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Ability of chlorhexidine, octenidine, polyhexanide and chloroxylenol to inhibit metabolism of biofilm-forming clinical multidrug-resistant organisms

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Abstract

Purpose: This in vitro study was designed to determine if standard antiseptics used for skin and environmental surface cleansing can disrupt the metabolic activity (as a measure of viability) of multidrug-resistant gram-negative bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* isolates within their native biofilms.

Methods: Sixty clinical isolates of multidrug-resistant bacteria were selected for testing in different chlorhexidine gluconate, octenidine, polyhexanide and chloroxylenol concentrations. Metabolic inhibition of biofilm for each clinical isolate was analysed using a biofilm viability assay.

Results: Chlorhexidine gluconate (mean = 83.8% ± 9.8%) and octenidine (mean = 84.5% ± 6.8%) showed the greatest efficacy against biofilms of the tested microorganisms, with the greatest efficacies against MRSA. The antiseptics demonstrated the least efficacy against biofilms of *Pseudomonas aeruginosa*.

Conclusion: Chlorhexidine gluconate and octenidine showed the greatest level of bacterial metabolic inhibition and were statistically equivalent. Polyhexanide was more effective than chloroxylenol, but both were inferior to chlorhexidine gluconate and octenidine against the tested organisms.

Keywords

Chlorhexidine, octenidine, multidrug resistant gram-negatives, hospital-acquired infection, MRSA, biofilm

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Introduction

Hospital-acquired infections (HAIs) are a common problem, complicated by increasing microbial antibiotic resistance and persistence despite efforts to establish best practices for the prevention of HAIs. In a large prospective cohort study, Lambert and colleagues found that 7% of patients admitted to an intensive care unit developed healthcare-associated pneumonia and 4% developed bloodstream infections (Lambert et al., 2011). The most common isolates were *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Antibiotic resistance was found in 75% of the *A. baumannii*, 11% of the *E. coli*, 22% of the *P. aeruginosa* and 34% of the *S. aureus* isolates (Lambert et al., 2011).

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The development of HAIs, particularly when combined with antibiotic resistance, leads to an increased length of stay in hospital and increased mortality (Kyaw et al., 2015). Lye and colleagues reported an excess hospital stay of 6.1 days in patients with gram-negative bacterial HAIs (Lye et al., 2012) and a study of vancomycin-resistant *Enterococcus* (VRE; *vanB* type) found VRE bacteraemia to be associated with longer lengths of stay and greater costs of hospitalisation (Cheah et al., 2013).

It is estimated that at least 59.5% of isolates from general infection episodes may be acquired as a result of cross-transmission from the environment (Agodi et al., 2007). Biofilm-forming organisms, such as *P. aeruginosa* and *S. aureus*, are less susceptible to decontamination efforts, owing to the protective effects of biofilm (Akinbobola et al., 2017; Alhede et al., 2014; Gandee et al., 2015). Persistence of these microorganisms within biofilm can lead to contamination of medical devices and to infection (Auler et al., 2010; Galli et al., 2016; Singhal et al., 2011; Wilson et al., 2012).

Biofilm-forming bacteria often exhibit antibiotic resistance. Extremely drug-resistant strains of *Pseudomonas*, as defined by Magiorakos and colleagues (Magiorakos et al., 2012), show significantly increased biofilm formation compared to non–antibiotic-resistant isolates, suggesting that biofilms enhance the fitness of *Pseudomonas* to persist in the environment (Kaiser et al., 2017). *Enterococcus* can also form biofilms associated with increased antibiotic resistance (Fallah et al., 2017), and methicillin-resistant *S. aureus* (MRSA) biofilms have been shown to be associated with the failure of antibiotic treatment and decolonisation measures (Gunther et al., 2017a). These findings underscore the importance of adequate antisepsis against biofilm-forming organisms.

Antisepsis with chlorhexidine gluconate (CHG) has been shown to be effective in reducing bacterial colonisation (Gunther et al., 2015). Several studies have documented that daily bathing with CHG reduces the risk of infection by drug-resistant bacteria in hospitalised patients (Climo et al., 2013; Huang et al., 2013; Lowe et al., 2017; Milstone et al., 2013; Mutters et al., 2015). Consistent application of standard infection control measures has been shown to reduce the likelihood of VRE infection in susceptible patients, resulting in the development of recommendations to adequately disinfect the environment (Mutters et al., 2013). In a study by Günther and colleagues, octenidine (OCT) and CHG inhibited MRSA within biofilms, but mupirocin, polyhexanide, and chloroxylenol did not show clinically relevant antimicrobial activity (Gunther et al., 2017b). The greatest bactericidal effects have been reported for CHG (log10 reduction of 9) (Gunther et al., 2017b).

