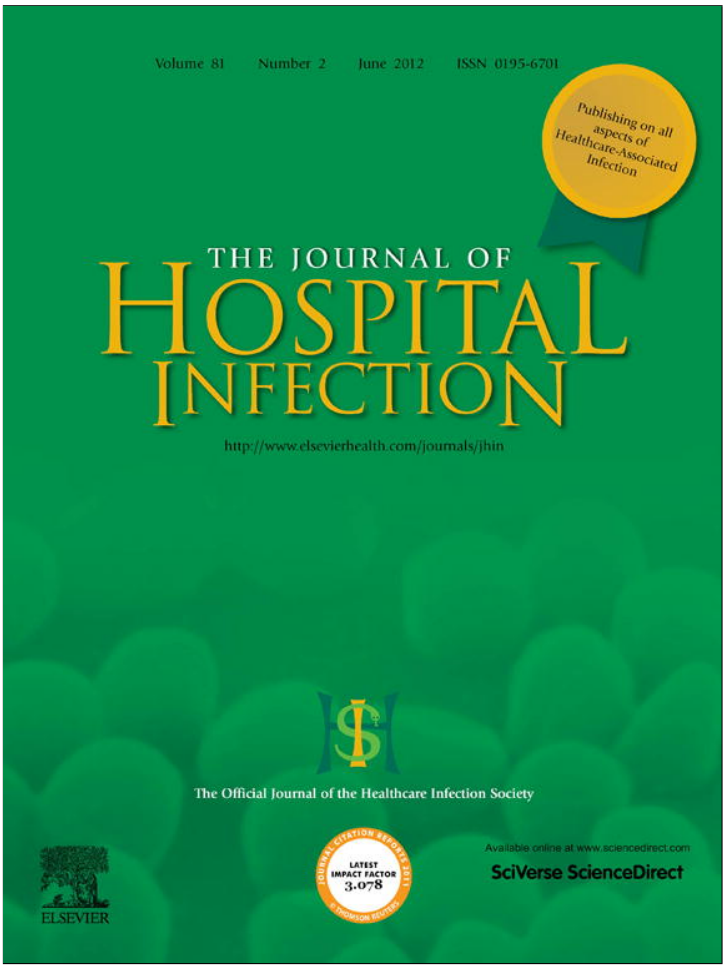

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Klebsiella pneumoniae susceptibility to biocides and its association with *cep*A, *qac*ΔE and *qac*E efflux pump genes and antibiotic resistance

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SUMMARY

Background: Although antiseptics are some of the most widely used antibacterials in hospitals, there is very little information on reduced susceptibility to these biocides and its relationship with resistance to antibiotics.

Aim: To determine the relationship between reduced susceptibility to biocides and the carriage of antiseptic resistance genes, *cep*A, *qac*ΔE and *qac*E, as well as identifying the role of efflux pumps in conferring reduced susceptibility.

Methods: Susceptibility was assessed for five biocides: chlorhexidine, benzalkonium chloride, Trigene, MediHex-4, Mediscrub; and for 11 antibiotics against 64 isolates of *Klebsiella pneumoniae*. Susceptibility to all compounds was tested by the agar double dilution method (DDM) and the effect of efflux pumps on biocides determined by repeating the susceptibility studies in the presence of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). The presence of the *cep*A, *qac*ΔE and *qac*E genes was identified by polymerase chain reaction.

Findings: The bacteria were not widely antibiotic resistant though a few showed reduced susceptibility to cefoxitin, chloramphenicol and rifampicin and later-generation cephalosporins but not to carbapenems. Biocide susceptibility, tested by DDM, showed that 50, 49 and 53 strains had reduced susceptibility to chlorhexidine, Trigene and benzalkonium chloride, respectively. The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were found in 56, 34 and one isolates respectively and their effects as efflux pumps were determined by CCCP (10 mg/L), which decreased the minimum inhibitory concentrations (MICs) of chlorhexidine and Medihex-4 by 2–128-fold but had no impact on the MICs of benzalkonium chloride, Trigene and Mediscrub.

