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In vitro activity of the new β-lactamase inhibitors relebactam and vaborbactam in combination with β-lactams against *Mycobacterium abscessus* complex clinical isolates

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Abstract

Pulmonary disease due to infection with *Mycobacterium abscessus* complex (MABC) is notoriously difficult to treat, in large part due to MABC's intrinsic resistance to most antibiotics, including β-lactams. MABC organisms express a broad-spectrum β-lactamase that is resistant to traditional β-lactam-based β-lactamase inhibitors but inhibited by a newer non-β-lactam-based β-lactamase inhibitor, avibactam. Consequently, the susceptibility of MABC to some β-lactams is increased in the presence of avibactam. Therefore, we hypothesized that two new non-β-lactam-based β-lactamase inhibitors, relebactam and vaborbactam, would also increase susceptibility of MABC to β-lactams. The objective of the present study was to evaluate the *in vitro* activity of various marketed β-lactams alone and in combination with either relebactam or vaborbactam against multidrug-resistant MABC clinical isolates. Our data demonstrate that both β-lactamase inhibitors significantly improved the anti-MABC activity of many carbapenems (including imipenem and meropenem) and cephalosporins (including cefepime, ceftaroline, and cefuroxime). As a meropenem/vaborbactam combination is now marketed and an imipenem/relebactam combination is currently in phase III trials, these fixed combinations may become the β-lactams of choice for the treatment of MABC infections. Furthermore, given the evolving interest in dual β-lactam regimens, our results identify select cephalosporins, such as cefuroxime, with superior activity in the presence of a β-lactamase inhibitor, deserving of further evaluation in combination with these carbapenem/β-lactamase inhibitor products.

Introduction

Mycobacterium abscessus (or *M. abscessus* subsp. *abscessus*), *M. massiliense* (or *M. abscessus* subsp. *massiliense*), and *M. bolletii* (or *M. abscessus* subsp. *bolletii*) comprise the *M. abscessus* complex (MABC) (1). These rapidly-growing nontuberculous mycobacteria, ubiquitous in the environment, are opportunistic human pathogens associated with a wide range of maladies, from localized skin lesions to systemic disease. Individuals with cystic fibrosis and other forms of bronchiectasis are especially vulnerable to MABC pulmonary disease, an infection that is notoriously difficult to eradicate due in large part to MABC's broad, intrinsic resistance to most antibiotics, including many anti-mycobacterial drugs (2-4). The paucity of effective treatment regimens has recently gained attention as the prevalence of MABC pulmonary disease is apparently increasing (5-7), justly highlighting the need for additional treatment options.

Like several other pathogenic and nonpathogenic mycobacteria, MABC organisms possess a constitutively expressed, broad-spectrum β-lactamase, Bla_{Mab}, which contributes to the intrinsic resistance of MABC to most β-lactam antibiotics (8-12). Several studies have indicated that Bla_{Mab} is not significantly inhibited by β-lactam-based β-lactamase inhibitors, namely clavulanate, tazobactam, and sulbactam (9, 13-15). In contrast, the non-β-lactam-based β-lactamase diazabicyclooctane (DBO) inhibitor avibactam does inhibit Bla_{Mab}, thereby reducing the minimum inhibitory concentration (MIC) of many β-lactams for MABC, especially carbapenems and cephalosporins, to clinically achievable concentrations (16-20). Avibactam is marketed solely in combination with the cephalosporin ceftazidime (trade name AVYCAZ® in the United States). However, ceftazidime has little or no demonstrable activity against MABC, even in combination with avibactam and against *M. abscessus* strains in which the gene encoding Bla_{Mab} has been entirely deleted (8, 9, 18). Thus, the current requirement to co-administer ceftazidime in order to potentiate the activity of other more effective β-lactams with avibactam

complicates this treatment strategy for MABC infections, as ceftazidime might only incur risk of adverse effects without perceived benefit.

Relebactam and vaborbactam are two newer non-β-lactam-based β-lactamase inhibitors developed for use with the carbapenems imipenem and meropenem, respectively (21). Whereas relebactam is a DBO β-lactamase inhibitor structurally related to avibactam, vaborbactam is a novel boronic acid-based inhibitor. While neither of these β-lactamase inhibitors are expected to be clinically available as sole formulations, both of the paired carbapenems have activity against MABC. Imipenem alone has good activity and is currently recommended as part of first-line treatments for MABC pulmonary disease (2, 3). Activity of meropenem, while comparatively less than imipenem when used alone, is increased comparable to that of imipenem in the presence of avibactam (8, 16, 18). As the meropenem/vaborbactam combination is already clinically available (trade name VABOMERE™ in the United States), and the imipenem/cilastatin/relebactam combination is currently being evaluated in multiple phase III clinical trials (ClinicalTrials.gov identifiers NCT02493764, NCT03583333, NCT03293485, NCT02452047), we set out to assess the impact of these β-lactamase inhibitors on the anti-MABC activity of a variety of β-lactam drugs. The objective of this study was to evaluate the activity of β-lactams alone and in combination with either relebactam or vaborbactam, against MABC, including multidrug-resistant (MDR) clinical isolates.

