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Effect of β-lactamase production and β-lactam instability on MIC testing results for *Mycobacterium abscessus*

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Objectives: Limited treatment options available for *Mycobacterium abscessus* infections include the parenteral β-lactam antibiotics cefoxitin and imipenem, which show moderate *in vitro* activity. Other β-lactam antibiotics (except meropenem) have no considerable *in vitro* activity, due to their rapid hydrolysis by a broad-spectrum β-lactamase (Bla_*Mab*). We here addressed the impact of β-lactamase production and β-lactam *in vitro* stability on *M. abscessus* MIC results and determined the epidemiological cut-off (ECOFF) values of cefoxitin, imipenem and meropenem.

Methods: By LC high-resolution MS (LC-HRMS), we assessed the *in vitro* stability of cefoxitin, imipenem and meropenem. *M. abscessus* ATCC 19977 strain and its isogenic bla_{Mab} deletion mutant were used for MIC testing. Based on MIC distributions for *M. abscessus* clinical strains, we determined ECOFFs of cefoxitin, imipenem and meropenem.

Results: A functional Bla_*Mab* increased MICs of penicillins, ceftriaxone and meropenem. LC-HRMS data showed significant degradation of cefoxitin, imipenem and meropenem during standard antibiotic susceptibility testing procedures. MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are influenced by incubation time.

Conclusions: The results of our study support administration of imipenem, meropenem and cefoxitin, for treatment of patients infected with *M. abscessus*. Our findings on *in vitro* instability of imipenem, meropenem and cefoxitin explain the problematic correlation between *in vitro* susceptibility and *in vivo* activity of these antibiotics and question the clinical utility of susceptibility testing of these chemotherapeutic agents.

Introduction

Mycobacterium abscessus is a dreadful and arduous to treat mycobacterial pathogen with a high level of innate resistance to most commercially available antibiotics, including the antituberculous agents.[1–5] Clinically relevant cases are predominantly associated with pulmonary infections in patients with cystic fibrosis or bronchiectasis and disseminated disease in immunocompromised individuals.[2–4] *M. abscessus* is also highly resistant to disinfectants and therefore it ordinarily causes skin and soft tissue infections following plastic surgery, tattooing or body piercing.[3,5–8] Several healthcare-associated outbreaks of *M. abscessus* infections, that have been reported worldwide, highlight the increasing medical importance of this MDR pathogen and the urgent need for reliable medication strategies.[3,4,9]

As *M. abscessus* clinical isolates are uniformly resistant to standard chemotherapeutic agents, so far, no reliable antibiotic regimen for *M. abscessus* pulmonary infections has been established. Antibiotic administration is empirical and heavily relies on *in vitro* antibiotic susceptibility testing (AST) by broth microdilution and definitive subspecies identification.[3,10] The clinical importance of *M. abscessus* subspecies identification (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) is attributed to the fact that *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* have a functional inducible erythromycin ribosome methyltransferase gene [*erm(41)*], which confers macrolide resistance, whereas *M. abscessus* subsp. *massiliense* has a non-functional *erm(41)*, leading to macrolide susceptibility, and thus *M. abscessus* subsp. *massiliense* infections have more favourable prognosis and treatment outcomes.[10–18] The American Thoracic Society recommends for the treatment of *M. abscessus* lung infections a combination therapy of an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to

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macrolides and the intravenous aminoglycoside drug amikacin, administered together with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[3,19–21] However, with the exception of the macrolide class, very limited data are present in the literature concerning the correlation between AST and the clinical efficacy of these recommended antimycobacterial drugs.[22] The clinical relevance of AST remains a controversy, particularly on account of technical problems associated with AST methods, reproducibility of AST results, significant discrepancies between *in vitro* susceptibility and *in vivo* activity of a given drug and solubility and stability issues of the drugs used.[23] Ideally, clinicians could take advantage of AST for *M. abscessus*, when the *in vitro* susceptibility to a drug is consistent with a clinically achievable drug exposure *in vivo*, resulting in favourable treatment outcomes. Accordingly, clinical susceptibility breakpoints must represent MIC distributions and zone diameter distributions for WT and resistant strains, resistance mechanisms, dosing regimens, drug pharmacokinetics, pharmacodynamics and epidemiological cut-off (ECOFF) values and must allow prediction of treatment outcomes.[22–25] CLSI is the only organization worldwide that has published AST guidelines for rapid growing mycobacteria[24] recommending susceptibility testing of amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline or minocycline, imipenem, linezolid, moxifloxacin, trimethoprim/sulfamethoxazole and tobramycin.[25] The breakpoint concentrations for these antibiotics have nonetheless been set on the basis of *in vitro* driven studies and their efficacy (except the new drugs linezolid and tigecycline) has been clinically evaluated by Wallace *et al.*[26] principally in patients with extrapulmonary disease. To date, EUCAST has not officially announced guiding principles for non-tuberculous mycobacteria susceptibility testing.

