International Journal of Antimicrobial Agents 52 (2018) 697–701

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

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

Short communication

Loss of activity of ceftazidime-avibactam due to MexAB-OprM efflux and overproduction of AmpC cephalosporinase in *Pseudomonas aeruginosa* isolated from patients suffering from cystic fibrosis

Hussein Chalhoub[a], Yolanda Sáenz[b], Wright W. Nichols[c], Paul M. Tulkens[a,*], Françoise Van Bambeke[a]

[a] *Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium*
[b] *Área de Microbiología Molecular, Centro de Investigación Biomédica de La Rioja (CIBIR), Logroño, Spain*
[c] *Consultant Microbiologist, Cambridge, MA, USA*

ARTICLE INFO

Article history:
Received 1 August 2017
Accepted 29 July 2018

Editor: Jeffrey Lipman

Keywords:
Avibactam
Ceftazidime
Cystic fibrosis
Pseudomonas aeruginosa
AmpC cephalosporinase
MexAB-OprM efflux pump

ABSTRACT

In *Pseudomonas aeruginosa* (*P. aeruginosa*) collected from cystic fibrosis (CF) patients, 24% resistance to ceftazidime-avibactam in isolates negative for carbapenemases and extended-spectrum β-lactamases (ESBLs) has previously been observed. The current study aimed to unravel the underlying mechanism(s). Using the laboratory strain PAO1 and derivatives thereof, with *ampC* expression induced by a sub-minimum inhibitory concentration (MIC) of imipenem, a higher MIC of ceftazidime-avibactam was found for those overexpressing MexAB-OprM (quantitative polymerase chain reaction (PCR) of *mexA*) and, to a lesser extent, MexEF-OprN (PCR of *mexE*), or without OprD expression (SDS-Page and Coomassie blue staining). This was ascribed to (i) an efflux of avibactam (efflux mutants) and (ii) a lack of avibactam penetration (OprD mutants), respectively. We then used 10 CF clinical isolates resistant to ceftazidime (MIC ≥ 128 mg/L) and with (i) variable basal levels of *ampC* overexpression, (ii) mutations in *mexA* or *mexB* inactivating to variable extent the MexAB-OprM transport capacity (assessed by extrusion of N-phenyl-1-naphthylamine [NPN]), and (iii) expression or not of *mexE* and of OprD porin. The reduction of ceftazidime MIC in the presence of avibactam was partially lost for isolates with large efflux activity of MexAB-OprM and/or increased *ampC* expression, but not significantly with *mexE* expression or lack of OprD (non-parametric and parametric tests). This identified MexAB-OprM as a main avibactam efflux transporter in *P. aeruginosa* that, together with *ampC* overexpression, reduced avibactam potency. Since about 30% of CF isolates show mutations in MexAB-OprM compromising efflux (Chalhoub, et al. Sci Reports 2017;7:40208), routine susceptibility testing of CF *P. aeruginosa* with ceftazidime-avibactam is warranted.

1. Introduction

Avibactam is a broad-spectrum inhibitor of β-lactamases approved in combination with ceftazidime for the treatment of Gram-negative infections with limited therapeutic options [1,2]. Since it shows in vitro activity against most class A and class C enzymes [3], including the chromosomally-encoded AmpC cephalosporinase of *Pseudomonas aeruginosa* (*P. aeruginosa*) [4], its activity was previously assessed against a large collection (n = 334) of *P. aeruginosa* isolates from patients suffering from cystic fibrosis (CF). This showed that 4 mg/L avibactam restored ceftazidime activity in 40% of the isolates but that 24% still showed minimum inhibitory concentration (MIC) values above the Food and Drug Administration (FDA) [1] and European Medicines Agency (EMA) [2] susceptibility breakpoint of ceftazidime-avibactam (≤ 8 mg/L), although scoring negative for carbapenemases or extended-spectrum β-lactamases (ESBLs) by genotypic testing [5,6].

