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Original article

In vitro activity of six biocides against carbapenemase-producing *Klebsiella pneumoniae* and presence of genes encoding efflux pumps☆

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ABSTRACT

Introduction: Acquisition of reduced susceptibility to biocides may contribute to the dissemination of high-risk (HR) clones of carbapenemase-producing *Klebsiella pneumoniae* (CP-Kp). The aim of this study was (a) to determinate the activity of biocides against CP-Kp, and (b) to analyse the relationship between biocide activity and the presence of efflux pumps.
Methods: The minimal inhibitory concentrations (MICs) of 6 biocides (sodium hypochlorite, chlorhexidine digluconate, benzalkonium chloride, povidone-iodine, ethanol and triclosan) were determined in triplicate at 25 °C and 37 °C in Mueller-Hinton broth (MHB) and M9 minimum medium, against 17 CP-Kp isolates representing different clones (HR and no-HR), sequence-types (STs) and carbapenemases. Efflux pumps genes were detected by whole genome sequencing (MiSeq).
Results: Median MICs were slightly higher at 37 °C than at 25 °C ($p \leq 0.05$), except for benzalkonium chloride, triclosan and ethanol. MIC medians were much higher in MHB than in M9, except for triclosan. No significant differences were observed in the median MICs, regarding the type of clone, ST or carbapenemase; *cepA*, *acrAB*, *kpnEF* and *oqxAB* genes were detected in all isolates, whereas *qacE* and *qacA* were not detected; *smvAR*, and *qacΔE* genes were detected in 94% and 47% of isolates, respectively.
Conclusions: Triclosan, chlorhexidine digluconate, benzalkonium chloride and ethanol were the most active biocides. The activity of some biocides is affected by temperature and growth media, suggesting that standardised procedures for biocide susceptibility testing based on MIC determination are required. This activity, in terms of MICs, are not related to the type of clone, ST, carbapenemase or the presence of the efflux pump genes.

Actividad *in vitro* de seis biocidas frente a *Klebsiella pneumoniae* productora de carbapenemasa y presencia de genes codificantes de bombas de expulsión

Palabras clave:
Klebsiella pneumoniae
Carbapenemasa

RESUMEN

Introducción: La adquisición de sensibilidad reducida a los biocidas puede contribuir a la diseminación de clones de alto riesgo (HR) de *Klebsiella pneumoniae* productor de carbapenemasa (Kp-PC).

Abbreviations: CP, carbapenemase-producing; Kp, *Klebsiella pneumoniae*; HR, high-risk; EP, efflux pump; MHB, Mueller-Hinton broth; RSB, reduced susceptibility to biocides; BEN, benzalkonium chloride; CHX, chlorhexidine digluconate; ETH, ethanol; POV, povidone-iodine; SOD, sodium hypochlorite; TRI, triclosan; WGS, whole genome sequencing; ST, sequence type.

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Clon de alto riesgo
Desinfectantes
Bombas de expulsión

El objetivo de este trabajo fue: (a) determinar la actividad de varios biocidas frente a Kp-PC, y (b) analizar la relación de dicha actividad con la presencia de genes codificantes de bombas de expulsión.
Métodos: Las concentraciones mínimas inhibitorias (CMI) de 6 biocidas (hipoclorito de sodio, digluconato de clorhexidina, cloruro de benzalconio, povidona yodada, etanol y triclosán) se determinaron por triplicado a 25 y 37 °C, tanto en caldo Mueller-Hinton (MHB) como en medio mínimo M9, frente a 17 aislados de Kp-PC representativos de diferentes clones (HR y no HR), secuenciotipos (ST) y carbapenemasas. Los genes de bombas de expulsión se detectaron mediante secuenciación masiva del genoma completo (MiSeq).
Resultados: Las medianas de las CMI fueron ligeramente superiores a 37 °C que a 25 °C, excepto para cloruro de benzalconio, etanol y triclosán. Las medianas de las CMI fueron considerablemente superiores en MHB que en M9, excepto para triclosán; *cepA*, *acrAB*, *kpnEF* y *oqxAB* se detectaron en todos los aislados, mientras que *qacE* y *qacA* no se detectaron; *smvAR* y *qacΔE* se detectaron en el 94% y en el 47% de los aislados, respectivamente.
Conclusiones: La actividad de algunos biocidas se afecta por la temperatura y el medio de crecimiento. Esta actividad, en términos de CMI, no se relaciona con el tipo de clon, ST, carbapenemasa, ni con la presencia de genes que codifican bombas de expulsión.