Results

Impact of culture medium on the *in vitro* growth of MABC clinical isolates

Clinical and Laboratory Standards Institute (CLSI) guidelines recommend the use of cation-adjusted Mueller-Hinton broth (CAMHB) for susceptibility testing of antimicrobials against rapidly-growing mycobacteria, including MABC; for MIC determination, the guidelines state that

cultures should be examined after 3 days of incubation, to be extended up to 5 days if growth of the non-drug-containing control sample is insufficient (22). Early in our work, we found that MABC clinical isolates in our collection, isolates resistant to almost all antimicrobials currently used to treat MABC infection (16), grow slowly in CAMHB, and that, on average, MIC values could not be determined until nearly 5 days of incubation (**Fig. S1A**). Such a long incubation period can be problematic when evaluating the activity of some β-lactams due to their innate instability in aqueous media, independent of the presence of β-lactamase enzymes (8, 23-25), which could potentially result in artificially high MIC values. The clinical strains grow better in Middlebrook 7H9 broth supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC) enrichment (**Fig. S1B**), a liquid laboratory medium for culturing mycobacteria (26-28). Therefore, Middlebrook 7H9 liquid medium was primarily used in this study.

Activity of β-lactams with relebactam or vaborbactam against *M. abscessus* ATCC 19977

We first evaluated the impact of relebactam and vaborbactam on the activity of β-lactams against a well-characterized MABC strain, *M. abscessus* ATCC 19977 (29). MICs of drugs representing the four major sub-classes of β-lactams (carbapenems, cephalosporins, monobactams, and penicillins) were determined in the presence and absence of relebactam or vaborbactam at 4 μg/ml (**Table 1**). Neither of these β-lactamase inhibitors exhibited antimicrobial activity on their own. The MICs of relebactam alone and vaborbactam alone were both >256 μg/ml, and neither drug inhibited bacterial growth in a disk diffusion assay (**Fig. S2**). As expected, without co-exposure to a β-lactamase inhibitor, the strain was able to grow in relatively high concentrations of all β-lactams tested, with imipenem having the lowest MIC at 8 μg/ml. Overall, the MICs of penicillins and the monobactam aztreonam were not affected by either β-lactamase inhibitor, although the MIC of amoxicillin did shift from >256 μg/ml to 32 μg/ml in the presence of relebactam. In contrast, all carbapenems and more than half of the cephalosporins tested exhibited decreased MICs in the presence of either relebactam or

vaborbactam. The magnitude of the MIC shift associated with each β-lactamase inhibitor was similar for most of these β-lactams, but for two of the carbapenems (tebipenem and ertapenem) and three of the cephalosporins (cefdinir, cefuroxime, and ceftaroline) the MICs associated with relebactam were one dilution lower than that associated with vaborbactam. Similar activity was also observed in a disk diffusion assay (**Fig. S2**).

Activity of β-lactams with relebactam or vaborbactam against MABC clinical isolates

The promising antimicrobial activity of the carbapenems and select cephalosporins, namely cefdinir, cefpodoxime, cefuroxime, cefepime, cefotaxime, cefoxitin, ceftaroline, and ceftriaxone, in combination with either relebactam or vaborbactam against the ATCC 19977 strain prompted us to evaluate the activity of these combinations against a collection of 28 MABC clinical isolates with MDR phenotypes (16). The MICs of the carbapenems and cephalosporins against each clinical isolate are presented in **Tables S1** and **S2**, respectively. With the exception of cefoxitin, the MICs of each β-lactam decreased in the presence of either β-lactamase inhibitor (**Table 2**). As observed with the ATCC 19977 strain, both β-lactamase inhibitors were mostly associated with MIC shifts of similar magnitude, although the MIC_{50}, MIC_{90}, and MIC range limits associated with relebactam were one or two dilutions lower than those associated with vaborbactam for tebipenem, biapenem, and meropenem. For tebipenem, 25/28 (89%) isolates has lower MIC values with relebactam compared to vaborbactam, and for biapenem and meropenem, 14/28 (50%) and 10/28 (36%) of the isolates, respectively, had lower MICs with relebactam compared to vaborbactam. For the carbapenems, the most striking shifts in MIC distribution were observed with tebipenem and ertapenem (**Fig. 1A**,**B**), while the lowest MIC values were obtained with imipenem, meropenem, biapenem, and doripenem (**Fig. 1C-F**). The MIC_{50} value for each of these carbapenems was 4-8 μg/ml in the presence of either β-lactamase inhibitor, suggesting that they have similar intrinsic activity in the absence of hydrolysis by Bla_{Mab}. For the cephalosporins, the largest shifts in MIC distribution associated

with the β-lactamase inhibitors were observed for ceftaroline, ceftriaxone, and cefuroxime (**Fig. 2A-C**), while the shifts were comparatively smaller for cefotaxime and cefepime (**Fig. 2D,E**). The MIC distribution of cefoxitin was not affected by the presence of either relebactam or vaborbactam (**Fig. 2F**). The lowest MICs in association with a β-lactamase inhibitor were observed with cefuroxime, cefepime, cefdinir, and ceftaroline.