The aim of the present study was to investigate possible mechanisms of resistance to the ceftazidime-avibactam combination in such isolates. We found that increased activity of the efflux pump MexAB-OprM (capable of extruding many drugs including β-lactam antibiotics and inhibitors of β-lactamases [7-9]) together with a high level of expression of *ampC* could explain this resistance, while the potential roles of OprD porin (originally described as facilitating the specific penetration of imipenem across the outer

* Corresponding author. Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Avenue Mounier 73 B1.73.05, 1200 Brussels, Belgium. Phone: +32-2-7647371.
E-mail address: tulkens@facm.ucl.ac.be (P.M. Tulkens).

https://doi.org/10.1016/j.ijantimicag.2018.07.027
0924-8579/

membrane of *P. aeruginosa* [10]) and of MexEF-OprN transporter remain unsettled.

2. Methods and Materials

Pseudomonas aeruginosa PAO1 and derivatives overproducing or defective in the expression of efflux pumps or OprD (see Table 1) were used as controls. Ten ceftazidime-resistant clinical isolates (Table 2) collected from patients suffering from CF in different countries (United Kingdom, Germany, and Belgium) were taken from our collection [5] to obtain a wide range of MICs of the ceftazidime-avibactam combination. These isolates had been previously characterized for genetic (clonality) relatedness (PFGE and/or MLST; see Table 2) and genotypically scored as negative for metallo-β-lactamases (VIM, IMP, NDM), serine carbapenemases (OXA-48, KPC), and the following ESBLs (BEL [BEL-1 to 3], PER [PER-1 to 5, 7], GES [GES-1 to 18], VEB [VEB-1 to 7], CTX-M [1, 2, 9], TEM, SHV, and OXA [1, 2, 9, 10, 18, 20, 23, 24, 30, 58, 198]) [6]. MICs were determined by microdilution in CA-MHB following the recommendations of Clinical and Laboratory Standards Institute (CLSI) [11] and using the interpretive criteria of both FDA and EMA (EUCAST (European Committee on Antimicrobial Susceptibility Testing)). To induce *ampC* expression, the reference strains and clinical isolates were grown until mid-log phase and then exposed to imipenem (0.25 mg/L; 1 h. For the BV1 strain, cefoxitin (500 mg/L; 1 h) was also used as an inducer). Cultures were sampled immediately before and after induction and pelleted for mRNA extraction. *ampC* was detected and quantified by real-time polymerase chain reaction (PCR) using published primers and amplification conditions [12] and the hydrolytic activity of its product towards cephalosporins was assessed by the Nordmann-Dortet-Poirel (NDP) ESBL test [13] in the presence of cefotaxime with phenol red as a pH indicator. The expression of genes encoding efflux pumps was assessed using previously published methods [14], with *mexA*, constitutively expressed in wild-type strains, quantified by real-time PCR and the inducible expression of *mexE* detected by PCR. The laboratory strains and clinical isolates used in the present study have previously been phenotypically tested for efflux activity of MexAB-OprM by measuring the extrusion of its fluorescent reference substrate *N*-phenyl-1-naphthylamine (NPN) as well as for mutations in *mexA* and *mexB* by sequencing [15]. The corresponding data were reproduced in this paper for the sake of completeness. OprD was detected by Coomassie Brilliant Blue staining after SDS-PAGE membrane protein separation [14]. Ceftazidime was procured as GLAZIDIM® (potency 88.2%; GlaxoSmithKline, Genval, Belgium) and used alone or combined with avibactam (investigational sample; potency 91.7%; AstraZeneca Pharmaceuticals; Waltham, MA). Statistical analyses were performed using GraphPad InStat version 3.10 (GraphPad Software, San Diego California USA, www.graphpad.com).