Introduction

Although *Klebsiella pneumoniae* (Kp) is part of the human intestinal microbiota, it can behave as an opportunistic nosocomial pathogen able to cause severe infections.[1]

High-risk (HR) clones of Kp are characterised by their enhanced ability to cause nosocomial outbreaks and to develop or acquire resistance to multiple antimicrobials, including carbapenems.[2] The horizontal transfer of plasmids coding for carbapenemase genes is one of the most important factors contributing to the success of these epidemic clones.[2] The dissemination of these carbapenemase-producing (CP)-HR clones has a major impact on the management of infections, due to the associated high morbidity and mortality, limited therapeutic options, and the difficulty of outbreaks control.[1] Loss or reduced expression of certain porins or overexpression of efflux pumps (e.g., acrAB-TolC), combined with other mechanisms have been shown to be involved in carbapenem resistance in Kp.[1]

Environmental contamination (e.g., wastewater drainage system) by carbapenem-resistant *Enterobacterales* has been described as an increasing occult reservoir for CP-Kp outbreaks, so that any factor that facilitates its persistence in the environment through tolerance development or acquisition of reduced susceptibility to biocides (RSB) could improve the success or epidemic behaviour of some CP-HR clones of Kp.[3–5]

The biocide concentrations used in hospitals are high enough to prevent bacterial growth. Nevertheless, inadequate biocide preparation or loss of effectiveness due to environmental conditions can result in the presence of residual or subinhibitory biocide concentrations that could favour (i) the persistence and selection of some CP-HR clones of Kp and/or (ii) the co-selection of some acquired antimicrobial resistance determinants, as has been observed in other nosocomial bacteria.[6–8]

The main mechanism involved in RSB are efflux pumps.[5,8] Efflux pumps can use only a specific biocide as substrate (e.g., chlorhexidine digluconate for CepA and SmvA) or several compounds, including different biocides and antimicrobials as substrates (e.g., chlorhexidine digluconate and benzalkonium chloride for QacA, QacE and QacΔE, and also triclosan for KpnEF and OqxAB).[9–12]

In Kp this information is scarce, but some efflux pumps, such as QacA, QacΔE, have been related to the development of reduced susceptibility or tolerance to chlorhexidine digluconate and benzalkonium chloride, among others.[9]

The aim of this study was (i) to determine the *in vitro* activity of some biocides commonly used in hospitals against representative HR and non-HR clones of CP-Kp; (ii) to evaluate the effect of some environmental conditions (temperature and type of growth medium) on the *in vitro* activity of biocides, and (iii) to analyse the relationship between presence of efflux pumps and the activity of the biocides tested.

Materials and methods

Bacterial isolates

Seventeen CP-Kp isolates representing different types of carbapenemases and clones (sequence types) circulating in Spanish hospitals during 2012–2015 were selected (Table 1). Eleven isolates were from a multicenter Spanish study performed at the Reference and Research for Resistance to Antibiotics Laboratory of Carlos III Health Institute, Madrid, and 6 isolates were from the Reference Laboratory for the Surveillance and Control of Nosocomial Infections and Prudent Use of Antimicrobials Program in Andalucía (PIRASOA Program; Hospital Universitario Virgen Macarena, Seville, Spain).[13]

