International Journal of Antimicrobial Agents 53 (2019) 868–872

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Repurposing azithromycin and rifampicin against Gram-negative pathogens by combination with peptide potentiators

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ARTICLE INFO

Article history:
Received 6 June 2018
Accepted 30 October 2018

Editor: Professor Jian Li

Keywords:
Antimicrobial peptide
Gram-negative bacteria
Multidrug resistance
Antibiotic adjuvant
Combination therapy
Antibiotic potentiation

ABSTRACT

Gram-negative bacterial pathogens are intrinsically resistant to several antibiotics that are not able to penetrate the cell envelope barrier. The aim of this study was to identify peptides that at low concentrations induce susceptibility to these antibiotics in multidrug-resistant (MDR) Gram-negative bacterial strains of clinical relevance. Pairwise screening of 34 diverse peptides and four antibiotics (erythromycin, linezolid, rifampicin and vancomycin) with primary activity against Gram-positive bacteria identified 4 peptides that at submicromolar concentrations conferred susceptibility to rifampicin or erythromycin in *Escherichia coli* ATCC 25922. The identified peptides exhibited synergy with azithromycin and potentiated clindamycin in MDR *E. coli* ST131 and *Klebsiella pneumoniae* ST258. The low cytotoxicity toward eukaryotic cells (IC_{50} > 50 μM) observed for two of these peptides (KLWKKWKKWLK-NH_2 and GKWKKILGKLIR-NH_2) prompted synthesis and evaluation of the corresponding all-D analogues (**D1** and **D2**), which retained similar synergistic antibacterial profiles. Low concentrations of **D1** and **D2** in combination with azithromycin and rifampicin inhibited growth of most clinical *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii* strains tested. These data demonstrate that combinatorial screening at low peptide concentrations constitutes an efficient approach to identify clinically relevant peptide–antibiotic combinations. *In vivo* pharmacokinetic/pharmacodynamic and toxicity studies are needed to further validate the use of the peptides identified in this study for repurposing azithromycin and rifampicin against Gram-negative pathogens.

1. Introduction

Due to the worldwide spreading of multidrug-resistant (MDR) Gram-negative bacterial clones, the World Health Organization (WHO) has ranked the development of new therapeutics to treat infections caused by Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as a critical priority [1]. Intrinsic antimicrobial resistance considerably limits the therapeutic options against these pathogens since several classes of available antibiotics cannot effectively penetrate the cell envelope barrier of Gram-negative bacteria [2]. Combination therapy represents an attractive approach for treating MDR infections as it typically reduces the required dose of the individual components and limits the risk of emergence of resistance [3,4]. Antimicrobial peptides that increase the therapeutic potency and expand the spectrum of antibiotics to include Gram-negative pathogens have potential use in combination therapy [3–5]. Although many reports have demonstrated synergistic peptide–antibiotic interactions, the clinical potential of such findings have rarely been studied systematically.

The aim of this study was to identify peptides that at low non-toxic concentrations render MDR Gram-negative bacterial pathogens susceptible to antibiotics to which they are intrinsically resistant. Following a systematic approach, a pairwise screening was designed based on the antibacterial activity of low concentrations of a diverse set of peptides in combination with four antibiotics with primary activity against Gram-positive bacteria. Subsequently, peptide-induced antibiotic susceptibility was confirmed, and cytotoxicity was then assessed for the top four antibiotic-potentiating peptides. This resulted in identification of

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https://doi.org/10.1016/j.ijantimicag.2018.10.025
0924-8579/

two lead peptides that displayed low cytotoxicity to different eukaryotic cell types and potentiated azithromycin and rifampicin against several Gram-negative bacterial species of clinical relevance.