3. Results and Discussion

Table 1 shows the data obtained with the control strains. In the absence of imipenem, the ceftazidime MIC was 2 mg/L for PAO1 (i.e. close to the value observed in other studies) [4,16]. This MIC was increased by one dilution in the strain overexpressing MexAB-OprM (PT629) and decreased by one dilution in the corresponding defective mutant (PAO1 mexAB), but was not changed by the overexpression of other efflux pumps (strains MutGr1, PAO-7H, or EryR) or by the defect in OprD porin (strain CC-03). The addition of avibactam reduced the MICs of all these strains by one (or two) dilution(s) in accordance with their low basal level of *ampC* expression. These measures were therefore repeated in the presence of a sub-MIC concentration (0.25 mg/L) of imipenem to induce the

Table 1
Control strains: description and main properties.

Name	Description/origin	MIC (mg/L)[a]				MexAB-OprM/ MexEF-OprN efflux system characteristics				Porin OprD	*ampC* relative expression[g]	
		CAZ		CAZ/AVI[b]		Log_2 fold reduction[c]	*mexA* or *mexB* mutations Protein (AA[a] length)*	MexAB-OprM Speed V_{max} (unit/sec)[d,*]	Relative expression			
		- IMI	+ IMI[h]	- IMI	+ IMI[h]				*mexA*[e]	*mexE*[f]	- IMI	+ IMI[h]
PAO1	reference strain, ATCC	2	32	1	0.125	8	MexA (383) and MexB (1046)	**-0.48**	1	-	+	1.0	1880
PAO1 mexAB[i]	PAO1 MexAB-OprM defective mutant	1	32	0.5	0.125	8	MexA (0) and MexB (0)	-0.12	1	-	+	1.1	2450
PT629[i]	PAO1 overproducing MexAB-OprM	4	32	2	**4**	3	MexA (383) and MexB (1046)	**-0.72**	**9.3**	-	+	2.0	4230 [j]
MutGr1[i]	PAO1 overproducing MexXY-OprM	2	32	1	0.125	8	MexA (383) and MexB (1046)	ND	1.5	-	+	1.8	2919
PAO-7H[i]	PAO1 overproducing MexEF-OprN	1	32	1	**2**	4	MexA (383) and MexB (1046)	ND	1	**+**	+	1.2	2637
EryR[i]	PAO1 overproducing MexCD-Opr]	2	32	1	0.125	8	MexA (383) and MexB (1046)	ND	1.2	-	+	0.6	3630
CC-03[i]	PAOD1 OprD-defective mutant	2	32	1	**4**	3	MexA (383) and MexB (1046)	ND	1.7	-	-	3.0	1390

* data from [15]
[a] CAZ, ceftazidime; IMI, imipenem; AVI, avibactam at 4 mg/L; AA, amino acids; ND, not determined
[b] values in bold denote MIC that, in the presence of 4 mg/L avibactam, remained higher than those measured in the absence of imipenem
[c] log_2 of the MIC ratio [CAZ+IMI]/[(CAZ/AVI)+IMI]
[d] kinetics of efflux of NPN (fluorescence arbitrary units/sec; see detailed methodology in [15])
[e] relative amount of *mexA* mRNA (quantitative PCR) compared with *P. aeruginosa* strain PAO1 basal expression. According to previous studies [14,17] isolates were considered borderline or positive for *mexA* overexpression (values highlighted in bold) when the corresponding mRNA level was between 2–3 or ≥ 3 and 5–10 or ≥ 10 fold higher than that of PAO1, respectively
[f] semi-quantitative PCR showing the expression of *mexE* as an inducible gene in *P. aeruginosa* as compared with the wild-type strain PAO1
[g] relative amounts of *ampC* mRNA (quantitative PCR) compared with *P. aeruginosa* strain PAO1 basal expression. A typical clinical isolate of *P. aeruginosa* for which the ceftazidime MIC was 128 mg/L was used as positive control for overexpression of *ampC* and showed a value of 4750 compared with the PAO1 non-induced reference strain
[h] Imipenem (0.25 mg/L; sub-MIC concentration) added to induce *ampC* expression, thereby increasing ceftazidime MIC above the FDA and EMA (EUCAST) susceptibility breakpoints (≤ 8 mg/L)
[i] see [15,18,19] for a more detailed description of these strains
[j] the high level of *ampC* overexpression of this strain is probably not the only reason for incomplete reversal of its resistance to ceftazidime by addition of avibactam since the EryR strain also shows a high level of *ampC* expression but becomes fully susceptible to ceftazidime in the presence of avibactam

Table 2
Clinical strains: description and main properties.