Susceptibility testing of biocides

Six biocides were selected for susceptibility testing; povidone-iodine (POV; Betadine®, MEDA Pharma SAU, Spain), sodium hypochlorite (SOD; Lejía Chari, Rubio Díaz Hnos, S.L., Spain), chlorhexidine digluconate (CHX; Sigma–Aldrich, San Luis, USA), benzalkonium chloride (BEN; Sigma–Aldrich, San Luis, USA), ethanol (ETH; Vaza Laboratorios, Spain), and triclosan (TRI; Irgasan, Sigma–Aldrich, San Luis, USA). Sterile distilled water was used to prepare biocide dilutions, except for TRI, where the solvent was 70% methanol (Panreac Química SAU, Barcelona, Spain). The MICs of biocides were determined by broth microdilution in 2 different media [Muller-Hinton Broth (MHB, Difco, Madrid, Spain) and minimum medium M9 (Sigma–Aldrich)], using serial 2-fold dilutions of each biocide as previously described.[14] Biocide minimum inhibitory concentrations (MICs) were also determined at 2 incubation temperatures (25 °C and 37 °C) for 16–20 h. *K. pneumoniae* subsp. *pneumoniae* (ATCC® 700603™) was used as control. All assays were performed in triplicate, expressing the MICs as the median of the 3 MIC values obtained for each biocide.

MICs within ± 1 $\log_2$ dilution step were considered identical. To verify that bacterial isolates are able to growth in any condition tested, 48 h growth curves were performed under these four conditions (25 °C and 37 °C; MHB and M9 medium) using an Infinite 200 PRO spectrophotometer (Tecan Trading AG®, Switzerland).

Table 1
Relevant characteristics of the 17 carbapenemase-producing *K. pneumoniae* isolates.

Isolate number	Sequence type[a]	Source[b]	High-risk clone	Genes coding for		MIC (mg/L)[c]		
				Carbapenemases	CTX-M type ESBLs	ETP	IMP	MEM
1	ST101	CNM	Yes	bla_{KPC-2}	$bla_{CTX-M-15}$	**>1**	**>8**	**>8**
2	ST258	PIRASOA	Yes	bla_{KPC-3}	No	**>1**	**>8**	**>8**
3	ST512	PIRASOA	Yes	bla_{KPC-3}	No	**>1**	**8**	**8**
4	ST11	CNM	Yes	bla_{VIM-1}	$bla_{CTX-M-15}$	**>1**	2	2
5	ST15	CNM	Yes	bla_{VIM-1}	No	**>1**	**8**	**8**
6	ST147	CNM	Yes	bla_{VIM-1}	$bla_{CTX-M-9}$	**>1**	**4**	**8**
7	ST11	CNM	Yes	$bla_{OXA-245}$	$bla_{CTX-M-15}$	**>1**	≤1	≤1
8	ST11	PIRASOA	Yes	bla_{OXA-48}	$bla_{CTX-M-15}$	**≤1**	≤1	≤1
9	ST15	PIRASOA	Yes	bla_{OXA-48}	$bla_{CTX-M-15}$	**>1**	**4**	≤1
10	ST37	PIRASOA	Yes	bla_{OXA-48}	No	**>1**	≤1	**4**
11	ST405	CNM	Yes	bla_{OXA-48}	$bla_{CTX-M-15}$	**>1**	≤1	≤1
12	ST340	CNM	No	bla_{VIM-1}	No	**>1**	2	2
13	ST437	CNM	No	$bla_{OXA-245}$	No	**>1**	≤1	≤1
14	ST13	CNM	No	bla_{OXA-48}	No	**>1**	2	≤1
15	ST16	CNM	No	bla_{OXA-48}	$bla_{CTX-M-15}$	**>1**	≤1	≤1
16	ST846	CNM	No	bla_{OXA-48}	$bla_{CTX-M-15}$	**>1**	**4**	**>8**
17	ST899	PIRASOA	No	bla_{OXA-48}	No	**>1**	2	2

[a] ST: sequence-type.
[b] CNM: National Center for Microbiology. PIRASOA: Reference Laboratory for the Surveillance and Control of Nosocomial Infections and Prudent Use of Antimicrobials Program in Andalucía.
[c] ETP: ertapenem, IMP: imipenem, MEM: meropenem. MICs associated to non-susceptibility appears in bold.

