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Interlaboratory reproducibility of a test method following 4-field test methodology to evaluate the susceptibility of *Clostridium difficile* spores

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SUMMARY

Background: Sporicidal surface disinfection is recommended to control transmission of *Clostridium difficile* in healthcare facilities. EN 17126 provides a method to determine the sporicidal activity in suspension and has been approved as a European standard. In addition, a sporicidal surface test has been proposed.
Aim: To determine the interlaboratory reproducibility of a test method for evaluating the susceptibility of a *C. difficile* spore preparation to a biocidal formulation following the 4-field test (EN 16615 methodology).
Methods: Nine laboratories participated. *C. difficile* NCTC 13366 spores were used. Glutaraldehyde (1% and 6%; 15 min) and peracetic acid (PAA; 0.01% and 0.04%; 15 min) were used to determine the spores' susceptibility in suspension in triplicate.
Findings: One-percent glutaraldehyde revealed a mean decimal $\log_{10}$ reduction of 1.03 with variable results in the nine laboratories (0.37–1.49) and a reproducibility of 0.38. The effect of 6% glutaraldehyde was stronger (mean: 2.05; range: 0.96–4.29; reproducibility: 0.86). PAA revealed similar results. An exemplary biocidal formulation based on 5% PAA was used at 0.5% (non-effective concentration) and 4% (effective concentration) to determine the sporicidal efficacy (4-field test) under clean conditions in triplicate with a contact time of 15 min. When used at 0.5% it demonstrated an overall $\log_{10}$ reduction of

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2.68 (range: 2.35–3.57) and at 4% of 4.61 (range: 3.82–5.71). The residual contamination on the three primarily uncontaminated test fields was <50 cfu/25 cm^2 in one out of nine laboratories (0.5%) and in seven out of nine laboratories (4%).

Conclusion: The interlaboratory reproducibility seems to be robust.

Introduction

Infections caused by *Clostridium difficile* are among the most common types of healthcare-associated infections in Germany and *C. difficile* infections are regarded as a worldwide disease [1,2]. Among the 2337 notified cases of *C. difficile* infection (CDI) in Germany in 2016 with a severe clinical course, 624 (28%) were lethal [3]. Based on the national surveillance system it was calculated that an average hospital in Germany will have 96 CDI cases per year with 70% of them acquired during hospitalization [4]. Such a high rate of nosocomial transmission requires review of all infection control measures to prevent transmission. Inanimate surfaces are one potential reservoir with average contamination rates of 30% [5,6]. A sporicidal surface disinfection is therefore one effective element in bundles to control nosocomial transmission and has been recommended both for the outbreak and the endemic setting [7–9]. Some products based on peroxides or aldehydes have been reported to be effective against spores of *C. difficile* in suspension tests [10,11]. Test protocols have been proposed already and existing norms such as EN 13704 or EN 14347, or modified norms such as EN 16615 or EN 13697, are used by manufacturers to support sporicidal activity claims of disinfectants. The enrichment of spores, the initial test suspension, the sensitivity of the spores, the interfering substances, the germination of treated spores with specified spore germinants, and the required decimal $\log_{10}$ reductions in the evaluation of sporicidal products often vary so that the results from the different efficacy tests are not comparable [12–14].

The European norm EN 17126 is an agreed standard to determine the sporicidal activity of disinfectants in a suspension test against *C. difficile* NCTC 13366 in the medical area [15]. The spore preparation is based on the Clospore method and has been used already in a ring trial in the UK [16,17]. EN 17126 is based on a test method originally proposed by the Association for Applied Hygiene as VAH method 18 [18]. The comparability of the bactericidal VAH and EN test procedures has been successfully confirmed by ring trials VAH 2011-2 and VAH 2013-4 [19]. Thus, some experimental differences such as the sequence of adding the test suspension and the interfering substance to the product and differences in the performance of the controls do not result in a significantly different assessment of disinfectants. One validation experiment in both test methods is to verify a specific susceptibility of the spore preparation to chemical substances with specific pass/fail criteria for glutaraldehyde (1% and 6%, v/v) and peracetic acid (0.01% and 0.04%, v/v). The lower concentration of each biocidal agent shall achieve only a poor sporicidal activity (<1.5 $\log_{10}$), whereas the higher concentration shall reveal a moderate sporicidal activity (≥1.5 $\log_{10}$) [15,18]. The interlaboratory reproducibility of this validation using a centrally produced spore suspension is currently unknown.