For a selection of β-lactams, MIC values with and without the β-lactamase inhibitors were also determined in CAMHB. For the ATCC 19977 strain, the MICs were determined after 3 days of incubation, while the MICs for clinical isolates could not be determined before day 4 or 5 of incubation (**Table S3**). For three of the clinical isolates (strains 2N, 11N, and JHHKB), adequate growth for determining MIC values did not occur within 5 days. With the exception of imipenem and cefdinir, the MIC_{50} values of the tested β-lactams were higher, both the presence and absence of relebactam or vaborbactam (**Table S4**), relative to the MIC_{50} values determined in Middlebrook 7H9 liquid medium (**Table 2**). The MIC values the ATCC 19977 strain also tended to be higher in CAMHB (**Table S3**).

Discussion

This study demonstrates that certain β-lactams, namely carbapenems and cephalosporins, exhibit improved activity against MABC in the presence of vaborbactam or relebactam, two new non-β-lactam-based β-lactamase inhibitors. Vaborbactam and relebactam reduced the MIC_{50}, MIC_{90}, and MIC range limits of meropenem and imipenem, respectively, by one or two dilutions in both 7H9 and CAMHB media. Since meropenem and imipenem MICs against MABC clinical isolates commonly lie at or around the recommended susceptibility breakpoints, these carbapenem/β-lactamase inhibitor combinations can be expected to improve PK/PD target attainment for the carbapenems and increase efficacy over either carbapenem alone. We recently observed similar reductions of carbapenem MICs with avibactam against the same

clinical isolates (16). However, as avibactam is currently marketed only with ceftazidime, a cephalosporin with poor intrinsic activity itself against MABC, the carbapenem/β-lactamase inhibitor combinations studied here appear more advantageous by not requiring combination of the carbapenem with ceftazidime/avibactam, which imposes the risk of ceftazidime-related side effects in order to gain the value of Bla_{Mab} inhibition. Further confirmation of these results against a larger number of clinical isolates might promote meropenem/vaborbactam or, pending future regulatory approval, imipenem/relebactam to become the β-lactams of choice against MABC infections.

The significant reductions in MIC values for carbapenems and cephalosporins against MABC in the presence of vaborbactam and relebactam provide indirect evidence that both of these β-lactamase inhibitors inhibit Bla_{Mab}, thus revealing the intrinsic antimicrobial activity of the paired β-lactams against their transpeptidase targets in MABC (30). Although carbapenems clearly have greater intrinsic potency than the cephalosporins against the MABC isolates tested here, vaborbactam and relebactam, like avibactam previously (16), also significantly reduced the MICs of a variety of marketed cephalosporins. A key question is whether these β-lactamase inhibitors increase the susceptibility of MABC to the cephalosporins to levels that are clinically relevant based on drug exposures that are achievable in patients.

One way to address this question is to compare the observed MICs in the context of CLSI interpretive categories and MIC breakpoints set for the antimicrobial susceptibility testing of these β-lactams (22, 31). Breakpoints for the interpretive categories of susceptible, intermediate, and resistant are not based solely on the natural MIC distribution, but also on microbiological and pharmacological data, and are “considered to be robust predictors of clinical outcome” (31). Among the drugs tested in this study, recommended breakpoints for rapidly-growing mycobacteria are currently only available for imipenem, meropenem, and cefoxitin

(**Table S5**), three β-lactams currently recommended for use for treatment of mycobacterial infections and expected to reach active concentrations in patients (2, 3). However, CLSI-recommended breakpoints for infections caused by other bacterial genera are available for all of the cephalosporins tested in this study (31). Although these cephalosporin breakpoints should not be used to predict susceptibility of MABC for clinical decision-making at this time, they can be considered as surrogate indicators of potential susceptibility to drug exposures that are achievable in patients. As previously observed for avibactam (16), vaborbactam and relebactam did not significantly affect the MICs of cefoxitin (MIC_{50} 32 μg/ml with or without either β-lactamase inhibitor) against our isolates (**Tables 1,2**), further confirming cefoxitin's stability in the presence of Bla_{Mab} (9), while also calling into question whether selected cephalosporins could be superior to cefoxitin for treatment of MABC if combined with an effective β-lactamase inhibitor. However, the addition of vaborbactam and/or relebactam decreased the MIC_{50} values of cefotaxime, ceftriaxone, cefuroxime and cefepime for MABC to concentrations at or within one dilution of the CLSI susceptible/intermediate breakpoints for these drugs against *Enterobacteriaceae* or anaerobic bacteria (**Table S5**), thus indicating that MABC may be susceptible to clinically achievable concentrations of these cephalosporins in the presence of vaborbactam or relebactam. With MICs consistently one dilution lower than cefoxitin in the presence of vaborbactam or relebactam, cefuroxime is particularly attractive given its narrower spectrum of activity, longer half-life and lower plasma protein binding compared to cefoxitin. Moreover, at a standard dose of 1.5 g every 8 hours, cefuroxime is expected to have higher probability than cefoxitin at a dose of 2 g every 8 hours of achieving adequate $T_{>MIC}$ even when MIC distributions for the two drugs are similar, as shown by Moine *et al*. (32). Further studies are needed to determine whether cefuroxime or an alternative cephalosporin might have superior efficacy compared to the currently recommended agent cefoxitin when combined with a β-lactamase inhibitor.

