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Determination of MIC Distribution and Epidemiological Cutoff Values for Bedaquiline and Delamanid in *Mycobacterium tuberculosis* Using the MGIT 960 System Equipped with TB eXiST

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Bedaquiline (Sirturo) and delamanid (Deltyba) have recently been approved by the regulatory authorities for treatment of multidrug-resistant tuberculosis (MDR-TB). Antimicrobial susceptibility testing is not established for either substance. On the basis of the use of the MGIT 960 system equipped with EpiCenter/TB eXiST, we determined a mean bedaquiline MIC for wild-type strains of 0.65 mg/liter (median, 0.4 mg/liter) and an epidemiological cutoff (ECOFF) of 1.6 mg/liter; for delamanid, a mean wild-type drug MIC of 0.013 mg/liter (median, 0.01 mg/liter) and an ECOFF of 0.04 mg/liter were determined.

Globally, 3.5% of new and 20.5% of previously treated tuberculosis (TB) cases were estimated to have represented multidrug-resistant TB (MDR-TB) in 2013 (1). Bedaquiline (Sirturo [formerly known as TMC207 and R207910]; marketed by Janssen Therapeutics, Titusville, NJ, USA) is the lead compound of a series of recently discovered diarylquinolines, first described in 2005 (2). The U.S. Food and Drug Administration (FDA) approved bedaquiline for the treatment of adults with MDR-TB in 2012 (3). Because of the new mechanism of action of bedaquiline—the compound acts via inhibition of mycobacterial ATP synthase (AtpE)—it has been postulated that antimicrobial susceptibility testing (AST) is not needed in patients who have never received bedaquiline (4). However, cross-resistance between bedaquiline and the antimycobacterial drug clofazimine through overproduction of the MmpL5 efflux pump has recently been described (5, 6). Thus, resistance may develop independently of treatment with bedaquiline (2, 7). Delamanid (Deltyba [previously known as OPC-67683]; marketed by Otsuka Novel Products GmbH, Munich, Germany) was approved by the European Medicines Agency (EMA) in April 2014. The mechanism of action of delamanid is incompletely understood; delamanid is suggested to inhibit production of methoxymycolic acid and ketomycolic acid (8). Similarly to the related drug PA-824, delamanid is a prodrug requiring activation by the mycobacterial F420 system, including the nitroreductase Ddn (Rv3547) (8–10). Delamanid resistance is thought to arise from mutations in the mycobacterial F420 genes (*ddn*, *fgd1*, *fbiA*, *fbiB*, and *fbiC*) associated with the prodrug's activation (8, 11). The spontaneous rate of delamanid resistance has been reported to be as high as 6.44×10^{-6} to 4.19×10^{-5}, emphasizing the need to protect delamanid with other active anti-TB drugs during therapy (9).

Initially, AST of bedaquiline was reported using radiometric Bactec 460TB (BD, Franklin Lakes, NJ, USA), production of which has since been discontinued (2). Reported MIC_{90}s for delamanid range from 0.006 mg/liter to 0.05 mg/liter (depending on the test system) across *Mycobacterium tuberculosis* isolates (8, 9, 12). Ten years after the drugs' discoveries, established protocols for automated *in vitro* AST of bedaquiline and delamanid are still not available. To establish procedures for bedaquiline and delamanid AST, we used well-characterized, fully drug-susceptible clinical *M. tuberculosis* strains of bedaquiline and delamanid treatment-naive patients, MDR-TB strains, and subsequent isolates of a well-characterized extensively drug-resistant (XDR) strain (6). It has been speculated that the phylogenetic lineage of the *M. tuberculosis* complex may affect innate drug susceptibility (13). To assess the phylogenetic diversity of the set of strains studied, all *M. tuberculosis* strains included underwent genotypic characterization by mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) analysis using a GenoScreen MIRU-VNTR typing kit (GenoScreen, Paris, France) according to the manufacturer's description. In order to determine the quality control (QC) MIC value, the pan-susceptible *M. tuberculosis* H37Rv reference strain was used. Details about the resistance patterns of the strains and genotypes are shown in Table S1 and S2 in the supplemental material. With the view to facilitating implementation in the routine laboratories, we used a semiautomated MGIT 960 system and EpiCenter software equipped with a TB eXiST module for quantitative drug susceptibility testing (14). The MGIT 960 platform is a fully automated system that uses a fluorescence-quenching-based oxygen sensor for growth detection. This system is widely used in routine laboratories for AST of *M. tuberculosis*. As bedaquiline and delamanid were not available to us as pure substances (supply of bedaquiline was denied; supply of delamanid would have been associated with unacceptable binding conditions), we decided to establish AST using tablet formulations. Based on the accompanying prescription information, the composition and drug content of the tablets were accessible. For