In addition to suspension tests it seems even more important to determine the sporicidal activity under practical conditions. In 2015, the 4-field test was published as the European Norm 16615 to determine the bactericidal and yeasticidal activity of surface disinfectants under practical conditions [20]. In analogy to EN 16615 the Association for Applied Hygiene recently proposed a similar 4-field test (VAH method 19) to determine the sporicidal activity of surface disinfectants [21]. The interlaboratory reproducibility of this sporicidal test, however, is also unknown. The aim of the study was therefore to determine the interlaboratory reproducibility of the validation of the susceptibility of a centrally prepared spore suspension and the interlaboratory reproducibility of the determination of the sporicidal activity of a biocidal formulation tested in the proposed sporicidal 4-field test.

Methods

Test organism and culture conditions

C. difficile NCTC 13366 was used in all experiments. It was chosen because of its clinical relevance (ribotype 027) and lower susceptibility to disinfectants compared with other strains of the species [22]. A spore suspension according to VAH method 18 or EN 17126 was prepared centrally at Bonn University and supplied to all laboratories [15,18]. Briefly, a subculture was prepared from the stock culture by streaking on to BHIYT-L agar plates. After anaerobic incubation of the plates for 48 h at 36°C an isolated colony was suspended in 5 mL pre-reduced Columbia broth and incubated in an anaerobic jar for 24 h at 36°C. Fifty microlitres of the culture were inoculated into 20 mL of pre-reduced Columbia broth and incubated anaerobically for 20 h at 36°C. The entire inoculum was then transferred into a 500 mL culture flask with the liquid sporulation medium. The flasks were incubated anaerobically for 10 days at 36°C. Finally, the cells were washed, vegetative cells and debris digested enzymatically with trypsin and lysozyme, and the spore suspension adjusted to a cell count of $1.5–5.0\times10^7$/mL. The spore suspension was visually checked under the microscope; slight debris was observed, and the purity was ~97%. Laboratories were advised to keep the spore suspension at 2–8°C.

Test products

Bioban™ GA 50 Antimicrobial (Dow Chemical Company Ltd, Staines, UK, 50% glutaraldehyde) was used as glutaraldehyde standard. Lerasept® spezial (Stockmeier Chemie GmbH & Co. KG, Bielefeld, Germany; 4.9% peracetic acid and 25.5% hydrogen peroxide) was used as peracetic acid standard. In order to validate the presented test protocol throughout the ring trial, an exemplary biocidal formulation based on 5% peracetic acid named 'product D' was used.

Determination of the susceptibility of the spore preparation

The susceptibility of the *C. difficile* spore suspension was determined in each laboratory in triplicate using a suspension test according to VAH method 18, which is equivalent to EN 17126 [15,18]. Briefly, 8 mL of the test product was thoroughly mixed with 1 mL of hard water and 1 mL of the test suspension ($1.5–5.0\times10^6$ cfu/mL) controlled at 20°C. Towards the end of the exposure time the tube content was mixed again. After the exposure time of 15 min an aliquot of 1 mL of the mixture was removed and transferred to a tube containing 9 mL of an appropriate neutralizer solution. The following neutralizers were used: polysorbate 80 (10 g/L) with glycine (20 g/L) in 0.25 M phosphate buffer for glutaraldehyde, and polysorbate 80 (10 g/L) with sodium thiosulphate (3 g/L) in 0.25 M phosphate buffer for peracetic acid. The suitability of the neutralizers for the test products was validated with *C. difficile* spores according to VAH method 18 [18]. After a neutralization time of 5 min the solution was mixed again and 1 mL taken out in duplicate. The 1 mL samples were poured into separate Petri dishes. Fifteen to 20 mL of melted BHIYT-L agar was added and cooled to 45°C. Plates were then incubated in anaerobic jars for five days at 36°C followed by counting the colonies per plate followed by calculating the number of cfu per millilitre on a $\log_{10}$ scale. The difference from the number of cells in the test mixture at the beginning of the contact time is reported as the $\log_{10}$ reduction. The susceptibility of the *C. difficile* test spores is considered to be validated if the mean $\log_{10}$ reduction is <1.5 with 1% glutaraldehyde and 0.01% peracetic acid and if the mean $\log_{10}$ reduction is ≥1.5 with 6% glutaraldehyde and 0.04% peracetic acid [15,18].