In the present study, we sought to determine if active ingredients found in standard antiseptics used for skin and environmental surface cleansing can disrupt the metabolic activity (as a measure of viability) of multidrug-resistant (MDR) gram-negative bacteria, MRSA and VRE isolates within their native biofilms.

Materials and methods

Bacterial strains

A range of clinical isolates of MDR bacteria was selected for testing in different concentrations of the active pharmaceutical ingredient (API) of CHG (Sage Products LLC, Cary, IL, USA), OCT (TCI, Eschborn, Germany), polyhexanide (POL; Fagron, Barsbüttel, Germany) and chloroxylenol (CLO; Sigma-Aldrich, Taufkirchen, Germany). Gram-negative isolates were classified as MDR, as described elsewhere (Magiorakos et al., 2012). Clinical isolates of MRSA and VRE collected at the Heidelberg University Hospital (Heidelberg, Germany) were also selected for testing. In total, 40 MDR gram-negative isolates (10 MDR *Klebsiella pneumoniae*, 10 MDR *A. baumannii*, 10 MDR *E. coli* and 10 MDR *P. aeruginosa*) were tested. A total of 20 MDR gram-positive isolates (10 MRSA and 10 VRE) were also tested, for an overall total of 60 test isolates. Isolates were collected from patients with a diagnosed infection caused by an MDR organism during their stay at the Heidelberg University Hospital during the period 2014–2016. All strains were isolated from clinical samples routinely collected in the microbiology laboratory and stored at –70 °C. The present study is thus descriptive of a bacterial collection of those isolates. Data collected from patients were anonymised and restricted to possible clinical symptoms of infection only. Therefore, according to the World Medical Association Declaration of Helsinki (1964, Helsinki, Finland; revised 2013, Fortaleza, Brazil), ethical approval was not required. Test organism suspensions contained approximately 2–5 $\times$ 10^9 bacterial cells per millilitre. A 1-mL suspension of each test organism was mixed with 1 mL sterile water of standardised hardness (WSH) with added antiseptic and incubated for six exposure times (15 s, 1 min, 3 min, 5 min, 10 min, and 20 min) at 37 °C.

Biofilm viability assay

The ability of CHG, OCT, POL and CLO to inhibit metabolism of each of the 60 biofilm-forming clinical isolates was determined by a biofilm viability assay, as described previously (Gunther et al., 2017b). In brief, each antiseptic was diluted in WSH with a hardness of 300 ppm (measured $CaCO_3$) containing 1.24 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 3.33 mM $NaHCO_3$ (all from Carl Roth, Karlsruhe, Germany), standardised to a pH of 7 $\pm$ 0.2 and used at a temperature of 25 °C. Antiseptics were prepared at a working concentration, according to the concentration of disinfectants in commercially available ready-to-use products as well as at half the standard concentration, the latter to mimic the effects of the antiseptic being further diluted (i.e.

possible dilution in a clinical setting). The tested concentrations of the antiseptics were 1% and 2% (w/v) for CHG, 0.05% and 0.1% (w/v) for OCT, and 0.02% and 0.04% (w/v) for POL. CLO was tested at concentrations of 0.24% and 0.48% (w/v) using 0.25% (w/v) Tween 80 (Carl Roth) as solubiliser rather than WSH. The used concentrations accurately reflect the concentrations of active pharmaceutical ingredients found in various commercially available products. However, we tested only the API of those products. Pure dilutions of the active components of disinfectants, without adjuvants, were used in the assay. The media and neutralisers used in this study were validated as previously described for their ability to effectively neutralise the disinfectants used here, before our experiments (Gebel et al., 2002; Gunther et al., 2017b).

To detect surviving bacteria, bacterial metabolic activity was measured by detecting the percentage of conversion of resazurin to resorufin at 620 nm with a Sunrise RC photometer (Tecan, Männedorf, Switzerland). The percentage reduction in conversion rate, compared between treated and untreated samples, was used as the determinant of bactericidal efficacy. This was measured every 60 min for up to 7 h for each sample during incubation at 37 °C. A control solution of bacteria exposed to WSH without antiseptic was used to define 100% viability or 0% inhibition by antiseptic.