Frequently used therapeutics for *M. abscessus* infections are the parenteral β-lactam antibiotics cefoxitin and imipenem,[24] which have moderate *in vitro* activity, with MIC values reported by Dubée *et al.*[27] for *M. abscessus* subsp. *abscessus* CIP 104536 of 32 and 8 mg/L, respectively. Meropenem, an ultra-broad-spectrum carbapenem closely related to imipenem,[28] displayed a lower MIC value (16 mg/L) than cefoxitin for *M. abscessus* subsp. *abscessus* CIP 104536.[27] Most of the other β-lactam antibiotics have no considerable *in vitro* activity due to their rapid hydrolysis by a broad-spectrum class A β-lactamase, encoded by *MAB_2875*, namely Bla_*Mab*, which was reported as the major determinant of β-lactam resistance in *M. abscessus*.[27,29] Determination of the kinetic parameters of this enzyme revealed that it can slowly hydrolyse imipenem and meropenem, while cefoxitin hydrolysis by Bla_*Mab* is immensely slow, as the methoxy group at cefoxitin's β-lactam ring was predicted to block the activity of class A β-lactamases.[27,29,30]

As imipenem, meropenem and cefoxitin are known to have limited *in vitro* stability,[31] we assessed by LC high-resolution MS (LC-HRMS) the *in vitro* stability of these β-lactams and, by exploiting a bla_{Mab} deletion mutant that we generated, we addressed the biological effect of β-lactam stability and β-lactamase production on MIC testing results after different periods of incubation. The direct impact of β-lactam stability on AST testing was further addressed by MIC determination of fresh and pre-incubated β-lactam antibiotics for the ampicillin-susceptible *Escherichia coli* XL1-Blue MRF′ strain. What is more, based on MIC distributions for *M. abscessus* clinical strains, mainly isolated from respiratory samples, we estimated ECOFFs of cefoxitin, imipenem and meropenem. Our results show that MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are severely influenced by stability issues, thus questioning the clinical utility of AST of cefoxitin, imipenem and meropenem, but not the use of these antibiotics in patients with *M. abscessus* infections.

Materials and methods

Bacterial strains and growing conditions

(i) *E. coli* strains were cultivated in LB medium or on LB agar plates, when necessary, containing either ampicillin (120 mg/L) or apramycin (50 mg/L), at 37 °C, overnight. For *E. coli* MIC testing and all cloning steps when constructing the bla_{Mab} deletion vector pSE-*katG-aac(3)IV*-Δ*MAB_2875*, *E. coli* XL1-Blue MRF′ (Stratagene, Switzerland) was used. (ii) *M. abscessus* strains were grown in Middlebrook 7H9-OADC-Tween 80 liquid medium or on LB agar plates, when needed, containing either apramycin (50 mg/L) or isoniazid (32 mg/L), at 37 °C, for 5 days. The *M. abscessus* ATCC 19977 type strain, the *M. abscessus* Δbla_{Mab} mutant strain and 62 clinical isolates of *M. abscessus* (33 *M. abscessus* subsp. *abscessus*, 17 *M. abscessus* subsp. *bolletii* and 12 *M. abscessus* subsp. *massiliense* isolates) were used throughout this study. The *M. abscessus* clinical isolates were mainly isolated from respiratory samples that were received at the Swiss National Center for Mycobacteria within the years 2007–14.

Definitive subspecies identification of clinical isolates

Subspecies identification of *M. abscessus* isolates (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) was based on the 16S rRNA, *rpoB* and *erm(41)* gene sequences.[12–14,32,33] Subspecies attribution for *M. abscessus* complex isolates was performed according to Tortoli *et al.*[12] The obtained sequences were analysed with the use of Lasergene SeqMan software (DNASTAR, USA), the SmartGene IDNS mycobacteria and *rpoB* databases (SmartGene, Switzerland), and the BLAST algorithm (http://blast.ncbi.nlm.nih.gov).

Antibiotics

Imipenem, meropenem, cefoxitin, ceftriaxone, ceftazidime, penicillin G, ampicillin, amoxicillin, amikacin, apramycin and isoniazid were bought from Sigma–Aldrich, Switzerland. All compounds were dissolved in H_2O, according to the manufacturer's recommendations, filter sterilized, aliquoted into stock solutions of 3–50 g/L and finally stored at −20 °C.

Deletion of MAB_2875 in M. abscessus

A 1.4 kbp HpaI/Pfl23II fragment from position 2926650 to 2928011 (5′bla_{Mab} flanking sequence) and a 1.5 kbp Pfl23II/Pfl23II fragment from position 2928642 to 2930147 (3′bla_{Mab} flanking sequence) were PCR amplified using genomic DNA from *M. abscessus* ATCC 19977 (5′-GAATTAGTTAACCAGGTGTGATCCAGATGCCCG-3′, 5′-GAATTTCGTACGGGCCGCCGAAATCCTTTTCC-3′ and 5′-GAAATACGTACGCCATCGTGATGGCGGTACTCAC-3′, 5′-GAATTTCGTACGGTGTCTACCAGTCCTTGCACACC-3′, respectively) and cloned into the pSE-*katG-aac(3)IV* vector with corresponding enzymes, resulting in the bla_{Mab} deletion vector pSE-*katG-aac(3)IV*-Δ*MAB_2875*. *M. abscessus* bla_{Mab} deletion mutant was generated using a double selection strategy described previously in detail by Rominski *et al.*[34,35] In brief, pSE-*katG-aac(3)IV*-Δ*MAB_2875* was transformed into electrocompetent *M. abscessus* ATCC 19977. Competent cells (100 μL) were mixed with 1–2 μg of plasmid DNA (supercoiled) and electroporated in a Bio-Rad Gene pulser II using 4 mm gap electroporation cuvettes and the settings: 2.5 kV, 1000 Ω and 25 μF.[36] Cells were resuspended after electroporation in 0.9 mL of 7H9-OADC-Tween 80 liquid medium and incubated for 5 h at 37 °C. Proper dilutions were eventually

