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Enterococcus hirae, *Enterococcus faecium* and *Enterococcus faecalis* show different sensitivities to typical biocidal agents used for disinfection

M. Suchomel[a,*], A. Lenhardt[a], G. Kampf[b], A. Grisold[c]

[a] *Institute for Hygiene and Applied Immunology, Medical University of Vienna, Kinderspitalgasse 15, 1090, Vienna, Austria*
[b] *Institute for Hygiene and Environmental Medicine, University Medicine Greifswald, Ferdinand-Sauerbruch-Strasse, 17475, Greifswald, Germany*
[c] *Diagnostic & Research Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Neue Stiftingtalstraße 6, 8010, Graz, Austria*

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Background: *Enterococcus faecium* and *E. faecalis* are known nosocomial pathogens. The bactericidal activity of biocidal agents used for disinfection, however, is determined with *E. hirae*.
Aim: To find out whether *E. hirae* is a suitable species to evaluate the efficacy of biocidal agents against the clinically relevant species *E. faecalis* and *E. faecium*.
Methods: The bactericidal activity was determined in suspension tests according to EN 13727 using *E. faecium* ATCC 6057, *E. faecalis* ATCC 47077 and *E. hirae* ATCC 10541. Glutaraldehyde, ethanol, benzalkonium chloride, peracetic acid and sodium hypochlorite were used with three exposure times per biocide. When major differences in the sensitivity of the three enterococcal species to the respective substance was found, two more replicates were performed. The number of colony-forming units (cfu) was transformed into decimal logarithms. Results from replicate experiments were described with means and standard deviations.
Findings: At a 5-min exposure time, *E. hirae* was found to be more tolerant to 0.2% glutaraldehyde and 0.0125% peracetic acid compared to *E. faecium* and *E. faecalis*, whereas it was more susceptible to 40% ethanol and 3% sodium hypochlorite. Only with 0.00125% benzalkoniumchloride (15 min) was the susceptibility of *E. hirae* between that of *E. faecium* and *E. faecalis*.
Conclusions: *E. hirae* is a suitable species when a bactericidal activity should be determined against enterococci with glutaraldehyde and peracetic acid. *E. hirae* may not be a suitable species for ethanol or sodium hypochlorite if the bactericidal activity should include the clinical pathogens *E. faecium* and *E. faecalis*.

* Corresponding author. Address: Institute of Hygiene and Applied Immunology, Medical University of Vienna, Kinderspitalgasse 15, 1090, Vienna, Austria. Tel.: +43 1 40160 33031; fax: +43 1 40160 933000.
E-mail address: miranda.suchomel@meduniwien.ac.at (M. Suchomel).

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Introduction

Nosocomial infections or hospital-acquired infections (HAIs) are a major patient safety issue in hospitals. The most frequent nosocomial infections are pneumonia (usually ventilator-associated), urinary tract infection (usually catheter-associated) and primary bloodstream infection (usually associated with the use of an intravascular device) [1]. Virtually every pathogen has the potential to cause infection in patients, but only a limited number of bacterial species is responsible for the majority of HAIs. Among them *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and enterococci are the most common [2]. Enterococci account for about 10% of hospital-acquired bacteraemia cases and are a major cause of sepsis worldwide [3]. HAIs caused by enterococci are difficult to treat due to acquired resistance to many classes of antibiotics [4]. Considering the severity of the consequences of nosocomial infections, such as morbidity, mortality, prolonged stay, costs, and treatment problems, it is all the more important that preventive measures in hospitals and other health facilities are fully effective [5]. Targeted disinfection, with effective procedures and correctly performed, is one of the most important measures to interrupt the transmission of pathogens in hospitals. In Europe, the microbicidal effectivity of any disinfection procedure must be evaluated and confirmed in accordance with national or international standards and norms *in vitro* and under practical conditions before it can be used in hospitals [6]. These efficacy tests are performed with defined test species that are considered to be the most resistant representatives of a whole range of human pathogenic microorganisms and, due to their role in nosocomial infections, also include enterococci. As part of the standardization efforts to determine the efficacy of disinfectants at European level, the enterococcal strain *Enterococcus faecium*, formerly used for chemical and chemo-thermal disinfection processes, was replaced by *E. hirae*. *E. faecium* is currently only used for testing thermal disinfection processes, such as for instance for testing laundry disinfection processes at temperatures above 60°C [6]. The differences in heat tolerance between the enterococcal species is already well described resulting in the use of *E. hirae* for testing chemical disinfectants and *E. faecium* for chemo-thermal and thermal processes [7,8]. Pidot *et al.* showed in 2018 that some recently isolated multi-drug-resistant *E. faecium* isolates are more tolerant to 23% isopropanol than older isolates, suggesting an adaptive cellular response [9]. Overall, the chemical susceptibility of two common clinical species (*E. faecalis* and *E. faecium*) and the commonly used test species (*E. hirae*) has not yet been sufficiently investigated [10–12]. The aim of this study was therefore to find out whether *E. hirae* is a suitable species to evaluate the efficacy of biocidal agents against the clinically relevant species *E. faecalis* and *E. faecium*. Therefore, the in-vitro bactericidal efficacy was determined of five substances from commonly used groups of biocidal agents (aldehydes, alcohols, surfactants, oxidizing agents and halogens) on *E. hirae*, *E. faecium* and *E. faecalis* according to the European Norm EN 13727 [13].