Statistical analysis

For descriptive purposes, arithmetic mean, SD, median, interquartile range and cumulative frequency were calculated, as appropriate. Categorical and continuous variables were analysed using either the Student's *t*-test or non-parametric tests, as appropriate. Normal distribution was tested with the Kolmogorov–Smirnov test. A P value of < 0.05 was considered to indicate statistical significance. Pooled efficacy was calculated to control for statistical outliers, usually found in biofilm testing, and to increase statistical robustness. Statistical analyses were performed using SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA).

Results

The pooled efficacy of all of the antiseptics to inhibit metabolic activity within biofilms is shown in Table 1. Control values were used to calculate pooled efficacy by subtracting control values and using them as baseline activity. Percent values indicate inhibition of bacterial metabolism within biofilms, ranging from 0% inhibition (least efficacy of tested antiseptic) to 100% inhibition (greatest efficacy of tested antiseptic). CHG (mean = 83.8% ± 9.8%) and OCT (mean = 84.5% ± 6.8%) showed the greatest efficacy against biofilms of the tested microorganisms, with no significant difference between the two antiseptics ($P = 0.81$) (Table 1). POL (mean = 65.4% ± 21.8%) showed a significantly greater efficacy against the tested organisms than CLO (mean = 53.4% ± 26.3%; $P < 0.05$); however, both were inferior to CHG and OCT.

The least efficacy of all of the antiseptics was observed against biofilms of *P. aeruginosa* (Table 2). The greatest efficacies of CHG and OCT were observed against MRSA. The greatest efficacy of POL was observed against VRE and the greatest efficacy of CLO was observed against *K. pneumoniae* (Table 2).

The use of lower or higher concentrations of OCT, CHG, POL and CLO had no significant effect on efficacy (Table 3).

The efficacies of OCT and POL were significantly greater against gram-positive bacteria than gram-negative bacteria. CLO showed greater efficacy against gram-negative bacteria than gram-positive bacteria. The efficacy of CHG did not differ significantly between gram-positive and gram-negative bacteria (Table 4). Overall, CHG and OCT had greater efficacy against both gram-positive and gram-negative bacteria than POL or CLO (Table 4).

Discussion

We tested commonly used antiseptics on the biofilm formations of clinical isolates of MDR gram-negative bacteria (*K. pneumoniae, A. baumannii, E. coli* and *P. aeruginosa*) and gram-positive bacteria (MRSA and VRE). We found that CHG (mean = 83.8% ± 9.8%) and OCT (mean = 84.5% ± 6.8%) showed the greatest efficacy against biofilms of the tested microorganisms. The implications of the present study relate to the effective selection of antiseptic agents to ensure adequate disinfection of the environment (Mutters et al., 2013) and reduction of bioburden, HAIs and device-related infections via antiseptic washing (Lambert et al., 2011; Melsen et al., 2013). One of the strengths of this particular study was that the biofilm-forming isolates were collected from a clinical setting and included multiple MDR strains to ensure clinical relevance. Prior studies have shown that antiseptics, including those tested in the present study, are effective against planktonic bacteria. However, pathogenic bacteria are mainly found in biofilms, as this is their natural state (Gunther et al., 2017a, 2017b; Kaiser et al., 2017).

CHG and OCT showed the greatest levels of bacterial metabolic inhibition and were statistically equivalent to each other. POL was more effective than CLO, but both were inferior to CHG and OCT. Our findings regarding CHG and OCT are similar to those of Gunther and colleagues, who reported that CHG and OCT were effective for disinfection of MRSA biofilms in vitro using the same method that we used for determining metabolic activity (Gunther et al., 2017b).

Prior studies have shown OCT and/or CHG to be effective against biofilm-forming organisms but used different methods for measuring the survival of microorganisms in

Table 1. Pooled efficacy of CHG, OCT, POL and CLO and mean efficacy to inhibit metabolic activity depicted per species, incubation time and concentration.*

