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# Reevaluation of the Critical Concentration for Drug Susceptibility Testing of *Mycobacterium tuberculosis* against Pyrazinamide Using Wild-Type MIC Distributions and *pncA* Gene Sequencing

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**Pyrazinamide (PZA) is a potent first-line agent for the treatment of tuberculosis (TB) with activity also against a significant part of drug-resistant *Mycobacterium tuberculosis* strains. Since PZA is active only at acid pH, testing for susceptibility to PZA is difficult and insufficiently reproducible. The recommended critical concentration for PZA susceptibility (MIC, 100 mg/liter) used in the Bactec systems (460 and MGIT 960) has not been critically evaluated against wild-type MIC distributions in clinical isolates of *Mycobacterium tuberculosis*. Using the Bactec MGIT 960 system, we determined the PZA MICs for 46 clinical *M. tuberculosis* isolates and compared the results to *pncA* sequencing and previously obtained Bactec 460 data. For consecutive clinical isolates ($n = 15$), the epidemiological wild-type cutoff (ECOFF) for PZA was 64 mg/liter (MIC distribution range, ≤8 to 64 mg/liter), and no *pncA* gene mutations were detected. In strains resistant in both Bactec systems ($n = 18$), the PZA MICs ranged from 256 to ≥1,024 mg/liter. The discordances between *pncA* sequencing, susceptibility results in Bactec 460, and MIC determinations in Bactec MGIT 960 were mainly observed in strains with MICs close to or at the ECOFF. We conclude that in general, wild-type and resistant strains were clearly separated and correlated to *pncA* mutations, although some isolates with MICs close to the ECOFF cause reproducibility problems within and between methods. To solve this issue, we suggest that isolates with MICs of ≤64 mg/liter be classified susceptible, that an intermediary category be introduced at 128 mg/liter, and that strains with MICs of >128 mg/liter be classified resistant.**

The increasing rates of multidrug-resistant (MDR; resistance to at least isoniazid [INH] and rifampin [RIF]) tuberculosis (TB) worldwide make accurate and reproducible drug susceptibility testing (DST) more important than ever. Drug susceptibility testing of the most important first-line anti-TB drugs rifampin and isoniazid usually does not represent any problems in the TB laboratory. *In vitro* testing of pyrazinamide (PZA) is, however, difficult and unreliable since the activity of PZA correlates with the acidity of the culture medium, making the drug most active at a pH of 5.5 and almost inactive at neutral pH (15, 20), at the same time that such a low pH is inhibitory to the *in vitro* growth of *Mycobacterium tuberculosis*. Attempts have been made to overcome the problem, usually by increasing the critical drug concentration as well as using alternative growth-promoting supplements (3, 10, 26). Another crucial factor for the reproducibility of the PZA DST is the size of the test inoculum. Zhang et al. showed that a large inoculum ($10^7$ to $10^8$ bacilli/ml) increased the pH of 5.5 to 7 in the culture medium, thereby inactivating the effect of PZA and producing false resistance results (29).

The 561-nucleotide (nt) *pncA* gene encodes the bacterial enzyme pyrazinamidase (PZase), which converts the prodrug pyrazinoic acid (POA) to PZA. DNA sequencing as well as a recently developed line probe assay, although not commercially available yet, provides an alternative method for rapid detection of *pncA* mutations conferring PZA resistance and has shown a high degree of correlation to the phenotypic DST results (13, 24).

The radiometric Bactec 460 system and the Bactec MGIT 960 system (Becton Dickinson Biosciences, Sparks, MD) are considered reference techniques for rapid testing of drug susceptibility of *M. tuberculosis* to the first-line anti-TB drugs. Both systems utilize an acidified culture medium at pH 6.0 and modified test protocols adapted for PZA DST. Comparisons of PZA susceptibility testing with the two techniques have been made, with inconsistent outcomes (5, 14, 18), and, unfortunately, many clinical TB laboratories do not perform DST to PZA due to the technical difficulties and poor reproducibility (5, 6, 11, 29). Consequently, PZA is not included in the WHO-coordinated quality assurance program for proficiency testing, whose task is, apart from offering external quality assurance, to evaluate the interlaboratory DST reproducibility of first and key second-line anti-TB agents.

