Journal of Hospital Infection 103 (2019) e88–e91

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevier.com/locate/jhin

Short report

Isopropanol at 60% and at 70% are effective against 'isopropanol-tolerant' *Enterococcus faecium*

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ARTICLE INFO

Article history:
Received 12 December 2018
Accepted 28 January 2019
Available online 1 February 2019

Keywords:
Isopropanol
E. faecium
Tolerance
Bactericidal activity
Four-field test
ST 796

SUMMARY

The bactericidal activity of isopropanol was determined against *Enterococcus faecium* ATCC 6057, ST 796 (isopropanol-tolerant strain) and *Enterococcus hirae* ATCC 10541 (EN 13727). Isopropanol at 60% and 70% were effective ($\geq$5.38 $\log_{10}$-reduction) in 15 s against all strains but 23% isopropanol was not ($<$0.99 $\log_{10}$-reduction in $\leq$15 min). Isopropanol at 70% was tested against *E. faecium* in the four-field test. Eight millilitres was not effective enough in 1 min ($<$5 $\log_{10}$-reduction), whilst 16 mL was effective ($\geq$5.85 $\log_{10}$-reduction). Healthcare workers can be reassured that 60% and 70% isopropanol with an appropriate volume are effective against *E. faecium*.

Introduction

The description of alcohol tolerance among Australian *Enterococcus faecium* strains to 23% isopropanol was recently described [1]. One of the strains, a novel clone of a *vanB E. faecium* ST 796, caused a large outbreak in Switzerland with 8% of invasive infections, mainly bloodstream infections. In addition, the tolerant strains were shown to resist a standard 70% isopropanol surface disinfection resulting in greater mouse gut colonization compared to isopropanol-sensitive *E. faecium*, and this tolerance was related to mutations in genes involved in carbohydrate uptake and metabolism [1]. Based on this finding the authors hypothesized that there will be skin surfaces in contact with alcohol-based hand rubs that do not receive the maximum biocide concentration or contact time required for effective killing [1].

These findings raised global concerns on the bactericidal efficacy of isopropanol used for hand disinfection and surface disinfection because a clinically relevant bacterial tolerance or

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https://doi.org/10.1016/j.jhin.2019.01.024

resistance to isopropanol has so far not been described [2]. Isopropanol at 75% is listed by the World Health Organization (WHO) as an essential medicine supporting its relevance as an antiseptic agent [3]. That is why the findings by Pidot *et al.* were associated with the concern that healthcare workers may regard hand rubs based on 70% isopropanol as becoming ineffective which may eventually result in a lower hand hygiene compliance [4]. In order to determine whether 60% or 70% isopropanol are bactericidal against an 'isopropanol-tolerant strain' their efficacies were evaluated in a suspension test according to EN 13727 and under practical conditions in the four-field test according to EN 16615.

Methods

Test strains

The new *E. faecium* strain ST 796 that emerged in Switzerland and Australia was provided by Sacha Pidot (Australian strain; The Doherty Institute for Infection and Immunity, University of Melbourne, Australia) and Jonas Marschall, Carlo Casanova and Walter Steiger (Swiss strain; Bern University Hospital, Switzerland). In addition, *Enterococcus hirae* ATCC 10541 and *E. faecium* ATCC 6057 were used.

Disinfectant solutions

Isopropanol was obtained from Carl Roth GmbH & Co. KG, Karlsruhe, Germany, and used at 70%, 60% and 23% (all v/v).

Suspension tests

Suspension tests in analogy to EN 13727 were performed using different types of organic load (no organic load, clean conditions with 0.03% serum albumin, dirty conditions with 0.3% serum albumin plus 0.3% sheep erythrocytes). The bactericidal activity was determined at 15-, 30- and 60-s exposure times. A combination of 3.0% polysorbate 80, 3.0% saponin, 0.1% histidine and 0.1% cysteine was used as a neutralizer. The suitability of the neutralizer was validated for 70% isopropanol with *E. faecium*. After neutralization for 5 min, serial dilutions were performed, aliquots of 1 mL were spread on tryptic soy agar plates and plates incubated at 36°C for 48 h. After incubation colonies were counted and the number of colony-forming units (cfu) per mL calculated and converted into a $\log_{10}$ value. All experiments were performed in triplicate. A $\log_{10}$-reduction $\geq$5.0 was required to demonstrate bactericidal activity.