Materials and methods

Test chemicals, test concentrations and exposure times

The test chemicals used in this study were pure biocides and included selected representatives from the five main active substance groups (aldehydes, alcohols, quaternary ammonium compounds, oxidizing agents and halogens): glutaraldehyde 25% (CAS-No. 111-30-8, Sigma Aldrich, Austria); ethanol absolute (CAS-No. 64-17-5, Merck, Germany); benzalkonium chloride 80% (CAS-No. 68424-85-1, Lonza Ltd., Switzerland); peracetic acid 39% (CAS-No. 79-21-0, Sigma Aldrich, Austria); sodium hypochlorite (6–14% active chlorine) (CAS-No. 7681-52-9, Merck, Germany).

The concentrations (volume percent concentrations, v/v %) and exposure times to be tested in this study were determined in advance by qualitative suspension tests and varied depending on the active agent. In this study, it was not the actual effective concentration of a disinfectant that was of interest, but the concentration and exposure time that could show a difference in sensitivity of the various enterococci. The biocidal agents were prepared as dilutions of the concentrates immediately before the experiments with water of standard hardness. Ethanol was tested as 25%, 40%, 50%, 60% and 70% solution and exposure times of 0.5, 1 and 5 min. The efficacy of glutaraldehyde was tested with concentrations of 0.10%, 0.15%, 0.20% and 0.25% and exposure times of 1, 5 and 15 min. The tested concentrations of benzalkonium chloride were 0.000625%, 0.00125% and 0.001875%, the exposure times 5, 15 and 30 min. Peracetic acid was tested with concentrations of 0.003125%, 0.00625%, 0.0125% and 0.025% within 1, 5 and 15 min and sodium hypochlorite was tested in 1, 5 and 15 min as 0.25%, 1.00%, 3.00% and 5.00% dilutions of the concentrate.

Neutralizing agents

Depending on the active agent, the tested chemicals required different neutralization. Therefore, prior to performing the suspension tests, appropriate neutralizing agents were identified for each species and chemical substance. The validation of the neutralizers, the neutralization controls and the non-toxicity controls were carried out according to EN 13727 [13]. The following were used: ethanol – no neutralizer necessary (dilution with pure broth was shown to neutralize any antimicrobial effect of remaining ethanol); glutaraldehyde and benzalkonium chloride – 3.0% polysorbate 80, 3.0% saponin, 0.1% histidine, 0.1% cysteine; peracetic acid – 3.0% polysorbate 80, 0.3% lecithine, 0.1% histidine, 3.0% saponin, 5.0% sodium thiosulfate; sodium hypochlorite – 3.0% polysorbate 80, 0.3% lecithine, 0.1% histidine, 5.0% sodium thiosulfate. All neutralizing agents used in this study were non-toxic to the tested enterococci.

Test strains

Tests standard reference strains were *E. hirae* (ATCC 10541), *E. faecium* (ATCC 6057) and *E. faecalis* (ATCC 47077) from the American Type Culture Collection. Bacterial test suspensions were prepared by transferring loopfuls of cells from a second subculture on agar plates into 10 mL of dilution fluid ((tryptone (1.0 g/L) and sodium chloride (8.5 g/L)) and mixing thoroughly. Cell counts were adjusted to 1.5–5.0 $\times$ 10^9 colony forming units (cfu) per millilitre.