Determination of the sporicidal activity of an exemplary biocidal formulation based on peracetic acid against C. difficile following EN 16615 methodology (4-field test)

PVC pieces (20×50 cm; Forex classic, thyssenkrupp Plastics GmbH, Essen, Germany) were prepared simulating a surface to be treated with a surface disinfectant [23]. Four areas of 5×5 cm were marked. The first field was contaminated with 50 µL of a mixture containing 0.9 mL of the test suspension ($1.5–5.0\times10^7$ cfu/mL) and 0.1 mL of the organic load (0.03% albumin; 'clean conditions'). The inoculum was spread with a glass spatula and allowed to dry at room temperature for up to 60 min. The test product D was diluted with water of standardized hardness to 0.5% and 4% (use dilutions). A standard wipe (16.5×30 cm, TORK Low-Lint Cleaning Cloth, Essity Professional Hygiene Germany GmbH, Mannheim, Germany) based on 55% cellulose and 45% polyethylene terephthalate was used. Each wipe was soaked for 30 min in 16 mL of the use dilutions of the disinfectant prior to the surface treatment following EN 16615. The volume of 16 mL was chosen to ensure complete soaking of the wipe in its size without any dripping. The unitary weight (granite block, 2.5 kg) was covered with parafilm on to the bottom. The soaked wipe, folded once, was placed on the protected area with parafilm and fixed with a rubber. The hand pushed the weight over the test surface without applying additional pressure. The wiping procedure was carried out across fields 1–4 within 1 s wiping procedure in the reverse direction (Figure 1). After the 15 min contact time, each test field was carefully swabbed using a cotton swab soaked with neutralizer (10 g/L polysorbate 80 with 3 g/L sodium thiosulphate and 0.25 g/L catalase in 0.25 M phosphate buffer). The suitability of the neutralizers for the test product was validated with *C. difficile* spores according to VAH method 18 [18]. 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 which was then vortexed for 1 min. After a 5 min neutralization time, 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 in neutralizer was prepared in addition. The samples were processed as described above for the suspension test. A $\log_{10}$ reduction of ≥4.0 is regarded as sufficient sporicidal activity. The numbers of cfu from the three other primarily uncontaminated test fields were also evaluated. A mean number of ≤50 cfu per 25 cm^2 was regarded as a sufficiently low residual contamination nevertheless demonstrating sporicidal activity. An additional experiment using water of standardized hardness instead of the surface disinfectant had to reveal a mean number of ≥10 cfu per 25 cm^2 on test fields 2–4 demonstrating the lack of sporicidal activity.

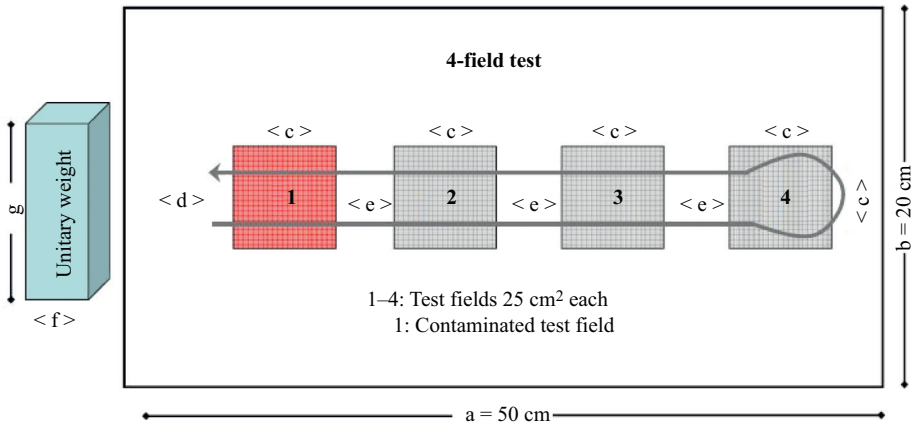

Figure 1. Scheme of the 4-field test. Test surface (20×50 cm) with four test fields (5×5 cm) and stipulated wiping route of the wiping cloth. a = 50 cm, b = 20 cm, c = 5 cm, d = 10 cm, e = 5 cm; dimensions of the unitary weight f×g at least 8.6 cm×12.1 cm.