2. Materials and methods

2.1. Media, antibiotics, bacterial strains and peptide synthesis

Bacteria were cultured in Luria–Bertani broth as well as cation-adjusted Mueller–Hinton agar (MHA) and Mueller–Hinton broth II (MHBII). All media and antibiotics were purchased from Sigma-Aldrich (Brøndbyvester, Denmark). ATCC reference strains included *Escherichia coli*, *Klebsiella pneumoniae*, *P. aeruginosa* and *A. baumannii*. MDR strains *E. coli* ST131 and *K. pneumoniae* ST258 are clinical isolates from urinary tract [6] and wound infections [7], respectively. A panel of β-lactam-resistant clinical isolates were provided by Prof. Laurent Poirel (Department of Medicine, University of Fribourg, Fribourg, Switzerland). Starting materials and solvents for peptide synthesis were purchased from commercial suppliers [Iris Biotech (Marktredwitz, Germany), Sigma-Aldrich and VWR (Søborg, Denmark)]. Peptides and all-D analogues were synthesised and analysed as previously described [8] (Supplementary Figs S6–S13). Peptide stock solutions were made in deionised water followed by dilution in MHBII.

2.2. Antimicrobial susceptibility testing

Bacterial susceptibility to compounds was determined by the microbroth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. In the screening, antibiotics and peptides were combined at fixed concentrations corresponding to the antibiotics' CLSI susceptibility breakpoint for *Staphylococcus* spp. [10]. Antibiotic minimum inhibitory concentrations (MICs) in the presence of peptide concentrations (0.5 μM or 1 μM) were determined as above. For growth curve assays, the MIC plates were prepared as above, and then the plates were incubated for 24 h at 37 °C with continuous shaking. The optical density at 600 nm was recorded at 10-min intervals.

2.3. Checkerboard assay

Synergy of peptide–antibiotic combinations was measured using a two-dimensional checkerboard assay [11] and CLSI guidelines [9]. The fractional inhibitory concentration index (FICI) was calculated and interpreted as previously reported [12].

2.4. Cellular viability and 50% inhibitory concentration (IC_{50})

Cell viability was determined in ATCC NIH 3T3 fibroblasts and HepG2 hepatocytes using the MTS/PMS assay as previously reported [13]. Peptide concentrations ranged from 0.1–500 μM. Relative cell viability was calculated according to Eq. 1 with 100% (Abs_{pos}) and 0% cell death (Abs_{neg}) defined as the absorbance values obtained after incubation of cells with sodium dodecyl sulfate (SDS) (0.2% w/v in medium) and with medium, respectively.

$$\text{Relative viability (\%)} = \frac{(Abs_{sample} - Abs_{pos})}{(Abs_{neg} - Abs_{pos})} \times 100\% \quad (1)$$

IC_{50} values were calculated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA) by fitting the relative viability of the cells to the concentration of the test compound using Eq. 2:

$$\text{Relative viability (\%)} = \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogIC}_{50} - \text{Log[peptide]}) \times \text{Hill slope}}} \quad (2)$$

with top and bottom values constrained to 100% and 0%, representing the mean of the highest and lowest observed values, respectively. Data were collected from technical triplicates.

2.5. Time–kill assay

Time–kill kinetics assays were performed in *K. pneumoniae* ATCC 13883 and *A. baumannii* ATCC 19606. Briefly, ca. 10^6 CFU/mL of log-phase cells were transferred to 15-mL round-bottom tubes and were incubated for 24 h at 37 °C with aeration in the presence or absence of antibiotic, peptide or their combination. At each time point, 100 μL of cells was serially diluted in sterile 0.9% NaCl and then 10 μL aliquots were plated on MHA in triplicate. The CFU/mL from each condition was calculated following 18–24 h of incubation at 37 °C. The detection limit was 10^2 CFU/mL. All time–kill curves represent the mean and standard deviation from biological duplicates. Synergy was defined as a $\geq$2 $\log_{10}$ CFU/mL decrease for the antibiotic–peptide combination relative to that of the individual compounds.