plated on selective agar and, after 5 days of incubation, single colonies were picked and restreaked on LB agar plates with appropriate antibiotics. Transformants were selected on LB agar plates containing apramycin and identified by *aac(3)IV* colony PCR (primers: 5′-CACCTTCTTCACGAGG CAGACCTC-3′ and 5′-GGTCTGACGCTCATGGAACTAGTAGG-3′). Isolation of genomic DNA was performed by phenol-chloroform-isoamyl alcohol extraction as described previously[37] and single crossover transformants were confirmed by Southern blot analysis with a 0.45 kbp SacI 3′bla_{Mab} DNA probe and subjected to counterselection on LB agar plates containing isoniazid. Single colonies were screened for deletion of *MAB_2875* by PCR (primers: 5′-GTACACCGTCTTCGGGACG-3′ and 5′-GAAAGTGCGAGTACGCGTCTG-3′) and their genotype was finally verified by Southern blot analysis using the same 0.45 kbp SacI 3′bla_{Mab} DNA probe. In this way, a 0.63 kbp region of the *MAB_2875* was deleted from the genome of *M. abscessus* ATCC 19977.

Nitrocefin test

For the detection of β-lactamase production in the *M. abscessus* ATCC 19977 type strain and the Δbla_{Mab} mutant strain we performed a chromogenic nitrocefin test (Becton Dickinson, USA). Tubes containing (i) pure nitrocefin solution, (ii) nitrocefin solution inoculated with five colonies of the *M. abscessus* ATCC 19977 type strain and (iii) nitrocefin solution inoculated with five colonies of the *M. abscessus* Δbla_{Mab} mutant were incubated for 1 h at room temperature before a photo was taken.

MIC assays

MIC assays were performed with the microdilution method and according to CLSI guidelines,[25] but with incubation of the 96-well microtitre plates at 37 °C, as described previously.[34,35] Directly after the preparation of the antibiotic stock solutions, working solutions were prepared by diluting the corresponding stock solutions in CAMHB (pH 7.4) (Becton Dickinson, Switzerland) to a concentration of 512 mg/L (final antibiotic concentration used for AST; 256 mg/L) and were subsequently stored at −80 °C. Shortly before the conduct of an MIC experiment (day 0), working solutions were thawed for the first time and were directly used. Alternatively, working solutions were thawed and pre-incubated for 1–7 days at 37 °C and their inhibitory effect was compared with freshly thawed antibiotics. Two-fold serial dilutions of the freshly thawed (and pre-incubated: for the pre-incubation MIC experiments) working solutions were prepared using CAMHB in sterile 96-well microtitre plates (Greiner Bio-One, Switzerland). Each 96-well microtitre plate included a positive growth control lacking antibiotic and a sterile negative control containing only CAMHB. To achieve a final inoculum titre of $1–5\times10^5$ cfu/mL, while final volume per well was 100 μL, bacterial suspensions of strains with smooth or rough phenotypes were adjusted to a McFarland standard of 0.5 and 3.0, respectively, and subsequently diluted in CAMHB. The proper titre of the inocula was confirmed by obtaining cfu counts on LB agar plates. MIC values were assessed by visual inspection after incubation at 37 °C for 16 h for *E. coli* strains and 3, 5, 7 and 12 days for *M. abscessus*. MIC assays for *E. coli*, *M. abscessus* ATCC 19977 and the *M. abscessus* bla_{Mab} deletion mutant were conducted in triplicate. MIC assays for *M. abscessus* complex clinical isolates were performed once.

LC-HRMS assays

In vitro antimicrobial stability of cefoxitin, imipenem and meropenem was evaluated using CAMHB and the maximum final antibiotic concentration used for AST (256 mg/L). A different test tube was prepared for each antibiotic and for every reading timepoint, i.e. 3, 5, 7 and 12 days. A day 0 control was used to define the 100% relative antibiotic concentration. The antibiotic-containing test tubes were incubated under the same temperature conditions as the microtitre plates used for AST (37 °C). Cefoxitin, imipenem and meropenem were quantified by LC-HRMS on a Q Exactive hybrid instrument (Thermo Fisher, Switzerland). Samples were precipitated using a precipitation solution consisting of methanol/acetonitrile (80/20, v/v), containing the corresponding stable-isotope labelled internal standards. After centrifugation at 11700 **g** for 10 min at 4 °C, 10 μL of the clear supernatant was injected into the turbulent flow online extraction system. As extraction column a Cyclone column (50×0.5 mm) was used and for analytical chromatography a Hypersil Gold C8 column (100×3 mm) was used. Mobile phases consisted of 10 mM ammonium acetate in water + 0.1% formic acid and 10 mM ammonium acetate in methanol/acetonitrile (50/50, v/v) + 0.1% formic acid. Samples were analysed in positive heated electrospray ionization mode and detection was done in full-scan mode with a resolution of 70000 full width at half maximum (calculated for *m/z* 200).