Statistical evaluation

Colony counts between 1 and 330 were used for calculation. Counts >330 were taken as 330. The evaluation was performed according to the Q/Hampel method described in DIN ISO 13528 using PROLab Standard version 2018.6.19.0 (QuoData GmbH, Dresden, Germany) [24]. The robust mean including 95% confidence interval and the tolerance limits for the laboratory mean values were calculated. A *z*-score ≤2.0 was considered acceptable to demonstrate 'satisfactory' performance whereas a *z*-score between 2.0 and 3.0 indicated 'questionable' performance [25]. The Kruskal–Wallis rank sum test was used to determine whether the sporicidal efficacy of product D depends on the laboratory. $P > 0.1$ was considered proof to demonstrate no influence of the laboratory.

Results

The number of *C. difficile* cells in test suspension was mostly within the set limit between 1.5 and 5.0×10^6/mL except in laboratories 5 (all validation experiments on spore susceptibility; 1.5×10^7/mL) and 7 (one validation experiment on spore susceptibility; 8.9×10^5/mL).

Susceptibility of the C. difficile spore preparation

A solution of 1% glutaraldehyde revealed an overall mean $\log_{10}$ reduction of 1.03 after 15 min with variable results in the nine laboratories (0.37–1.49; Table I). The reproducibility between the laboratories was 0.38. The mean $\log_{10}$ reduction for each of the nine laboratories was <1.5. A solution of 6% glutaraldehyde revealed an overall mean $\log_{10}$ reduction of 2.05 in 15 min with variable results in the nine laboratories (0.96–4.29). The reproducibility between the laboratories was 0.86. The mean $\log_{10}$ reduction for seven of the nine laboratories was ≥1.5. Two *z*-scores were ≥2.0 (2.03 in laboratory 1 with 6% glutaraldehyde, −2.01 in laboratory 9 with 1% glutaraldehyde).

Similar results were found with peracetic acid. A solution of 0.01% peracetic acid revealed an overall mean $\log_{10}$ reduction of 0.90 in 15 min with variable results in the nine laboratories (0.41–1.73; Table I). The reproducibility between the laboratories was 0.45. The mean $\log_{10}$ reduction for seven of the nine laboratories was <1.5. A solution of 0.04% peracetic acid revealed an overall mean $\log_{10}$ reduction of 2.71 in 15 min with variable results in the nine laboratories (1.80–3.95; Table I). The reproducibility between the laboratories was 0.71. The mean $\log_{10}$ reduction for each of the nine laboratories was ≥1.5. All *z*-scores were <2.0.

Sporicidal activity of a product against C. difficile in a proposed 4-field test (following EN 16615 methodology)

The test product at 0.5% led to an overall reduction of *C. difficile* spores of 2.68 in all nine laboratories (range: 2.35–3.57; Table II). The residual contamination on the three primarily uncontaminated test fields was <50 cfu/25 cm^2 in one laboratory and ≥50 cfu/25 cm^2 in eight laboratories (range: 58–706 cfu/25 cm^2). The reproducibility between the laboratories was 0.30 $\log_{10}$ and the repeatability within the laboratories was 0.17 $\log_{10}$. The *z*-scores for the mean $\log_{10}$ reductions of the contaminated test field were <2 in eight out of nine laboratories (exception: 2.82 in laboratory 7).

Table I
Mean decimal $\log_{10}$ reduction obtained with glutaraldehyde and peracetic acid (all v/v) in 15 min against spores of *Clostridium difficile* according to EN 17126

Laboratory	Mean $\log_{10}$ reduction (SD)			
	Glutaraldehyde		Peracetic acid	
	1%	6%	0.01%	0.04%
1	0.91 ± 0.18	4.29 ± 0.13	1.59 ± 0.12[a]	3.88 ± 0.20
2	1.13 ± 0.46	1.95 ± 0.74	0.75 ± 0.02	2.42 ± 0.20
3	1.14 ± 0.20[b]	2.46 ± 0.05[b]	0.76 ± 0.31	3.26 ± 0.94
4	1.02 ± 0.12	1.81 ± 0.38	0.64 ± 0.24	2.39 ± 0.28
5	0.97 ± 0.04	1.20 ± 0.08[c]	0.91 ± 0.73	2.27 ± 0.27
6	1.49 ± 0.54	2.52 ± 0.96	0.88 ± 0.17	2.26 ± 0.66
7	1.44 ± 0.13	2.28 ± 0.15	1.73 ± 0.38[a]	3.95 ± 0.15
8	0.70 ± 0.41	1.92 ± 1.10	0.57 ± 0.06	2.48 ± 0.24
9	0.37 ± 0.22	0.96 ± 0.33[c]	0.41 ± 0.17	1.80 ± 0.16

[a] Does not meet the acceptance criterion of <1.5 $\log_{10}$.
[b] Based on n = 2.
[c] Does not meet the acceptance criterion of ≥1.5 $\log_{10}$.