3. Results and discussion

To identify peptide-induced antibiotic susceptibility, a pairwise combinatorial screening of 34 peptides and four antibiotics with poor activity against Gram-negative bacteria (rifampicin, erythromycin, vancomycin and linezolid) was performed by assaying growth inhibition of *E. coli* ATCC 25922. Since potentiation of antibiotics is a frequent characteristic of cationic peptides, screening at low peptide concentrations (1 μM or 0.5 μM) and at clinically relevant antibiotic concentrations [10] would identify the most potent antibiotic potentiators, thus expediting the discovery of peptides with potential clinical utility. Three peptides (**1**, **2** and **3**) exhibited growth inhibition in combination with rifampicin or erythromycin (Supplementary Fig. S1). A fourth peptide (**4**) was selected for further analysis owing to its ability to enhance susceptibility to both rifampicin and erythromycin at 0.5 μM (Supplementary Fig. S1). All four peptides had a low MIC of 2 μM against *E. coli* ATCC 25922. These peptides were all short (9–13 residues), highly cationic and possessed similar hydrophobicity as estimated from their retention in reversed-phase analytical high-performance liquid chromatography (HPLC) (Table 1) [14–16]. None of the 34 screened peptides induced susceptibility to linezolid or vancomycin.

The ability of the four identified peptides to induce antibiotic susceptibility in two epidemic MDR clones with high clinical relevance (*E. coli* ST131 and *K. pneumoniae* ST258) was evaluated by determining the MICs of rifampicin, erythromycin, clindamycin and azithromycin in combination with low concentrations ($\leq$1 μM) of peptide. In the presence of subinhibitory concentrations of peptide (Table 1), the MICs of the antibiotics were reduced considerably, resulting in synergistic peptide–antibiotic combinations with estimated FICIs ranging from 0.02 for rifampicin to 0.38 for azithromycin (Table 2; Supplementary Table S1). The reductions in antibiotic MICs ranged from 8-fold for azithromycin in combination with peptides **1** and **4** to $\geq$500-fold for rifampicin in the presence of peptides **2** and **3**. All four peptides reduced the MICs of rifampicin and azithromycin to below susceptibility breakpoints [10] in both strains. For clindamycin, the most favourable interactions were observed for peptides **2** and **3** in *E. coli* ST131, with a reduction of the MICs below the resistance breakpoint [10]. In *K. pneumoniae* ST258, the clindamycin MICs reached the resistance breakpoint. Susceptibility to erythromycin was not achieved, most likely due to the high MICs of this macrolide in the two strains (256 μg/mL and 512 μg/mL, respectively). Consequently, azithromycin was chosen as the representative macrolide for further analyses. For the above combinations that reduced the antibi-

Table 1
Peptide sequences, physicochemical characteristics, cytotoxicity and minimum inhibitory concentrations (MICs) in *Escherichia coli* and *Klebsiella pneumoniae*

Peptide	Sequence	Length (residues)	MW (g/mol)	Molecular mass (Da)		Net charge[a]	Retention time (min)	Cytotoxicity (μM)[b]		MIC (μM)		
				Calc.	Obs.			NIH 3T3	HepG2	*E. coli* ATCC 25922	*E. coli* ST131	*K. pneumoniae* ST258
1	KLWKKWKKWLK-NH_2	11	2369.17	1571.01	1571.02	+7	6.73	51 ± 35	105 ± 21	2	2	64
2	GKWKKILGKLIR-NH_2	12	2121.92	1438.97	1438.97	+6	7.14	143 ± 21	175 ± 38	2	2	32
3	KKWRKWLKWLAKK-NH_2	13	2710.50	1798.15	1798.16	+8	6.93	20 ± 13	19 ± 3	2	2	4
4	KWRRWIRWL-NH_2	9	1968.85	1398.84	1398.84	+5	7.42	43 ± 16	34 ± 8	2	2	4
D1	klwkkwkkwlk-NH_2	11	2369.17	1571.01	1571.00	+7	6.66	ND	ND	2	2	64
D2	gkwkkilgklir-NH_2	12	2121.92	1438.97	1438.97	+6	7.16	ND	ND	2	2	32
D3	kkwrkwlkwlakk-NH_2	13	2710.50	1798.15	1798.14	+8	6.89	ND	ND	2	2	4
D4	kwrrwirwl-NH_2	9	1968.85	1398.84	1398.84	+5	7.38	ND	ND	2	2	4

MW, molecular weight; ND, not determined.
[a] At pH 7.4.
[b] 50% inhibitory concentration (IC_{50}) (mean ± standard deviation); n= 3.