Conclusion: There was a close link between carriage of efflux pump genes, *cep*A, *qac*ΔE and *qac*E genes and reduced biocide susceptibility, but not antibiotic resistance, in *K. pneumoniae* clinical isolates.

Introduction

Klebsiella pneumoniae causes about 8% of hospital-acquired infections (nosocomial infections) including pneumonia, wound infections, diarrhoea and urinary tract infections.[1,2] The severity of these infections has increased as outbreaks have

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occurred with multiply-resistant strains often producing extended-spectrum beta-lactamase enzymes (ESBLs), with associated increases in morbidity and mortality especially in intensive care units (ICUs), neonatal units and surgical wards.[1–6]

Hand contamination of hospital staff such as nurses and doctors is one of the most common modes of transmission of *K. pneumoniae* (17% ICU staff). Casewell and Phillips found that in order to combat these infections, the staff frequently washed their hands with cationic biocides (such as chlorhexidine), which produces a 98–100% reduction in the number of patients infected with *K. pneumoniae*.[7] Recently, a considerable effort has been made to improve standards of infection control within hospitals, leading to the increased use of biocides as disinfectants and antiseptics. In specific cases judicious biocide use has led to considerable reductions in numbers of *K. pneumoniae*; however, the general trend is that infections caused by multi-resistant *K. pneumoniae* are increasing, and this raises concerns that intensive exposure of hospital pathogens to biocides may result in the emergence of resistance not just to themselves but also to antibiotics.[8–12]

It is difficult to define the clinical relevance of biocide resistance as the concentrations used clinically are often higher than those required to inhibit the organism. However, a series of mitigating factors, such as the local dilution policy, presence of organic matter, formation of biofilms and length of exposure, could comprise the effective concentration of the biocide so that resistance may become a problem.[13,14] With reference to biocides, it is therefore more appropriate to consider reductions in susceptibility rather than increases in resistance.

This study investigates the correlation between biocide susceptibility and its association with both the efflux pump genes, which have been linked to antiseptic 'resistance' (*cep*A, *qac*ΔE, *qac*E), and resistance to later-generation anti-Gram-negative antibiotics.[15]

Methods

Bacterial strains

Sixty-four strains of *K. pneumoniae* were isolated from different infection sites at Edinburgh Royal Infirmary from 2006 to 2008. Isolates were identified at the Infirmary by Vitek2 and confirmed by the API 20E strip (bioMérieux, Basingstoke, UK).

Minimum inhibitory concentration (MIC)

The antibiotics used were cefotaxime, cefoxitin, colistin, chloramphenicol, gentamicin, polymyxin B, rifampicin and trimethoprim (Sigma, Poole, UK), ceftazidime (GlaxoSmith-Kline, Brentford, UK), imipenem (Merck, Sharp & Dohme, Huddleston, UK), and meropenem (AstraZeneca, Luton, UK). The common hospital biocides used were 1% chlorhexidine gluconate, a member of the biguanide family, and 1% benzalkonium chloride, a quaternary ammonium compound (QAC), both supplied from Sigma. The commercial biocide preparation Trigene was tested; this has polymeric biguanide hydrochloride as the main active component. The cationic biocide MediHex-4, containing 4% chlorhexidine gluconate, and Mediscrub, containing 1% triclosan, were obtained from Medichem International (Sevenoaks, Kent, UK).[16]

Susceptibility to antibiotics was determined by double dilution method following the guidelines of the British Society for Antimicrobial Chemotherapy.[17,18] MICs were determined using the agar dilution method on Iso-Sensitest (IST) agar (Oxoid Ltd, Basingstoke, UK) and the agar plates were incubated in air at 37°C for 18–20 h.[17,18] *Escherichia coli* NCTC 1048, *Pseudomonas aeruginosa* NCTC 10662 and *Staphylococcus aureus* NCTC 6571 were employed as control reference strains for determination of MIC.