Recent studies suggest that combining different classes of β-lactams against MABC (30, 33) and other bacteria (34, 35) may be synergistic due to targeting a wider spectrum of enzymes that appear to be uniquely relevant to synthesis of peptidoglycan in MABC (36, 37). For example, Kumar *et al*. recently demonstrated synergy between doripenem and cefdinir against MABC (30). Remarkably, despite its poor activity when tested alone or in combination with avibactam, ceftazidime demonstrates synergy with either imipenem or ceftaroline against MABC, whether avibactam is present or not (38). These studies suggest that a dual-β-lactam combination may be superior to a single β-lactam for treatment of MABC pulmonary disease. In this context, our finding that several marketed cephalosporins exhibit greater activity than ceftazidime in the presence of vaborbactam and relebactam indicates that administering a carbapenem/β-lactamase inhibitor combination together with a more intrinsically active cephalosporin, such as cefuroxime, may have superior activity compared to the same carbapenem combined with ceftazidime/avibactam or another cephalosporin or the same carbapenem/β-lactamase inhibitor alone.

Another interesting finding of our study is that the MICs of the carbapenems and cephalosporins, alone and in combination with relebactam or vaborbactam, against the ATCC 19977 strain were often similar to MICs against the MABC clinical isolates (**Tables S1, S2**). For all of the 15 drugs tested, the MIC for the ATCC 19977 strain fell within one dilution of the MIC_{50} for the clinical isolates (**Tables 1, 2**). Thus, the MDR clinical isolates, which were isolated from patients with cystic fibrosis and therefore likely previously exposed to β-lactam drugs, were still largely vulnerable to those cephalosporins and carbapenems with activity against MABC transpeptidase targets. These findings also suggest that the widely used and well-characterized *M. abscessus* strain ATCC 19977 (29) is a suitable model strain for the evaluation of β-lactam activity, with or without β-lactamase inhibitors, and therefore can serve as a valuable tool for

studying the molecular mechanisms of intrinsic susceptibility and resistance of MABC to β-lactams.

CLSI guidelines state that broth MIC testing of rapidly-growing mycobacteria, including MABC, should be performed using CAMHB (22), but we found that our MABC clinical isolates grew poorly in this medium, allowing MIC reading to be done after 4, 5, or even 6 days of incubation (**Fig. S1, Table S3**). Due to concerns that the inherent instability of β-lactams may lead to artificially high MIC values after such prolonged incubation periods (8, 23-28), we relied primarily on Middlebrook 7H9 liquid medium for the broth MIC assays in this study. However, we also evaluated a selection of carbapenems and cephalosporins in CAMHB, and found that, in general, the MICs were indeed higher in CAMHB compared to Middlebrook 7H9 medium (**Tables 2, S4**). We also observed that MICs against strain ATCC 19977, which grew equivalently in either type of media, tended to be higher in CAMHB, indicating that media-specific factors may influence the anti-MABC activity of β-lactams. There are other reports of higher β-lactam MICs against strain ATCC 19977 in CAMHB compared to in Middlebrook 7H9 liquid medium (16, 18, 19). As CAMHB is recommended for use in clinical microbiology laboratories for antimicrobial susceptibility testing of MABC isolates from patient samples, it will be important to understand the basis of these apparent media-dependent discrepancies in MABC susceptibility to β-lactams. Ultimately, the imperative is to understand which MICs in any given medium correlate with *in vivo* activity. Preclinical animal models suitable for testing the antimicrobial activity of β-lactams against MABC would be useful to address this issue, but the absence of a well-qualified animal model is currently a critical missing link in our ability to translate *in vitro* activity to clinical utility.