ECOFF determination

MIC data for 62 *M. abscessus* complex clinical isolates were collected and the resistance population analysis charts were calculated using the integrated histographical analysis tool of Microsoft Excel. Median MIC_{50} values of cefoxitin, imipenem and meropenem for the *M. abscessus* complex clinical strains were calculated using SPSS software. All ECOFF values were determined by the eyeball method.[38,39]

Results and discussion

Generation of the M. abscessus MAB_2875 deletion mutant

Similarly to our previously published techniques,[34,35] we intended to generate an *M. abscessus* Δ*MAB_2875* mutant, which would enable us to address directly the role of the β-lactamase production of *M. abscessus* on MIC testing results of β-lactam antibiotics. The bla_{Mab} deletion mutant was constructed by transformation of *M. abscessus* ATCC 19977 with plasmid pSE-*katG-aac(3)IV*-Δ*MAB_2875* applying an apramycin-positive selection[40] and a *katG*-dependent isoniazid counterselection strategy (Figure 1a).[34,35] Deletion of *MAB_2875* was confirmed by Southern blot analysis (Figure 1b). While this study was ongoing, a Δ*MAB_2875* mutant was also generated by Dubée *et al.*,[27] but with different cloning, genetic manipulation and selection strategies.

M. abscessus bla_{Mab} deletion mutant is deficient in β-lactamase production

To assess the β-lactamase production of the *M. abscessus* Δbla_{Mab} mutant, we performed a chromogenic nitrocefin test. The cephalosporin nitrocefin changes colour from yellow to red in the presence of β-lactamases that hydrolyse the amide bond in its β-lactam ring.[41] As Figure 2 shows, the tube containing yellow pure nitrocefin solution (Figure 2a) did not change colour. The tube containing nitrocefin solution inoculated with *M. abscessus* ATCC 19977 (Figure 2b) changed colour and became red, while the tube containing nitrocefin solution inoculated with the *M. abscessus* Δbla_{Mab} mutant (Figure 2c) stayed yellow, confirming that the *M. abscessus* bla_{Mab} deletion mutant is deficient in β-lactamase production. These results align with the findings from Dubée *et al.*,[27] who reported Bla_*Mab* as the single determinant of β-lactam resistance in *M. abscessus*.

AST of M. abscessus ΔMAB_2875

To explore the role of Bla_*Mab* in *M. abscessus* β-lactam resistance, MIC values for the *M. abscessus* ATCC 19977 type strain and the *M. abscessus* Δbla_{Mab} mutant were determined with the microdilution method for the β-lactam antibiotics, imipenem,