Molecular typing and resistome

Whole genome sequencing (WGS) was performed using the MiSeq system (Illumina®, San Diego, CA, USA).[15] Assignment of isolates to sequence type (ST) and characterisation of the resistome were determined using the nucleotide sequences of the genomes obtained by next-generation sequencing and the MLSTFinder 2.0 (https://cge.cbs.dtu.dk/services/MLST) and ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/), respectively. Clones that caused at least four recognised outbreaks and were reported in ≥10 countries were classified as HR clones. The emerging clone ST405 was also considered as HR.[1]

Detection of genes coding for efflux pumps

Gene annotation was performed with the RAST server (http://rast.nmpdr.org/). The genes coding for efflux pumps were searched from annotated genes or by manual search. The absence of genes not detected in some isolates by WGS (*qacΔE* and *smv*A) was confirmed by conventional PCR using specific primer pairs.

Statistics

Quantitative non-parametric variables were compared using the Mann-Whitney *U* test and Pearson's chi-squared, as appropriate, using IBM SPSS Statistics 18 (IBM Corporation, Armonk, NY). Differences of $p < 0.05$ were considered statistically significant.

Results

As shown in Table 2, the median MICs (mg/L) of the biocides tested at 37 °C in MHB were 1094 for SOD, whereas for the remaining biocides ranged between 1.9–0.5 (TRI), 19.5–1.2 (CHX), 15.6–7.8 (BEN), 35.6–4.5 (ETH), and 3125–1562 (POV). Using M9 medium (Table 3), the median MICs were 1 for SOD, whereas for the remaining biocide ranged between 0.08–0.04 (CHX), 0.2–0.1 (BEN), 0.28–0.07 (ETH), 1.9–0.1 (TRI), and 24.4–12.2 (POV).

The median MICs at 25 °C in MHB were the same as or slightly lower than those determined at 37 °C (Table 2), and they ranged between 1.9–0.05 (TRI), 9.8–2.4 (CHX), 31.2–7.8 (BEN), 35.6–4.5 (ETH), 1094–547 (SOD), and 3125–1562 (POV). In contrast, using M9 medium (Table 3), the median MICs at 25 °C were lower than those determined at 37 °C. The median MICs of BEN was 0.1, whereas for the remaining biocides ranged between 0.08–0.04 (CHX), 1–0.5 (SOD), 1.9–0.05 (TRI), 0.28–0.07 (ETH), and 24.4–6.1 (POV).

The median MICs were higher at 37 °C than 25 °C, except for BEN, ETH and TRI, whose median MICs at 25 °C were identical to those determined at 37 °C. The median MIC values of the latter at both temperatures were: 7.8 in MHB ($p = 0.41$) and 0.1 in M9 medium ($p = 0.07$) for BEN; 8.9 in MHB ($p = 0.14$) and 0.1 in M9 medium ($p = 0.54$) for ETH; and 0.5 in MHB ($p = 0.08$) and 0.5 at 25 °C and 0.2 at 37 °C in M9 medium ($p = 0.23$) for TRI. Considering dilution steps in MIC values instead of median MIC values, it was observed that MIC values were within $\pm 2 \log_2$ dilution steps for CHX tested in MHB and within $\pm 1 \log_2$ dilution steps for the remaining biocides (Tables 2 and 3).

With respect to the type of growth medium used (MHB or M9 medium), the differences observed in the MIC medians of the biocides were statistically significant for all of them (higher MIC medians in MHB than M9), except for TRI, whose MIC median was 0.5 in MHB and M9, both at 37°C (p = 0.47), and 0.5 in MHB and 0.2 in M9 medium at 25°C ($p = 0.55$). Considering dilution steps in MIC values instead of median MIC values, it was observed that MIC values were not within $\pm 1 \log_2$ dilution step, except for TRI in most isolates (Tables 2 and 3).