In conclusion, the data presented in this study demonstrate that the β-lactamase inhibitors vaborbactam and relebactam markedly improve the anti-MABC activity of many carbapenems

and cephalosporins and should improve the clinical utility of some of these β-lactams. Each of these β-lactamase inhibitors is currently formulated with a carbapenem: vaborbactam with meropenem and relebactam with imipenem, and our data suggest that these fixed combinations will be more useful for the treatment of MABC disease than either carbapenem alone, especially in the case of meropenem. Moreover, the combinations of meropenem/vaborbactam and, if it is approved in the future, imipenem/relebactam, would make it unnecessary to combine the carbapenems with the marketed ceftazidime/avibactam combination in order to gain effective β-lactamase inhibition. Hence, they may quickly become the preferred β-lactams for MABC lung disease. Finally, given the burgeoning interest in combining β-lactams from different classes to obtain synergistic effects against mycobacteria, our findings suggest several cephalosporins whose potency is enhanced by Bla_{Mab} inhibition that are worthy of combining with a fixed carbapenem/β-lactamase inhibitor combination. Cefuroxime may be of particular interest given its intrinsic potency and relatively narrow spectrum of activity. Future studies should examine the efficacy of such dual β-lactam/β-lactamase inhibitor combinations at clinically relevant exposures in preclinical models of MABC infection.

MATERIALS AND METHODS

Bacterial strains. *M. abscessus* strain ATCC 19977 was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained per the provider's instructions. The MABC clinical isolates were obtained from the Johns Hopkins Hospital Clinical Microbiology Laboratory as previously described (16) and were maintained in the laboratory similarly as the ATCC 19977 strain. These isolates, from predominantly the sputum of patients with cystic fibrosis, are resistant to most antimicrobials currently available for treatment of MABC lung disease. Their drug resistance profiles were previously described (16).

296 **β-lactams and β-lactamase inhibitors.** Faropenem, tebipenem, and biapenem were
297 purchased from Octagon Chemicals Limited (Hangzhou, China). Doripenem hydrate, cefdinir,
298 cefoxitin sodium salt, ceftazidime hydrate, cloxacillin sodium salt monohydrate, dicloxacillin
299 sodium salt monohydrate, flucloxacillin, and oxacillin sodium salt were purchased from Sigma-
300 Aldrich (St. Louis, MO, USA). Ertapenem sodium salt, cefixime trihydrate, cefpodoxime free
301 acid, cefuroxime sodium salt, cephalexin monohydrate, cefazolin sodium salt, cefoperazone
302 sodium salt, cefotaxime sodium salt, ceftriaxone disodium salt hemi(heptahydrate), cephalothin
303 sodium salt, moxalactam sodium salt, aztreonam, and amoxicillin trihydrate were purchased
304 from Research Product International (Mount Prospect, IL, USA). Imipenem monohydrate,
305 meropenem trihydrate, and cefepime dihydrochloride monohydrate were purchased from
306 Carbosynth (San Diego, CA, USA). Ceftaroline was manufactured by Astra-Zeneca.
307 Vaborbactam was purchased from MedChem Express (Monmouth Junction, NJ, USA).
308 Relebactam was purchased from Advanced ChemBlocks, Inc. (Burlingame, CA, USA). The
309 purity of all compounds was >95%. Drug powders were stored at either 4°C or 20°C, per the
310 manufacturers' instructions to ensure stability. Drugs were dissolved in dimethyl sulfoxide or
311 deionized water, again per the manufacturers' instructions, on the day of use in MIC or disk
312 diffusion assays.
313
314 **Media.** Middlebrook 7H9 broth supplemented with 0.5% (v/v) glycerol, 10% (v/v) Middlebrook
315 OADC enrichment, and 0.05% (v/v) Tween 80, was used as the growth medium for all routine
316 culturing of MABC strains, including from frozen stocks. Two types of MIC assay media were
317 used in this study. 7H9 assay medium consisted of Middlebrook 7H9 broth supplemented with
318 0.5% (v/v) glycerol and 10% (v/v) Middlebrook OADC enrichment, without Tween 80; CAMHB
319 assay medium consisted only of Mueller-Hinton II broth powder. 7H11 agar supplemented with
320 0.5% (v/v) glycerol and 10% (v/v) Middlebrook OADC enrichment was used for disk diffusion
321 assays. Middlebrook 7H9 broth powder, Middlebrook OADC enrichment, 7H11 agar, and

CAMHB were purchased from Becton, Dickinson, and Co., (Hunt Valley, MD, USA). Glycerol and Tween 80 were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Growth curves. MABC strains were grown from frozen stock in growth medium, shaking, at 37°C, until the cultures reached log-phase growth (optical density at 600 nm [OD_{600}] between 0.4 to 0.7). The bacterial suspensions were then adjusted to an OD_{600} of 0.01 using 7H9 and CAMHB MIC assay media, in a total volume of 15 mL in 50-mL polystyrene tubes. The cultures were incubated without agitation at 30°C, and the OD_{600} of each culture was measured after 2, 3, 5, and 7 days of incubation.

