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SUSCEPTIBILITY

# *In Vitro* Bedaquiline and Clofazimine Susceptibility Testing in *Mycobacterium abscessus*

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**ABSTRACT** Bedaquiline and clofazimine are increasingly used to treat infections with *Mycobacterium abscessus*. We determined distributions of MICs by broth microdilution for bedaquiline and clofazimine for 61 *M. abscessus* clinical isolates using different media and incubation times. We show that incubation time and growth media critically influence the MIC. Our data will aid in defining future clinical breakpoints for *in vitro* susceptibility testing for bedaquiline and clofazimine in *M. abscessus*.

**KEYWORDS** ECOFF, MIC, *Mycobacterium abscessus*, antibiotic susceptibility testing, bedaquiline, clofazimine, epidemiological cutoff

Increasing numbers of infections due to *Mycobacterium abscessus* are being reported, presenting mostly as pulmonary disease in patients with cystic fibrosis (CF), bronchiectasis, or chronic obstructive pulmonary disease (COPD) and as skin and soft tissue infections following trauma or surgery (1, 2). *M. abscessus* belongs to the rapidly growing mycobacteria and consists of the three subspecies *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (3). *M. abscessus* naturally exhibits extensive drug resistance (4), and treatment requires individual multidrug regimens that are based on *in vitro* susceptibility testing and clinical expertise (5, 6). Treatment options are limited especially for macrolide-resistant *M. abscessus*, which is common due to expression of the inducible 23S rRNA methyltransferase Erm(41) (7). Recently approved drugs for combination therapy of multidrug-resistant tuberculosis, such as bedaquiline, a diarylquinoline antibiotic, and clofazimine, a key drug in therapy for leprosy, have been increasingly used for the treatment of infections with nontuberculous mycobacteria, particularly for *M. abscessus* (8–10). Procedures for *in vitro* susceptibility testing of these two drugs in *M. abscessus* have not yet been standardized. Bedaquiline and clofazimine were not included in commercial microdilution panels until recently, when Thermo Fisher Scientific (Waltham, MA) released the RAPMYCO2 Sensititre plate with clofazimine. Accordingly, no clinical breakpoints have been defined by the Clinical and Laboratory Standards Institute (CLSI) or by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to separate bedaquiline and clofazimine susceptible from resistant *M. abscessus* strains. The frequency of *M. abscessus* strains categorized as clofazimine resistant in different studies varies between 0% and 95%, with a pooled rate of *in vitro* resistance of 16% (95% confidence interval, 4.0% to 16%) (11). Determination of MIC distributions and epidemiological cutoff values (ECOFF) is a prerequisite for assignment of clinical breakpoints (12).

In this study, we determined the MICs of bedaquiline and clofazimine for the type strain *M. abscessus* ATCC 19977 and 61 *M. abscessus* nonduplicate (one isolate per patient) clinical isolates from the years 2008 to 2013 that were isolated at or submitted to our laboratory and for which antibiotic susceptibility testing was requested. Fifty-one (84%) *M. abscessus* isolates were of respiratory origin, and six isolates (10%) were of nonrespiratory origin. For four isolates (6%), the clinical origin was unknown. Thirty-



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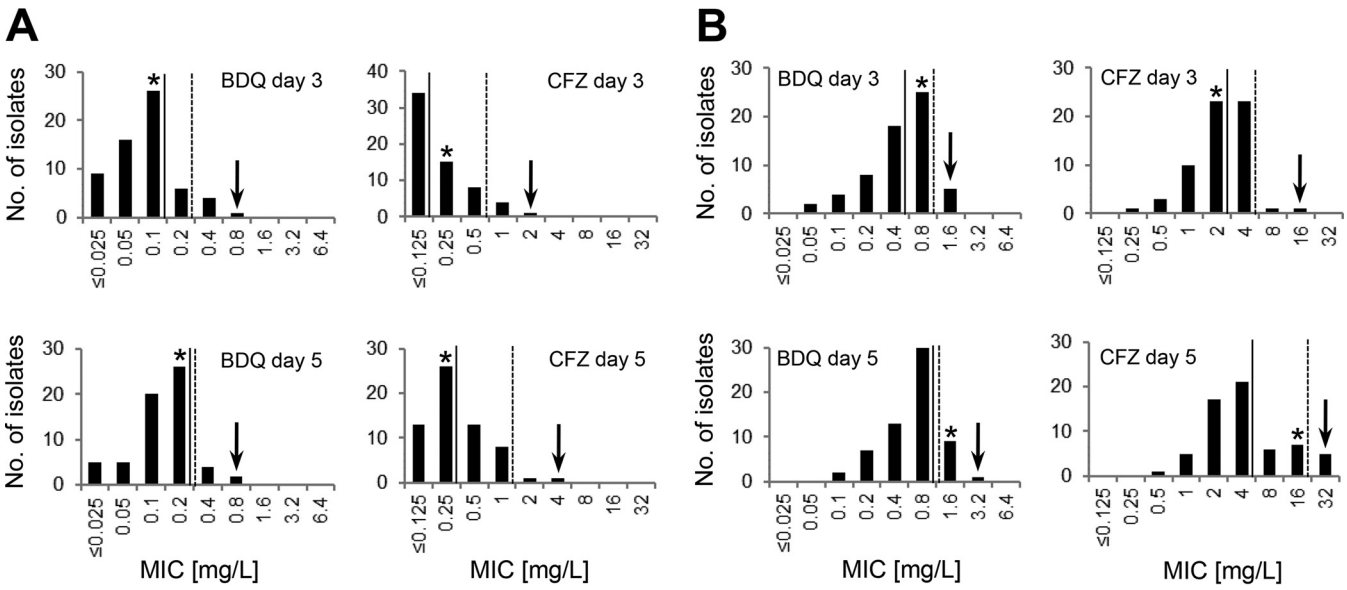