The test product at 4% led to an overall reduction of *C. difficile* spores of 4.61 in all nine laboratories (range: 3.82–5.71; Table II). Two laboratories failed to show a 4 $\log_{10}$ reduction in field 1. The residual contamination on the three test fields without a contamination was <50 cfu/25 cm^2 in seven out of the nine laboratories and ≥50 cfu/25 cm^2 in two laboratories (163 and 277 cfu/25 cm^2). Whereas the mean of 163 cfu/25 cm^2 was based on three values ≥50 cfu/25 cm^2 (140, 158, 190 cfu/25 cm^2), the mean of 277 cfu/25 cm^2 was explained by an outlier (2, 8, 822 cfu/25 cm^2). The reproducibility between the laboratories was 1.14 $\log_{10}$ and repeatability within the laboratories was 0.44 $\log_{10}$. The *z*-scores for the mean $\log_{10}$ reductions of the contaminated test field were <2 in all laboratories. The mean $\log_{10}$ reduction of the effective product concentration was significantly influenced by the laboratory ($P = 0.046$; Kruskal–Wallis test).

The water control led to a reduction of *C. difficile* spores between 2.18 and 3.20 $\log_{10}$ (Table II). The residual contamination on the three non-contaminated test fields was ≥10 cfu/25 cm^2 in all laboratories (range: 11–421 cfu/25 cm^2).

Discussion

The determination of a sporicidal activity of chemical disinfectants requires a robust test method. One aspect is a consistent and reproducible susceptibility of the spores to chemical substances. In the present study we showed for the first time that a *C. difficile* spore suspension has an overall equivalent susceptibility to two concentrations of glutaraldehyde and peracetic acid in nine different laboratories based on a centrally prepared spore suspension which was provided throughout the ring trial to all laboratories. This finding allows confidence that the test method with a reproducibility between 0.38 and 0.86 is suitable to determine the susceptibility of *C. difficile* spores, also to other biocidal

Table II
Mean decimal $\log_{10}$ reduction obtained with product D (0.5% and 4%) and the water control in 15 min against spores of *Clostridium difficile* according to a proposed 4-field test

Laboratory	Mean $\log_{10}$ reduction (SD) on test field 1			Mean cfu/25 cm^2 on test fields 2–4		
	0.5%	4%	Water control	0.5%	4%	Water control
1	2.67 ± 0.04	3.82 ± 0.26	2.39 ± 0.05	58	26[a]	61
2	2.53 ± 0.31	4.08 ± 1.02[b]	2.18 ± 0.05	344	277	402
3	2.90 ± 0.34	4.47 ± 0.31[b]	2.20 ± 0.13	62	6[a]	266
4	2.35 ± 0.06	4.13 ± 0.90[b]	2.24 ± 0.10	230	12[a]	249
5	2.84 ± 0.52	3.87 ± 0.25	2.84± 0.05	372	163	184
6	2.63 ± 0.21	5.51 ± 0.99[b]	2.83 ± 0.15	706	0[a]	421
7	3.57 ± 1.02	4.19 ± 0.66[b]	3.20 ± 0.43	59	22[a]	11
8	2.45 ± 0.22	5.71 ± 0.27[b]	2.19 ± 0.14	73	29[a]	291
9	2.66 ± 0.08	5.67 ± 0.12[b]	n.d.	33[a]	0[a]	267

n.d., not done.
[a] Meets the acceptance criterion of <50 cfu/25 cm^2.
[b] Meets the efficacy criterion of ≥4.0 $\log_{10}$.

agents or to disinfectant products. However, even though a centrally prepared spore suspension was used, two laboratories did not meet the acceptance criterion with the active control 6% glutaraldehyde, and two other laboratories did not meet the acceptance criterion with the non-active control 0.01% peracetic acid. Other factors apart from chemical susceptibility of the spore preparation, such as handling details, may account for the failed acceptance criteria in the respective laboratories.