	Incubation time	MRSA		*Klebsiella pneumoniae*		*Acinetobacter baumannii*		*Escherichia coli*		VRE		*Pseudomonas aeruginosa*		Pooled efficacy (%)	SD (%)	*P*
CHG	Concentration	1%	2%	1%	2%	1%	2%	1%	2%	1%	2%	1%	2%	–83.8		
	15 s	–88.7	–55.3	–73.9	–65.1	–78.3	–82.2	–65	–75.1	–77.1	–61.8	–72.6	–76.9			
	1 min	–95.4	–93.2	–74.4	–79.9	–86.4	–91.7	–92.1	–91.3	–83.7	–81.9	–71.1	–71.8			
	3 min	–95.8	–95.3	–73.2	–76.5	–88.1	–92.9	–91.8	–91.6	–85	–83.4	–70.9	–70.5		9.8	0.81†
	5 min	–95.9	–95.5	–86.1	–87.4	–89.4	–93.1	–92.1	–92.1	–85.4	–83.8	–72.4	–71.5			
	10 min	–96	–95.5	–85.6	–79.7	–91.4	–93.4	–92.1	–92.2	–85.4	–84.3	–72.2	–70.3			
	20 min	–95.8	–95.6	–84.5	–89.3	–92.9	–93.6	–92.2	–91.9	–84.9	–84.4	–73.5	–74.2			
OCT	Concentration	0.05%	0.1%	0.05%	0.1%	0.05%	0.1%	0.05%	0.1%	0.05%	0.1%	0.05%	0.1%	–84.5		
	15 s	–94.5	–94.6	–77.3	–80.7	–62	–67.3	–86.2	–81.3	–81	–82.4	–68	–76.7			
	1 min	–94.7	–94.7	–81.6	–86.1	–86	–87.7	–84.4	–88	–84.3	–84.3	–73.7	–76.7			
	3 min	–94.7	–94.7	–82.3	–85.9	–83.6	–89	–87.9	–83.6	–84.4	–84.9	–75.2	–78		6.8	0.81†
	5 min	–94.6	–94.6	–83.3	–82.6	–87.3	–89.3	–84.4	–84.5	–85.1	–85	–82.2	–80.5			
	10 min	–94.8	–94.6	–81.6	–86.3	–86	–90.7	–73.4	–76.5	–84.7	–85.6	–84.4	–81.3			
	20 min	–94.2	–94.3	–84.3	–86.3	–89.2	–87.1	–83.4	–77.5	–83.7	–84.3	–85.9	–82.3			
POL	Concentration	0.02%	0.04%	0.02%	0.04%	0.02%	0.04%	0.02%	0.04%	0.02%	0.04%	0.02%	0.04%	–65.4		
	15 s	–43.6	–52.6	–8.9	–12.9	–29.9	–39.5	–22.2	–31.2	–78.1	–76	–17.5	–21.3			
	1 min	–58.3	–61.4	–19.4	–39.4	–65.2	–77.3	–26	–50.2	–79.6	–80.3	–37	–41.9			
	3 min	–62.3	–67	–46.9	–73.5	–86.2	–86.2	–49.6	–68.2	–82.8	–83.5	–55.9	–60.1		21.8	<0.05‡
	5 min	–67.7	–72.4	–65.3	–78.8	–88.1	–88.4	–62.6	–81.9	–82.8	–83.4	–60.1	–65.8			
	10 min	–68.7	–70.7	–77.6	–82.9	–88.8	–88.9	–76.7	–87.8	–83	–83.3	–63.9	–71.8			
	20 min	–79.4	–81.2	–70.3	–87.1	–87.2	–87.8	–81.5	–90.5	–83	–83.8	–66.3	–74.7			
CLO	Concentration	0.24%	0.48%	0.24%	0.48%	0.24%	0.48%	0.24%	0.48%	0.24%	0.48%	0.24%	0.48%	–53.4		
	15 s	–3.3	–6.4	–28.5	–45.6	–22.3	–21.3	–22.3	–31.6	–8.9	–36.3	–15.7	–20.3			
	1 min	–9.3	–19	–48	–66.5	–47.8	–46	–39.5	–52.1	–11.8	–51.3	–19.6	–27.7			
	3 min	–15.6	–28.8	–66	–81.3	–66.9	–63.7	–64.4	–77.7	–29.2	–66.8	–37.6	–41.3		26.3	<0.05‡
	5 min	–19	–33.7	–76.2	–87	–71.9	–72	–82.1	–88.9	–35.8	–75.9	–44.1	–50.7			
	10 min	–31.9	–46.2	–83.8	–90.8	–75.3	–77.1	–83.6	–86	–53.6	–83.3	–57.8	–62.2			
	20 min	–48.5	–65	–89.2	–91.5	–80	–82.2	–90.9	–95.1	–69.1	–84.1	–68.1	–72.4			

