Susceptibility of vancomycin-resistant and -sensitive *Enterococcus faecium* obtained from Danish hospitals to benzalkonium chloride, chlorhexidine and hydrogen peroxide biocides

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Abstract

**Purpose.** In Danish hospitals, the number of infections caused by vancomycin-resistant *Enterococcus faecium* (VRE *faecium*) has dramatically increased in recent years. Hospital disinfectants are essential in eliminating pathogenic microorganisms, and reduced susceptibility may contribute to hospital-associated infections. We have addressed whether clinical VRE *faecium* display decreased biocide susceptibility when compared to vancomycin-sensitive *Enterococcus faecium* (VSE *faecium*) isolates.

**Methodology.** In total 12 VSE *faecium* and 37 VRE *faecium* isolates obtained from Danish hospitals over an extended time period were tested for susceptibility towards three commonly applied biocides, namely benzalkonium chloride, chlorhexidine and hydrogen peroxide.

**Results.** For benzalkonium chloride, 89% of VRE *faecium* strains had a minimal inhibitory concentration (MIC) of 8 mg l⁻¹, whereas for VSE *faecium*, only 25% of the strains had an MIC of 8 mg l⁻¹. For chlorhexidine, the MIC of 95% of VRE *faecium* strains was 4 mg l⁻¹ or higher, while only 33% of VSE *faecium* strains displayed MIC values at the same level. In contrast, both VRE and VSE *faecium* displayed equal susceptibility to hydrogen peroxide, but a higher minimal bactericidal concentration (MBC) was found for the former. The efflux activity was also assessed, and this was generally higher for the VRE *faecium* strains compared to VSE *faecium*.

**Conclusion.** VRE *faecium* from Danish hospitals demonstrated decreased susceptibility towards benzalkonium chloride and chlorhexidine compared to VSE *faecium*, where the use of chlorhexidine is particularly heavy in the hospital environment. These findings suggest that biocide tolerance may characterize VRE *faecium* isolated in Danish hospitals.

INTRODUCTION

Vancomycin-resistant enterococci (VRE) are among the pathogens most frequently responsible for hospital-associated infections around the globe, such as urinary tract infections, bacteraemia, endocarditis and wound infections [1]. In particular, two species are of importance, namely *Enterococcus faecium* and *Enterococcus faecalis*. While infections with *E. faecalis* were previously the more common, the number of cases involving vancomycin-resistant *E. faecium* (VRE *faecium*) has increased dramatically in recent years [1, 2]. In Denmark, the number of nosocomial infections associated with VRE *faecium* began to increase in 2010, with 54 cases in 2012, 248 in 2013, 303 in 2014 and, most recently, 368 cases in 2015 [3]. The majority of *E. faecium* isolates from Danish hospital outbreaks have sequence types (ST) belonging to the Bayesian Analysis of Population Structure (BAPS) subgroups; CTs, cluster types; EtBr, ethidium bromide; MBC, minimal bactericidal concentration; MH, Mueller Hinton; MIC, minimal inhibitory concentration; MLST, MultiLocus Sequence Typing; ST, sequence types; TS, Trypticase soy; VRE, vancomycin-resistant *Enterococcus*. Three supplementary tables and two supplementary figures are available with the online version of this article.
Structure subgroups BAPS 2–1 and 3–3 (e.g. sequence types ST18, ST117, ST80, ST192 and ST203) [4, 5]. Thus, within a few years VRE faecium has been become a serious medical challenge.

Vancomycin is a cell wall-active glycopeptide that is used in the treatment of Gram-positive bacterial infections, including those caused by enterococci [6]. In enterococci, nine types (vanABCDEGLMN) of glycopeptide resistance determinants have been reported [7, 8]. Worldwide, the vanA and vanB genes are most prevalent [7, 8]. Accordingly, all isolates of VRE faecium from a large outbreak at a Danish hospital carried vanA even though they belonged to six different clonal complexes of E. faecium [9]. Similarly, vanA was located on plasmids and present in all isolates of VRE faecium obtained from multiple hospital outbreaks in Denmark in the period 2012–2013 [2]. In 2015, 368 vanA E. faecium were detected in Danish hospitals and were subdivided into 33 cluster types (CTs). Thus, the number of cases with VRE faecium has increased in Denmark in the period 2005–2015, with several vanA E. faecium clones involved [4].

Biocides are cleaning and disinfecting agents that control the growth of or kill bacteria, and they are widely used on hard surfaces and as skin antiseptics [10]. Reduced susceptibility to biocides has been associated with the activity of multidrug-resistance efflux pumps such as EmeA, EfrAB and Smr, and with qac genes [11, 12]. In enterococci, only a few studies have addressed biocide susceptibility. In a study of E. faecalis from human, food and animal sources, only four isolates out of 585 carried either qacA/B or smr, and of these only a single strain displayed an elevated minimum inhibitory concentration (MIC) towards a quaternary ammonium compound [13]. Biocide tolerance of E. faecium has recently been studied in the pork production chain, where one strain out of 12 isolates with qacA/B showed increased tolerance to chlorhexidine. This strain carried qacA/B [14]. Schwaiger et al. confirmed that E. faecium from animals, food and humans is generally susceptible to biocides [15].