Table 2
Minimum inhibitory concentrations (MICs) of azithromycin (AZM), erythromycin (ERY), rifampicin (RIF) and clindamycin (CLI) in *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST258 exposed to low concentrations of peptides **1–4**

Antibiotic	MIC (μg/mL)[a]										Clinical breakpoint (μg/mL)[b]	
	E. coli ST131 with 0.5 μM of peptide					*K. pneumoniae* ST258 with 1 μM of peptide						
	1	**2**	**3**	**4**	None	**1**	**2**	**3**	**4**	None	≤S	≥R
AZM	**1**	**≤0.25**	**0.25**	**1**	8	**2**	**1**	**1**	**2**	32	2	8
ERY	4	4	1	4	256	8	8	4	8	512	0.5	8
RIF	**0.25**	**≤0.03**	**≤0.03**	**0.25**	4	**0.125**	**0.06**	**≤0.03**	**0.6**	16	1	4
CLI	≥8	1	2	4	>64	>8	4	4	4	>64	0.5	4

S, susceptible; R, resistant.
[a] MICs equal to or below the susceptibility breakpoint are in bold.
[b] Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints for *Staphylococcus* spp. [10].

otic MICs below the resistance breakpoints, synergy was confirmed by checkerboard assays (Supplementary Table S2).

As a preliminary evaluation of the toxicity, and thus potential for clinical application, the cytotoxicity for peptides **1–4** was determined in two relevant eukaryotic cell lines (Table 1). Peptides **1** and **2** exhibited low cytotoxicity with IC_{50} values >50 μM in mouse fibroblasts (NIH 3T3) and >100 μM in human hepatocytes (HepG2), whilst peptides **3** and **4** reduced cell viability with IC_{50} values of 19–43 μM (Table 1). Regardless, the peptide–antibiotic combinations were non-toxic at synergistic concentrations (Supplementary Fig. S2).

All-D analogues of the four selected peptides (hereafter denoted as D-peptides **D1–D4**) were synthesised and tested for their ability to induce susceptibility of MDR Gram-negative pathogens to azithromycin, rifampicin and clindamycin. The all-D analogues retained the MICs of the corresponding L-forms (Table 1) and exhibited synergy with the antibiotics in MDR *E. coli* ST131 and *K. pneumoniae* ST258 (Supplementary Table S2). As peptides **1** and **2** alone had significantly lower cytotoxicity compared with peptides **3** and **4**, it is likely that **D1** and **D2** will retain better safety profiles compared with **D3** or **D4**; hence the first two D-peptides were studied further.

The activity of **D1** and **D2** was further tested in combination with the same three antibiotics (azithromycin, rifampicin and clindamycin) using a collection of reference and clinical isolates of *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*. Overall, the MICs of rifampicin and azithromycin were reduced to below their respective susceptibility breakpoints in three reference strains when co-exposed to sub-MICs of peptides **D1** and **D2**, whilst the MIC of azithromycin was reduced to two-fold above the susceptibility breakpoint in *P. aeruginosa* (Supplementary Table S3). Similarly, the MICs of clindamycin were below the resistance breakpoint for *K. pneumoniae* and *A. baumannii*. In *P. aeruginosa*, the peptide–antibiotic combinations were overall not synergistic, and only borderline synergy was observed for combinations with rifampicin (Supplementary Table S3). Overall, the antibacterial activity of the D-peptide–antibiotic combinations against the reference strains reflected the activity observed against the clinical isolates (Supplementary Table S4). Most (83%) and ≥50% of the isolates, except for *P. aeruginosa*, were inhibited by the D-peptides in combination with rifampicin and azithromycin, respectively (Supplementary Table S4).

The above synergistic combinations were further investigated in growth curve assays, which showed that neither peptide nor antibiotic individually inhibited the growth of *E. coli* or *K. pneumoniae* at the concentration present in the synergistic combination (Supplementary Fig. S3A–J). However, *A. baumannii* growth was retarded in the presence of each antibiotic or peptide **D2** alone (Supplementary Fig. S3K–P).