One obvious reason for the poor reproducibility and also the disagreements between the two Bactec systems may be the critical concentration itself, which may result in discrepancies for isolates with a PZA MIC close to the critical concentration because of the normal method variation of 1 MIC dilution step. The definition of the critical concentration is the lowest concentration of drug that will inhibit 95% (90% for pyrazinamide) of wild-type strains of *M. tuberculosis* that have never been exposed to drugs (28) while at the same time not inhibiting clinical strains of *M. tuberculosis* that are considered to be resistant (e.g., from patients who are not responding to therapy). Apart from automatically defining up to

Received 11 October 2011 Returned for modification 2 November 2011
Accepted 21 December 2011
Published ahead of print 27 December 2011
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doi:10.1128/AAC.05894-11

10% of the wild-type strains resistant, this definition likely introduces reproducibility problems since the critical concentration will cut the upper range of the MIC distribution of the wild-type population.

Considering these shortcomings, there is a clear need to reassess the critical concentration for DST of PZA against *M. tuberculosis*. Current standards (www.eucast.org/definitions) for setting susceptibility testing breakpoints require the definition of the so-called wild-type MIC distribution, i.e., the normal (Gaussian) distribution that is formed by the MICs when consecutive bacterial strains coming from treatment-naïve patients are tested against serial 2-fold antibiotic dilutions. Strains with MICs inside the wild-type distribution by definition lack phenotypically detectable acquired mutational resistance mechanisms (www.eucast.org/definitions), and the highest MIC within the wild type has been labeled the epidemiological breakpoint or the epidemiological cutoff (ECOFF). However, the ECOFF is not always the same as the breakpoint defining clinically relevant susceptibility. To define a clinical breakpoint, the ECOFF is related to pharmacokinetic and pharmacodynamic (PK/PD) and clinical outcome data in order to set clinical breakpoints according to the well-known SIR (susceptible, intermediary, resistant) system. However, in the case of tuberculosis, PK/PD and clinical outcome data for individual drugs are difficult to obtain since patients are given multidrug regimens. For this reason, the determination of clinical breakpoints will have to rely mainly on wild-type MIC distributions.

We have earlier reported wild-type MIC distributions for the other first-line drugs (22), the second-line aminoglycosides, cyclic peptides, and fluoroquinolones (1, 12), as well as for seven other per oral second-line anti-TB agents (21). The scientific basis for defining critical breakpoints for *M. tuberculosis* in terms of MIC distributions has been weak, and we showed that even though most of the current critical concentrations were correctly defined in relation to the ECOFF, some were likely to be erroneous, potentially leading to poor reproducibility and suboptimal treatment. Since PZA is a potent first-line anti-TB agent in the effective treatment of drug-susceptible (S) as well as about 50% of MDR TB strains (4), it is crucial to test the activity of this drug at a relevant and well-defined susceptibility breakpoint concentration.

Thus, the objective of this study was to critically reevaluate the critical concentration used in the Bactec 960 MGIT system by establishing the wild-type MIC distribution and ECOFF for PZA and comparing the results to those of *pncA* sequencing.

## MATERIALS AND METHODS

**Strains.** From the Karolinska University Hospital, 15 fully drug-susceptible consecutive clinical *M. tuberculosis* isolates were used as the wild-type sample. Additionally, 31 clinical *M. tuberculosis* isolates were selected from the national strain collection at the Swedish Institute for Communicable Disease Control (SMI). Of these, 21 had been determined to be resistant (R) and 10 susceptible to 100 mg/liter PZA using the Bactec 460 system kit (Becton Dickinson Biosciences, Sparks, MD). All 46 strains had been tested with Bactec 460 for susceptibility to the first-line drugs INH, RIF, ethambutol (EMB), and streptomycin (SM), following the test instructions of the manufacturer. Full Bactec 460 DST data are shown in Table 1.

**MIC determinations with Bactec MGIT 960.** The determination of PZA MICs was performed with the Bactec MGIT 960 system using the recommended standard protocol provided by the manufacturer (Becton Dickinson Biosciences, Sparks, MD). The fully drug-susceptible *M. tuberculosis* H37Rv reference strain was included as a control for the MIC determinations in all separate test runs in the Bactec MGIT 960 system.

The DST inoculum was prepared from bacterial growth on Löwenstein-Jensen egg medium that was not older than 3 weeks. Briefly, two 1-$\mu$l loops of bacteria were suspended in 3 ml of phosphate-buffered saline (PBS) in a small glass tube with glass beads. Homogenization of the bacterial suspension was made using vortex or an ultrasound water bath to disperse clumps. Thereafter, the suspension was left to sediment for 20 min and the upper 2 ml was transferred to a new tube and left to sediment for another 15 min.