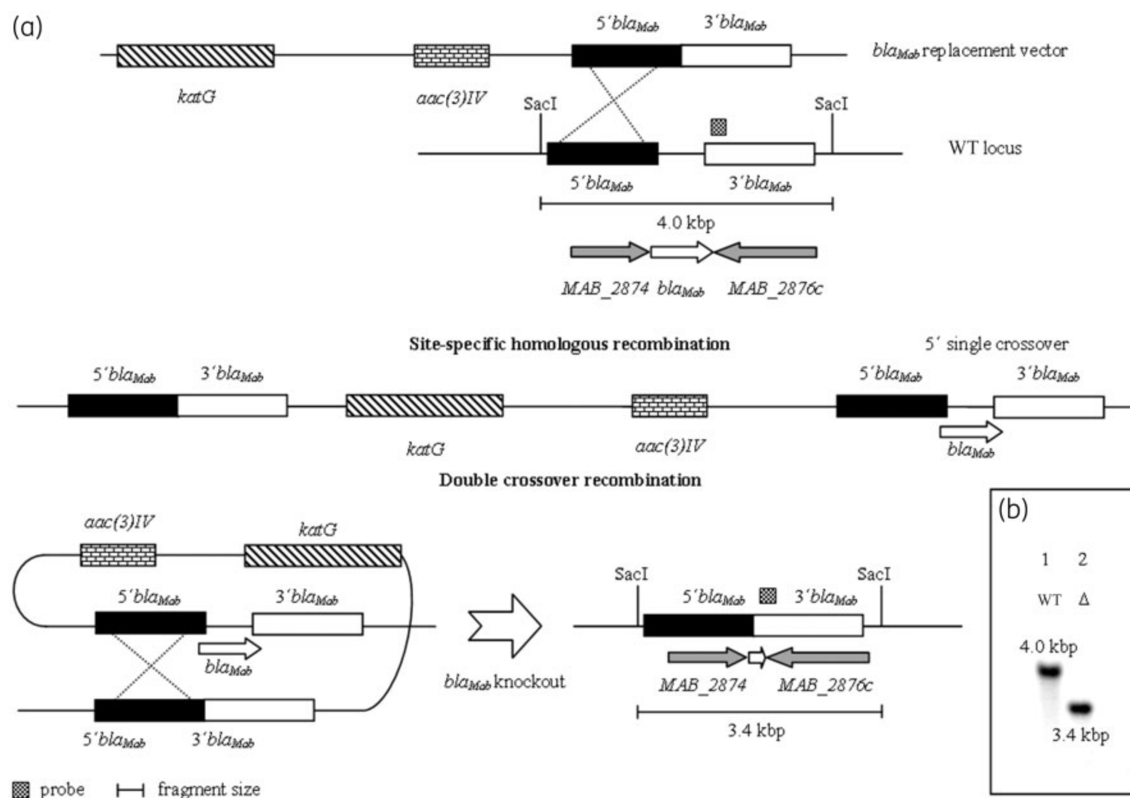

Figure 1. Genotypic analyses of the *M. abscessus* bla_{Mab} locus. (a) Schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirmed the deletion of *MAB_2875* from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1) and *M. abscessus* transformant with pSE-*katG-aac(3)IV*-Δ*MAB_2875* targeting vector after KatG-dependent isoniazid counterselection (2) was digested with SacI and probed with a 0.45 kbp fragment from the 3′ bla_{Mab} flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 4.0 kbp fragment of the WT parental strain and to a 3.4 kbp fragment of the *M. abscessus* Δbla_{Mab} mutant (Δ).

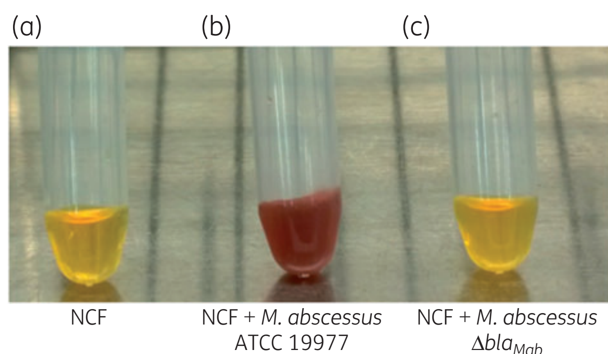

Figure 2. Chromogenic nitrocefin solution test results after incubation for 1 h at 37 °C. (a) Tube containing pure nitrocefin solution. (b) Tube containing nitrocefin solution inoculated with *M. abscessus* ATCC 19977 WT. (c) Tube containing nitrocefin solution inoculated with the *M. abscessus* Δbla_{Mab} mutant. NCF, nitrocefin. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

meropenem, cefoxitin, ceftriaxone, ceftazidime, penicillin G, ampicillin and amoxicillin, and a control antibiotic from the class of aminoglycosides, i.e. amikacin (Table 1). As formerly reported,[27] *M. abscessus* ATCC 19977 was highly resistant to all β-lactam antibiotics tested except imipenem, meropenem and cefoxitin. Deletion of bla_{Mab} significantly reduced the MIC values of all penicillins tested (penicillin G, ampicillin and amoxicillin) and the cephalosporin ceftriaxone. MIC values of ≥256 mg/L of the oxyimino-cephalosporin ceftazidime for both *M. abscessus* ATCC 19977 and the *M. abscessus* Δbla_{Mab} mutant suggest that ceftazidime is not active against *M. abscessus*. Cefoxitin MIC values of 32 mg/L (day 3) for both *M. abscessus* ATCC 19977 and the Δbla_{Mab} mutant (Table 1) agree with earlier reported data,[27] corroborating previous molecular modelling and structural analyses,[42] which delineated that substitutions of the β-lactam ring by a methoxy group in cefoxitin inhibits the activity of class A β-lactamases.[30] As expected, amikacin MICs were independent of the *bla* genotype.

To date, none of the recent studies on β-lactam susceptibility testing of *M. abscessus* CIP 104536 and the *M. abscessus* Δbla_{Mab} mutant has reported MIC data obtained after 3 days of incubation.[27,29,30,43,44] Indeed, CLSI guidelines[25] for imipenem MIC testing for rapid growing mycobacteria recommends a maximum incubation period of 3 days and state that 'the reported breakpoints for imipenem are considered tentative'. Our observation that imipenem, meropenem and cefoxitin MIC values rise substantially with the incubation time for both the *M. abscessus* ATCC 19977 strain and the *M. abscessus* Δbla_{Mab} mutant (Table 1), in correlation with the fact that certain antibiotics undergo partial degradation

under *in vitro* testing conditions,[45] led us to investigate further the effect of the β-lactam stability on MIC testing procedures.