No statistically significant differences were observed between the median MICs of biocides tested against isolates belonging to HR clones and those belonging to non-HR clones. With respect to the type of carbapenemase produced, no statistically significant differences were observed, except for VIM-1 producers which showed higher median MICs of BEN than OXA-48-producing isolates (37 °C and 25 °C; $p = 0.02$ and $p = 0.01$, respectively) or KPC-producing isolates (25 °C, $p = 0.02$).

The efflux pump genes *cepA*, *acrAB*, *kpnEF* and *oqxAB* were detected in all the isolates tested, whereas *qacE* and *qacA* were not detected at all (Table 4). *smvAR* was detected in all isolates, except for the one belonging to the OXA-48-producing ST15 clone (Table 4). Finally, the distribution of the efflux pump gene *qacΔE* was more variable (47% of isolates) (Table 4). No significant differences were observed between the presence or absence of *smvAR* and *qacΔE* and the median MICs of the biocides tested or the type

Table 2
Median MIC values (mg/L) of 6 biocides tested at 37 °C and 25 °C in MHB against 17 carbapenemase-producing *K. pneumoniae* isolates.

K. pneumoniae isolate	Median MIC (mg/L) determined in MHB											
	Povidone-iodine		Sodium hypochlorite		Chlorhexidine digluconate		Benzalkonium chloride		Ethanol		Triclosan	
	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C
ST101/KPC-2	1562	1562	1094	1094	19.5	9.8	7.8	7.8	8.9	8.9	0.5	0.3
ST258/KPC-3	3125	3125	1094	1094	4.9	4.9	7.8	7.8	8.9	8.9	1.9	0.5
ST512/KPC-3	1562	3125	1094	1094	19.5	4.9	7.8	7.8	8.9	8.9	0.5	0.3
ST11/VIM-1	1562	1562	1094	547	19.5	9.8	15.6	31.2	17.8	35.6	0.5	0.3
ST15/VIM-1	1562	1562	1094	547	2.4	2.4	15.6	15.6	8.9	8.9	0.3	0.05
ST147/VIM-1	3125	1562	1094	1094	4.9	4.9	15.6	15.6	17.8	17.8	1.9	1.9
ST11/OXA-245	3125	1562	1094	547	9.8	4.9	7.8	7.8	8.9	17.8	0.5	0.5
ST11/OXA-48	3125	1562	1094	1094	4.9	4.9	7.8	7.8	8.9	17.8	0.5	0.5
ST15/OXA-48	3125	3125	1094	1094	1.2	4.9	7.8	7.8	8.9	17.8	0.5	0.5
ST37/OXA-48	3125	3125	1094	1094	4.9	2.4	7.8	15.6	8.9	8.9	0.3	0.1
ST405/OXA-48	1562	1562	1094	547	19.5	4.9	7.8	7.8	8.9	8.9	0.5	0.5
ST340/VIM-1	3125	3125	1094	1094	19.5	4.9	7.8	15.6	8.9	8.9	0.9	0.5
ST437/OXA-245	1562	1562	1094	547	9.8	2.4	7.8	7.8	8.9	17.8	0.9	0.9
ST13/OXA-48	3125	1562	1094	1094	19.5	4.9	7.8	7.8	8.9	8.9	0.5	0.3
ST16/OXA-48	3125	1562	1094	1094	19.5	4.9	7.8	7.8	8.9	17.8	0.5	0.5
ST846/OXA-48	3125	1562	1094	1094	19.5	4.9	15.6	15.6	35.6	17.8	1.9	0.5
ST899/OXA-48	3125	1562	1094	1094	9.8	9.8	7.8	7.8	4.5	4.5	0.1	0.1

Table 3
Median MIC values (mg/L) of 6 biocides tested at 37 °C and 25 °C in M9 medium against 17 carbapenemase-producing *K. pneumoniae* isolates.