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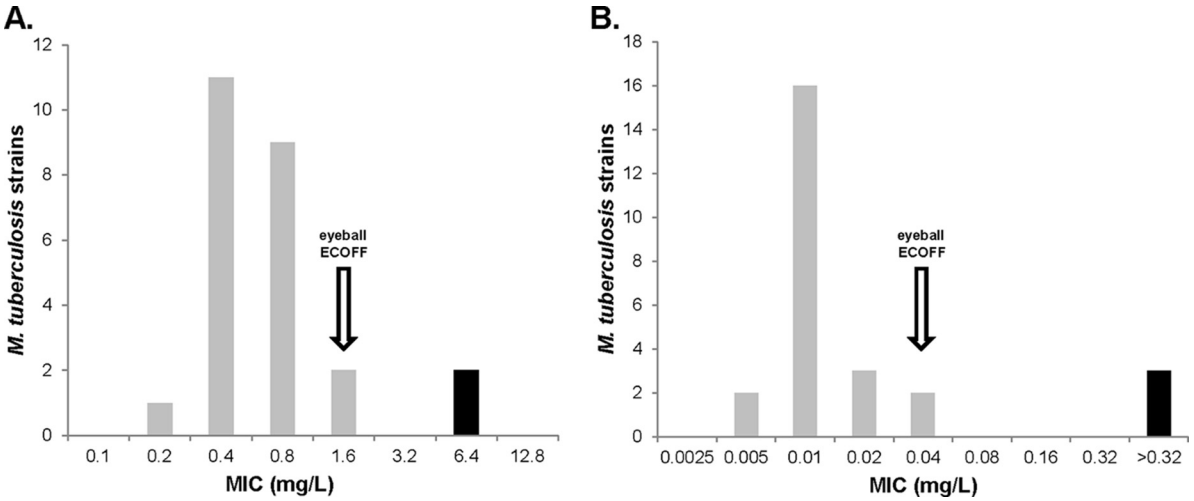

FIG 1 Distribution analysis of MIC values for bedaquiline (A) and delamanid (B). Proposed ECOFF values are marked with an arrow. Wild-type isolates are indicated by gray bars; resistant isolates are indicated by black bars.

bedaquiline, the tablet contained 100 mg active compound as well as colloidal anhydrous silica, croscarmellose sodium, hypromellose 2910, lactose monohydrate, magnesium stearate, corn starch, microcrystalline cellulose, and polysorbate 20 (15). For delamanid, one tablet contained 50 mg of active compound and, according to the summary of product characteristics as provided by the producer, hypromellose phthalate, povidone, all-rac-α-tocopherol, cellulose, microcrystalline, sodium starch glycolate (type A), carmellose calcium, colloidal hydrated silica, magnesium stearate, lactose monohydrate, hypromellose, macrogol 8000, titanium dioxide, talc, and yellow iron oxide (E172) (16). After the tablet was ground, the powder was dissolved in dimethyl sulfoxide (DMSO; Sigma D5879) and stored in small aliquots at −80°C. Test concentrations were obtained by serial 2-fold dilutions in DMSO. After thawing, stock solutions were used for same-day experiments. The stabilities of stock solutions for both drugs were assessed in parallel. For AST, MGIT tubes supplemented with 0.8 ml of oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton Dickinson) were inoculated with 0.2 ml of the drug in DMSO solution and 0.5 ml of the test strain suspension (final DMSO concentration, 2.4%). For preparation of the drug-free-growth control tube, the organism suspension was diluted 1:100 with sterile saline solution, and then 0.5 ml was inoculated into the tube (for proportion testing) containing 2.4% (vol/vol) DMSO. The bacterial suspensions were prepared from MGIT subcultures. Results were interpreted as follows. At the time when the growth unit (GU) value for the drug-free control tube was >400, the strain was categorized as resistant (R) if the GU of the drug-containing tube was ≥100. If the GU value for the drug-containing tube was <100 at this time point, the strain was categorized as sensitive (S). The MIC of each strain was defined as the lowest drug concentration that was categorized as sensitive per the definition above. According to EUCAST (the European Committee on Antimicrobial Susceptibility Testing), the epidemiological cutoff (ECOFF) value is the MIC value identifying the upper limit for the wild-type population (17). The ECOFF can be estimated by visual inspection of a histographic population analysis of the tested strains (eyeball method) or calculated statistically (18, 19). We used visual inspection and a receiver operating characteristic (ROC) curve-based method to determine the ECOFF (20). Drug stability was tested in four series of 11 different drug concentrations in MGIT tubes using *M. tuberculosis* H37Rv as the test strain. For the first series, no preincubation was chosen. For the second series, MGIT tubes were preincubated without a bacterial inoculum for 1 week. For the third series, a preincubation time of 2 weeks was chosen. And for the fourth series, a preincubation time of 3 weeks was chosen. The results of all measurements were compared. No difference in susceptibility patterns for all series was detected for bedaquiline. For delamanid, MIC values 1 dilution higher were measured after 2 weeks and MIC values 2 dilutions higher were measured after 3 weeks, indicating a stability issue.