Name/patient's code, country	Clonality (PFGE/MLST)	MIC (mg/L)[a,b]			MexAB-OprM/ MexEF-OprN efflux system characteristics					*ampC*[f]	Porin OprD
					mexA or *mexB* mutations[*]		MexAB-OprM Speed V_{max} (unit/sec)[e,*]	Relative expression			
		CAZ	CAZ/AVI [c]	Log_2 fold reduction[d]	Gene	Protein (AA length)		*mexA*[f]	*mexE*[g]		
CF53/DP, UK	CA/ST146 (MDR LES clone[b]	256	4	6	*mexA*: C360G nonsense mutation	Truncated MexA (119)	-0.02	0.1	+	**6.8**	+
143-1/143, DE	WI/ND[b]	128	8	4	*mexA*: Δ 2 nt (837-838) nonstop mutation	MexA (0)	-0.02	1.1	-	4.7	+
135-1/135, DE	H/ST2254 (new ST)	128	8	4	*mexB*: G1261_C1262insG + Δ 2 nt (1947-1948)	Truncated MexB (719)	-0.10	1	-	4	-
BV1/DC, UK [h]	CA/ST146	128	8	6	*mexB*: Δ 8 nt (1555-1562) nonsense mutation	Truncated MexB (672)	-0.08	0.8	+	**10.9**	-
CF16/RC, UK	CA/ST146	256	**16**	4	*mexB*: Δ 154 nt (85-239) nonsense mutation	Truncated MexB (30)	-0.12	1	-	**12.7**	+
CF19/LS, UK	CA/ST146	512	**16**	5	*mexA*: C360G nonsense mutation	Truncated MexA (119)	-0.02	0.4	+	**12.7**	+
191-4/191, DE	CK/ND[b]	1024	**16**	6	*mexA*: G82T nonsense mutation	Truncated MexA (27)	-0.01	0.3	-	**10.8**	+
208-3/208, DE	H/ST2254	128	**16**	3	*mexA* : missense mutation G616T *mexB*: Δ 1 nt (T1854) + Δ 2 nt (1947-1948)	MexA (383); 30 AA substitutions in MexB (1045)	**-0.32**	0.7	+	3.7	+
208-2/208, DE	H/ST2254	256	**64**	2	*mexA* : missense mutation G616T *mexB*: Δ 1 nt (T1889) + Δ 2 nt (1947-1948)	MexA (383); 19 AA substitutions in MexB (1045)	**-0.25**	1.4	-	**25.5**	-
128/DAF69, BE	YY/ST958 (MDR clonal complex ST111)	1024	**512**	1	*mexB*: conservative missense mutation C1126G	1 AA substitution L376V in MexB (1046)	**-0.58**	**2.2**	-	**49.7**	-