Efflux pump inhibitor and reduced biocide susceptibility

Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was purchased from Sigma. In order to determine whether an efflux pump was active, these pump inhibitors were dissolved in small volumes of dimethyl sulphoxide, and the volume made up with distilled water. This was then added to IST agar at 10 mg/L in plates containing increasing concentrations of biocide for minimum inhibitory concentration (MIC) determination. A decrease in biocide MIC indicated the presence of an efflux pump.

Polymerase chain reaction (PCR)

The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were detected by PCR with the primers and the annealing temperatures described below *cep*A primer pairs were designed to amplify 1051 base pair (bp) F5′CAACTCCTTCGCCTATCCCG3′, R5′TCAGGTCAGACCAAACGGCG3′ with annealing temperature 66°C for 30 s.[19] The *qac*ΔE and *qac*E primer pairs F5′GCCCTACACAAATTGGGAGA3, R5′CTGCGGTACCACTGCCAC AA3′; F5′GCCCTACACAAATTGGGAGA3′, R5′TTAGTGGGCACTT GCTTTGG3′ were designed to amplify 370 and 350 bp respectively, all having the same annealing temperature of 49°C for 40 s.[20] The PCR products were analysed on 1.5% of agarose (Fisher Scientific, Loughborough, UK). The gel was stained with GelRed and gel image was taken in tag image file format (JPEG files) with the Diversity Database software image capturing system (Bio-Rad, Hemel Hempstead, UK).

Results

MICs of antibiotics and biocides

The susceptibility of the 64 isolates was determined by measuring the MIC of antibiotics and biocides. Some isolates were reported resistant to cefotaxime, ceftazidime, chloramphenicol, trimethoprim and rifampicin (Table I); however, most isolates were sensitive to third-generation cephalosporins and all were sensitive to imipenem (MIC $\leq$2 mg/L) and meropenem (MIC $\leq$0.12 mg/L) (Table I).

The ranges of MIC for each biocide are shown in Table I. In 51 of the 64 *K. pneumoniae* isolates, there was a considerable decrease in susceptibility to chlorhexidine, benzalkonium chloride and Trigene with MICs ranging from 32 to 128 mg/L and high MIC_{50} values (Table I). However, Medihex-4 showed a moderate level with MICs from 8 to 16 mg/L in 53 isolates; these values were about four-fold lower than for chlorhexidine reflecting the four-fold increase in chlorhexidine concentration

Table I
Minimum inhibitory concentrations (MICs) of biocides and antibiotics against 64 *Klebsiella pneumoniae* isolates

Antibacterial agent	MIC (mg/L)		
	Range	MIC_{50}	MIC_{90}
Biocide			
Chlorhexidine	4–128	32	64
Benzalkonium chloride	16–64	32	64
Trigene	16–64	32	64
MediHex-4	2–16	8	16
Mediscrub	0.12–128	0.12	0.5
Antibiotics			
Cefotaxime	0.03–128	0.06	128
Ceftazidime	0.12–128	0.25	64
Cefoxitin	2–128	8	32
Imipenem	0.12–1	0.25	1
Meropenem	0.015–0.06	0.015	0.03
Colistin	2–8	8	8
Gentamicin	0.12–64	0.25	0.5
Chloramphenicol	4–128	4	8
Trimethoprim	0.12–128	0.5	128
Rifampicin	8–64	32	64
Polymyxin B	0.5–8	1	1

in this commercial product. Fifty-five isolates had MICs of Mediscrub $\leq$4 mg/L (Table I).