In 1997, one study showed that hydrogen peroxide was ineffective for all VRE faecium strains tested, but three quaternary ammonium compounds were highly effective [16]. Here, we examined the biocide susceptibility of current hospital-associated enterococci in Denmark, and found that clinical VRE faecium may be associated with reduced susceptibility to benzalkonium chloride and chlorhexidine compared to VSE faecium isolates.

**METHODS**

**Bacterial strains**

For the present study, 12 vancomycin-susceptible E. faecium and 37 VRE faecium isolates were obtained from strain collections at Statens Serum Institut. The isolates were all clinical samples obtained from Danish patients, and were chosen to represent an extended time period and cover major subtypes (Table S1, available in the online version of this article). The VSE faecium isolates belonged to nine MultiLocus Sequence Typing (MLST) groups, including both BAPS 3–3 subgroups and non- BAPS 3–3 subgroups [17, 18, unpublished data]. Furthermore, 37 VRE faecium isolates were included belonging to four ST types, all of which were part of the BAPS 2–1 and 3–3 subgroups [2, 5]. In regard to VRE faecium, the presence of vanA was confirmed by polymerase chain reaction (PCR) [19]. Only one isolate per patient was included. Each frozen sample was directly inoculated onto a blood agar base (Oxoid, Roskilde, Denmark) supplemented with 5 % horse blood and incubated at 37 °C for 24 h, before being prepared as a new frozen stock solution with brain–heart infusion broth (BHI, Oxoid, Roskilde, Denmark) and glycerol (50 %). Strains were inoculated on Mueller–Hinton (MH) agar (Sigma-Aldrich, Denmark) plates supplemented with 5 % of horse blood and incubated at 37 °C for at least 24 h before each experiment.

**Biocides**

Three biocides were used in this study, namely benzalkonium chloride (10 mg l⁻¹; Sigma-Aldrich, Germany), chlorhexidine digluconate (20 mg l⁻¹; Brenntag Nordic A/S, Denmark) and hydrogen peroxide (30 mg l⁻¹; Sigma-Aldrich, Germany). All biocides were stored according to the manufacturers’ instructions.

**MIC and minimum bactericidal concentration (MBC)**

MICs were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) standards for antimicrobial susceptibility testing [20–22]. Briefly, overnight cultures in MH broth were diluted to 10⁴ c.f.u. ml⁻¹ for each strain and added to wells containing various concentrations of biocide with MH broth. After mixing the content of each well using a pipette, the plates were incubated for 24–48 h at 37 °C. Bacterial growth was checked visually after 24 h. MICs were determined as the lowest concentration without growth. For the MBC test, 10 µl from each well of the microtitre plate assay were spotted on MH agar (Oxoid, UK) and incubated at 37 °C. The lowest concentration without growth after 48 h of incubation was determined as the MBC.

**Gradient plate assay**

Twenty millilitres of Trypticase soy (TS) agar (Oxoid, Roskilde, Denmark) containing 10 mg l⁻¹ of benzalkonium chloride was poured onto square plates (120×120 mm) at a 5-degree angle. After solidification, the same amount of pure TS agar was poured on top, resulting in a gradient increase of the biocide concentration from one side of the plate to the other [23–25]. Overnight cultures in TS broth (Oxoid, UK) were inoculated with a sterilized cotton applicator in a straight line, and incubated for 24 h at 37 °C. Growth lengths for each strain were measured after incubation.
Efflux activity assay
The ethidium bromide (EtBr) Agar Cartwheel method was used [26] for assessing the efflux pump activity of VSE faecium and VRE faecium. Overnight cultures grown in TS broth were inoculated with a sterilized cotton applicator on TS agar containing 0.05% EtBr. After incubation at 37°C for 24 h, plates were examined under UV light and fluorescence was recorded.

Time–kill studies
Two VSE faecium strains with low MIC and MBC levels for benzalkonium chloride and chlorhexidine, and two VRE faecium strains with high MIC and MBC levels for the same biocides were chosen for the time–kill study. Overnight cultures of the selected strains were grown in BHI broth, and 100 μl from each overnight culture were then added to flasks containing 10 ml of saline solution (0.9% NaCl) with and without biocides. The flasks were incubated in a 37°C water bath while shaking at 150 r.p.m. Samples (100 μl) was taken at specific time intervals, diluted immediately with saline solution, spotted on BHI agar and incubated for 24 h at 37°C. Colony-forming units (c.f.u.) were determined the following day.