[*] data from [15]
[a] all MICs were measured in the presence of 0.25 mg/L imipenem (sub-MIC concentration) in order to induce *ampC* expression
[b] CAZ, ceftazidime; AVI, avibactam at 4 mg/L; AA, amino acids; LES, Liverpool Epidemic Strain; MDR, multi-drug resistant; ND, not determined
[c] values in bold denote MICs that remained high > EUCAST and CLSI susceptibility breakpoints (≤ 8 mg/L) for clinical isolates) in the presence of 4 mg/L avibactam
[d] log_2 of the MIC ratio [CAZ+IMI]/[(CAZ/AVI)+IMI]
[e] kinetics of efflux of NPN (fluorescence arbitrary units/sec; see detailed methodology in [15])
[f] relative amount of *mexA* and *ampC* mRNA compared with *P. aeruginosa* strain PAO1 basal expression. According to previous studies [14,17], isolates were considered borderline or positive for *mexA* and *ampC* overexpression (values highlighted in bold) when the corresponding mRNA level was between 2–3 or ≥ 3 and 5–10 or ≥ 10 fold higher than that of PAO1, respectively. These measurements were made without induction of *ampC* with imipenem
[g] semi-quantitative PCR seeking the expression of *mexE* as an inducible gene in *P. aeruginosa* as compared with the wild-type strain PAO1. PAO1 mutant overexpressing MexEF-OprN efflux pump was used as an internal control
[h] when exposed to cefoxitin (500 mg; 1 h), this strain strongly overexpressed *ampC* (ratio to non-induced PAO1 basal expression: 81.6) and the MIC of ceftazidime rose from 128 to 256 mg/L. The addition of increasing concentrations of avibactam (0.5 to 32 mg/L) caused a progressive decrease of the ceftazidime MIC down to 4 mg/L when non-induced and to 8 mg/L when induced with cefoxitin

expression of the AmpC cephalosporinases. All control strains overexpressed *ampC* in the presence of imipenem. As expected, the MIC of ceftazidime increased to 32 mg/L for all strains in the absence of avibactam and the presence of imipenem. Adding avibactam reduced the ceftazidime MIC to 0.125 mg/L for PAO1 and its derivatives defective for MexAB-OprM or overexpressing MexXY-OprM (MutGr1) or MexCD-OprJ (EryR) (this value was lower than for the non-induced strain, which was interpreted as resulting from a synergy between ceftazidime-avibactam and imipenem, possibly due to the ability of avibactam to inhibit the hydrolysis of imipenem in AmpC-induced cells). Ceftazidime MIC was reduced to 2 mg/L in the derivative overexpressing MexEF-OprN (PAO-7H), and to 4 mg/L in the derivative overexpressing MexAB-OprM. This was possibly due to active efflux of avibactam (it was previously shown that the MIC of imipenem was not modified by the overexpression of efflux pumps in PAO1 [14]). The MIC of ceftazidime-avibactam was also 4 mg/L for the OprD-defective mutant (CC-03), which was consistent with absence of this porin impairing the diffusion of imipenem into the cell, thereby preventing the synergy with avibactam.

Table 2 shows the data obtained with the 10 CF clinical isolates. When induced by exposure to imipenem (0.25 mg/L), all these isolates (i) showed ceftazidime MICs $\geq$ 128 mg/L in the absence of avibactam, (ii) presented variable levels of *ampC* overexpression, and (iii) were all positive in the colorimetric phenotypic test detecting the enzymatic activity of cephalosporinases. They also had natural mutations in *mexA* or *mexB*, which impaired the efflux activity of MexAB-OprM to different extents, allowing determination of the respective contribution of MexAB-mediated efflux and of other potential mechanisms of resistance to avibactam. Four isolates (CF53, 143-1, 135-1, BV1) became susceptible to ceftazidime (MIC $\leq$ 8 mg/L) in the presence of avibactam (4 mg/L). They all presented a very slow NPN efflux (associated with severe deletions in *mexA* or *mexB*) and a borderline (see caption of Table 2 for definition) overexpression of *ampC*. CF53 and BV1 expressed *mexE*, and CF53 and 143-1 were positive for OprD. Three other isolates (CF16, CF19, 191-4) showed resistance to ceftazidime-avibactam (MIC 16 mg/L). These also showed slow NPN efflux (due to severe truncations in MexA or MexB) and OprD expression, but overexpressed *ampC*. A critical role of *ampC* overexpression in reducing avibactam activity is best seen when comparing the MICs of the isogenic isolates CF53 and CF19 that only differ by the expression level of this enzyme. In the isogenic isolates 208-3 and 208-2, avibactam reduced the ceftazidime MIC from 128 and 256 mg/L to 16 and 64 mg/L, respectively. Despite mutations in *mexA* and *mexB*, these isolates still extruded NPN but at a slower rate than PAO1. The low resistance level of isolate 208-3 could be attributed to the fact that, although expressing *mexE,* it showed low *ampC* overexpression and expressed OprD. Conversely, the high resistance level of isolate 208-2 may result from a high overexpression of *ampC* perhaps combined with a loss of the OprD porin. The last isolate (128) remained highly resistant to ceftazidime in the presence of avibactam. It showed a fast NPN efflux compared with PAO1 (associated with *mexA* overexpression), a high overexpression of *ampC*, and no expression of OprD. A statistical analysis of the changes in MIC caused by addition of avibactam vs. the expression of *mexE* or the presence of OprD revealed no significant association using either nonparametric (Mann-Whitney; $p = 0.33$ and $p = 0.23$, respectively) or parametric (unpaired *t*-test with Welch correction: $p = 0.31$ and $p = 0.18$, respectively) analysis.