Prior to inoculation of the Bactec MGIT culture tube, the bacterial suspension was adjusted to a McFarland turbidity of 0.5 and diluted in PBS according to the PZA test protocol from the manufacturer.

To define the MIC for all strains, stock solutions (672 to 21,504 mg/liter) of PZA (Sigma-Aldrich, Germany) were made in Bactec MGIT 960 PZA test medium (pH 6.0) and heated by surrounding the container with hot tap water to be completely dissolved. By adding 100 $\mu$l of the stock solutions to the MGIT culture tube, test concentrations ranging from 8 to 256 mg/liter were achieved. Furthermore, 200 $\mu$l and 400 $\mu$l (for final volume adjustment for 400 $\mu$l, 300 $\mu$l of the culture medium was removed from the MGIT tube before adding the drug solution) of the 21,504-mg/liter stock solution were added to receive test concentrations of 512 and 1,024 mg/liter PZA, respectively. The critical test concentration of 100 mg/liter provided by the Becton Dickinson PZA kit was also included for all strains.

The MIC determinations were performed in the Bactec MGIT system for the 46 strains, and the test concentrations ranged from 8 to 100 mg/liter or 100 to 1,024 mg/liter, depending on whether the strains had previously been defined to be sensitive or resistant to PZA. To evaluate the reproducibility of the MGIT PZA MIC test, the drug-susceptible *M. tuberculosis* H37Rv (ATCC 25618 and CCUG 37357) reference strain was included and tested for susceptibility to 8 to 100 mg/liter PZA in 13 separate runs.

The MIC was determined to be the lowest concentration to which the growth value (GU) of the PZA-containing culture tube was $<$100 when the 1:10-diluted drug-free control had reached a GU of 400.

**Pyrazinamidase test.** To analyze the PZase activity in three strains with *pncA* mutations and PZA MICs of $\leq$128 mg/liter as well as one PZA-resistant strain with no *pncA* gene mutation, we followed a modified PZase test protocol of the classical Wayne's test (27) described by Singh et al. (25). The fully drug-susceptible *M. tuberculosis* H37Rv (ATCC 25618) strain was included as a PZase-positive control, and a clinical isolate of *M. bovis* (BTB11-396) and a highly PZA-resistant clinical isolate of *M. tuberculosis* (BTB00-042; MIC, $>$1,024 mg/liter, with a His51Gln mutation in *pncA*) were used as PZase-negative controls. Briefly, one full loop of bacterial growth was inoculated on the surface of two PZase agar medium tubes and incubated at 37°C for 4 and 7 days, respectively. After incubation, 1 milliliter of freshly prepared 1% ferrous ammonium sulfate (Sigma-Aldrich, Germany) was added to one of the PZase agar tubes, which were observed over 4 h for the appearance of a pink band (PZase positivity) in the subsurface agar. For strains with negative results at day 4, the procedure was repeated on the 7-day tube.

**Sequencing of *pncA*.** According to Jureen et al. (13), the 561-nt *pncA* gene, along with surplus regions of approximately 200 nt up- and downstream of the gene, was sequenced using the pncA_F3 (AAGGCCGCGA TGACACCTCT) and pncA_R4 (GTGTCGTAGAAGCGGCCGAT) primers. These primers were used in a standard PCR to give a template for the subsequent sequencing reactions. The pncA_F3 and pncA_R4 primers, as well as the P3-F (ATCAGCGACTACCTGGCCGA) and P4-R (GA TTGCCGACGTGTCCAGAC) primers, were used to subdivide the PCR fragment into two overlapping bidirectional sequencing reaction fragments. The sequencing reactions were performed using a BigDye Terminator cycle sequencing kit and a 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, CA). Retrieved sequences were then analyzed with the (ClustalW) vector NTI Advance (version 9) software (InfoMax, Inc.) using the wild-type H37Rv strain's *pncA* gene (Rv2043c) as the master sequence.