**FIG 1** MIC distributions of bedaquiline (BDQ) and clofazimine (CFZ) for *M. abscessus* complex isolates ($n$ = 61) determined in CAMHB without (A) and with (B) 10% OADC and read after 3 and 5 days of incubation at 37°C. $MIC_{50}$ (continuous line), $MIC_{90}$ (broken line), and tentative ECOFF values (arrow) are indicated as well as MIC values of the type strain *M. abscessus* ATCC 19977 (*).

one (51%) *M. abscessus* isolates were recovered from patients with CF, 19 (31%) *M. abscessus* isolates were recovered from non-CF patients, and for 11 (18%) isolates, the CF status of the patient was not known. The isolates were selected to comprise the three *M. abscessus* subspecies and include *M. abscessus* subsp. *abscessus* ($n$ = 32), *M. abscessus* subsp. *bolletii* ($n$ = 17), and *M. abscessus* subsp. *massiliense* ($n$ = 12). Subspecies assignment was done by combined 16S rRNA gene, *rpoB*, and *erm*(41) sequence analysis (13). MIC determination was conducted by in-house broth microdilution according to CLSI guidelines, except for the incubation temperature, which was set at 37°C (14). Bedaquiline (Adooq, Irvine, CA) and clofazimine (Sigma-Aldrich, St. Louis, MO) were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in cation-adjusted Mueller-Hinton broth (CAMHB) (Merck, Darmstadt, Germany) to final concentrations of 0.025 mg/L to 6.4 mg/L for bedaquiline and 0.125 mg/L to 32 mg/L for clofazimine (2-fold serial dilutions with a maximum final DMSO concentration of 6.25% [vol/vol]). $MIC_{50}$ and $MIC_{90}$ values of 0.1 mg/L and 0.2 mg/L, respectively, for bedaquiline and of ≤0.125 mg/L and 0.5 mg/L, respectively, for clofazimine were calculated when growth was judged after 3 days of incubation in CAMHB in polystyrene plates (Greiner Bio-One, Monroe, NC) (Fig. 1A). The exact definition of the $MIC_{50}$ but not of the $MIC_{90}$ value of clofazimine was hampered by the limits of the test range. Tentative ECOFFs of 0.8 mg/L for bedaquiline and of 2 mg/L for clofazimine were assigned by visual inspection of the histograms (eyeball method) (15, 16). We did not observe significant differences between the three *M. abscessus* subspecies. For *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*, the $MIC_{50}$ values for bedaquiline were 0.05 mg/L, 0.1 mg/L, and 0.1 mg/L, respectively, and the $MIC_{90}$ values were 0.1 mg/L, 0.2 mg/L, and 0.4 mg/L. Clofazimine $MIC_{50}$ values of ≤0.125 mg/L, ≤0.125 mg/L, and 0.25 mg/L were found for *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*. The clofazimine $MIC_{90}$ value was 0.5 mg/L for all three subspecies. The MIC of the type strain *M. abscessus* ATCC 19977 was determined at 0.1 mg/L for bedaquiline and at 0.25 mg/L for clofazimine. For a subset of 12 isolates (four each of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*), the MIC of bedaquiline and clofazimine was also determined at 30°C. For bedaquiline, an $MIC_{50}$ of 0.025 mg/L and an $MIC_{90}$ of 0.05 mg/L was observed; for clofazimine, the $MIC_{50}$ and $MIC_{90}$ values were ≤0.125 mg/L and 0.5 mg/L, respectively. For the CLSI quality control strain *Mycobacterium peregrinum* ATCC 700686, we determined the MIC of bedaquiline to be