K. pneumoniae isolate	Median MIC (mg/L) determined in M9 medium											
	Povidone-iodine		Sodium hypochlorite		Chlorhexidine digluconate		Benzalkonium chloride		Ethanol		Triclosan	
	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C	25 °C
ST101/KPC-2	12.2	12.2	1	1	0.08	0.04	0.1	0.1	0.14	0.14	0.3	0.1
ST258/KPC-3	12.2	6.1	1	1	0.08	0.04	0.1	0.1	0.14	0.07	1.9	0.9
ST512/KPC-3	12.2	12.2	1	1	0.08	0.04	0.1	0.1	0.14	0.07	0.9	0.5
ST11/VIM-1	12.2	6.1	1	1	0.08	0.04	0.1	0.1	0.14	0.14	0.3	0.3
ST15/VIM-1	12.2	6.1	1	0.5	0.08	0.04	0.1	0.1	0.14	0.14	0.1	0.3
ST147/VIM-1	12.2	6.1	1	0.5	0.08	0.08	0.1	0.1	0.14	0.14	1.9	1.9
ST11/OXA-245	12.2	6.1	1	1	0.08	0.04	0.2	0.1	0.14	0.14	0.5	0.5
ST11/OXA-48	24.4	12.2	1	0.5	0.08	0.04	0.1	0.1	0.14	0.28	0.5	0.5
ST15/OXA-48	12.2	6.1	1	0.5	0.04	0.04	0.1	0.1	0.07	0.07	0.5	0.3
ST37/OXA-48	12.2	12.2	1	1	0.08	0.08	0.1	0.1	0.14	0.14	0.1	0.05
ST405/OXA-48	24.4	12.2	1	1	0.08	0.04	0.2	0.1	0.28	0.28	0.1	0.1
ST340/VIM-1	24.4	12.2	1	1	0.08	0.04	0.1	0.1	0.14	0.14	1.9	0.9
ST437/OXA-245	12.2	12.2	1	0.5	0.08	0.04	0.1	0.1	0.14	0.07	1.9	0.9
ST13/OXA-48	24.4	6.1	1	0.5	0.08	0.04	0.1	0.1	0.07	0.07	0.1	0.05
ST16/OXA-48	12.2	12.2	1	0.5	0.08	0.08	0.1	0.1	0.14	0.14	0.5	0.5
ST846/OXA-48	24.4	24.4	1	0.5	0.08	0.04	0.2	0.1	0.28	0.28	0.9	0.3
ST899/OXA-48	24.4	12.2	1	1	0.08	0.04	0.1	0.1	0.14	0.14	0.1	0.05

Table 4
Efflux pump genes detected in 17 carbapenemase-producing *K. pneumoniae* isolates.

K. pneumoniae isolate	Efflux pump genes							
	cepA	*acrAB*	*kpnEF*	*oqxAB*	*qacE*	*qacA*	*qacΔE*	*smvAR*
ST101/KPC-2	+	+	+	+	–	–	–	+
ST258/KPC-3	+	+	+	+	–	–	+	+
ST512/KPC-3	+	+	+	+	–	–	+	+
ST11/VIM-1	+	+	+	+	–	–	+	+
ST15/VIM-1	+	+	+	+	–	–	+	+
ST147/VIM-1	+	+	+	+	–	–	+	+
ST11/OXA-245	+	+	+	+	–	–	–	+
ST11/OXA-48	+	+	+	+	–	–	+	+
ST15/OXA-48	+	+	+	+	–	–	–	–
ST37/OXA-48	+	+	+	+	–	–	–	+
ST405/OXA-48	+	+	+	+	–	–	–	+
ST340/VIM-1	+	+	+	+	–	–	+	+
ST437/OXA-245	+	+	+	+	–	–	–	+
ST13/OXA-48	+	+	+	+	–	–	–	+
ST16/OXA-48	+	+	+	+	–	–	+	+
ST846/OXA-48	+	+	+	+	–	–	–	+
ST899/OXA-48	+	+	+	+	–	–	–	+

+: detected; –: not detected.

of clone (HR or non-HR). Nevertheless, significant differences were observed between the presence of *qacΔE* and the presence of OXA-48-like ($p = 0.01$) and VIM-1 carbapenemase ($p = 0.02$).