Suspension test

The bactericidal activity of the five biocidal agents against enterococci was determined in a quantitative suspension test based on the European Norm EN 13727 [11]. Experiments were carried out using the dilution neutralization method. In a first step, all reagents were equilibrated to a temperature of 20°C (±1°C). One millilitre of test suspension was added to 1 mL of bovine albumin solution (0.3 g/L; clean conditions), mixed and incubated for 2 min at 20°C (±1°C). Then, 8 mL of the biocidal agent was added, and after the selected exposure time, 1 mL of the mixture was transferred to a tube containing 8 mL of an appropriate neutralizing solution and 1 mL of sterile water. After neutralization (5 min ±10 s or 10 s ± 1 s for contact times less than or equal to 10 min), 10-fold dilutions were made into tubes containing dilution fluid and neutralizing agents. Afterwards 0.1-mL and 1-mL aliquots of these solutions were plated on to tryptic soy agar (TSA; Caso agar®, Merck) and incubated at 36°C (±1°C) for 48 h. Each experiment was accompanied by a water control without the disinfectant. The water control was used to determine the cfu per mL without the action of a biocidal agent and is regarded as a survival control. The number of cfu per plate was counted after incubation and the number of cfu per millilitre was calculated, transformed into decimal logarithms, and the log reduction factors ($\log_{10}$ RF) were calculated.

In addition, those concentration and exposure time combinations that showed major differences in the sensitivity of the three enterococcal species to the respective substance were repeated twice. These data are described with means and standard deviations. Statistical analyses were performed using SPSS software, version 22.0 (IBM Corp, NY, USA). Significance of differences in mean $\log_{10}$ RFs between the three enterococcal species was determined by one-way analysis of variances (ANOVA). *Post hoc* test of differences was performed using Games–Howell tests. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Our results are presented in the Tables I–VI and explained here. The numbers in brackets represent $\log_{10}$-reductions. Ethanol at ≥50% led to $\log_{10}$-reductions ≥5 against all three enterococcal species, whereas concentrations ≤40% were not sufficiently effective with $\log_{10}$-reductions <5 (Table I). Significant differences in sensitivity between the three species were found with 40% ethanol at 5 min. *E. faecium* (1.56 ± 0.49) proved to be the most tolerant species to ethanol, followed by *E. faecalis* (4.04 ± 0.06). *E. hirae* was the most sensitive species (6.81 ± 0.45) (Table VI).

Glutaraldehyde was effective against all three species at 0.1% in 15 min and at 0.25% in 5 min (Table II). Major differences in sensitivity between the three species were found at 0.20% and 5 min (Table VI). *E. faecalis* was significantly less sensitive (6.09 ± 0.22) than the other two strains at this concentration and exposure time, *E. faecium* (5.31 ± 0.30) and *E. hirae* (4.17 ± 0.06).

Benzalkonium chloride at 0.001875% was effective with ≥5 $\log_{10}$ in 15 min against all three species (Table III). Significant differences in sensitivity of the three enterococcal species were seen at 0.00125% and 15 min (Table VI). *E. faecalis* proved to be the most tolerant species towards benzalkonium chloride (2.00 ± 0.18), *E. hirae* was more susceptible (3.88 ± 0.13) and *E. faecium* reacted most sensitively (5.13 ± 0.14).

Table I
Reduction ($\log_{10}$) in *Enterococcus hirae, E. faecium* and *E. faecalis* viability after exposure to ethanol under clean conditions

Strain	Exposure time (min)	Concentration of ethanol (v/v)				
		25%	40%	50%	60%	70%
E. hirae	0.5	1.16	1.26	7.16	7.26	7.16
	1	1.29	1.16	7.29	7.16	7.29
	5	1.25	7.31	7.25	7.31	7.25
E. faecium	0.5	1.25	1.36	5.44	7.36	7.25
	1	1.29	1.34	5.55	7.34	7.29
	5	1.21	1.24	7.21	7.24	7.21
E. faecalis	0.5	1.36	1.40	7.36	7.40	7.36
	1	1.43	1.35	7.43	7.35	7.43
	5	1.46	4.11	7.46	7.39	7.46

Table II
Reduction ($\log_{10}$) in *Enterococcus hirae, E. faecium* and *E. faecalis* viability after exposure to glutaraldehyde under clean conditions

Strain	Exposure time (min)	Concentration of glutaraldehyde (v/v)			
		0.10%	0.15%	0.20%	0.25%
E. hirae	1	1.56	1.25	1.25	1.56
	5	1.35	2.99	4.18	6.15
	15	7.46	7.24	7.24	6.99
E. faecium	1	1.23	1.20	1.20	1.23
	5	1.23	3.24	5.63	7.23
	15	7.16	7.24	7.24	7.24
E. faecalis	1	1.49	1.63	1.63	1.49
	5	1.52	3.97	6.32	7.52
	15	7.49	7.43	7.43	7.34