**TABLE 1** Pyrazinamide MICs in Bactec MGIT 960 system correlated to *pncA* gene sequencing data and previously obtained Bactec 460 results for 46 *M. tuberculosis* strains

| Strain group | MGIT PZA MIC (mg/liter) | No. of strains | *pncA* amino acid change | Previous BACTEC 460 result[c] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | PZA | INH | RIF | EMB | SM |
| Consecutive fully susceptible strains | ≤8 | 1 | None | S | S | S | S | S |
| | 16 | 4 | None | S | S | S | S | S |
| | 32 | 8 | None | S | S | S | S | S |
| | 64 | 2 | None | S | S | S | S | S |
| Nonconsecutive strains with MICs less than or equal to ECOFF (≤64 mg/liter) | 16 | 1 | None | S | S | S | S | S |
| | 32 | 2 | None | S | S | S | S | S |
| | 32 | 1 | None | S | S | R | S | S |
| | 32 | 1[a] | Ser65Ser | S | R | R | S | S |
| | 64 | 3 | None | S | S | S | S | S |
| | 64 | 1 | None | R | R | S | S | R |
| | 64 | 1 | None | S | R | S | S | S |
| | 64 | 1[a] | Thr47Ala | R | R | R | R | R |
| Nonconsecutive strains with MICs greater than ECOFF (>64 mg/liter) | 128 | 1[a] | None | S | S | S | S | S |
| | 128 | 1[b] | Phe58Leu | R | R | R | S | S |
| | 256 | 1[a] | None | R | R | R | R | R |
| | 256 | 1 | Ser65Ser/frameshift | R | R | R | S | S |
| | 256 | 1 | Ile6Thr | R | R | R | R | S |
| | 256 | 1 | Val125Phe | R | R | R | S | S |
| | 512 | 1 | Pro54Leu | R | R | R | S | S |
| | 512 | 1 | Phe58Leu | R | R | R | S | R |
| | 1,024 | 1 | Gly132Ala | R | R | R | R | R |
| | 1,024 | 1 | Leu172Pro | R | R | R | R | S |
| | 1,024 | 1 | Val155Gly | R | R | R | S | R |
| | >1,024 | 1 | Val155Gly | R | R | R | S | S |
| | >1,024 | 1 | Met175Thr | R | R | R | R | R |
| | >1,024 | 1 | Pro54Ser | R | R | R | R | S |
| | >1,024 | 1 | Gly132Ser | R | R | R | R | S |
| | >1,024 | 1 | Frameshift | R | R | R | S | R |
| | >1,024 | 1[b] | His51Gln | R | S | S | S | S |
| | >1,024 | 1 | Arg123Pro | R | S | S | S | S |
| | >1,024 | 1 | 4-amino-acid in-frame insertion | R | R | R | S | S |
| | >1,024 | 1 | Thr142Lys | R | R | R | R | S |

[a] PZase positive.
[b] PZase negative.
[c] INH, isoniazid (0.1 mg/liter); RIF, rifampin (1 mg/liter); EMB, ethambutol (2.5 mg/liter); SM, streptomycin (1 mg/liter); PZA, pyrazinamide (100 mg/liter); R, resistant; S, susceptible.

## RESULTS AND DISCUSSION

**MIC determinations were reproducible.** The reproducibility of the Bactec MGIT 960 PZA test was investigated by analyzing the drug-susceptible *M. tuberculosis* reference strain H37Rv in all 13 separate rounds of MIC determinations. The MIC of H37Rv strain ATCC 25618 showed a variation of 1 MIC step at the most (32 to 64 mg/liter, $n = 6$), whereas the MIC of H37Rv strain CCUG 37357 ranged from 16 to 32 mg/liter ($n = 6$), and was determined to be 8 mg/liter in 1 of 7 tests. To ensure a proper quality control during MIC determinations for PZA, the inclusion of an internal quality control which should have an MIC within predefined limits is highly recommended.

**MICs of consecutive strains formed a normal distribution with a tentative ECOFF for S of ≤64 mg/liter.** The MIC distribution of the 15 consecutive strains which were susceptible against the first- and second-line drugs (22) (Table 1; Fig. 1) using the Bactec 960 MGIT system ranged from ≤8 to 64 mg/liter, suggesting a tentative ECOFF of susceptibility of ≤64 mg/liter. All consecutive strains lacked *pncA* mutations and showed a narrow, Gaussian MIC distribution. However, quality-controlled MIC wild-type data from other laboratories are necessary, according to the guidelines of the EUCAST, in order to make a final definition of the ECOFF.

