Brief Communication

Bactericidal activity of five antiseptics on *Klebsiella pneumoniae* and its relationship to the presence of efflux pump genes and influence of organic matter

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Klebsiella pneumoniae is responsible for about 8% of hospital-acquired infections. Biocides, including quaternary ammonium compounds (QACs) and biguanides, are frequently used in hospitals and there is evidence that their use has reduced hospital-acquired infections significantly.[1] The routine application of biocides could result in decreased susceptibility over time, which may eventually lead to the development of resistance to themselves and antibiotics.[2] This study assessed the bactericidal activity of five biocides, both under ideal conditions and in the presence of organic matter. The efficacy of biocide action was compared with the association of efflux pump genes that have been linked to antiseptic resistance (*cep*A, *qac*Δ*E*, *qacE*).[3]

Six isolates of *K. pneumoniae* isolated from patients in the Royal Infirmary Edinburgh were chosen for their varying sensitivity to chlorhexidine (CHX). The common hospital biocides used were 1% CHX gluconate, a member of biguanide family, and 1% benzalkonium chloride (BZK), a QAC, both were supplied from Sigma (Poole, UK), The commercial biocide preparations Trigene, the main component being polymeric biguanide hydrochloride, the cationic biocide Medihex-4 (MH-4), containing 4% CHX gluconate, and Mediscrub (MS), containing 1% polymeric biguanide hydrochloride, were obtained from Medichem International (Kent, UK).[4] The exact details and composition of the proprietary products may be found with reference to their accompanying literature.

Susceptibility studies were performed with each biocide; if it was a mixture the result was expressed as the concentration of the principal component, as listed above. Minimum inhibitory concentrations (MICs) were determined by the agar dilution method on Iso-Sensitest agar (Oxoid Ltd, Basingstoke, UK) and the agar plates were incubated in air at 37°C for 24 hours.[5] Minimum bactericidal concentrations (MBCs) were defined as the lowest concentration of the biocide required to kill 99.9% of the original bacteria within a given time (in this case 3 minutes).[6,7] Evaluation of MBC of the five disinfectants against *K. pneumoniae* was conducted according to the European standard test method EN 1040 on Tryptone Soya Agar (Oxoid).[7] One hundred μl of $\sim 10^8$ cfu/ml bacterial suspension was added to 900 μl biocide solution and the mixture was maintained at 20–22°C (room temperature) for 3 minutes, 100 μl of this mixture was transferred to 900 μl of neutralizer solution comprising 10% Tween 80, 0.1% histidine and 0.5% sodium thiosulphate (Sigma, Poole, UK), 3% lecithin soybean (MP Biomedicals, LLC Cambridge, UK) in Phosphate buffer saline (PBS, pH 7.4). This mixture was kept at 20–22°C for 3 minutes and diluted 1 : 10 serially with PBS and 100 μl aliquots of the diluted mixture was spread onto Tryptone Soya Agar plates and incubated at 37°C for 18–24 hours.[7] As a control, the toxicity of the neutralizer to the bacterial activity was evaluated by adding 100 μl of bacterial suspension to 900 μl of neutralizer.[7] In both cases, care was taken to measure the sensitivity to the active component of each antiseptic, as stated by the manufacturer, and this is the figure quoted in the results.

Time killing test were performed to assess the bactericidal effects of CHX (360 mg/l) following the European standard test method EN 1040, with assay times of 1, 3, 5 and 10 minutes.[7] This test was repeated to evaluate bactericidal activity, under conditions that simulated the presence of organic matter, in this case by using bovine serum albumin (BSA) (Roche Diagnostics Ltd, Burgess Hill, UK). The test was repeated with BSA (1, 3, and 10%) added complying with the European standard test method EN 1276.[7]