RESULTS
Decreased biocide susceptibility of VRE faecium clinical strains
In total 33 out of 37 (89%) of VRE faecium strains showed MIC values of 8 mg l⁻¹ for benzalkonium chloride. For VSE faecium, 3 out of 12 (25%) had an MIC of 8 mg l⁻¹ while the remainder (75%) had an MIC value of 4 mg l⁻¹ or lower (Fig. 1a). A similar result was seen when MBC was determined, where 36 out of 37 (97%) of VRE faecium strains had an MBC of 8 mg l⁻¹ or higher (Fig. 1b). For benzalkonium chloride, both MIC and MBC values indicated

![Graphs showing benzalkonium chloride MIC and MBC distribution for VSE and VRE strains.](image)

Fig. 1. Benzalkonium chloride MIC (a) and MBC (b) distribution for VSE and VRE strains.
a significant difference \( (P<0.0001) \); the chi-square test was used as a statistical test) between all VRE and VSE \textit{faecium} strains. This difference in susceptibility was also reflected when growth was observed on TSA agar plates carrying gradient concentrations of benzalkonium chloride [23–25], where the decreased susceptibility of VRE \textit{faecium} strains was evidenced as growth at greater concentrations of benzalkonium chloride than was apparent for VSE \textit{faecium} strains (Table S2). These results indicate a significant difference in benzalkonium chloride susceptibility between VSE and VRE \textit{faecium} strains, with most of the former being more susceptible than the latter.

For chlorhexidine, the MIC for 95% of the VRE \textit{faecium} strains (35 out of 37) was 4 mg l\(^{-1}\) or higher, while only 33% of VSE \textit{faecium} strains (4 out of 12) yielded MIC values at the same level (Fig. 2a). The MBC for 95% of the VRE \textit{faecium} strains (35 out of 37) was 4 mg l\(^{-1}\) or higher, compared to 50% of VSE \textit{faecium} strains at the same level (Fig. 2b). For chlorhexidine, both MIC and MBC results indicated a significant difference \( (P=0.0003 \text{ and } P=0.0013, \text{ respectively, by the chi-square test}) \) between all VRE and VSE \textit{faecium} strains. For hydrogen peroxide, we observed a significant difference in susceptibility only between VRE and the VSE \textit{faecium} at an MBC level where 75% of VRE \textit{faecium} showed MBC values of 70 mg l\(^{-1}\) or higher compared to only 25% of VSE \textit{faecium} with those values (Fig. 3a, b).

**Decreased biocide susceptibility correlates with efflux (pump) activity**

Using the EtBr Agar Cartwheel method, we assessed the accumulation of EtBr in 12 VSE \textit{faecium} strains and 37 VRE \textit{faecium} strains. As shown in Fig. 4a, most VSE \textit{faecium} strains showed various degrees of fluorescence when exposed to UV, while the majority of VRE \textit{faecium} strains displayed no fluorescence. According to fluorescence levels, strains were ranked for their efflux activity as high, medium or low efflux activity (Fig. 4a, b). In total 95% of VRE \textit{faecium} strains (35 out of 37) showed a high level of efflux
activity as they displayed no fluorescence, indicating that EtBr does not accumulate in these strains, and 2.5 % showed medium active efflux. For VSE faecium strains, only 17 % showed medium active efflux with the remaining strains displaying only a low level of efflux activity. Thus, reduced susceptibility to biocides correlates with increased efflux activity.

Enhanced survival in the presence of biocides correlates with decreased susceptibility

To examine the susceptibility of strains to biocide killing, two VSE faecium strains (VSE 216, VSE 361) with low MIC levels and two VRE faecium strains (VRE 483, VRE 537) with high MIC levels towards benzalkonium chloride and chlorhexidine were chosen (Table S3). Importantly, the VRE faecium strains with MIC values of 8 mg l⁻¹ showed a reduced level of killing by 10 mg l⁻¹ benzalkonium chloride compared to the VSE faecium strains, with MIC values of 2 mg l⁻¹ (Fig. S1). The difference in killing rate is significant (two-tailed t-test) between VRE and VSE faecium, and was apparent after only a short time exposure (15 min). Susceptibility to chlorhexidine-mediated killing was investigated at 100 mg l⁻¹ and, in general, the reduction in both VSE and VRE faecium strains was much lower than that for benzalkonium chloride. However, VSE faecium strains (MIC value of 1 mg l⁻¹) again showed somewhat greater reduction in killing rates than VRE faecium strains at all time intervals (Fig. S2), and a significant difference was observed in killing rates between VRE and VSE faecium at 15 and 30 min.