Effect of β-lactam stability on E. coli MIC testing

To determine experimentally whether the results of the *M. abscessus* MIC testing of β-lactams after 3, 5 and 7 days of incubation clearly reflect β-lactamase production or are affected by instability of the antibiotics tested, we performed an MIC experiment using the *E. coli* XL1-Blue model strain, which produces no β-lactamases, and fresh and pre-incubated (at 37 °C, for 1–7 days) β-lactam antibiotics. In that way, all MIC results could be obtained after 16 h of incubation of the microtitre plates and putative MIC differences between the fresh and the pre-incubated antibiotics could be directly correlated to instability of the tested antibiotics. Our results show that pre-incubation at 37 °C affects MIC results of imipenem, meropenem and to a lesser extent cefoxitin and ceftazidime (Figure 3a and b), whereas the MIC results of ceftriaxone, penicillin G, ampicillin, amoxicillin and as expected the control stable aminoglycoside antibiotic amikacin are not influenced by pre-incubation (Figure 3b, c and d). By assuming that drug degradation follows an exponential decay process and using the data displayed in Figure 3, we calculated the half-life values of imipenem as 0.75 days, of meropenem as 1.5 days and of cefoxitin as 3 days. We therefore conclude that the increased MIC values of the pre-incubated antibiotics, already before day 3, reflect instability of the antibiotics. Reading of *M. abscessus* MICs at day 3 and later gives a *de facto* misleading impression for the true extent of activity of imipenem, meropenem and cefoxitin.

Table 1. AST results of *M. abscessus* WT and Δbla_{Mab} mutant (broth microdilution method)

	MIC (mg/L)							
	M. abscessus ATCC 19977				*M. abscessus* Δbla_{Mab}			
Antibiotic	day 3	day 5	day 7	day 12	day 3	day 5	day 7	day 12
Imipenem	2	4	8	64	1	2	4	64
Meropenem	8	16	32	256	2	4	4	16
Cefoxitin	32	32	64	128	32	32	32	128
Ceftriaxone	64	64	64	64	8	16	16	16
Ceftazidime	>256	>256	>256	>256	256	>256	>256	>256
Penicillin G	>256	>256	>256	>256	2	4	4	4
Ampicillin	>256	>256	>256	>256	4	4	8	8
Amoxicillin	>256	>256	>256	>256	2	4	4	4
Amikacin	1	2	4	8	1	2	4	8

In vitro antimicrobial stability of cefoxitin, imipenem and meropenem by LC-HRMS

Based on our results suggesting that incubation of cefoxitin, imipenem and meropenem at 37 °C gives rise to misleading MIC

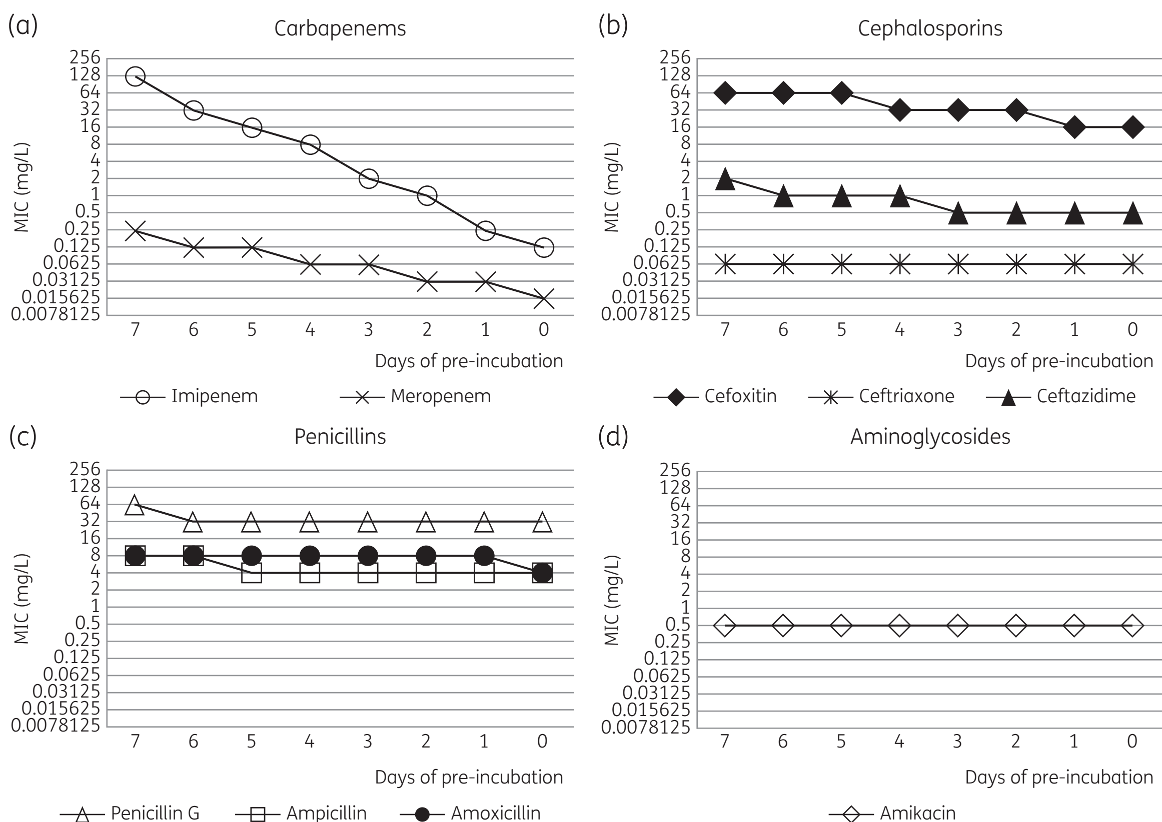

Figure 3. MIC values for *E. coli* XL1-Blue strain of fresh and pre-incubated (at 37 °C, for 1–7 days) β-lactam antibiotics. (a) Carbapenems: imipenem and meropenem. (b) Cephalosporins: cefoxitin, ceftriaxone and ceftazidime. (c) Penicillins: penicillin G, ampicillin and amoxicillin. (d) Aminoglycoside (control): amikacin.