Antiseptic resistance genes

In order to determine whether the decreased susceptibility could be correlated with specific resistance genes, PCR was used to screen for the antiseptic resistance genes *cep*A, *qac*E and *qac*ΔE, which may have an important role in decreasing susceptibility to antiseptics.[21] Fifty-six isolates contained the *cep*A gene and, in every case, the size of gene fragment was 1051 bp. The *qac*E and *qac*ΔE genes were amplified by PCR; the *qac*ΔE gene was detected in 34 isolates; and *qac*E was found in only one isolate. Thirty-two isolates had both *cep*A and *qac*ΔE, whereas just one isolate had both *cep*A and *qac*E gene and six isolates had no efflux pump genes (Table II).

Table II associates the individual levels of chlorhexidine, Trigene and benzalkonium chloride susceptibility with the presence of individual antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E. Medihex-4 was not tested as its main component is 4% chlorhexidine; the strains were susceptible to Mediscrub, so this biocide was also excluded. High MICs (32–128 mg/L) of chlorhexidine, Trigene and benzalkonium chloride were found in 50, 49 and 53 isolates respectively. In each case the *cep*A gene was present, while *qac*ΔE and *qac*E antiseptic resistance genes were found in 31, 33 and 34 isolates for chlorhexidine, Trigene and benzalkonium chloride respectively.

The antiseptic resistance genes *cep*A, *qac*ΔE and *qac*E were detected in 56, 34 and one isolates respectively, 32 isolates had both *cep*A and *qac*ΔE and one isolate had both *cep*A and *qac*E whereas six isolates had no antiseptic resistance genes. The association between carriage of the *cep*A, *qac*ΔE and *qac*E genes with significant reductions in biocide susceptibility of the clinical *K. pneumoniae* isolates was only partial, e.g. 51 of 56 isolates had efflux pump genes and had significant reductions in susceptibility (MIC 32–128 mg/L) to chlorhexidine, Trigene and benzalkonium chloride; however, six isolates did not have antiseptic resistance genes, but four of these six isolates had high MICs (32–64 mg/L) of chlorhexidine, Trigene and benzalkonium chloride.

Efflux pump inhibitors and reduced biocide susceptibility

The effects of CCCP (10 mg/L) on the MICs of the biocides was examined. It had no impact on benzalkonium chloride, Trigene and Mediscrub (data not shown); however, it reduced the MICs of chlorhexidine and Medihex-4 by between two- and 128-fold (Tables III and IV). In the case of chlorhexidine, this was associated, except in five cases, with the presence of the *cep*A gene. The presence of CCCP had slightly less effect on Medihex-4 and this was associated, except in eight cases, with the presence of the *cep*A gene. The *qac*ΔE gene was associated with less than half of the strains showing the reduction of chlorhexidine and Medihex-4 MICs.

Table II
Individual minimum inhibitory concentrations (MICs) of chlorhexidine, Trigene and benzalkonium chloride in the presence of *cep*A, *qac*ΔE and *qac*E genes

Biocide	MIC (mg/L)	No. of strains	Antiseptic resistance genes					
			*cep*A	*qac*ΔE	*qac*E	*cep*A + *qac*E	*cep*A + *qac*ΔE	NO *cep*A + *qac*ΔE
Chlorhexidine	4	1						1
	8	1	1					
	16	5	5	4			4	1
	32	34	28	20			19	4
	64	20	19	9	1	1	8	
	128	3	3	1			1	
Trigene	16	9	7	1	1	1	1	2
	32	41	39	24			24	2
	64	14	10	9			7	2
Benzalkonium chloride	16	3	3	1			1	
	32	48	43	27	1	1	25	3
	64	13	10	6			6	3

Table III
Effect of CCCP (10 mg/L) on the minimum inhibitory concentration (MIC) of chlorhexidine correlated with the presence of *cep*A and *qac*ΔE genes

No. of strains	Fold reduction of chlorhexidine MIC in 10 mg/L CCCP	*cep*A	*qac*ΔE
1	8	−	−
1	8	+	−
3	8	+	+
1	16	−	−
3	16	+	−
3	16	+	+
11	32	+	−
2	32	−	+
15	32	+	+
1	64	−	−
7	64	+	−
9	64	+	+
2	128	+	−
1	128	+	+

CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

Discussion

The increasing incidence of *K. pneumoniae* infections in hospitals worldwide has led to greater awareness of the hazards of nosocomial infection and efforts to improve infection control using appropriate hygiene measures.