Discussion

Since RSB may contribute to certain dissemination of CP-Kp clones, understanding how environmental conditions, such as temperature and the presence of organic material, could affect the biocidal activity and explore the mechanisms capable of reducing susceptibility in CP -Kp (e.g., efflux pumps) could be useful for the design of new infection prevention and control strategies. The present study evaluated (i) the effect of different growth temperatures (25 °C and 37 °C) and growth medium conditions (MHB and M9 medium) on the antimicrobial activity of 6 biocides tested against representative CP-Kp isolates belonging to HR or non-HR clones and (ii) the association between MICs of biocides and the presence of some efflux pumps able to extrude biocides.

Our results indicate that, based on the median MICs, the biocides with higher *in vitro* activity are TRI, CHX, BEN, and ETH, and this activity is much higher in M9 medium than MHB, except for TRI (similar in M9 and MHB). The impact of culture medium on MIC values has been described for various techniques such as microdilution and disk diffusion.[16,17] The organic nutrients contained in MHB may inactivate or reduce the activity of these biocides, as was observed by Kawamura-Sato et al. in *Acinetobacter baumannii*.[18] Our results suggest that the activity of the biocides analysed, with the exception of TRI, may be reduced in environments containing organic matter.

Regarding temperature effects on biocide *in vitro* activity, our results show that POV, CHX and SOD were more active (lower MIC medians) at 25 °C than 37 °C. This finding is partially in agreement with the results of other studies, such as the ones obtained by Lambert et al., who observed that the MIC of the biocides they tested were affected by the conditions under which the technique was performed, such as temperature.[19] It is important to highlight that differences in biocide activity were only significant for CHX tested in MHB, considering the number of dilution steps of difference in MIC values instead of median MICs, so the impact of the temperature range we tested on biocide activity seems to be low. In any case, the higher *in vitro* activity of some biocides at 25 °C may be related to higher chemical degradation of active compounds at 37 °C than 25 °C, or to better growth of Kp at 37 °C than at 25 °C.[20] Regarding the results obtained for TRI, the absence of temperature effects would be in line with the results obtained by Kim et al., who observed that temperature increase (from 22 to 40 °C) when using a soap with TRI does not significantly improve its antibacterial activity.[21] Our results indicate that TRI is the biocide less affected by changes in growth medium or the temperatures we tested.

With respect to type of clone (HR or non-HR) or the type of carbapenemase produced, no statistically significant differences in median MICs were observed, suggesting that high-risk clones or the resistance determinants analysed are not related to susceptibility or tolerance to biocides. One exception to this was VIM-1 producers which showed higher median MICs of BEN tested on MHB than OXA-48-producing isolates (37 °C and 25 °C) or KPC-producing isolates (25 °C). This could be explained by the possible co-mobilisation of the $bla_{\text{VIM-1}}$ carbapenemase gene and biocide tolerance genes like *qacΔE*, both detected in class I integrons according to Pitout et al., and Guo et al., respectively.[2,22] This association could not be established in our isolates because of the relatively reduced length of contigs containing $bla_{\text{VIM-1}}$ and those containing *qacΔE*. The use of longer contigs generated by other genetic platforms (i.e.; PacBio) would be helpful in this context.