results, we intended to address the *in vitro* stability of these β-lactams, by quantifying them using LC-HRMS. Tubes containing CAMHB and the maximum antibiotic concentration used for AST (256 mg/L) were incubated at 37 °C. The 100% relative antibiotic concentration of each antibiotic was defined by a day 0 control tube. On days 3, 5, 7 and 12 the concentrations of cefoxitin, imipenem and meropenem were quantified. Data obtained by the LC-HRMS method, clearly show degradation of cefoxitin, imipenem and meropenem. Particularly, the relative concentration on day 3 for imipenem dramatically dropped to 3.3%, whereas for cefoxitin and meropenem it dropped to 36.6% and 35.6%, respectively (Figure 4e, j and o). By using the results of this assay, the calculated half-life of imipenem was 0.6 days, that of meropenem was 2 days and that of cefoxitin was 2.1 days. These findings agree with those deducted from the *E. coli* MIC testing and confirm that the MIC values of cefoxitin, imipenem and meropenem for *M. abscessus* on day 3 and later are misleading, as concentration of the active compound is lowered. Particularly for imipenem, MIC_{50} on day 3 was 16 mg/L (Figure 4j). However, growth inhibition of half of the clinical isolates was in reality achieved by considerably lower drug amounts, as the true active relative concentration of imipenem shrank to 3.3% by day 3 due to the short half-life of this antibiotic. Consequently, due to instability of imipenem, meropenem and cefoxitin, *M. abscessus* MIC determination after 3 days of incubation does not reflect the potency of these three compounds, particularly as clinical administration[3] of these drugs is daily and in multiple doses. These data, on the one hand, question the utility of susceptibility testing of imipenem, meropenem and cefoxitin, but, on the other hand, support administration of these parenteral β-lactam antibiotics as part of combinational regimens for the treatment of *M. abscessus* infections.

ECOFF values of cefoxitin, imipenem and meropenem for M. abscessus complex

According to EUCAST, for the determination of clinical susceptibility breakpoint values and improvement of the MIC interpretation for clinical isolates, ECOFFs need to be defined to separate the WT population from any non-WT strains with acquired drug resistance mechanisms to the chemotherapeutic agents in question.[46] We therefore endeavoured to estimate ECOFF values of cefoxitin, imipenem and meropenem by visual inspection of the MIC histographic distribution analysis (eyeball method)[36,37] for 62 *M. abscessus* complex clinical strains isolated from respiratory samples, after 3, 5, 7 and 12 days of incubation at 37 °C (Figure 4). The results show that MIC distributions for our collection of *M. abscessus* complex isolates of cefoxitin, imipenem and meropenem are close to the breakpoints established by CLSI and that the eyeball-estimated ECOFF values did not show a separation of the *M. abscessus* complex isolates into two distinct subgroups, WT and resistotype. ECOFFs were set at the highest MIC value observed among the isolates tested and all of these isolates are therefore classified in the WT subgroup. According to this classification, a putative clinical isolate with an additional β-lactam resistance mechanism would have a very high MIC value (higher than the corresponding ECOFF) that would be neither detected by standard AST laboratory procedures nor relevant for clinical interpretation. Interestingly, the presence or absence of a functional or non-functional β-lactamase in *M. abscessus* could not be predicted by the MIC distribution for the clinical isolates, as the MIC values determined for *M. abscessus* Δbla_{Mab} (Table 1) fall in the MIC distribution for the 'WT' subgroup of clinical isolates of all three β-lactams (Figure 4).

Furthermore, our results show that MIC and MIC_{50} values of cefoxitin, imipenem and meropenem are greatly influenced by incubation time; the longer the incubation period, the higher the MIC for the individual strains, the MIC_{50} and the estimated ECOFF values (Figure 4). The presented MIC distribution bars, as well as the MIC_{50} and ECOFF lines, would represent valid data for MIC, MIC_{50} and ECOFF values, only if the drugs were 100% stable. However, according to the LC-HRMS results, cefoxitin, meropenem and particularly imipenem were proven as unstable compounds and thus MIC values for the *M. abscessus* complex clinical isolates of these three β-lactams on day 3 and later are certainly overestimated, as already discussed. If the concentration of the biological active form of the drug could have been kept constant during the AST procedures, we expect that the position of the MIC distribution bars and MIC_{50} and ECOFF lines would be shifted to the left in all MIC distribution analysis graphs (Figure 4). Our observations highlight the impact of cefoxitin, imipenem and meropenem stability issues on MIC, MIC_{50} and ECOFF determination for *M. abscessus* complex clinical isolates.