**MICs less than or equal to or greater than the ECOFF correlated well to the absence or presence of mutations in the *pncA* gene.** Among a total of 20 strains with MICs above the ECOFF (MICs > 64 mg/liter), all strains had mutations in the *pncA* gene (Table 1). As reported in previous studies, mutations were distributed along the whole *pncA* gene (2, 13, 17, 19, 23). Although this study tested a limited number of PZA-resistant strains, it was obvious that a variety of *pncA* mutations rather than one specific mutation conferred high-level resistance to PZA (Table 1). In contrast, *pncA* mutations were lacking in 24/26 strains with MICs equal to or below the ECOFF (MICs ≤ 64), including the 15 consecutive fully susceptible strains. One strain with an MGIT MIC of 32 mg/liter had a silent mutation (Ser65Ser). Another strain pre-

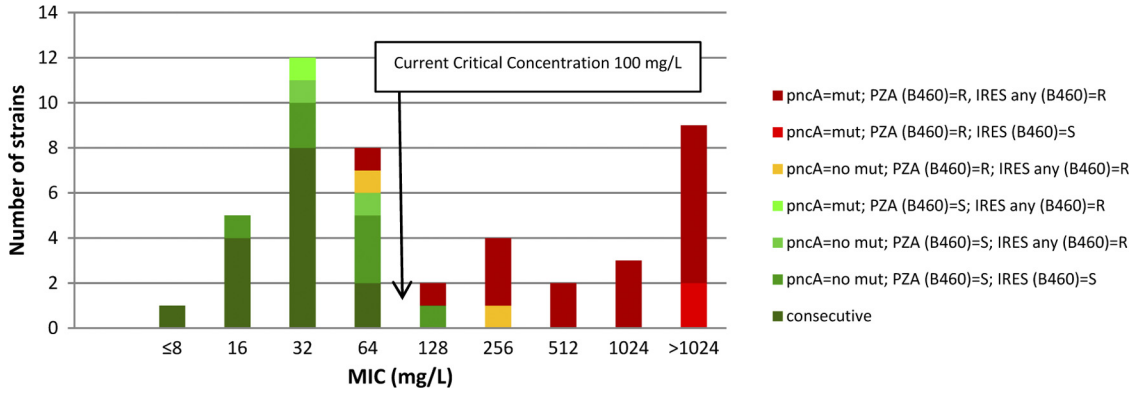


**FIG 1** MIC distribution of PZA determined by Bactec MGIT 960 for 46 *M. tuberculosis* strains compared to the presence or absence of *pncA* mutations (mut) and to previously obtained Bactec 460 (B460) susceptibility results. IRES stands for isoniazid, rifampin, ethambutol, streptomycin. One isolate with an MIC of 32 mg/liter had a silent *pncA* mutation.

viously defined to be resistant to first-line drugs, including PZA, with an MIC of 64 mg/liter, had a Thr47Ala mutation. Both of these strains showed a positive PZase test, indicating that these *pncA* mutations are silent with regard to PZase production. Interestingly, the Thr47Ala strain was of the *M. tuberculosis* Beijing genotype, for which this specific mutation has been suggested to confer PZA resistance below the critical breakpoint concentration, although in the upper range of the wild-type distribution, with variable results between 50 and 100 mg/liter (7, 16). The clinical relevance of such mutations in PZase-positive isolates with MICs belonging to the wild-type distribution remains to be shown in clinical studies but is likely to be low. One strain had a PZA MIC of 256 mg/liter but no *pncA* mutation and a positive PZase test, confirming that although most PZA-resistant strains show mutations in *pncA*, there are also other, unknown resistance mechanisms (4). This has been summarized in a recent meta-analysis, where the sensitivity of *pncA* sequencing was close to 90% (4). Yet another strain with a Phe58Leu mutation had an MGIT MIC of 128 mg/liter on two occasions (and was also susceptible to 100 mg/liter). This strain had previously been defined to be resistant to PZA with the Bactec 460 system and also showed a PZase-negative test. Hence, although there is doubtless a correlation between mutations in *pncA* and phenotypic DST, there seems to be a reproducibility problem around the ECOFF. In these cases, the introduction of an intermediary (I) category may help to compensate for methodological problems.