MIC broth microdilution assays. MIC assays were performed in 96-well, round-bottom plates. MABC strains were grown from frozen stock in growth medium, shaking, at 37°C, until the cultures reached log-phase growth. The bacterial suspensions were then adjusted to 1×10^5 to 5×10^5 colony forming units per milliliter in assay medium, and 100 µl of this bacterial suspension was added to each well, except for the no bacteria controls. Drug stock solutions prepared from powder were diluted in assay medium to achieve the appropriate concentrations in a final assay volume of 200 µl. Vaborbactam and relebactam were used at a final concentration of 4 µg/ml (31, 39), a concentration readily achievable for both β-lactamase inhibitors in human plasma (40, 41). Plates were sealed and incubated, undisturbed, at 30°C for at least 72 hours or until there was sufficient growth in the drug-free bacterial growth control wells (22). The MIC was defined as the lowest concentration of β-lactam that prevented growth as observed by the naked eye. Two biological replicates of all MIC assays were performed. MIC_{50} and MIC_{90} were defined as the MIC at which at least 50% and 90% of the clinical MABC strains were inhibited, respectively.

Disk diffusion assays. MABC strain ATCC 19977 was grown from frozen stock to mid-log phase in growth medium until mid-log phase (OD_{600} at least 0.5). If necessary, bacterial suspensions were adjusted to an OD_{600} of 0.5, and 1 ml of the suspension was spread over 7H11 agar in a 100 mm-diameter petri dish. All drugs were prepared from powder in dimethyl sulfoxide at 10 mg/ml, further diluted in Middlebrook 7H9 broth to 1 mg/ml, and the drug solutions were applied to paper disks, 7 mm in diameter, which had been prepared from Whatman qualitative filter paper, grade 1 (Sigma-Aldrich, St. Louis, MO, USA) and sterilized prior to use. For the β-lactams, 20 µl, delivering 20 µg of drug, were applied to each disk. For the β-lactamase inhibitors alone, either 20 or 4 µl, delivering 20 or 4 µg, respectively, were applied to the disks. When used in combination with a β-lactam, 4 µl, delivering 4 µg of either relebactam or vaborbactam were applied to the appropriate disks. The disks were then air-dried and transferred using sterile forceps to the MABC-covered agar plates. The disks were pressed lightly on the agar surface to ensure contact with the bacteria. Plates were sealed in plastic bags and incubated for 4 days at 37°C.

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TABLE 1 MIC values of β-lactams with and without β-lactamase inhibitors[a] against *M. abscessus* strain ATCC 19977

	MIC (μg/ml) in 7H9 media[b]		
β-lactam tested	Alone	With relebactam	With vaborbactam
Oral (carba)penems			
Faropenem	128	32	32
Tebipenem	256	8	16
Parenteral carbapenems			
Biapenem	16	4	4
Doripenem	16	4	4
Ertapenem	>256	16	32
Imipenem	8	4	4
Meropenem	16	4	4
Oral cephalosporins			
Cefdinir	64	16	32
Cefixime	>256	256	256
Cefpodoxime	>256	64	64
Cefuroxime[c]	256	16	32
Cephalexin	>256	256	256
Parenteral cephalosporins			
Cefazolin	>256	>256	>256
Cefepime	32	16	16
Cefoperazone	>256	>256	>256
Cefotaxime	256	64	64
Cefoxitin	32	32	32
Ceftaroline	>256	16	32
Ceftazidime	>256	>256	>256
Ceftriaxone	>256	64	64
Cephalothin	>256	>256	>256
Moxalactam	256	128	128
Monobactam			
Aztreonam	>256	>256	>256
Penicillins			
Amoxicillin	>256	32	>256
Cloxacillin	>256	>256	>256
Dicloxacillin	>256	>256	>256
Flucloxacillin	>256	>256	>256
Oxacillin	>256	>256	>256

[a] Relebactam and vaborbactam were each used at a fixed concentration of 4 μg/ml.
[b] Assay medium was Middlebrook 7H9 broth supplemented with 0.5% (v/v) glycerol and 10% (v/v) Middlebrook OADC enrichment; MIC values were determined after 72 hours of incubation at 30°C.
[c] Cefuroxime is available in both oral and parenteral formulations.