Four-field tests

The four-field tests were performed according to EN 16615. Briefly, polyvinyl chloride (PVC) pieces (20 × 50 cm; Forex classic, thyssenkrupp Plastics GmbH, Essen, Germany) were prepared simulating a surface to be treated with a surface disinfectant. Four areas of 5 × 5 cm were marked. The first field was contaminated with 0.05-mL of a mixture containing the test suspension ($1.5–5.0 \times 10^9$ cfu/mL) and the organic load (0.03% albumin; 'clean conditions') resulting in a total colony count on test field 1 of 6.75×10^7 to 2.25×10^8 cfu/mL (mean: 8.02 ± 0.13 cfu/mL). The inoculum was spread using a glass spatula and allowed to dry at room temperature for up to 60 min.

A standard wipe (16.5 × 30 cm) based on 55% cellulose and 45% polyethylene terephthalate (PET) was used. Each wipe was soaked for 30 min in 8 or 16 mL of the isopropanol solution prior to the surface treatment. The soaked wipe was weighed (Sartorius BP 2100 S, Göttingen, Germany). A granite block weighing 2.5 kg was placed on top of the soaked wipe. The block was pushed from the side with the contaminated test field in a smooth 1-s motion across the whole test area followed by moving back in another 1-s motion. The used wipe was weighed, and the difference from the soaked wipe regarded as the released volume.

After the 15-min contact time, each test field was carefully swabbed using a cotton swab soaked with neutralizer. A combination of 3.0% polysorbate 80, 3.0% saponin, 0.1% histidine and 0.1% cysteine was used as a validated neutralizer. The mean recovery of a control field after the maximum exposure time without any treatment was 7.42 ± 0.24 cfu/mL indicating a good recovery rate of the sampling method. The swab was then put into a vial containing 5 mL of neutralizer. With a second dry swab the entire test field was carefully swabbed once more until the test field was visibly dry. This swab was put into the same neutralizer vial that was then vortexed for 1 min. After 5 min neutralization time, two aliquots of 1 mL were taken out in duplicate and poured into separate Petri dishes.

For the sample obtained from the contaminated test field a 1:10 dilution was prepared in addition. Melted tryptic soy agar (15–20 mL) was added and cooled to 45°C. Plates were then incubated for 24 h at 36°C followed by counting colonies per plate. The numbers of cfu from the contaminated test field were transformed to the number of cfu per mL on a $\log_{10}$ scale. The difference from the number of cells obtained from an untreated control field was described as the $\log_{10}$ reduction. A $\log_{10}$ reduction of $\geq$5.0 was regarded as adequate bactericidal activity. The numbers of cfu from the three other test fields were also evaluated in order to measure any cross-contamination through wiping to originally non-contaminated surfaces. A mean number of $\leq$50 cfu per 25 cm^2 was regarded as a sufficiently low residual contamination demonstrating adequate bactericidal activity.

Data presentation and statistical evaluation

Experiments were performed in triplicate. Means with standard deviations were calculated.

Results

Isopropanol at 60% and 70% (both v/v) was highly effective in suspension tests after only 15 s against *E. hirae* and all *E. faecium* strains including the purportedly isopropanol-tolerant strain (Table I). The $\log_{10}$-reduction was consistently >5.0 with different types of organic loads. Isopropanol at 23% (v/v), however, revealed only poor bactericidal activity against *E. hirae* and all *E. faecium* strains in up to 15 min (<0.99 $\log_{10}$).