**Discrepancies between Bactec 460 and Bactec 960 MGIT DST results may be explained by a critical concentration near the wild-type MIC distribution.** Using the current critical concentration (100 mg/liter) to distinguish between susceptible and resistant strains, the agreement between the methods was 100% for MICs of ≤32 mg/liter ($n = 20$) and in strains with MICs of >128 mg/liter ($n = 18$) (Table 1; Fig. 1). Overall, the discrepancy in the classification of S and R between Bactec 960 MGIT and Bactec 460 was 6.5% (3/46). There were three resistant strains in Bactec 460 which were susceptible in Bactec 960 MGIT at the current critical concentration. All these isolates had MICs at or very close to the current critical concentration (64 to 100 mg/liter). One of these isolates had no mutation in the *pncA* gene, whereas the other two had Thr47Ala and Phe58Leu mutations, respectively. Thus, since all strains with disagreements had MICs close to the current breakpoint (100 mg/liter), it indicates that it is highly likely that the poor reproducibility is caused by the close relationship between the breakpoint and the wild-type distribution. This is further illustrated by the data from a study comparing the performance between Bactec 460 and Bactec 960 MGIT, showing a disagreement in 24 out of 743 strains, where Bactec 960 MGIT showed resistance, whereas Bactec 460 indicated susceptibility. None of these 24 strains had mutations in the *pncA* gene (5). Upon retesting, MGIT showed that 14 of the 24 isolates were susceptible, indicating that the isolates had an MIC close to the breakpoint, and the conclusion was that Bactec 460 should be the reference assay without any further discussion of the problems of the breakpoint itself. However, it is well-known that not all PZA-resistant isolates have resistance mutations in *pncA* (19). We have recently described a similar problem with the critical concentration for ethambutol (22), where there is an overlap between the wild-type and resistant strains. Thus, the discrepancies between laboratories for DST of PZA and ethambutol are most likely due to a poor definition of the breakpoint in relation to the wild-type MIC distribution rather than any of the methods being more "gold standard" or some laboratories being superior to the other. There is a well-known laboratory variation of 1 MIC dilution step which may explain the discrepancies for both ethambutol and PZA. This argument is also supported by a recent meta-analysis, which concludes that there were no differences in test performances between the Bactec systems for PZA (4).

**Reevaluation of the pyrazinamide breakpoint using wild-type MIC distributions related to PK/PD data.** To our knowledge, there are no studies to confirm the critical concentration for PZA against clinical outcome, but the definition, rather, is based on the fact that the concentration for 90% of the wild-type strains should be below the critical concentration which will make 10% of wild-type strains resistant and cause obvious reproducibility problems. This could be avoided by introducing an intermediary category at an MIC of 128 mg/liter, with an MIC of ≤64 mg/liter used to define susceptible isolates and an MIC of >128 mg/liter used to define resistant isolates. Our suggestion to increase the cutoff for resistance to >128 mg/liter is supported by other groups, such as Heifets et al. (9) and Zhang et al. (29), which also suggest a higher cutoff for resistance at 200 to 300 mg/liter, although they used Bactec 460. Our system does not support increasing the cutoff point to MIC levels higher than 128 mg/liter in the MGIT system, at least not without support from clinical out-

come studies. Considering PK/PD simulations derived from mouse models, it has been suggested that the doses used today could not reach a treatment efficacy over 50 mg/liter (8). This is confirmed by pharmacokinetic studies in humans, where a serum concentration of 41 mg/liter was reached with a daily dosing strategy of 1.5 g, whereas 66.1 mg/liter was reached by doses of 3 g in adults (30). In fact, the target area under the concentration-time curve/MIC for the currently used doses of 15 to 30 mg/liter could be reached only for 15 to 53% of adults in Monte Carlo simulations, and the authors suggest considering an increase in the dose to 60 mg/liter, although there are concerns of liver toxicity in the elderly population (8).

**Conclusions.** By the use of MIC determinations in the MGIT system, we could define a tentative wild-type MIC distribution for PZA at ≤64 mg/liter in strains without *pncA* mutations. Provided that other investigators can confirm our findings, testing of PZA in MGIT should first be done at 64 mg/liter. This could be performed by diluting the commercially available stock solution. If a strain is susceptible to that concentration, no further testing is necessary. Then, in order to account for methodological variations, we suggest that strains resistant to 64 mg/liter should be tested for resistance 128 mg/liter and that an intermediary (I) category be used if the isolate is resistant to 64 mg/liter and susceptible to 128 mg/liter. Consequently, resistance is defined as an MIC of >128 mg/liter. However, to keep the time of testing to a minimum, strains could preferably be screened at both 64 and 128 mg/liter simultaneously to report the PZA result as S or R only when the same results are seen for both concentrations. This strategy is likely to increase the accuracy and the reproducibility of PZA testing between laboratories.

## ACKNOWLEDGMENTS

This work was partly funded by EC project FP7-HEALTH-2007-B-223681 (TB PAN-NET) and the research council of South East Sweden (FORSS).

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