The susceptibility of 211 clinical isolates of *Mycobacterium tuberculosis* complex (201 *M. tuberculosis* and 10 *Mycobacterium bovis* isolates) to pyrazinamide (PZA) was assessed by the nonradiometric Bactec MGIT 960 system (M960). Detection of PZA resistance was followed by a repeat testing using a reduced inoculum (RI) of 0.25 ml instead of 0.5 ml. According to the first M960 analysis, resistance was observed in 55 samples. In the RI assay, 32 samples turned out to be susceptible and 23 proved to be resistant (58.2% false positivity). The Bactec 460 assay confirmed as resistant those strains detected by the RI assay, while discrepant results were found susceptible. Mutation analysis performed on 13 *M. tuberculosis* isolates detected *pncA* mutations in 11 samples. On the basis of our data, we suggest using the RI assay to confirm all PZA resistance results obtained with the standard M960 assay. Further studies are required to confirm our findings.

Pyrazinamide (PZA) is a first-line drug currently being used for the treatment of both drug-susceptible and drug-resistant tuberculosis (TB) (1). PZA (a nicotinamide analog) is a produg that requires conversion into its active moiety, pyrazinonic acid, by the mycobacterial enzyme pyrazinamidase (PZase) in order to be effective against *Mycobacterium tuberculosis* (2, 3). Loss of PZase activity is a common finding in PZA-resistant clinical isolates (4, 5), and mutations in *pncA*, the gene coding for PZase, are now regarded as the major mechanism of PZA resistance (6–8). In addition, the drug is active in an acid medium only, thus making drug susceptibility testing (DST) in the clinical laboratory more demanding (9, 10). Aiming to overcome the problem of frequent uninterpretable results because of poor growth, attempts have been made to set up a slightly acidified (pH 6), supplement-enriched medium able to support bacterial growth as well as to preserve PZA activity (11, 12). In this context, the Bactec 460 radiometric system (Becton, Dickinson and Company, Sparks, MD), currently the “gold standard” method for PZA susceptibility (13), has been withdrawn from the market, and most clinical laboratories have already replaced or soon will replace it with the nonradiometric Bactec MGIT 960 (M960) system (Becton, Dickinson and Company, Sparks, MD). Although both methods utilize an acidified, 7H9-like broth and a modified proportion method with a critical concentration of 100 μg/ml, some recently published papers have reported unprecedented false resistance rates with the M960 system (14–16). Alternatively, PZA susceptibility may also be determined by testing cultured strains for PZase activity or by detecting *pncA* mutations. However, these methods also have their shortcomings: the PZase test lacks sensitivity (resistant strains may be PZase positive) (5), and *pncA* mutations are highly diverse and widely scattered throughout the gene and regulatory region, thus limiting the chances to develop a simple and rapid commercial assay. Moreover, not all PZA-resistant *M. tuberculosis* isolates have mutations in the *pncA* gene (17), and another gene (*rpsA*), coding for ribosomal protein S1, has recently been involved in a newly described mechanism of low-level PZA resistance (18). The aim of the present study was to develop a method able to rule out major errors recently reported with the M960 system when testing the susceptibility of *M. tuberculosis* to PZA.

A total of 211 clinical isolates collected over a 2-year period from January 2010 to December 2011 were studied. Of these isolates, 201 were *M. tuberculosis* and 10 were *Mycobacterium bovis*. Microorganisms were identified to both the complex and species levels by standard procedures (19, 20). Fully susceptible *M. tuberculosis* strain ATCC 27284 and PZA-resistant *M. tuberculosis* strain ATCC 35828 were used as reference strains.

PZA susceptibility testing using the MGIT 960 system was performed according to the manufacturer’s instructions (Becton, Dickinson and Company, Sparks, MD) (21). Briefly, a positive MGIT tube obtained 1 to 2 days after the instrument had recorded a positive signal was used as the test inoculum. The test tube containing 0.1 ml of PZA solution in order to achieve the recommended critical concentration of 100 μg/ml was inoculated with 0.5 ml of the inoculum, while a drug-free control tube was inoculated with the same volume of a 1:10 dilution of the seed suspension. Tubes were placed in the MGIT 960 instrument and monitored until the control tube flagged positive. At that time, the PZA test tube was read as either resistant (≥100 growth units [GU]) or susceptible (<100 GU). PZA susceptibility testing with a reduced inoculum (RI) was performed as described above, except for the inoculum volume, which was reduced in both test and control tubes from 0.5 ml to 0.25 ml.