We tested 10 wild-type, fully drug-susceptible *M. tuberculosis* strains isolated between 2011 and 2014 from bedaquiline and delamanid treatment-naive patients. Due to the limited amount of bedaquiline and delamanid available, we chose 6 to 10 concentrations for AST (see Tables S1 and S2 in the supplemental material). For bedaquiline, the MIC arithmetic mean for the wild-type strain was 0.54 mg/liter and the median was 0.4 mg/liter. *M. tuberculosis* H37Rv had a drug MIC of 0.4 mg/liter. In an analysis of 12 MDR and pre-XDR isolates, the arithmetic mean of the drug MIC was 0.77 mg/liter and the median of the MIC was 0.8 mg/liter ($P > 0.05$ for fully drug-susceptible strains versus MDR strains [nonsignificant difference between medians]). The overall drug MIC arithmetic mean for the susceptible phenotype was 0.65 mg/liter; the overall median was 0.4 mg/liter. Two XDR isolates with a bedaquiline-associated resistance mutation (Rv0678 fMet1Ala) that also confers cross-resistance to clofazimine had a drug MIC of 6.4 mg/liter (6). Using the eyeball method for ECOFF determination, a value of 1.6 mg/liter can be supposed (Fig. 1A). This eyeball-derived ECOFF was confirmed by a ROC-based method at a >90% specificity level. The 10 strains from treatment-naive patients showed delamanid MIC values between 0.005 and 0.04 mg/liter. The delamanid MIC arithmetic mean for the wild-type strain was 0.016 mg/liter; the median was 0.01 mg/liter. *M. tuberculosis* H37Rv had a drug MIC of 0.01 mg/liter. The 12 MDR and pre-XDR strains had drug MIC values between 0.005 and 0.04 mg/liter. The overall drug MIC arithmetic mean for the susceptible phenotype was 0.013 mg/liter; the overall median was 0.01 mg/

liter. A total of 3 XDR isolates from a patient with acquired delamanid resistance (case report in preparation) showed drug MIC values of >0.32 mg/liter (see Table S2). We propose an eyeball-derived ECOFF of 0.04 mg/liter (Fig. 1B). The ROC curve methodology could not be applied for delamanid, due to the lack of exact drug MIC values for the three resistotype isolates.

The published MIC values for *M. tuberculosis* H37Rv (bedaquiline MIC, 0.03 mg/liter; delamanid MIC, 0.002 mg/liter) are considerably lower than those found in our study (2, 21). This probably reflects a systematic difference in methodology. Both bedaquiline and delamanid show extensive protein binding; i.e., pharmacokinetics/pharmacodynamics (PK/PD) data indicate a plasma protein-bound fraction of >99.9% (9, 22). It has been shown that the bedaquiline MIC increases in the presence of 5% bovine serum albumin (22). Previous studies determined drug susceptibility mostly in the absence of albumin (21). The albumin content in the MGIT 960 test tube resulting from addition of OADC (this study) or of MGIT growth supplement as supplied by BD was approximately 4% (wt/vol), comparable to the physiological plasma protein concentration. A challenge in AST for both substances is their poor solubility in water, a complication known for other antimycobacterial drugs such as ethionamide. Corresponding compounds have to be dissolved in DMSO as a solvent, and the growth control has to contain the same amount of DMSO to control for any possible effect on bacterial growth. In general, AST is done using pure substances as provided by the manufacturer. For this study, tablet formulations had to be used, because both producing companies denied the supply of the substances or were unwilling to provide the compound without extensive binding conditions for use and data publication. This is a policy not previously seen for new antimicrobials entering the market, as AST should be established and verified independently by expert laboratories (17, 23). The development and periodic revision of AST guidelines as part of drug development require close cooperation between academic experts, funding agencies, pharmaceutical companies, and regulatory authorities, as has occurred for antivirals in the past (24).

Our study had several limitations. Most notably, the limited amount of compound available precluded the analysis of a larger strain collection to more precisely determine the ECOFF. The proposed ECOFFs might change slightly with increasing sample size and a finer resolution of drug concentration scaling. In addition, given that bedaquiline and delamanid have entered the market only recently, *M. tuberculosis* isolates with acquired resistance are barely accessible. We established AST (see Tables S1 and S2 in the supplemental material) using a phylogenetically diverse strain set as shown by MIRU-VNTR analysis (see Fig. S1) in order to measure the variation in the "wild-type" MIC distribution and to maximize the chance of identifying genotypes that might be intrinsically resistant (13). Further studies evaluating *in vitro* laboratory drug MICs using pure compounds and PK/PD and clinical data from a large number of drug-susceptible and drug-resistant strains are required to define clinical breakpoints (17, 23).

Despite all these limitations, our report provides valid AST results. We propose ECOFF values based on population analysis and the eyeball method, which allow discrimination between wild-type and resistotype populations. Our study shows the feasibility of MGIT 960 equipped with TB eXiST for AST of bedaquiline and delamanid in the routine clinical laboratory.

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