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Identification of a *Mycobacterium tuberculosis* Strain with Stable, Low-Level Resistance to Isoniazid

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We have identified a potential quality control strain of *Mycobacterium tuberculosis* to monitor isoniazid susceptibility testing. This strain (strain A) has a stable phenotypic low-level resistance to isoniazid, has a mutation of C (−15) → T in the *inhA* promoter region, and gave consistent susceptibility test results in 141 laboratories.

Among the primary drugs used to perform susceptibility testing of *Mycobacterium tuberculosis*, isoniazid (INH) is often used in two concentrations, frequently showing low-level resistance (to 0.1 or 0.2 μg/ml) and being fully susceptible to higher INH concentrations. A low-level resistance quality control (QC) strain for monitoring the accuracy of susceptibility testing, especially with low INH concentrations, has not yet been identified (6). The available highly resistant strain H37Rv is not recommended as a QC strain, since it is unable to detect efficiency of low-level resistance and does not provide information to discriminate the quality of test media, drug strength, or technical performance in the testing process. It is also believed that low-level INH resistance is often not stable during long-term storage, subculturing, and freezing-thawing of the cultures. This study was conducted to identify a potential QC strain with stable low-level resistance to INH which is stable on several subculturing and freezing cycles.

Three low-level INH-monoresistant *M. tuberculosis* clinical isolates (A, B, and C) were selected from the CDC *M. tuberculosis* Drug Susceptibility Testing Performance Evaluation Program (PEP) and subcultured on Middlebrook 7H10 agar to obtain isolated colonies. To purify one low-level INH-resistant isolate from each subculture, the selected colonies were tested for MICs with twofold dilutions of INH, starting with 0.05 μg/ml, in 7H12 BACTEC broth and by agar dilution method. The selected low-level INH-resistant subcultures from each strain were inoculated into Middlebrook 7H9 broth and then subcultured on Lowenstein-Jensen slants. These cultures were sent to two laboratories for confirmation by MIC testing using agar dilution and broth dilution (7H12 BACTEC broth) methods (1, 2). The MICs for the three strains ranged from 0.1 to 0.4 μg/ml in BACTEC 7H12 broth and from 0.2 to 0.8 μg/ml by the agar dilution method. The MIC for the susceptible control strain H37Rv tested at the same time was 0.1 μg/ml, and the MIC for the resistant control strain ATCC 35822 was ≥1.0 μg/ml by the agar dilution method (7H10 Middlebrook agar).

Strains A, B, C, and D (a fully susceptible strain) were sequenced for detecting genes associated with INH resistance. Three laboratories performed studies on the stability of low-level INH resistance with all four cultures. Susceptibility testing was performed using a bacterial suspension made from a freshly grown culture. The bacterial suspension was then frozen at −70 ± 10°C, thawed a few days later, subcultured again, and tested for susceptibility to INH. This cycle of freezing, thawing, subculturing, and testing was repeated three times for each culture at each of the three sites. Routine BACTEC 460TB susceptibility testing (S. Siddiqi, BACTEC 460TB system product and procedure manual, Becton Dickinson Diagnostic Instrument Systems, Inc., Sparks, Md.) at concentrations of 0.1 and 0.4 μg of INH/ml was performed after each cycle. Additionally, one laboratory also performed susceptibility testing to 0.2 and 1.0 μg of INH/ml by the agar proportion (AP) method (2, 4). Strain D (the susceptible control) was tested concurrently with the resistant strains. Strains A, B, and C and a susceptible control D strain were sent to 141 laboratories under the CDC-PEP (shipment 2002-06).

The results we obtained indicated that the MICs of INH for the three strains ranged from ≤0.1 to 0.4 μg/ml when tested in the BACTEC 7H12 broth and from 0.2 to 0.8 μg/ml by the agar dilution method. The MIC for strain D, the susceptible control, was 0.1 μg/ml. All three laboratories reported stable results of resistance for strains A, B, and C to 0.1 μg of INH/ml in BACTEC 460TB broth through three cycles of freezing, thawing, and subculturing. However, strains B and C were unstable after the freeze-thaw-subculture cycles to the higher concentrations of INH, as determined by the BACTEC 460TB and AP methods. Only strain A was consistently susceptible to the higher INH concentrations by both the BACTEC 460TB and AP methods. Control strain H37Rv was stable through the freeze-thaw-subculture cycles with both low and high concen-

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TABLE 1. DNA sequence results for *M. tuberculosis* low-level INH-resistant strains[a]

Strain	*inhA* promoter	*inhA* ORF	*katG* codon 315 region
A	C (−15) → T	WT	WT
B	WT	WT	Ser 302 → Arg (AGC → CGC)
C	WT	WT	WT
D	WT	WT	WT

[a] Abbreviations: ORF, open reading frame; WT, wild type.

trations of INH. In some instances, growth indices in both control and INH test vials were lower than expected in BACTEC 460TB, which was possibly due to the effect of repeated freeze-thaw-subculture cycles.

Testing in 141 laboratories under the CDC-PEP showed that 99% of the laboratories detected low-level INH resistance in strain A by the BACTEC 460TB method, while only 75 and 73% of laboratories detected this level of resistance in strains B and C, respectively. All laboratories reported control strain D as susceptible to the low INH concentration by both methods. Strain A has a C (−15) → T mutation in the *inhA* promoter (5), while strain B has a Ser 302 → Arg mutation, which has not been reported previously (3, 7). INH resistance mutations were not detected in strain C (Table 1).

A critical element for ensuring the accuracy of susceptibility testing of *M. tuberculosis* is selecting a reference strain that is genetically stable and for which the MIC is within the range of drug concentrations frequently used in laboratories. A pan-susceptible strain, *M. tuberculosis* H37Rv, is now the single QC strain used for susceptibility testing by most U.S. laboratories. For QC, some laboratories also use mono- or multiresistant mutants of H37Rv or clinical isolates, but these strains are usually resistant to very high drug concentrations and are not suitable for QC purposes (6). Strain A has a C (−15) → T mutation in the *inhA* promoter, which is a well-established mechanism of low-level INH resistance (5). On the other hand, variable results for strains B and C were revealed by the participants in the CDC-PEP study.

Strain A may be used to monitor detection of low-level INH resistance in *M. tuberculosis* susceptibility testing procedures and for QC of new lots of INH or the test medium. Strain A is available from the American Type Culture Collection as *M. tuberculosis* ATCC BAA-812. It should be stored by preparing a homogenous suspension from a freshly grown culture on solid culture medium (i.e., Lowenstein-Jensen or Middlebrook), adjusted to the optical density of a McFarland no.1 standard, and frozen in small (1.0- to 1.5-ml) aliquots at −70°C (4). As with other QC strains, refreezing after thawing is not recommended (BACTEC 460TB system product and procedure manual, Becton Dickinson Diagnostic Instrument Systems, Inc.).

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