PZA testing using the radiometric Bactec 460 system was determined according to the instructions provided by the manufacturer (Becton, Dickinson and Company, Sparks, MD). Briefly,
actively growing cultures exhibiting a growth index (GI) of greater than 300 were used as the inoculum source for the test. For each strain, two Bactec PZA medium vials were inoculated, one of which contained PZA (100 µg/ml) and polyoxylethylene stearate (POES) and the other of which was used as a control with POES only. The vials were incubated, and daily GIs were read on the Bactec 460 instrument until the GI of the control vial was ≥200. At that time, a strain was considered to be resistant if the GI of the PZA vial was >11% of the GI for the control vial, susceptible if <9%, and borderline in case of GI values ranging from 9 to 11% (22).

In order to evaluate the extent of PZA resistance, concentrations of 300 and 900 µg/ml were applied to those strains showing resistance to the critical concentration of 100 µg/ml. Moderate resistance was defined as a MIC of 300 µg/ml, while resistance as a MIC of ≥900 µg/ml was considered severe (12).

For amplification and sequencing of the pncA and rpsA genes, bacterial strains were grown on Löwenstein-Jensen slants at 37°C for 3 weeks. Colonies were suspended in 300 µl of distilled water, heated at 95°C for 20 min, incubated in an ultrasonic bath for 15 min, and centrifuged at 13,000 × g for 15 min. Five microliters of supernatants containing genomic DNA was used for PCR. A region of 932 bp containing the pncA gene was amplified in a C 1000 thermal cycler (Bio-Rad, Hercules, CA) using pncA_F3 (5′-AAG GCCGCGATGACCTCTT-3′) and pncA_R4 (5′-GTGTCGTA GAAGCGGCCGAT-3′) primers, with an initial denaturation step for 3 min at 95°C and 30 cycles as follows: 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by a final 5-min step at 72°C (23). A region of 1,530 bp containing the rpsA gene (18) was amplified using forward (5′-GTCCCTACGGCACCACCTG-3′) and reverse (5′-GTCAAGCGGATCGAGCAT-3′) primers under the PCR conditions described above. Automated DNA sequencing was performed on a 3730 DNA analyzer and with 3730 Data Collection v.3 software (Life Technology, Paisley, United Kingdom) using the same primers and sequences aligned to wild-type H37Rv pncA with the ClusterW program.

After the standard M960 analysis, we observed PZA resistance in 55 out of 211 strains (26.1%). However, in a second M960 test which employed the RI procedure, 32 out of the 55 strains proved to be susceptible and resistance could be confirmed in 23 cases (10.9%), indicating a major error rate of 58.2% with the standard M960 test. Radiometric MICs of the presumptive true-positive

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**TABLE 1** Comparison of phenotypic and genotypic methods to detect pyrazinamide resistance in *M. tuberculosis* complex isolates

<table>
<thead>
<tr>
<th>Mycobacterial species</th>
<th>No. of strains</th>
<th>No. of PZA-resistant isolates by:</th>
<th>No. of isolates with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard inoculum</td>
<td>Reduced inoculum</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fully susceptible</td>
<td>169</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td>Non-MDR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>MDR</td>
<td>9</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>211</td>
<td>55</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> MDR, multidrug resistant. One strain failed to grow in MGIT 960 (M960) PZA medium. It was shown to be susceptible when tested with Bactec 460.

<sup>b</sup> ND, not done.

<sup>c</sup> Of the 10 *M. bovis* strains, only one (recovered from a human source) was investigated for pncA mutations.
In addition, we routinely used disposable Pasteur pipettes to seed cultures. Agreement with results obtained with the gold standard method was solved by changing the inoculum rate. Our data show that a simple reduction from 0.5 ml to 0.25 ml allowed a clear separation of results.

To our knowledge, this is the first study in which major errors obtained testing PZA susceptibility with the M960 system have been solved by changing the inoculum rate. We data show that a simple reduction from 0.5 ml to 0.25 ml allowed a clear separation between true- and false-resistant isolates, as demonstrated by full agreement with results obtained with the gold standard method. In addition, we routinely used disposable Pasteur pipettes to seed the inoculum, which was dispensed regardless of any time-wasting procedure, such as leaving the seed MGIT to settle for 15 min and then taking the inoculum from the top of the seed tube instead of deeper down, close to the sediment. In case of severe resistance, our results were also in agreement with pncA mutation analysis, thus suggesting that molecular detection of PZA resistance is going to be the way forward for clinical laboratories assoon as rapid line probe assays are commercially available.

In conclusion, due to the potential for major errors during PZA testing with the M960 assay, laboratories should consider retesting all PZA-resistant isolates to provide accurate and reliable susceptibility results. We suggest that any M. tuberculosis clinical isolate reported as PZA resistant by the standard M960 test should undergo a repeat DST using the RI of 0.25 ml. In case of confirmation, a pncA gene mutation analysis should be performed: otherwise, the isolate should be reported as susceptible.

### References