**TABLE 1** *In vitro* MIC studies for bedaquiline and clofazimine for *M. abscessus* complex

| Study[a] | No. of isolates | Method | Medium[b] | MIC range (mg/L) | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) |
|---|---|---|---|---|---|---|
| Bedaquiline | | | | | | |
| This study | 61 | Broth microdilution | CAMHB | ≤0.025 to 0.8 | 0.1 | 0.2 |
| This study | 61 | Broth microdilution | CAMHB OADC | 0.05 to 1.6 | 0.4 | 0.8 |
| Asami et al., 2021 (24) | 70 | Broth microdilution | CAMHB | 0.06 to 0.25 | 0.13 | 0.25 |
| Chew et al., 2021 (25) | 211 | Broth microdilution | CAMHB | 0.008 to 0.25 | 0.06 | 0.12 |
| Gumbo et al., 2020 (26) | 20 | Broth microdilution | NA | 0.25 to 1 | 1 | 1 |
| Sarathy et al., 2020 (27) | 12 | Broth microdilution | 7H9 ADC | 0.08 to 0.42[c] | 0.28[c] | 0.36[c] |
| Sorayah et al., 2019 (28) | 17 | Broth microdilution | 7H9 ADC | 0.02 to 0.38[c] | 0.06[c] | 0.21[c] |
| Viljoen et al., 2019 (29) | 18 | Broth microdilution | CAMHB | 0.032 to 0.128 | 0.064 | 0.128 |
| Kim et al., 2019 (30) | 132 | Broth microdilution | CAMHB | ≤0.016 to 0.5 | 0.062 | 0.125 |
| Brown-Elliott and Wallace, 2019 (31) | 104 | Broth microdilution | CAMHB | 0.003 to 0.5 | 0.06 | 0.12 |
| Li et al., 2018 (32) | 197 | Broth microdilution | CAMHB | 0.007 to 1 | 0.062 | 0.125 |
| Dupont et al., 2018 (33) | 30 | Broth microdilution | CAMHB | 0.031 to 0.125 | 0.062 | 0.125 |
| Vesenbeckh et al., 2017 (21) | 20 | Agar dilution | 7H10 OADC | 0.12 to 1 | 0.5 | 1 |
| Pang et al., 2017 (34) | 381 | Broth microdilution | CAMHB | 0.016 to >16 | 0.13 | >16 |
| Clofazimine | | | | | | |
| This study | 61 | Broth microdilution | CAMHB | ≤0.125 to 2 | ≤0.125 | 0.5 |
| This study | 61 | Broth microdilution | CAMHB OADC | 0.25 to 16 | 2 | 4 |
| Asami et al., 2021 (24) | 70 | Broth microdilution | CAMHB | 0.25 to 1 | 0.5 | 1 |
| Chew et al., 2021 (25) | 211 | Broth microdilution | CAMHB | 0.008 to 1 | 0.12 | 0.25 |
| Kwak et al., 2021 (18) | 40 | Broth microdilution | 7H9 OADC | 0.031 to 16 | 4 | 8 |
| Luo et al., 2018 (35) | 40 | Broth microdilution | CAMHB | 0.031 to >8 | 4 | >8 |
| Shen et al., 2018 (36) | 20 | Broth microdilution | CAMHB | 0.25 to 128 | NA | 32 |
| Schwartz et al., 2018 (37) | 17 | Broth microdilution | CAMHB | 0.38 to 3 | 1.5 | 3 |
| Kim et al., 2015 (38) | 57 | Broth microdilution | CAMHB | ≤1 to ≥4 | NA | ≤1 |
| Singh et al., 2014 (39) | 67 | Broth microdilution | CAMHB | 2 to 8 | 2 | 8 |
| van Ingen et al., 2012 (40) | 390 | Broth microdilution | CAMHB | NA | ≤0.5 | 1 |
| Shen et al., 2012 (41) | 117 | Broth microdilution | CAMHB | 0.03125 to 2 | 0.25 | 0.5 |

[a]Selected studies based on PubMed search including the keywords "*Mycobacterium*," "*abscessus*" and "bedaquiline" or "clofazimine." Studies that analyzed >10 *M. abscessus* complex isolates are shown.
[b]7H9 ADC, Middlebrook 7H9 broth 0.2% glycerol, 0.05% Tween 80, 10% ADC (albumin-dextrose-catalase); 7H9/7H10 OADC, Middlebrook 7H9 broth/7H10 agar 10% OADC (oleic acid albumin dextrose catalase); CAMHB, cation-adjusted Mueller-Hinton broth without/with 10% OADC; NA, not available.
[c]MIC calculated in milligrams per liter from original data in $\mu$M (molecular weight of bedaquiline, 555.5 g/mol).