Conclusions

Taken together, our study confirmed Bla_*Mab* as the major determinant of innate β-lactam resistance in *M. abscessus* and addressed experimentally the biological effect of β-lactamase production and β-lactam stability on *M. abscessus* MIC testing results after different incubation periods. Our results show that MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are immensely influenced by incubation time. LC-HRMS data and MIC determination of pre-incubated drugs for the fast growing model organism (*E. coli*) proved significant degradation of cefoxitin, imipenem and meropenem during standard AST procedures, explaining the problematic correlation between *in vitro* susceptibility of these three β-lactams and their *in vivo* activity. Our findings critically question the clinical utility of cefoxitin, imipenem and meropenem susceptibility testing, but further support administration of these chemotherapeutic agents for the treatment of *M. abscessus* infections.

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Transparency declarations

None to declare.

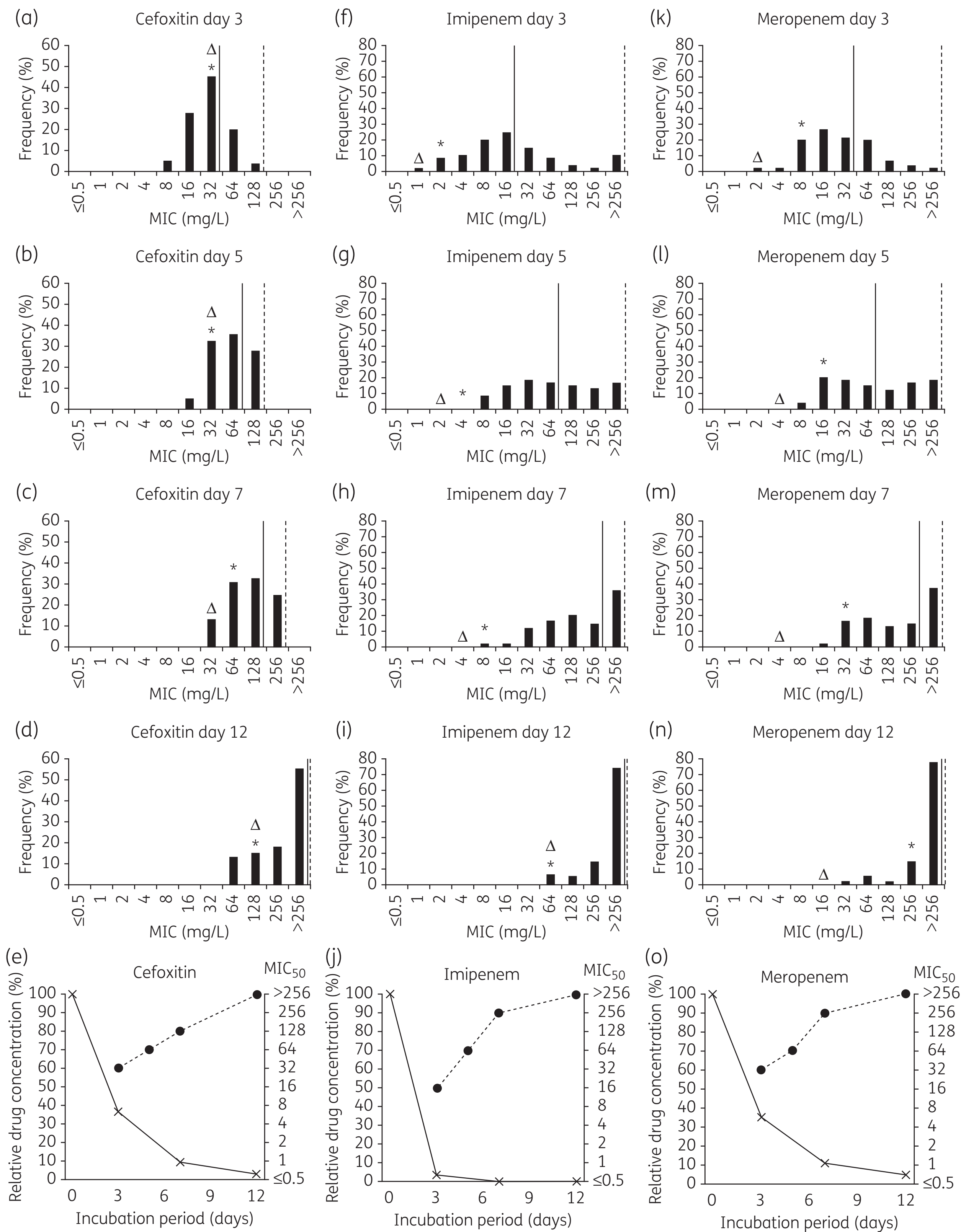

Figure 4. MIC distributions of cefoxitin (a–d), imipenem (f–i) and meropenem (k–n) for *M. abscessus* complex strains ($n = 62$). MIC_{50} values are indicated by a black vertical continuous line, whereas ECOFF values are indicated by a black vertical broken line. *, MIC values for the *M. abscessus* ATCC 19977 type strain; Δ, MIC values for the *M. abscessus* Δbla_{Mab} mutant. Drug stability of cefoxitin (e), imipenem (j) and meropenem (o) in comparison with median MIC_{50} values. (e, j and o) Crosses connected with a black continuous line show relative drug concentrations and filled circles connected with a black broken line show MIC_{50} values.

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