Peptide **D2** was studied further to understand the bactericidal kinetics of **D2**–antibiotic combinations. This peptide was chosen based on its high potency in synergistic combinations. Time–kill experiments with **D2** in combination with antibiotics and alone were performed with reference strains of *A. baumannii* and *K. pneumoniae* that served as the representative of Enterobacteriaceae. All **D2**–antibiotic combinations exerted synergistic bactericidal effects in the time–kill assay (Fig. 1). Moreover, at sub-MIC levels of **D2** (i.e. ≤2 μM), all antibiotic concentrations were below their respective susceptibility breakpoints, except for clindamycin in *K. pneumoniae*.

Time–kill kinetics of the antibiotics, **D2** and their combinations were compared to examine whether the **D2**–antibiotic combinations were able to enhance the rate and efficiency of killing relative to either component individually. In both species, faster killing kinetics were achieved for the **D2** combinations with clindamycin and rifampicin than for either antibiotic alone (Supplementary Fig.

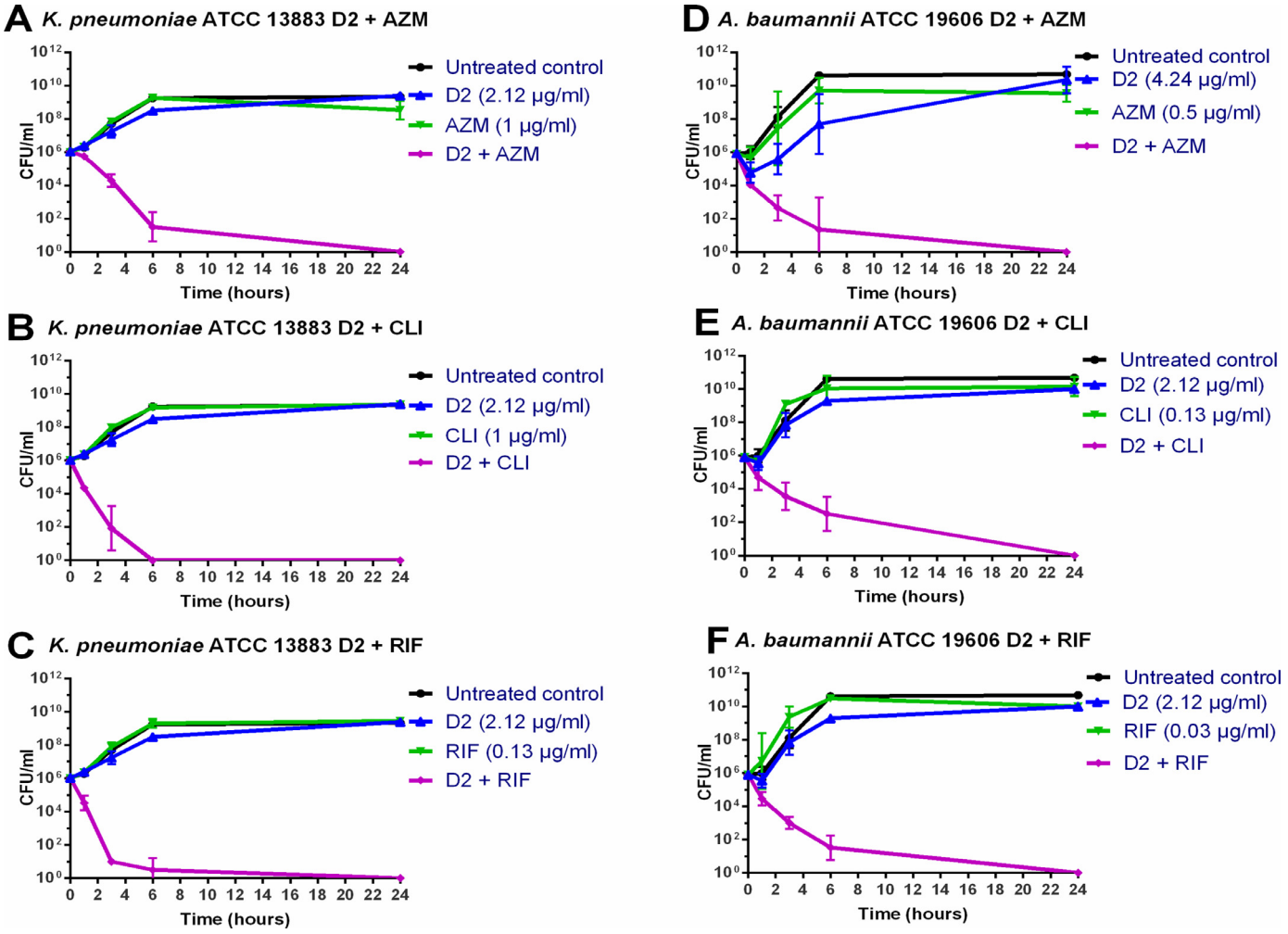

Fig. 1. Peptide **D2**–antimicrobial combinations kill bacteria synergistically. Time–kill kinetics for azithromycin (AZM), rifampicin (RIF) and clindamycin (CLI) as individual compounds and in combination with **D2** are presented for (A–C) *Klebsiella pneumoniae* ATCC 13883 and (D–F) *Acinetobacter baumannii* ATCC 19606, including only **D2** and untreated control. Curves of the synergistic combination and the untreated control are also depicted in Supplementary Fig. S4.

S4B,C,E,F). The **D2**–azithromycin combination also exhibited faster killing kinetics than azithromycin alone in *A. baumannii* (Supplementary Fig. S4D), whilst the combination displayed similar kinetics in *K. pneumoniae* (Supplementary Fig. S4A). However, **D2** did not exhibit efficient killing in *K. pneumoniae* (Supplementary Fig. S5) even at concentrations eight-fold above the MIC (Supplementary Table S5).