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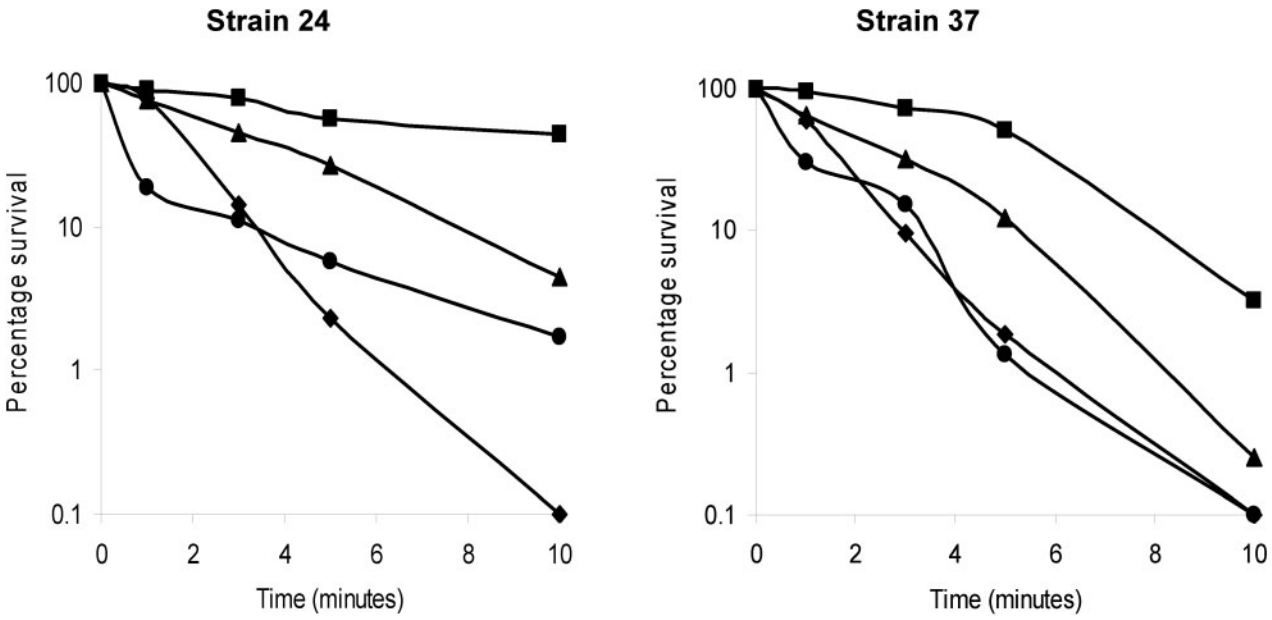

Figure 1 The effect of time on the bactericidal effect of chlorhexidine (360 mg/l) against *K. pneumoniae* strains 24 and 37 and the impact of increasing concentrations of bovine serum albumin. The viability of strains 24 and 37 was monitored against time in the presence of chlorhexidine at 40% of the MBC (♦). The experiment was repeated in the presence of bovine serum albumin at concentrations of 1% (●), 3% (▲), and 10% (■).

The *cep*A, *qac*ΔE, and *qac*E antiseptics resistance genes were amplified by PCR. The primers pairs of Fang *et al.* were used to identify the *cepA* gene, which encodes a cation efflux pump associated with CHX resistance[8] and the *qacΔE* and *qac*E genes were amplified by the primer pairs of Kazama *et al.*[9]

All strains were sensitive to cefotaxime, ceftazidime, imipenem and meropenem; only strain 52 was resistant to cefoxitin. Strains 24 and 52 were resistant to pipericillin/tazobactam and only the latter could be considered multi-antibiotic resistant (Table 1). The MICs of the biocides showed that, in 4, 5, and 6 *K. pneumoniae* isolates, there was reduced susceptibility to CHX, Trigene and BZK respectively with MICs ranging from 32 to 128 mg/l (Table 1). However, MH-4 showed little variation in MICs (8–16 mg/l) in all isolates. On the other hand, five of the six isolates were susceptible to MS (MICs ≤4 mg/l) (Table 1). PCR detected the characteristic 1051 bp fragment of the amplified *cep*A gene in five isolates whereas the *qacΔE* gene was detected in two isolates; both also had the *cep*A gene. One isolate had neither gene (Table 1). Most of the strains carrying the *cepA* gene had reduced susceptibility to CHX but it was not possible to relate susceptibility to the other antiseptics to the carriage of either the *cepA* or *qacΔE* genes.