TABLE 2 MIC values of β-lactams with and without β-lactamase inhibitors[a] against 28 MDR MABC clinical isolates

β-lactam tested	MICs (μg/mL) in 7H9 media[b] for MABC clinical isolates[c]								
	Alone			With relebactam			With vaborbactam		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Oral (carba)penems									
Faropenem	64 - >256	128	256	16 – 64	32	64	16 – 64	32	64
Tebipenem	64 - >256	256	>256	4 – 64	8	32	8 – 256	32	64
Parenteral carbapenems									
Biapenem	8 - 128	32	128	2 – 16	4	16	4 – 32	8	16
Doripenem	16 - 256	32	128	2 – 16	4	16	4 – 32	4	16
Ertapenem	128 - >256	256	>256	16 – 256	32	128	16 – 256	32	256
Imipenem	4 – 64	16	32	2 – 16	8	16	2 – 32	8	16
Meropenem	8 - >256	32	128	2 – 32	4	16	4 – 64	8	32
Oral cephalosporins									
Cefdinir	32 - 256	64	128	8 – 64	16	64	8 – 128	16	64
Cefpodoxime	64 - >256	256	>256	32 – 128	64	64	32 – 128	64	128
Cefuroxime[d]	16 - >256	128	>256	8 – 32	16	32	8 – 32	16	32
Parenteral cephalosporins									
Cefepime	16 – 128	32	64	8 – 64	16	64	8 – 64	16	64
Cefotaxime	32 – 256	128	256	16 – 64	32	64	16 – 128	32	64
Cefoxitin	16 – 128	32	64	16 – 64	32	64	16 – 64	32	64
Ceftaroline	256 - >256	>256	>256	8 – 256	16	64	8 – 128	32	64
Ceftriaxone	128 - >256	>256	>256	16 - 128	32	128	16 - 128	64	128

[a] Relebactam and vaborbactam were each used at a fixed concentration of 4 μg/ml.
[b] Assay medium was Middlebrook 7H9 broth supplemented with 0.5% (v/v) glycerol and 10% (v/v) Middlebrook OADC enrichment; MIC values were determined after 72 hours of incubation at 30°C.
[c] MIC values for each strain are provided in **Table S1** (carbapenems) and **Table S2** (cephalosporins).
[d] Cefuoxime is available in both oral and parenteral formulations.

