, the low number of resistant samples included was a limitation to obtain definite conclusions about the real clinical usefulness of AID line probe assay.

It is of note that the sensitivity of any molecular method depends on the knowledge about the genes and mutations involved in drug resistance and, additionally, on the prevalence of these mutations in each geographical setting. The sensitivity is also subject to the proportion of wild-type
and mutant DNA: molecular methods are able to detect resistance if the ratio of mutant DNA is 10% or more, while phenotypic DST reports resistance if 1% of the bacilli in the population grows in presence of the tested drug.7 Moreover, the possibility of obtaining a valid result with a genotypic method when testing clinical samples further depends on the amount of mycobacteria present, considering that is more difficult to detect DNA in smear negative or smear 1+ samples. In consequence, culture for M. tuberculosis isolation and subsequent phenotypical DST must be performed in order to confirm the molecular results, especially when wild type patterns indicating drug susceptibility are obtained. Finally, new insights on the molecular mechanisms involved in drug resistance are needed, as the sensitivity of molecular methods, especially to detect resistance to FQ and EMB, is usually lower.

In conclusion, AID line probe assay shows a good performance and can be a useful tool to detect resistance to first- and second-line drugs directly in clinical samples in a short turnaround time. The rapid identification of the susceptibility/resistance pattern would facilitate adjusting treatment and consequently improving the clinical management of TB patients.

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