Drug susceptibility tests (DSTs) for *Mycobacterium tuberculosis* require at least 7 days of incubation. Drugs that are unstable at 37°C, such as ertapenem, are likely to be degraded before killing or inhibiting slow-growing bacteria. This would alter the MICs of these drugs, including ertapenem, leading to falsely high MICs. Here, we describe a new strategy we developed to perform DSTs and measure MICs for such unstable compounds.

Ertapenem, a β-lactam agent of the carbapenem class, has shown promising clinical results and favorable pharmacokinetics against *Mycobacterium tuberculosis* (1, 2). The scourge of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), a global problem, has increased the urgency for the use of carbapenems, such as ertapenem, meropenem, and faropenem (2–4). Recently, the first phase 2 study (NCT02349841) to evaluate early bactericidal activity of ertapenem on storage following reconstitution in Middlebrook 7H9 broth to the desired temperature (5). Degradation of ertapenem on storage following reconstitution and dilution is temperature dependent, and the proposed in-use shelf life is 6 h at room temperature or 24 h at 2°C to 8°C (6). *M. tuberculosis* has a doubling time of at least 24 h under the best of circumstances (7, 8). *M. tuberculosis* cell division is particularly slow; FtsZ, a protein responsible for initiating cell division and recruiting proteins for formation of new cell walls, is known to have a polymerization rate that is at least 20 times slower in *M. tuberculosis* than in *Escherichia coli*, for example (9). In *M. tuberculosis* at low pH, the replication rate is up to 10 to 20 times slower; kill of such semidormant bacteria is defined as a sterilizing effect (7, 8, 10). Thus, microbial killing and inhibition of growth by the most effective of antibiotics are slow and take place over several days, especially by β-lactams that depend on cell wall turnover.

Drug susceptibility tests (DSTs) for *M. tuberculosis* using Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) approved methods require at least 7 days of incubation (11). Drugs such as ertapenem, already appearing unstable at 37°C (2), are likely to be degraded before killing or inhibiting slow-growing bacteria, especially semidormant *M. tuberculosis*. This would be expected to alter the MICs of ertapenem, leading to falsely high MICs and false resistance. Here, we saw the rapid decline of ertapenem during DSTs; therefore, we developed a new strategy to perform DSTs and measure MICs for such unstable compounds.

Ertapenem (Sigma) was first dissolved in purified water and subsequently diluted in Middlebrook 7H9 broth to the desired drug concentrations of 5.0 and 50 mg/liter, respectively. The two solutions were incubated at 37°C. After 0, 5, 8, 24, 32, and 48 h, three samples were collected from each solution and immediately stored at −80°C until further analysis. All samples were then fully thawed at room temperature and analyzed in duplicate using fully validated assays (12). The calibration curve of ertapenem was linear over a range of 0.1 to 125 mg/liter, and the correlation coefficient was 0.999. The coefficients of variation between the replicates for each concentration at each time point were 2.7% to 11.2%. Figure 1 shows the decrease in ertapenem concentration in the solution at 37°C. After 5 h of incubation, ertapenem concentration was reduced by 45.3% and 40.7% in comparison with the initial concentrations of 5 and 50 mg/liter, respectively. After 48 h, the concentrations were 20.1% and 26.8% of the time-zero concentrations.

*M. tuberculosis* H37Ra (ATCC 25177) was used in the MIC and dose-response experiments. For each experiment, one stock vial was thawed and bacteria grown to a logarithmic growth phase (log-phase growth) in Middlebrook 7H9 broth enriched with 10% oleic acid-albumin-dextrose-catalase for 4 days at 37°C under shaking conditions and 5% CO2. For semidormant bacteria under acidic conditions, the day 4 culture was inoculated in Middlebrook 7H9 acidified to a pH of 5.8 by means of citric acid, as described previously (7). Sterilizing effect MICs were identified using the broth dilution and the resazurin colorimetric assay (11, 13). *M. tuberculosis* in acidified Middlebrook 7H9 broth was exposed to the following ertapenem concentrations, in triplicate: 0, 0.075, 0.15, 0.3, 0.6, 1, 1.25, 2, 2.5, 4, 5, 8, 16, 32, and 64 mg/liter. The cultures were incubated at 37°C with 5% CO2 for 7 days. In
one set, each replicate received a daily 50% supplementation of ertapenem concentration at volumes of $0.001\%$ vol/vol added to the samples and plates, which were then further incubated for 24 h at $37^\circ C$ under 5% CO$_2$. The ertapenem MIC without daily ertapenem supplementation was 64 mg/liter, while that with supplementation was 0.6 mg/liter.

Day 7 cultures described above that were not used for resazurin assays were washed twice in normal saline to prevent drug carry-over and were subsequently spread on Middlebrook 7H10 agar and incubated for 3 weeks at $37^\circ C$ for enumeration of CFU counts. Inhibitory sigmoid maximum effect ($E_{\text{max}}$) curves for concentration versus CFUs per milliliter under sterilizing effect conditions are shown in Fig. 2. Comparison of the two regressions, with the null hypothesis that the maximal kill ($E_{\text{max}}$) or efficacy and concentration mediating 50% of $E_{\text{max}}$ (EC$_{50}$) or potency revealed a ratio of probabilities of 7.46 and a difference in corrected Akaike information criteria scores of 4.02, which means that the efficacy and potency differed with the supplementation. The EC$_{50}$ was 1.41 mg/liter without ertapenem supplementation and 0.19 mg/liter with daily supplementation. The efficacy was $0.751 \log_{10}$ CFU/ml without daily supplementation versus $2.38 \log_{10}$ CFU/ml with supplementation. Thus, ertapenem displays potential for a sterilizing effect that would otherwise be masked by not accounting for the degradation.

Here, we first show that ertapenem degrades considerably, at rates of $20$-fold the doubling times of $M. tuberculosis$ under acidic conditions. This effect is striking when the MICs with and without supplementation are compared. The MICs in the absence of supplementation would be considered in the resistance range by EUCAST breakpoints (14). Second, we show that ertapenem supplementation brings it well within the susceptibility range, suggesting that most of the published MICs for this drug are likely falsely high, and rates of resistance are likely falsely elevated. In addition, since many people have used MICs to choose which of the carbapenems would be better suited for treatment of XDR-TB, good drugs may have been discarded because of the artifactual manner in which current MIC measurements are performed for unstable molecules. Third, we show that ertapenem is likely to have a good sterilizing effect in tuberculosis. Follow-up hollow-fiber studies for a sterilizing effect have been completed in order to identify the ertapenem dose that can be used against both drug-resistant and drug-sensitive $M. tuberculosis$ (our unpublished data).

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