Table III
Reduction ($\log_{10}$) in *Enterococcus hirae, E. faecium* and *E. faecalis* viability after exposure to benzalkonium chloride under clean conditions

Strain	Exposure time (min)	Concentration of benzalkonium chloride (v/v)		
		0.000625%	0.00125%	0.001875%
E. hirae	5	1.42	2.16	5.65
	15	1.34	3.53	6.50
	30	1.38	5.08	7.34
E. faecium	5	1.19	2.75	5.47
	15	1.38	5.15	7.39
	30	1.30	5.42	7.23
E. faecalis	5	1.45	1.45	3.59
	15	1.28	1.85	5.33
	30	1.31	2.61	6.22

Table IV

Reduction (log_{10}) in *Enterococcus hirae*, *E. faecium* and *E. faecalis* viability after exposure to peracetic acid under clean conditions

Strain	Exposure time (min)	Concentration of peracetic acid (v/v)			
		0.003125%	0.00625%	0.0125%	0.025%
E. hirae	1	1.16	1.16	1.31	7.31
	5	1.17	1.17	1.23	7.23
	15	1.16	1.16	1.27	7.27
E. faecium	1	1.20	1.20	1.27	7.27
	5	1.11	1.11	7.13	7.13
	15	1.11	1.11	7.02	7.02
E. faecalis	1	1.35	1.35	4.36	7.13
	5	1.38	7.08	7,18	7,18
	15	1.39	7.39	7,37	7,37

Table V

Reduction (log_{10}) in *Enterococcus hirae*, *E. faecium* and *E. faecalis* viability after exposure to sodium hypochlorite under clean conditions

Strain	Exposure time (min)	Concentration of sodium hypochlorite (v/v)			
		0.25%	1%	3%	5%
E. hirae	1	1.26	1.20	3.25	7.15
	5	1.38	1.27	5.63	7.34
	15	1.39	1.22	7.30	7.14
E. faecium	1	1.40	1.09	1.22	7.15
	5	1.31	1.19	1.24	7.23
	15	1.30	1.17	1.25	7.26
E. faecalis	1	1.45	1.48	1.42	7.34
	5	1.66	1.43	4.04	7.37
	15	1.61	1.48	7.42	7.48

Data obtained with peracetic acid show a variable picture. Only a concentration of 0.025% peracetic acid was able to reduce all enterococcal species by at least 5 log_{10} within 1 min. Peracetic acid at 0.0125% reduced *E. faecium* and *E. faecalis* by more than 5 log_{10} steps in 5 min but not *E. hirae*, not even in 15 min (Table IV). Differences in sensitivity of the three enterococcal species were best seen at 0.0125% and 5 min (Table VI). Whereas *E. faecium* and *E. faecalis* showed comparable sensitivities against peracetic acid (6.61 ± 0.55 and 6.88 ± 0.26), *E. hirae* was not sufficiently reduced (1.69 ± 0.42).

Sodium hypochlorite led to log_{10}-reductions >5 in all three species at concentrations higher than 5%, whereas the 1% solution was not effective enough (Table V). Major differences in sensitivity were observed at 3% at 5 min (Table VI). *E. faecium* proved to be most resistant species against sodium hypochlorite (1.60 ± 0.39), followed by *E. faecalis* (4.33 ± 0.27) and *E. hirae* (5.49 ± 0.32).

Discussion

The data show that the testing of disinfectants based upon a culture collection *E. hirae* strain alone may not represent the sensitivity of other collection *Enterococcus* spp. with more clinical relevance. At a 5-min exposure time, the current EN 13727 test species *E. hirae* was found to be more tolerant to 0.2% glutaraldehyde and 0.0125% peracetic acid compared to *E. faecium* and *E. faecalis,s* whereas it was more susceptible to 40% ethanol and 3% sodium hypochlorite. Only with 0.00125% benzalkoniumchloride (15 min) was the susceptibility of *E. hirae* between that of *E. faecium* and *E. faecalis*. Based on these data *E. hirae* is a suitable species when bactericidal activity needs to be determined against enterococci with the biocidal agents glutaraldehyde and peracetic acid. It may, however, not be a suitable species for ethanol at 40% or sodium hypochlorite at 3% if the bactericidal activity includes the clinical pathogens *E. faecium* and *E. faecalis*.

Ethanol and other alcohols such as iso-propanol or n-propanol are typically used for hand disinfection or surface disinfection. An ethanol concentration of 40% will not be found in alcohol-based hand rubs because the bactericidal efficacy will be too low to fulfill European efficacy standards such as EN 1500. Even hand rubs based on 60% or 70% often fail to meet the EN 1500 efficacy requirements although the alcohols are effective against *E. faecium* and *E. faecalis* [11,14–16]. In that respect, it is of concern that the use of *E. hirae* may yield a sufficient efficacy against enterococci although *E. faecium* and *E. faecalis* are less susceptible.