*Percent values indicate inhibition of bacterial metabolism within biofilms, ranging from 0% inhibition (least efficacy of tested antiseptic) to 100% inhibition (greatest efficacy of tested antiseptic). Red squares indicate the least antiseptic efficacy (bacterial inhibition), approaching 0% (darkest hue of red). Yellow squares indicate greater antiseptic efficacy than the hues of red but lesser efficacy than the hues of green (lightest to darkest hue of yellow, or lesser to greater efficacy within this category. Green squares indicate the greatest antiseptic efficacy, approaching 100% (darkest hue of green).
†OCT compared to CHG, Mann–Whitney *U* test.
‡POL compared to CLO, Mann–Whitney *U* test.
CHG, chlorhexidine gluconate; CLO, chloroxylenol; MRSA, methicillin-resistant *Staphylococcus aureus*; OCT, octenidine; POL, polyhexanide; VRE, vancomycin-resistant *Enterococcus*.

Table 2. Mean efficacy to inhibit metabolic activity depicted per species.

		Antiseptic			
		CHG	OCT	POL	CLO
		Mean efficacy (%)	Mean efficacy (%)	Mean efficacy (%)	Mean efficacy (%)
Bacteria	MRSA	−91.5	−94.6	−65.4	−27.2
	VRE	−81.8	−84.1	−81.6	−50.5
	Klebsiella pneumoniae	−79.6	−83.2	−55.3	−71.2
	Acinetobacter baumannii	−89.5	−83.8	−76.1	−60.5
	Escherichia coli	−88.3	−82.6	−60.7	−67.9
	Pseudomonas aeruginosa	−72.3	−78.7	−53.0	−43.1

CHG, chlorhexidine gluconate; CLO, chloroxylenol; MRSA, methicillin-resistant *Staphylococcus aureus*; OCT, octenidine; POL, polyhexanide; VRE, vancomycin-resistant *Enterococcus*.

Table 3. Mean efficacy to inhibit metabolic activity depicted per concentration.

				Mean efficacy		*P**
				(%)	SD (%)	
Antiseptic	CHG	Concentration	High	−83.5	10.7	0.32
			Low	−84.2	9.0	
	OCT	Concentration	High	−85.0	6.3	0.87
			Low	−84.0	7.3	
	POL	Concentration	High	−69.0	20.3	0.45
			Low	−61.7	22.8	
	CLO	Concentration	High	−59.1	24.9	0.58
			Low	−47.7	26.8	

*Student's *t*-test.
CHG, chlorhexidine gluconate; CLO, chloroxylenol; OCT, octenidine; POL, polyhexanide.

Table 4. Mean efficacy to inhibit metabolic activity depicted per gram type and antiseptic.

				Mean inhibition (%)	*P**
Antiseptic	CHG	Gram	Positive	−86.6	0.09
			Negative	−82.4	
	OCT	Gram	Positive	−89.4	<0.05
			Negative	−82.1	
	POL	Gram	Positive	−73.5	<0.05
			Negative	−61.3	
	CLO	Gram	Positive	−38.9	<0.05
			Negative	−60.7	

*Student's *t*-test.
CHG, chlorhexidine gluconate; CLO, chloroxylenol; OCT, octenidine; POL, polyhexanide.

biofilm (Amalaradjou and Venkitanarayanan, 2014; Buckingham-Meyer et al., 2007; Silva et al., 2013; Zelichenko et al., 2013). Most methods of assessing survival of bacteria in biofilm are based on detecting bacterial growth after disrupting the biofilm and harvesting the bacteria, measuring the mass of the biofilm or imaging the affected surface (e.g. endotracheal tubes). The method used in the present study uses photometric measurement of the rate of conversion of resazurin to resorufin as a reflection of the metabolic activity of residual bacteria in a biofilm (Gunther et al., 2017b). The percentage reduction in conversion rate, compared between treated and untreated samples, was used as the determinant of bactericidal efficacy. This method offers a way to detect persistent bacteria that may not have been detected using biofilm disruption, culture or other techniques.

A limitation of the present study is the small sample size, which might limit external validity. An additional limitation of this study is that the results of an in vitro study cannot be directly extrapolated to demonstrate clinical relevance in humans. In vitro studies serve as an important first step in research, but in vivo studies and additional research are needed to determine clinical relevance. Biofilm formation was assessed on representative isolates to ensure that isolates with greater biofilm production were selected. Thus, the tested isolates should reflect those that would cause a significant burden in the healthcare setting.

Conclusions

We found that the APIs of CHG and OCT showed the greatest level of bacterial metabolic inhibition and were statistically equivalent to each other in this regard. POL was more effective than CLO, but both were inferior to CHG and OCT.

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