MBCs were calculated for the isolates using the differences between the proportion of surviving colonies and a control (bacterial suspension was treated with PBS only). The MBC of Trigene was the same for all strains at 1800 mg/l of the active component (polymeric biguanide hydrochloride). The MBCs of CHX ranged from <360 mg/l to 900 mg/l. whereas those for MH4, between <36 mg/l to 360 mg/l, and BZK, at 90 mg/l, were lower. The

Table 1 Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) of antiseptics, and the carriage of the *cep*A and *qacΔ*E efflux genes in *K. pneumoniae*

Strain no.	Antiseptic												Antibiotic resistance
	MIC (mg/l)					MBC (mg/l)					Efflux genes		
	CHX	Tri	MH-4	MS	BZK	CHX	Tri	MH-4	MS	BZK	*cep*A	*qac*ΔE	
14	4	16	8	0.12	32	<360	1800	<36	9000	90	−	−	
16	8	32	8	0.5	64	<360	1800	<36	9000	90	+	−	
24	128	64	8	0.5	32	900	1800	360	9000	90	+	+	CAR, DO, E, PRL
37	128	32	8	0.12	32	900	1800	360	9000	90	+	−	CAR, E, DO
52	128	64	16	0.5	64	<360	1800	<36	9000	90	+	−	CAR, CHL, CXT, DO, E, NV, PRL, TCG, TMP
64	64	64	16	>128	32	<360	1800	<36	9000	90	+	+	CAR

Note: CHX, Chlorhexidine; Tri, Trigene; MH-4, MediHex-4; MS, MediScrub; BZK, Benzalkonium chloride; CAR, Carbenicillin; CHL, Chloramphenicol; CXT, Cefoxitin; DO, Doxycycline; E, Erythromycin; NV, Novobiocin; PRL, Piperacillin/Tazobactam; TCG, Tigecycline; TMP, Trimethopim.

MBC for Mediscrub was the highest for each strain at 9000 mg/l. The presence of the two efflux genes had no influence on the MBC values (Table 1).

CHX is the active component of many proprietary antiseptics. The activity of CHX was measured at 40% of the lowest determined figure for the MBC (360 mg/l), in order to obtain a drop in viability that could be precisely measured over time. Even at these concentrations CHX had the ability to reduce the viability of both *K. pneumoniae* strains 24 and 37 within 10 minutes (Fig. 1). In both cases, the rate of kill was logarithmic in relation to time. When BSA was added, the ability of CHX to kill was reduced; virtually eliminated for strain 24 in 10% BSA though its effect was considerably less in strain 37 (Fig. 1). It is interesting to note that the presence of a small concentration of BSA (1%) initially promoted the killing effect; this is not considered to be an artefact because it was found to be repeatable with both strains.

There is very little in published literature about the effects of CHX and other antiseptics on clinical strains of *K. pneumoniae*. Koljalg *et al.*[10] found a correlation between CHX and antibiotic susceptibility in a mixed collection of clinical isolates. In our study, all strains showed reduced susceptibility to BZK and Trigene, the MIC of polymeric biguanide hydrochloride, the main active component, was measured. On the other hand all, except strain 64, were susceptible to Mediscrub, again the MIC of polymeric biguanide hydrochloride was measured but this activity may have been boosted by the surfactant sodium lauryl sulphate. Mediscrub is used for washing hands and the surfactants, present to reduce the surface tension, clearly are also having an effect on the viability of the bacterial cells. The main component of Medihex-4 is CHX gluconate and it was the MIC of this compound that was measured; however, the MICs of Medihex-4 were lower than that of the CHX preparation. Medihex-4 also contains cocamidopropyl betaine and sodium lauryl sulphate as surfactants and these may contribute to the reduced MICs found with this preparation.

Although most of the strains had reduced susceptibility to CHX, this could not be correlated with multiresistance to antibiotics. There was no correlation of reduced susceptibility to CHX with the presence of the *qacΔE* gene but all the strains showing reduced CHX susceptibility did possess the *cepA* gene.

The MICs of the polymeric biguanide hydrochloride antiseptics, Mediscrub and Trigene, were much lower than the MBCs, in some cases by 10 000-fold. This strongly indicates that these antiseptics are primarily bacteriostatic in their action against *K. pneumoniae* and to obtain a rapid kill, large quantities would have to be used. This suggests that, in clinical concentrations, they have little capacity to eradicate *K. pneumoniae*. Although the MIC of BZK was high, the MBC was not much higher indicating that once the concentration threshold of inhibiting the bacterium had been reached, a three-fold increase was sufficient to kill the bacteria quickly.

Similarly the differential between the MICs and the MBCs of the two CHX compounds was low and a 5-fold increase above the MICs of CHX was sufficient to elicit a rapid bactericidal response, which would be important in clinical practice. The presence of organic matter, represented here by BSA, suggests that CHX is very sensitive to its presence though the extent of this will vary from strain to strain. In conclusion, clinical *K. pneumoniae* may not respond to common biocides, which may not kill the bacteria especially in the presence of organic matter.

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