The presence of specific efflux pumps has been related to decreased susceptibility or tolerance to biocides.[23] Our results indicate that the presence of genes encoding efflux pumps *cepA*, *acrAB*, *kpnEF*, and *oqxAB* does not appear to be related to the median MIC or biocide activity observed, or to the type of clone (HR or non-HR), since these genes were detected in all isolates analysed, which is in agreement with previous studies performed in CP-Kp and in non-CP-Kp.[10,22,24–26]

The prevalence of the cationic efflux pump SmvAR, described as an important cationic biocide efflux pump in Kp by Wand et al., is unknown in CP-Kp but is expected to be high, as we observed in our collection of isolates.[7] In our study, the *smvAR* gene was detected in all isolates except in the one belonging to the OXA-48-producing ST15 clone, suggesting that the presence of this efflux pump gene is not related to type of clone.

The gene encoding the efflux pumps qacΔE was present in 47% of the isolates tested, and its presence or absence was not related to the activity of some of the biocides that are substrates of these pumps, type of carbapenemase produced or type of clone. The prevalence of the *qacΔE* gene in our study is similar to that previously described by Guo et al.[22] The association between *qacΔE* and $bla_{\text{VIM-1}}$ genes could be partially explained if both genetic determinants are carried out in the same plasmid.[2,22]

Finally, the *qacE* and *qacA* genes were not detected in our study. The absence of *qacE* is in agreement with the low prevalence (1/64) previously described by Abuzaid et al.[9] The absence of *qacA* is in agreement to that previously described by Chen et al., but is in contrast to that previously described by Guo et al., who showed a prevalence of *qacA* gene of 41% or Vijayakumar et al., who describe a prevalence of 44.4%.[22,27,28] This difference can probably be explained by differences in the methodology or in criteria used to select the isolates.

Regarding the effect of these genes on the values of biocide median MICs, our results are in consonance with those of Vijayakumar et al., who did not find significant differences between the *in vitro* activity of the biocides tested and the presence of *cepA* or *qacE* genes.[28] On the other hand, these findings do not agree with the results of the study performed by Abuzaid et al., who observed a close association between RSB and the presence of *cepA*, *qacΔE* or *qacE* in Kp clinical isolates.[9] This could be explained by the presence of additional efflux pumps other than QacA and QacΔE and/or by differences in the expression levels of these pumps in CP-Kp, as previously described by Yazgan et al.[26]

There is currently no proposed or recommended gold standard procedure or assay agreed between experts for biocide susceptibility testing. Multiple non-standardised methodologies are used for biocide susceptibility testing, such as minimum inhibitory concentration assay, quantitative suspension tests, qualitative suspension tests and carrier tests.[29] This variability of methods makes it difficult to compare results from different studies and obtain clear conclusions. Biocide activity tested by mean of MIC determination can be controversial since in practice much higher biocide concentrations are used, and also because high MIC values are not always related with a decreased lethal effect as reported by Lear et al. but in spite of this methodological difficulty, many studies and reports on biocide tolerance and resistance are based on broth microdilution results.[30] Nevertheless, MIC determination allowed us to evaluate the impact that different laboratory conditions (culture medium and temperature) could have on the method used to assess the biocidal activity and reflects the need for standardisation.

One limitation of our study is the relative limited number of clones of CP-Kp (e.g., absence of ST307) and type of carbapenemases (e.g., absence of NDM) included, however they represent the most prevalent clones in Spain at the time this work was designed. Another important limitation is the lack of information regarding the mRNA expression level of the efflux pump genes investigated.

This could be relevant as the MICs of biocides may be related to the expression levels of some efflux pump genes.

In conclusion, our results suggest that the biocides with the highest activity against the CP-Kp isolates tested were CHX, BEN, ETH and TRI. Biocide activity is slightly affected by temperature (lower at 37 °C than 25 °C) whereas a great impact is observed regarding the type of growth medium (lower in MHB than in M9 medium), suggesting that organic matter can reduce the activity of most of the biocides studied. Moreover, biocide activity is not related to the clone type (HR vs no-HR), the ST or the type of carbapenemase produced neither to the presence or absence of the efflux pumps genes studied. The implementation of standardised and reproducible methods for *in vitro* biocide susceptibility testing are necessary for the application of effective disinfecting protocols, particularly in the context of nosocomial surveillance programs.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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