Biocides are currently considered to be an essential component of infection control strategies in hospitals and Block and Furman have observed a significant inverse relationship between the intensity of chlorhexidine use and overall susceptibility of organisms (*S. aureus*, coagulase-negative staphylococci, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter baumannii* and *Candida albicans*) to this antiseptic.[16,22] Koljalg *et al.* found a strong correlation between chlorhexidine and antibiotic susceptibility in MICs among 70 distinct clinical isolates of Gram-negative bacteria, comprising *Escherichia coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *S. aureus* (not MRSA), *Streptococcus pyogenes* and *Enterococcus faecalis*. They noted that non-fermentative bacteria tolerated chlorhexidine at high concentrations better than the Enterobacteriaceae and that Gram-positive cocci, especially *S. pyogenes*, were the most susceptible.[23]

In order to examine the effect on one species in detail, in this study the efficacy of five biocides, extensively used in hospitals, was screened against 64 clinical isolates of *K. pneumoniae*. Mediscrub was the most effective biocide against *K. pneumoniae*, with MICs between 0.12 and 0.5 mg/L, followed by MediHex-4; chlorhexidine, benzalkonium chloride and Trigene were the least effective.

These results may be explained in part by the fact that most of these unrelated *K. pneumoniae* strains carried the *cepA* efflux pump gene. The MIC assays performed in the presence of CCCP showed that there was a considerable decrease in the MICs of chlorhexidine for almost all the strains and this was usually associated with the presence of *cepA*. On the other hand, there were reductions in the MICs of MediHex-4, but these were not so great as with chlorhexidine, and were not significantly associated with the presence of either the *cepA* or *qac* genes. This suggests that efflux pumps, as yet undefined, are capable of exporting MediHex-4 but this does not play an important role in MediHex-4 susceptibility in these *Klebsiella* strains. The lack of influence of CCCP on the MICs of benzalkonium chloride, Trigene and Mediscrub suggests that other resistance genes are more important, particularly for benzalkonium chloride and Trigene. It was interesting to note though that for both these antiseptics there was a high proportion of strains, with reduced susceptibility, that had both the *cepA* and *qacΔE* genes.

It is interesting to note that the proportion of reduced biocide susceptibility was high and the carriage of biocide resistance genes was also high. This suggests that the *Klebsiella* population in the Infirmary has been exposed to a considerable quantity of biocides and that reduced susceptibility is creeping into the population. However, the clinical importance of this may be doubtful as the concentration of biocides used is usually far greater than the MICs, even of the strains with reduced susceptibility. This is likely to be become more important when other factors are taken into account, such as biofilm formation, which already compromise the activity of the biocide.[13,14] Furthermore, this population was not noticeably resistant to the antibiotics tested and these results are in contrast with those of Koljalg *et al.*; however, in that study only a very small proportion of the strains tested were *Klebsiella* spp.[23] There is evidence in other species that biocide resistance genes can be closely linked to antibiotic resistance genes and, if that occurs in this species in the future, the use of biocides could select for antibiotic-resistant strains.[24]

Table IV
Effect of CCCP (10 mg/L) on the minimum inhibitory concentration (MIC) of Medihex-4 (MH-4) correlated with the presence of *cep*A and *qac*ΔE genes

No. of strains	Fold reduction of MH-4 MIC in 10 mg/L CCCP	*cep*A	*qac*ΔE
1	2	+	+
2	4	−	−
4	4	+	−
1	8	−	−
1	8	−	+
6	8	+	+
2	16	−	−
1	16	−	+
11	16	+	−
21	16	+	+
1	32	−	−
7	32	+	−
5	32	+	+
1	64	+	+

CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

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Conflict of interest statement
None declared.

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