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 prodrug that requires conversion into its active moiety, pyrazinoic 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(Becton, Dickinson and Company, Sparks, MD), currently the “gold standard” method for PZA susceptibility (13), has been withdrawn from the market, and most clinical laboratories have already replaced or soon will replace it with the nonradiometric Bactec MGIT 960 (M960) system (Becton,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,

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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 polyoxyethylene 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 GCCGCGATGACCTCTCT-3′) and pncA_R4 (5′-GTGTCGTA GAAGCGGCCGATGACACCTCT-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′-GTCAGCCCGATGCCCAGCAT-3′) and reverse (5′-GTCAGCCCGATGCCCAGCAT-3′) primers under the PCR conditions described above. Automated DNA sequencing was performed on a 3730 DNA analyzer and with 3730 Data Collection v3 software (Life Technology, Paisley, United Kingdom) using the same primers and sequences wild-type H37Rv pncA with the ClustalW 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 strains showed that all of these except two exhibited severe resistance (>900 μg/ml), while a screening test applied to about 30% of discrepant isolates (10 out of 32), whose preliminary resistance was not confirmed with the RI assay, was negative (Table 1). Finally, follow-up testing of 14 true-resistant isolates (excluding 9 M. bovis isolates) showed the presence of pncA mutations in 12 of them (Table 2). The remaining two strains (showing a moderate degree of resistance) were further investigated for mutations in the rpsA gene, which did not reveal any nucleotide change upon sequencing analysis.

Three phenotypic methods of PZA susceptibility testing were used in this study, with 211 isolates being tested by the standard M960 method, 55 by the RI assay, and 33 by the radiometric Bactec assay. When the test sensitivity and specificity for the above methods were calculated, assuming the radiometric system as the golden standard, the values were 100% and 100%, respectively, for the RI assay and 100% and 85.4%, respectively, for the standard M960 method. Cumulatively, the identification of PZA resistance within the pncA and rpsA genes exhibited a sensitivity and specificity of 92% and 100%, respectively. The overall accuracy of the RI assay for detecting PZA resistance was 100% (Fig. 1).

The nonradiometric M960 assay was shown to overreport PZA resistance, possibly because of several differences between the inoculum used in the M960 method and that used in the Bactec 460 assay. In fact, due to a different ratio of inoculum to medium, the bacillary concentration is more than 2.5 times greater in the M960 assay than that in the radiometric system (14). Such a large amount of actively growing bacilli may significantly increase the pH of the culture medium, thereby inactivating the effect of PZA (24). Moreover, there is an important variability in the concentration of the inoculum used in the Bactec M960 assay according to the day of test setup. For days 1 and 2 after the culture flags positive, there is no dilution of the MGIT seed vial, but for days 3 to 5, the inoculum becomes very heavy and must be diluted 1:5. However, even after dilution, large clumps are likely to crowd the inoculum, thus exceeding the rate of 10⁶ CFU/ml required for a correct DST. In this context, a repeat testing using a 0.5-ml inoculum is discouraged because, besides poor reproducibility, it is likely to confirm false resistance results in as many as ≈50% of cases (14).

Alternatively, a different approach may lie in reassessing the critical concentration for PZA susceptibility testing. Heifets has

**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>Radiometric MIC of &gt;900 μg/ml</th>
<th>pncA mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M960</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standard inoculum</td>
<td>Reduced inoculum</td>
<td>Bactec 460</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fully susceptible</td>
<td>169</td>
<td>33</td>
<td>5</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>
<td>2</td>
</tr>
<tr>
<td>MDR</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>211</td>
<td>55</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12&lt;sup&gt;c&lt;/sup&gt;</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.
Inoculum (14), which was dispensed regardless of any time-
in addition, we routinely used disposable Pasteur pipettes to seed
agreement with results obtained with the gold standard method.

To our knowledge, this is the first study in which major errors
obtained testing PZA susceptibility with the M960 system have
suggested that a resistance breakpoint of 300 μg/ml may be more
appropriate than 100 μg/ml (9, 12) with the Bactec radiometric
method, and Zhang et al. considered a cutoff of 200 μg/ml closer
to the theoretical MIC at pH 6.0 predicted from the Henderson-
Hasselbach equation (24), while Werngren et al., using the M960
system, proposed to classify clinical isolates as susceptible, inter-
mEDIATE, or resistant according to MIC values of <64 μg/ml, 128
μg/ml, and >128 μg/ml, respectively (25).

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. Our 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 (14), which was dispensed regardless of any time-


testing procedure, such as leaving the seed MGIT to set 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 resis-
tance is going to be the way forward for clinical laboratories as
soon as rapid line probe assays are commercially available (26, 27).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Drug resistance pattern</th>
<th>Radiometric MIC (μg/ml)</th>
<th>pncA mutation</th>
<th>rpsA mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Site</td>
<td>Nucleotide change</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>PZA</td>
<td>300</td>
<td>NAa</td>
<td>None (wild type)</td>
</tr>
<tr>
<td>512151</td>
<td>PZA</td>
<td>900</td>
<td>NAa</td>
<td>None (wild type)</td>
</tr>
<tr>
<td>551184</td>
<td>PZA</td>
<td>&gt;900</td>
<td>152</td>
<td>A→G</td>
</tr>
<tr>
<td>588707</td>
<td>PZA</td>
<td>&gt;900</td>
<td>464</td>
<td>T insertion</td>
</tr>
<tr>
<td>589876</td>
<td>PZA</td>
<td>&gt;900</td>
<td>464</td>
<td>T insertion</td>
</tr>
<tr>
<td>480717</td>
<td>SM, INH, RMP, PZA</td>
<td>&gt;900</td>
<td>61</td>
<td>G insertion</td>
</tr>
<tr>
<td>373592</td>
<td>SM, INH, RMP, EMB, PZA</td>
<td>&gt;900</td>
<td>428</td>
<td>C→G</td>
</tr>
<tr>
<td>530619</td>
<td>SM, INH, RMP, EMB, PZA</td>
<td>&gt;900</td>
<td>287</td>
<td>A→T</td>
</tr>
<tr>
<td>586492</td>
<td>SM, INH, RMP, EMB, PZA</td>
<td>&gt;900</td>
<td>291</td>
<td>T deletion</td>
</tr>
<tr>
<td>589209</td>
<td>SM, INH, RMP, PZA</td>
<td>&gt;900</td>
<td>347</td>
<td>T→C</td>
</tr>
<tr>
<td>589210</td>
<td>SM, INH, RMP, PZA</td>
<td>&gt;900</td>
<td>34</td>
<td>G→A</td>
</tr>
<tr>
<td>589212</td>
<td>INH, PZA</td>
<td>&gt;900</td>
<td>289</td>
<td>G→T</td>
</tr>
<tr>
<td>589216</td>
<td>INH, PZA</td>
<td>&gt;900</td>
<td>289</td>
<td>G→T</td>
</tr>
<tr>
<td>M. bovis 521587</td>
<td>PZA</td>
<td>&gt;900</td>
<td>169</td>
<td>C→G</td>
</tr>
</tbody>
</table>

| a | PZA, pyrazinamide; SM, streptomycin; INH, isoniazid; RMP, rifampin; EMB, ethambutol. |
| b | NA, not applicable. |
| c | ND, not done. |

FIG 1 Diagnostic algorithm for detecting pyrazinamide resistance.

REFERENCES