The situation is different in surface disinfection. Many low-alcohol products are available for immediate use in the patient environment, often as presoaked tissues [17]. Low alcohol concentration has the advantage of a better compatibility with plastic surfaces which are now commonly found in healthcare such as mobile phones or tablet computers [18]. Based on our data obtained with suspension tests, it seems to be possible that low-alcohol surface disinfectants which are effective against *E. hirae* do not provide the same level of bacterial killing against *E. faecium* or *E. faecalis*. In 2014 a dramatic increase in infections caused by vancomycin-resistant enterococci was described [19]. The reasons for the increase are still

Table VI

Mean (N=3) log_{10} reduction (±standard deviation) in *Enterococcus hirae*, *E. faecium* and *E. faecalis* viability after exposure to biocidal agents and Games–Howell test of log_{10} cfu comparisons between the tested enterococcal species

	Ethanol	Glutaraldehyde	Benzalkonium chloride	Peracetic acid	Sodium hypochlorite
	40%/5 min	0.20%/5 min	0.00125%/15 min	0.0125%/5 min	3%/5 min
E. hirae	6.81 ± 0.45	4.17 ± 0.06	3.88 ± 0.13	1.69 ± 0.42	5.49 ± 0.32
E. faecium	1.56 ± 0.49	5.31 ± 0.30	5.13 ± 0.14	6.61 ± 0.55	1.60 ± 0.39
E. faecalis	4.04 ± 0.06	6.09 ± 0.22	2.00 ± 0.18	6.88 ± 0.26	4.33 ± 0.27
E. hirae – E. faecium	<0.001	0. 035	0.019	0.001	<0.001
E. faecalis – E. hirae	0.013	0.005	0.004	<0.001	0.021
E. faecium – E. faecalis	0.022	0.051	<0.001	0.749[a]	0.002

[a] Not significant.

unknown. But it is known that *Enterococcus* spp. can survive on inanimate surfaces between 4 days and 4 months [20]. It is therefore important to ensure a sufficient bactericidal efficacy of alcohol-based surface disinfectant against *Enterococcus* spp. However even with higher concentrations of alcohol it is essential to apply a sufficient volume. Approximately 10% of the solution is released during wiping when a soaked tissue is used [16,21]. It has been shown previously that the application of a low volume of an effective alcohol results in failure to meet the efficacy requirements [16].

Sodium hypochlorite at 3% was also more effective in 5 min against *E. hirae* and less effective against *E. faecalis* and *E. faecium*. It is a biocidal agent commonly used in many countries for surface disinfection [22]. Our findings with *E. faecalis* appear plausible because sodium hypochlorite at 2.5% has been described to achieve at least 5 $\log_{10}$ against ATCC 35550 (10 min) and ATCC 29212 (20 min) [23,24]. The very low effect of 3% sodium hypochlorite even in 15 min against *E. faecium* is of concern and should be followed up with more research on the possible implications for its use in healthcare.

In this study, only used culture collection strains from each of the three *Enterococcus* spp. were used in order to compare the susceptibility of potential test strains for disinfectant efficacy testing. No *Enterococcus* spp. clinical isolates were used. That is why it was not possible to evaluate whether the different biocidal agents would reveal a similar bactericidal activity against clinical isolates of each of the three enterococcus species.

Another limitation of our study was that all experiments were carried out using a low organic load described as clean conditions. That is why it was not possible to describe whether similar or other results would be obtained under dirty conditions. Clean conditions were chosen because they reflect the majority of applications of these agents. Alcohol-based hand rubs are applied to clean hands, ethanol is a typical biocidal agent used for hand disinfection. Instrument disinfectant should be used on cleaned instruments, glutaraldehyde, benzalkonium chloride and peracetic acid are typical agents used for instrument disinfection. Surface disinfection is often performed without prior cleaning, benzalkonium chloride and sodium hypochlorite are typical agents used for surface disinfection. With sodium hypochlorite it has been described before that the bactericidal efficacy will be impaired in the presence of organic load [25].

E. hirae is a suitable species when bactericidal activity is determined against enterococci with glutaraldehyde and peracetic acid. *E. hirae* may not be a suitable species for ethanol at 40% or sodium hypochlorite at 3% if the bactericidal activity includes the clinical pathogens *E. faecium* and *E. faecalis*.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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