0.0031 to 0.0125 mg/L (median, 0.0063 mg/L) and the MIC of clofazimine to be 0.0625 to 0.25 mg/L (median, 0.125 mg/L) for 10 replicates (incubation at 30°C). Incubation of *M. peregrinum* ATCC 700686 at 37°C resulted in comparable MIC ranges (median MIC for bedaquiline, 0.0125 mg/L, and for clofazimine, 0.125 mg/L). CLSI has not yet published quality control concentration ranges for *M. peregrinum* ATCC 700686 for bedaquiline and clofazimine (17).

Reported bedaquiline and clofazimine MIC distributions for *M. abscessus* vary between laboratories and definition of uniform ECOFF values has not been possible so far (Table 1). In particular, reported $MIC_{50}$ and $MIC_{90}$ values for clofazimine vary from 0.12 mg/L to 4 mg/L and from 0.25 mg/L to 32 mg/L, respectively. A recent study of *M. abscessus* pulmonary disease demonstrated that clofazimine MICs of ≤1 mg/L were most often associated with sputum conversion to negative in patients who were being treated with a clofazimine-containing drug regimen. The effect was more pronounced when MIC values were ≤0.25 mg/L, while no culture conversion was observed for MIC values exceeding 2 mg/L (18). Of note, MIC determination in this study by Kwak et al. was conducted in 7H9 broth supplemented with oleic acid, albumin, dextrose, and catalase (OADC), which is not recommended by CLSI, and showed a bimodal distribution of MICs (18). Most likely, for *M. abscessus* as for *Mycobacterium tuberculosis*, the bedaquiline and clofazimine MIC values are affected by *in vitro* culture conditions, which may account for the diverging MIC results obtained in different studies (19, 20).

We observed an influence of incubation time on MIC. Prolonged incubation of 5 days resulted in slightly increased $MIC_{50}$ values of 0.2 mg/L for bedaquiline and of 0.25 mg/L for clofazimine (Wilcoxon signed-rank test, bedaquiline [$Z = -5.1594$, $P =$

<0.00001] and clofazimine [Z = −3.2911, *P* = 0.001]) (Fig. 1A). All isolates showed sufficient growth to be read at days 3 and 5. We next investigated a putative influence of media composition by including 10% OADC (Becton, Dickinson, Franklin Lakes, NJ). OADC is a growth supplement that contains oleic acid, albumin, dextrose, and catalase and is used in combination with Middlebrook 7H9 broth or CAMHB for standard drug susceptibility testing of *M. tuberculosis* and slowly growing nontuberculous mycobacteria (14). Media containing OADC were also used for resistance testing of rapidly growing nontuberculous mycobacteria (18, 21), although this is not recommended by CLSI (17). Addition of 10% OADC to CAMHB increased the $MIC_{50}$ of bedaquiline from 0.1 mg/L to 0.4 mg/L (Z = −6.6573, *P* = <0.00001) and the $MIC_{50}$ of clofazimine from ≤0.125 mg/L to 2 mg/L (Z = −6.8463, *P* = <0.00001) when the MIC results were determined at day three of incubation (Fig. 1B). *M. abscessus* ECOFFs shifted from 0.8 mg/L to 1.6 mg/L for bedaquiline and from 2 mg/L to 16 mg/L for clofazimine in the presence of OADC. These findings suggest that including OADC to the growth medium increases the MICs for bedaquiline and clofazimine in isolates of *M. abscessus*. Bedaquiline and clofazimine show high plasma protein binding capacities of >99%, and consequently, the protein content of the culture medium is likely to affect the MIC results as has been previously discussed for *M. tuberculosis* and MICs to bedaquiline (20). Drug instability during prolonged incubation has been shown to affect MIC values for mycobacteria, particularly the MIC of beta-lactam antibiotics (22). Bedaquiline and clofazimine are, however, stable over time as has been demonstrated for *in vitro* susceptibility testing of *M. tuberculosis* (20, 23). For *M. abscessus*, the increased MIC values observed at 5 days versus 3 days of incubation are most probably the result of increased visible growth due to bacteriostatic rather than bactericidal effects around MIC.

In summary, we show that incubation time and composition of the growth medium critically influence the MICs of bedaquiline and clofazimine in *M. abscessus* while temperature (37°C versus 30°C) has little effect. With the increased use of bedaquiline and clofazimine for treatment of *M. abscessus* infections, there is a need for standardized MIC testing and interpretation guidelines. In the meantime, it is important that laboratory testing conditions of bedaquiline and clofazimine are precisely documented. Uniform testing conditions are a prerequisite for data comparison and the definition of ECOFF and clinical breakpoints. Beyond that, clinical trials are needed to establish the correlation between *in vitro* MIC results and clinical response.

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