DISCUSSION

We assessed the biocide susceptibility of 37 VRE faecium isolates from Danish hospitals towards three biocides – benzalkonium chloride, chlorhexidine and hydrogen peroxide – and compared susceptibility to that of 12 VSE faecium isolates. In accordance with previous findings, VRE faecium and VSE faecium were found to be equally susceptible to hydrogen peroxide [16], although 75 % of VRE faecium showed MBC values of 70 mg l⁻¹ or higher compared to only 25 % for VSE.
faecium. In contrast, we found a statistically significant reduced susceptibility of VRE faecium to both benzalkonium chloride and chlorhexidine compared to VSE faecium at both MIC and MBC levels. Previously, no difference in susceptibility has been reported for these biocides [27] indicating either variation in biocide susceptibility among VRE faecium strains or that the biocide susceptibility of VRE faecium strains has decreased over time.

In regard to VSE faecium, 70% of the strains had MIC and MBC values of 4 mg l⁻¹ or lower towards benzalkonium chloride. These values are in agreement with those reported by Morrissey et al. [28] from a study of 53 clinical VSE faecium isolates collected worldwide between 1986 and 2009. For VRE faecium it has been reported that strains isolated from humans yielded MIC levels of 4 to 6 mg l⁻¹ for chlorhexidine and 5 to 6 mg l⁻¹ for benzalkonium [26], while
isolates from wastewater effluents yielded MIC levels of 2 mg l\(^{-1}\) or lower for chlorhexidine [29]. In both cases the susceptibility reported was greater than that observed in the present study, suggesting that VRE \textit{faecium} obtained from Danish hospitals are more tolerant to biocides in comparison to that reported in the literature.

Importantly the enhanced tolerance of VRE \textit{faecium} to benzalkonium chloride and chlorhexidine was also reflected in reduced biocidal killing when compared to VSE \textit{faecium}. The recommended concentration of these agents in everyday use is 0.1%, which is lower than that used in hospitals 0.2–0.3%). The highest concentration used in this study was 256 mg l\(^{-1}\), which is equal to 0.02%. These results imply that survival of VRE \textit{faecium} is superior to that of VSE \textit{faecium} in regard to two key cleaning and disinfection agents commonly used in hospitals; and that the selective advantage in the presence of these agents may increase the prevalence of VRE \textit{faecium} in hospitals.

Exposure to non-antibiotic antimicrobial agents can select for bacterial adaptations that result in decreased susceptibility to biocides, specifically among antibiotic-resistant isolates [30, 31]. In enterococci little is known of susceptibility to biocides, but in \textit{Staphylococcus aureus} reduced susceptibility to chlorhexidine and quaternary ammonium compounds has been related to the presence of the \textit{qacA/B} efflux pump [31, 32], which has also been detected in enterococci [13]. We observed decreased biocide susceptibility of the majority of VRE \textit{faecium} strains, accompanied by high efflux activity as monitored by ethidium bromide efflux. Our results corroborate reports that efflux pumps decrease the intracellular concentration of toxic compounds, including biocides, and these have been shown to reduce the efficacy of many biocides including phenolics, parabens, quaternary ammonium compounds and intercalating agents [33–37]. More recently, point mutations in a two-component signal transduction system led to increased chlorhexidine tolerance [34], indicating that tolerance to biocides can be obtained by multiple mechanisms. Also a recently published paper found that two genes of a two-component regulatory system, \textit{chtR} and \textit{chtS}, are important in bacterial adaptation to changes in the environment, and have been implicated in orchestrating cellular responses that lead to increased tolerance to antimicrobials (disinfectants) in enterococci [38]. Future studies will address the genetic basis for the decreased benzalkonium chloride and chlorhexidine susceptibility of VRE \textit{faecium} strains from Danish hospitals, and whether there is a genetic linkage to vancomycin resistance.

**Conclusion**

This study shows that there is a statistically significant difference in the susceptibility of benzalkonium chloride and chlorhexidine between VRE \textit{faecium} and VSE \textit{faecium} isolates obtained from Danish hospitals, with the former tolerating higher concentrations of these compounds than the latter. This decreased susceptibility correlates with active efflux activity, suggesting that decreased biocide susceptibility is mediated by increased efflux activity. While the recommended concentrations of benzalkonium chloride and chlorhexidine in everyday use are much higher than those examined here, lower effective concentrations of biocides may appear as a consequence of, for example, improper cleaning. Based on our findings, we speculate that benzalkonium chloride and chlorhexidine tolerance could be a contributory factor to the overall incidence of VRE \textit{faecium} infection in Danish hospitals.

**Funding information**

This work was funded by the Saudi Food and Drug Authority (SFDA).

**Acknowledgements**

Thanks go also to Jette Kjeldgaard and Mara Baldry for providing technical assistance.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

No experimental work was conducted on either humans or animals in this study.

**References**