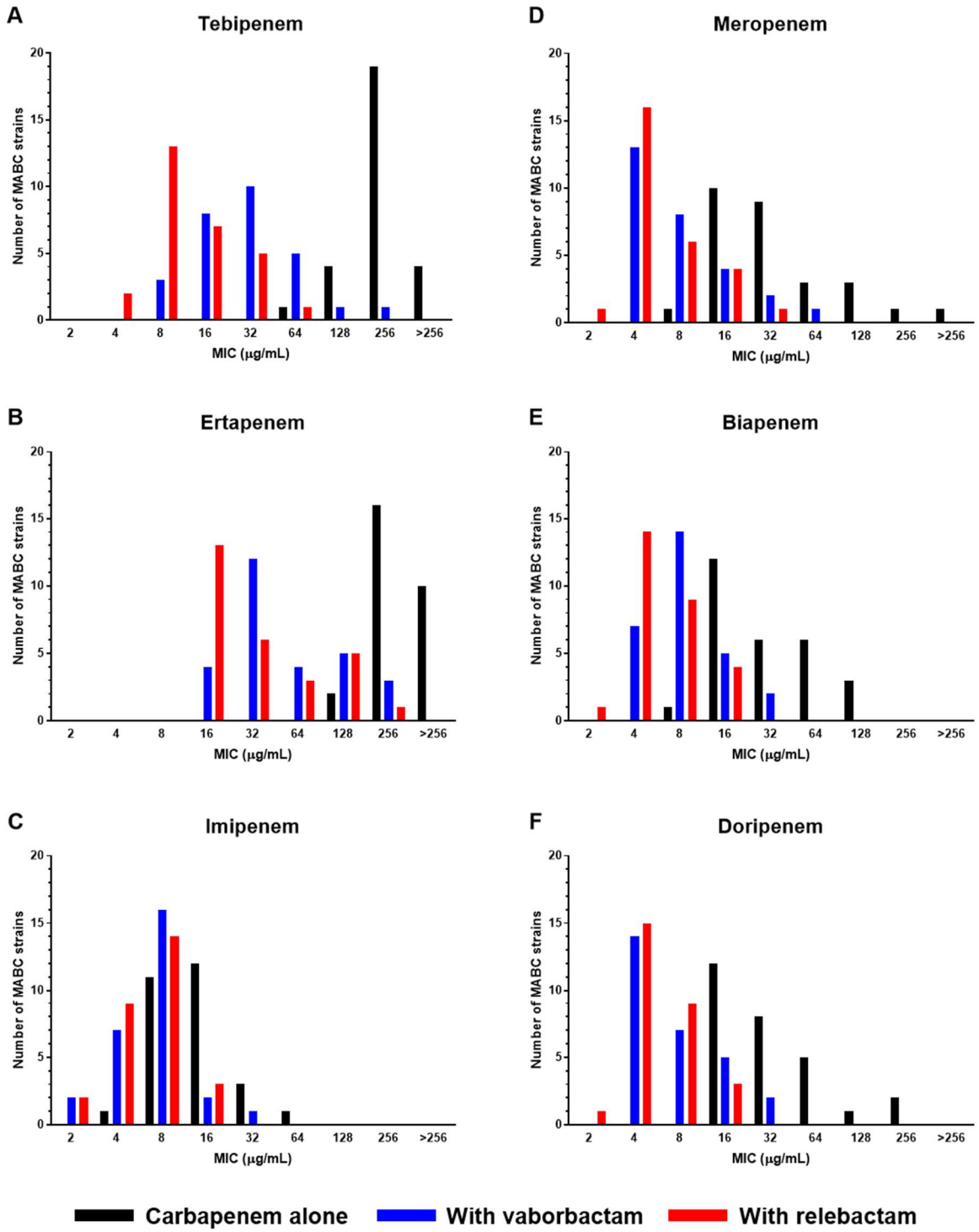

FIG. 1 MIC distributions of carbapenems, alone and in combination with 4 μg/ml relebactam or vaborbactam, against 28 MABC clinical isolates.

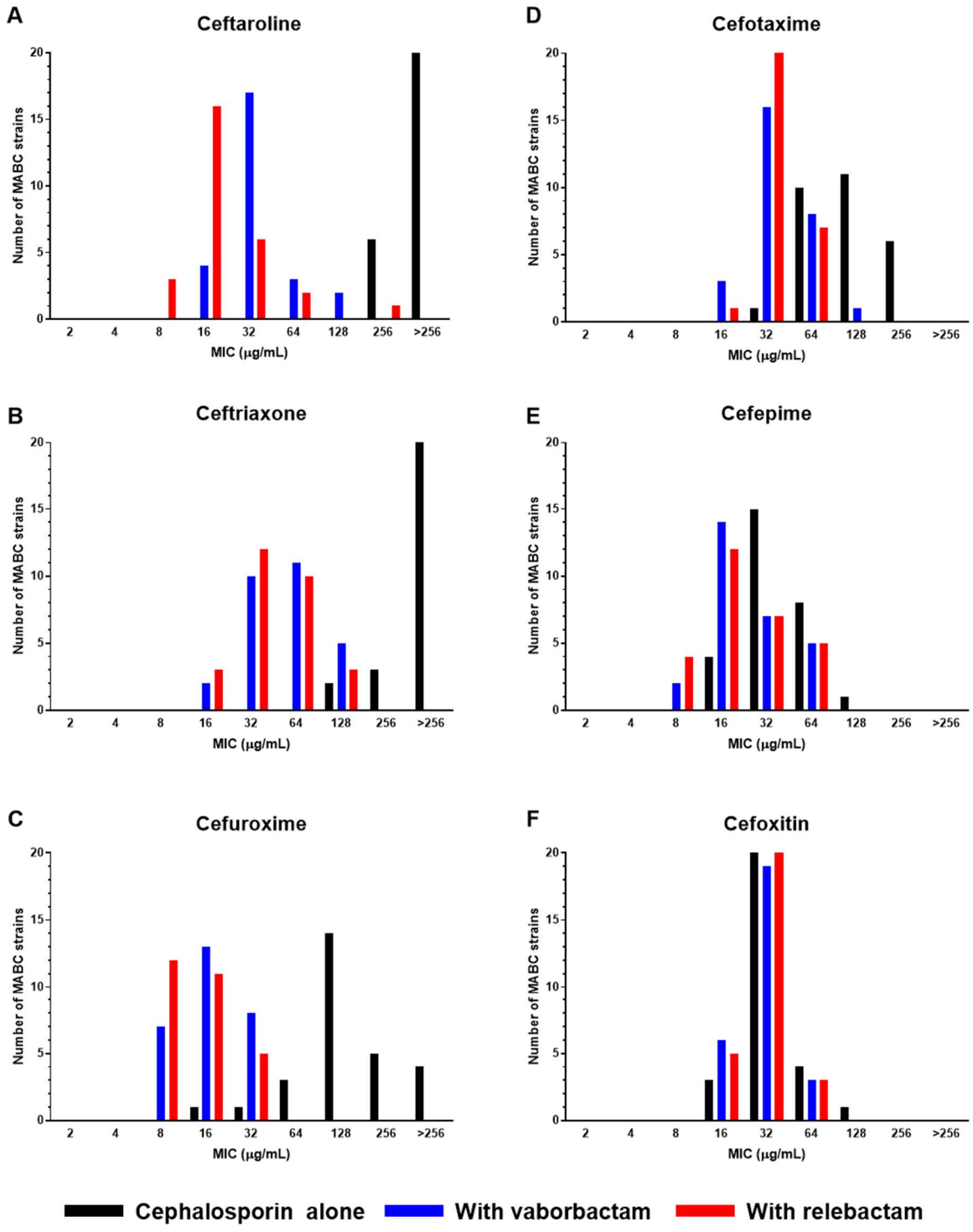

FIG. 2 MIC distributions of cephalosporins, alone and in combination with 4 µg/ml relebactam or vaborbactam, against 28 MABC clinical isolates.