The approach developed in this study, which combines combinatorial screening at low compound concentrations with cytotoxicity testing, can be used to expedite discovery of clinically relevant peptide–antibiotic combinations. This approach enabled rapid identification of two peptides (**1** and **2**) that at low, non-toxic sub-MIC levels were able to circumvent intrinsic resistance to azithromycin and rifampicin in multiple Gram-negative species of clinical relevance, including epidemic MDR clones. Furthermore, the all-D peptide analogues induced susceptibility to rifampicin and azithromycin and reduced the MICs of clindamycin by >500-fold. These findings may help mitigate the lack of novel antibiotics effective against Gram-negative species by opening new avenues to repurpose these antibiotics for the treatment of infections caused by MDR Gram-negative pathogens.

Peptides **1** and **2** as well as their all-D analogues (**D1** and **D2**) exhibited substantial synergy with rifampicin, azithromycin and clindamycin in *K. pneumoniae* and *A. baumannii* (Supplementary Tables S2 and S3) at low (≤1 μM) non-toxic peptide concentrations. The present study constitutes the first report on antibiotic synergy of these peptides, whilst their antimicrobial activity, cytotoxic and haemolytic properties have been reported previously [17,18]. Notably, according to these studies peptides **1** and **2** do not exhibit haemolytic activity at concentrations up to 200 μM. Use of the analogue **D2** appears to be particularly promising for antibiotic potentiation since **D2**–antibiotic combinations displayed synergistic bactericidal activity (Fig. 1) and faster killing kinetics than each individual component (Supplementary Fig. S4). Importantly, *in vivo* pharmacokinetic/pharmacodynamic and toxicity studies are needed to fully assess the clinical potential of these findings.

In conclusion, intrinsic resistance to azithromycin and rifampicin in Gram-negative bacteria can be overcome by low peptide concentrations that are non-toxic to eukaryotic cells. The two peptide leads identified in this study merit further investigation as antibiotic potentiators for repurposing azithromycin and rifampicin against MDR Gram-negative pathogens.

Acknowledgment

The authors thank Prof. Laurent Poirel (Department of Medicine, University of Fribourg, Fribourg, Switzerland) for contributing the panel of clinical isolates used in the study.

Funding

This study was supported by the University of Copenhagen Research Centre for Control of Antibiotic Resistance (UC-Care) and by a grant from Zoetis.

Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.10.025.

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