In the four-field test, 70% isopropanol was effective against all tested strains with mean $\log_{10}$ reductions $\geq$5.85 when applied with 16 mL for 1 min (Table II). The application of 8 mL, however, yielded a lower efficacy with mean $\log_{10}$ reductions between 4.05 and 4.74. The mean bacterial transfer to test

Table I
Mean log_{10}-reduction of *Enterococcus faecium* and *Enterococcus hirae* by exposure to isopropanol at various concentrations in suspension tests

Test strains	Concentration of isopropanol (v/v)	Exposure times	Mean log_{10}-reduction
E. faecium ATCC 6057	23%	5 min	0.99 ± 0.27*
			0.82 ± 0.29**
		15 min	0.86 ± 0.11*
	60%	15 s	5.56 ± 0.29**
			5.47 ± 0.37***
		30 s	5.91 ± 0.02**
			5.80 ± 0.21***
		60 s	5.91 ± 0.02**
			5.96 ± 0.20***
	70%	15 s	5.89 ± 0.10**
			5.90 ± 0.13***
		30 s	5.89 ± 0.10**
			5.96 ± 0.11***
		60 s	5.89 ± 0.10**
			5.86 ± 0.12***
E. faecium ST 796 (Australia)	23%	5 min	0.84 ± 0.09**
			0.91 ± 0.27*
		15 min	1.62 ± 0.31*
	60%	15 s	5.45 ± 0.19**
			5.57 ± 0.30***
		30 s	6.13 ± 0.03**
			6.04 ± 0.04***
		60 s	6.13 ± 0.03**
			6.04 ± 0.04***
	70%	15 s	5.38 ± 0.69**
			5.65 ± 0.20***
		30 s	6.14 ± 0.23**
			5.84 ± 0.01***
		60 s	6.14 ± 0.23**
			5.84 ± 0.01***
E. faecium ST 796 (Switzerland)	23%	5 min	0.79 ± 0.20**
			0.77 ± 0.06*
		15 min	0.80 ± 0.12*
	60%	15 s	5.65 ± 0.53**
			5.92 ± 0.02***
		30 s	5.94 ± 0.03**
			5.92 ± 0.02***
		60 s	5.94 ± 0.03**
			5.92 ± 0.02***
	70%	15 s	5.95 ± 0.02**
			5.83 ± 0.02***
		30 s	5.95 ± 0.02**
			5.83 ± 0.02***
		60 s	5.95 ± 0.02**
			5.83 ± 0.02***
E. hirae ATCC 10541	60%	15 s	5.99 ± 0.03**
			5.67 ± 0.44***
		30 s	5.83 ± 0.29**
			6.02 ± 0.02***
		60 s	5.99 ± 0.03**
			6.02 ± 0.02***
	70%	15 s	5.95 ± 0.03**
			5.56 ± 0.45***
		30 s	5.95 ± 0.03**
			5.93 ± 0.05***
		60 s	5.72 ± 0.41**
			5.93 ± 0.05***

*No organic load; **clean conditions (0.03% serum albumin); ***dirty conditions (0.3% serum albumin plus 0.3% sheep erythrocytes).

fields 2–4 was low (≤11 cfu/25 cm^2) with all treatments and strains.

Discussion

Based on these data this study did not detect a reduced bactericidal efficacy of 60% or 70% isopropanol against *E. faecium*, as also reported by Pidot *et al.* [1]. An increased bacterial tolerance to isopropanol at low concentrations is uncommon. In *Escherichia coli* it was shown that low-level exposure to variable isopropanol concentrations up to 2.7% for up to 24 days reduced the susceptibility of the six tested strains to isopropanol substantially. But no minimum inhibitory concentration maximum values were described after adaptation, and the stability of the lower susceptibility is also unknown [5].

The probability that bacterial species may develop tolerance to isopropanol used in hand hygiene is even lower, provided the concentration of the alcohol is high enough and the applied volume large enough to ensure the required bactericidal efficacy [6]. A limitation of this study and of that by Pidot *et al.* was that no hand inoculation model was used for testing, e.g. according to EN 1500. A previous study has shown isopropanol at 60% has effective bacterial killing on hands artificially contaminated with a clinical isolate of *E. faecalis* with mean log_{10}-reductions between 5.03 (15 s) and 6.07 (30 s) [7]. However, a similar study of two vancomycin-resistant *E. faecium* isolates showed more variable clearance from the hands of human volunteers [8].