The European Summary of Product Characteristics and the US label of ceftazidime-avibactam mention that resistance mechanisms that could affect this combination include decreased outer membrane permeability, active efflux, and β-lactamase enzymes refractory to inhibition by avibactam [1,2]. First, this study showed that *ampC* overexpression reduces avibactam potency. Second, it identified MexAB-OprM as the main efflux transporter of avibactam in *P. aeruginosa*, further contributing to the resistance of strains overexpressing this transporter if they also overexpress AmpC cephalosporinases. This conclusion is partly in contrast with a previous report [16], which showed that avibactam restored ceftazidime activity to a large proportion of *P. aeruginosa* isolated from bloodstream infections, including those with AmpC hyperproduction or overexpression of MexAB-OprM [16]. While the different origin of the strains may explain this divergence, the level of expression of *ampC* or *mexAB* was not mentioned for individual isolates in that study, making the comparison with the current data difficult. Yet, the authors also came to the conclusion that MICs remain more elevated in isolates combining *ampC* and *mexAB* overexpression. Third, the role of OprD in avibactam entry inside the periplasmic space remains to be unambiguously established because previous studies have reported no effect of loss of OprD on the activity of avibactam in combination with ceftazidime [4,16]. However, any impact of its absence on avibactam activity may become significant only when combined with other resistance mechanisms.

In a broader context, this study has also demonstrated the existence of CF isolates (up to 30% in the collection [15]) in which the MexAB-OprM transporter is inactive due to severe mutations. These should therefore remain susceptible to ceftazidime-avibactam, suggesting that systematic susceptibility testing of this combination could be rewarding in the clinical handling of these patients. Lastly, the data open the way to studies examining how an increase in the avibactam concentration could help to counteract these resistance mechanisms.

Acknowledgements

Thanks to AstraZeneca Pharmaceuticals, Waltham, MA, USA, for providing us with avibactam. (AstraZeneca's rights to ceftazidime-avibactam were acquired by Pfizer in December 2016), O. Denis (*Hôpital Erasme, Université libre de Bruxelles*, Brussels, Belgium), J.S. Elborn and M.M. Tunney (Queen's University Belfast, Belfast, United Kingdom), and B.C. Kahl (University Hospital of Münster, Münster, Germany) for the kind gift of the clinical isolates, and V. Mohymont for expert technical assistance.

Declarations

None.

Funding

This work was supported in part by the Belgian *Région Wallonne* and by the Belgian *Fonds de la Recherche Scientifique* (grant J.0018.17). H.C. received a *Bourse du Patrimoine* of the *Université catholique de Louvain* for the performance of this work. F.V.B. is *Maître de Recherches* of the Belgian *Fonds de la Recherche Scientifique* (F.R.S.-FNRS).

Competing interests

W.W.N. is a former employee of and current shareholder of AstraZeneca PLC. P.M.T. and F.V.B. have received research grants from AstraZeneca PLC for other works than those presented here. The other authors have nothing to declare.

Ethical approval

Not required.

References

[1] Anonymous. AVYCAZ (ceftazidime and avibactam) for injection. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/206494s003lbl.pdf. [accessed 28th July 2017].

[2] Anonymous. Zavicefta Summary of Product Characteristics. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/004027/WC500210234.pdf. [accessed 6th April 2017].

[3] Bush K. A resurgence of β-lactamase inhibitor combinations effective against multidrug-resistant Gram-negative pathogens. Int J Antimicrob Agents 2015;46:483–93 PM26498989.

[4] Mushtaq S, Warner M, Livermore DM. In vitro activity of ceftazidime+NXL104 against *Pseudomonas aeruginosa* and other non-fermenters. J Antimicrob Chemother 2010;65:2376–81 PM20801783.

[5] Chalhoub H, Tunney M, Elborn JS, Vergison A, Denis O, Plesiat P, et al. Avibactam confers susceptibility to a large proportion of ceftazidime-resistant *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis patients. J Antimicrob Chemother 2015;70:1596–8 PM25587996.

[6] Mustafa MH, Chalhoub H, Denis O, Deplano A, Vergison A, Rodriguez-Villalobos H, et al. Antimicrobial Susceptibility of *Pseudomonas aeruginosa* Isolated from Cystic Fibrosis Patients in Northern Europe. Antimicrob Agents Chemother 2016;60:6735–41 PM27572406.

[7] Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J Bacteriol 1993;175:7363–72 PM8226684.

[8] Van Bambeke F, Glupczynski Y, Plesiat P, Pechere JC, Tulkens PM. Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. J Antimicrob Chemother 2003;51:1055–65 PM12697642.

[9] Li XZ, Zhang L, Srikumar R, Poole K. Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1998;42:399–403 PM9527793.

[10] Trias J, Nikaido H. Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. J Biol Chem 1990;265:15680–4 PM2118530.

[11] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 27th Informational Supplement. CLSI document M100-S27. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.

[12] Juan C, Moya B, Perez JL, Oliver A. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. Antimicrob Agents Chemother 2006;50:1780–7 PM16641450.

[13] Nordmann P, Dortet L, Poirel L. Rapid detection of extended-spectrum-beta-lactamase-producing Enterobacteriaceae. J Clin Microbiol 2012;50:3016–22 PM22760052.

[14] Chalhoub H, Sáenz Y, Rodriguez-Villalobos H, Denis O, Kahl BC, Tulkens PM, et al. High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations. Intern J Antimicrob Ag 2016;48:740–3 PM28128097.

[15] Chalhoub H, Pletzer D, Weingart H, Braun Y, Tunney MM, Elborn JS, et al. Mechanisms of intrinsic resistance and acquired susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients to temocillin, a revived antibiotic. Sci Rep 2017;7:40208 PM28091521.

[16] Torrens G, Cabot G, Ocampo-Sosa AA, Conejo MC, Zamorano L, Navarro F, et al. Activity of ceftazidime-avibactam against clinical and isogenic laboratory *Pseudomonas aeruginosa* isolates expressing combinations of most relevant beta-lactam resistance mechanisms. Antimicrob Agents Chemother 2016;60:6407–10 PM27480848.

[17] Cabot G, Ocampo-Sosa AA, Tubau F, Macia MD, Rodriguez C, Moya B, et al. Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. Antimicrob Agents Chemother 2011;55:1906–11 PM21357294.

[18] Mesaros N, Glupczynski Y, Avrain L, Caceres NE, Tulkens PM, Van Bambeke F. A combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*. J Antimicrob Chemother 2007;59:378–86 PM17289770.

[19] Juan C, Conejo MC, Tormo N, Gimeno C, Pascual A, Oliver A. Challenges for accurate susceptibility testing, detection and interpretation of beta-lactam resistance phenotypes in *Pseudomonas aeruginosa*: results from a Spanish multicentre study. J Antimicrob Chemother 2013;68:619–30 PM23143898.