Results from the four-field test indicate a strong bactericidal activity of 70% isopropanol against *E. faecium* ST 796 whereas the application of the same type of alcohol against the same strain was not sufficient to prevent transmission to mice as reported previously [1]. There are, however, some relevant differences. The major difference is probably the amount of alcohol solution used for surface disinfection. In the current study it was either 8 or 16 mL in a standard wipe of 16.5 × 30 cm which is a volume that ensures complete wetting of the surface in the experimental setting of the four-field test. In the mouse experiments by Pidot *et al.*, a volume of 0.85 mL was applied to a sterile filter paper of 4 × 4 cm for treatment of a surface of 450 cm^2 (15 × 30 cm) [1]. When this volume was used on the described filter paper, the Bonn research team observed that only 0.15 g of the solution was released during surface treatment and that the treated surface was not completely covered with a thin liquid film indicating an insufficient volume for effective surface disinfection.

In addition, it has been shown recently with 11 different surface disinfectants that the treatment of a surface of

Table II
Efficacy of isopropanol at 70% (v/v) in 1 or 5 min on surfaces contaminated with *E. faecium* strains according to EN 16615

Test strain	Volume per tissue	Exposure time	Type of organic load	Released volume (mean)	Mean $\log_{10}$-reduction on test field 1 (contaminated)	Mean cfu per 25 cm^2 on test fields 2–4 (non-contaminated)*
E. faecium ATCC 6057	8 mL	1 min	Clean conditions	0.88 mL	4.74 ± 0.28	0
	16 mL	1 min	Clean conditions	1.86 mL	6.74 ± 1.15	11
	16 mL	1 min	Dirty conditions	1.94 mL	≥7.45	0
		5 min		1.87 mL	≥7.23	0
E. faecium ST 796 (Australia)	8 mL	1 min	Clean conditions	0.77 mL	4.65 ± 0.84	0
	16 mL	1 min	Clean conditions	1.98 mL	7.54 ± 0.06	7
	16 mL	1 min	Dirty conditions	1.82 mL	≥7.68	6
		5 min		1.87 mL	6.96 ± 0.40	0
E. faecium ST 796 (Switzerland)	8 mL	1 min	Clean conditions	1.07 mL	4.05 ± 0.06	3
	16 mL	1 min	Clean conditions	1.86 mL	6.58 ± 1.45	7
	16 mL	1 min	Dirty conditions	1.81 mL	≥7.92	6
		5 min		1.88 mL	5.85 ± 0.53	0

cfu, colony-forming units.
* Measures any cross-contamination by wiping to originally non-contaminated surfaces.

approximately twice the size (929 cm^2) using a wipe, releases on average 10% of the liquid (range: 5–13%) [9]. Treating larger surfaces releases larger volumes up to 45%. Regarding the applied volume in the study by Pidot *et al.* it is therefore a reasonable to assume that an estimated volume of approximately 0.085 mL was applied to the surface of 450 cm^2. In the current study the surface of 1000 cm^2 was treated with mean volumes of 1.81–1.98 mL when 16 mL were applied and 0.77–1.07 mL when 8 mL were applied. These volumes are closer to a routine surface disinfection in healthcare with approximately 1 mL per 1000 cm^2 [10] which is typically reflected in the four-field test with an application of 1.3 mL per 1000 cm^2. These data support the use of isopropanol at an appropriate concentration for some healthcare items such as stethoscopes or other small surfaces with multiple hand or skin contacts.

Healthcare workers can be reassured that isopropanol used at 60% or 70% (v/v) for the appropriate contact time and with a sufficient volume is effective against *E. faecium*. Further testing using hand inoculation would be useful to support these findings.

Conflict of interest statement

Günter Kampf was employed until 2016 by Bode Chemie GmbH, Hamburg, Germany, a manufacturer of chemical disinfectants. The other authors have no conflicts of interest.

Funding sources

The study was funded by the Association of Applied Hygiene (VAH), Bonn, Germany.

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