The ring trial results obtained with the newly developed sporicidal 4-field test (EN 16615 methodology adapted to spores) indicated that a 0.5% concentration of peracetic acid-based formulation used to validate the test method shows in all nine laboratories consistently a mean $\log_{10}$ reduction <4.0. When used at 4% the overall $\log_{10}$ reduction was >4.0 in seven laboratories; two laboratories reported a mean $\log_{10}$ reduction just below 4.0. A second parameter to determine a sporicidal activity in the presented 4-field test is to demonstrate that the sporicidal surface disinfection limits the carry-over, i.e. spreading of spores to previously non-contaminated areas during surface wiping. When applying the chosen non-effective concentration (i.e. 0.5%) of the exemplary biocidal formulation, the test method demonstrated a significant carry-over effect of >50 bacterial spores per 25 cm^2 in eight of nine laboratories. When the chosen effective concentration (i.e. 4%) was applied, seven laboratories reported no carry-over effect of >50 bacterial spores. However, two laboratories reported values of >50 bacterial spores, where it is noteworthy that the non-acceptable carry-over effect in laboratory 2 is based on one result of 822 cfu, clearly an outlier value. In laboratory 5 all three values were >50, which requires further evaluation at a later stage.

The reproducibility between the laboratories was 0.30 $\log_{10}$ for the non-active (0.5%) and 1.14 $\log_{10}$ for the active concentration (4%). The ring trial with *Staphylococcus aureus* shows a reproducibility of 0.50–0.54 $\log_{10}$ for a non-active product and 0.80–1.16 for the active concentration [26]. The reproducibility of this sporicidal ring trial is therefore comparable with results obtained with a bactericidal ring trial according to EN 16615 (4-field test) and confirms the robustness of the sporicidal 4-field test. The repeatability of the bactericidal 4-field test also shows comparable results that vary up to 0.19 $\log_{10}$ for the non-active and 0.67 $\log_{10}$ for the active product concentration.

Data obtained with the effective concentration of product D (4%) showed that the means of the laboratories were between 3.82 and 5.71. An additional analysis revealed that the laboratory itself has a significant impact on the overall result ($P = 0.046$). This is in line with the finding that even though the spore preparation used throughout this ring trial was centrally prepared, some laboratories did not meet the acceptance criteria when testing the reference substances using a quantitative suspension test. Installing a reference test substance within a test may be an option to identify and explain inter- and intralaboratory variances and thereby to further increase robustness. The aim of the study was not to evaluate the necessary number of tests to support a sporicidal claim; this would require further evaluation. Our data indicates, however, that data from a single laboratory may not sufficiently represent the true sporicidal efficacy of products, although some interlaboratory variability will always be found even in accepted standards, especially at borderline concentrations of biocidal agents or products. The interlaboratory variability may be different with other products already. Nevertheless, a simple approach to reduce the risk of a sporicidal product claim based on a single favourable report despite a true sporicidal efficacy of the product below the 4 $\log_{10}$ requirement is to provide two test reports from different laboratories. In addition, further research will be helpful to further reduce interlaboratory variability of disinfectant efficacy tests in general.

Both the sporicidal suspension test according to VAH method 18 or respectively EN 17126 and the proposed sporicidal 4-field test require reduction of ≥4 $\log_{10}$ of the *C. difficile* spores [15,18]. In 2015, Fraise *et al.* proposed a reduction requirement of ≥5 $\log_{10}$ reduction in a suspension test [17]. Perez *et al.* even used a 6 $\log_{10}$ reduction in their study [27]. It is known that approximately one-third of the inanimate surfaces around a patient with CDI are contaminated with *C. difficile* although higher rates of up to 90% have been reported [5,6,28,29]. One study indicates that in a *C. difficile* outbreak situation surfaces may be contaminated with up to 5.1 cfu per 25 cm^2 [30]. Weber *et al.* reported in 2013 that surfaces were mostly contaminated with <1 to 2 $\log_{10}$ *C. difficile*. Two studies reported >2 $\log_{10}$ *C. difficile* cells on surfaces, and one study

that sampled several sites with a sponge found up to 1300 colonies [31]. No other studies were found on the number of *C. difficile* cells on inanimate surfaces in the patient environment. The few available data indicate that 4 $\log_{10}$ reduction on contaminated surfaces may well be sufficient for a sporicidal efficacy. Although it would be desirable to justify a minimum sporicidal activity with more epidemiological data, that is currently not possible.

In conclusion, the interlaboratory reproducibility of the determination of spore susceptibility and the sporicidal efficacy in a 4-field test seems to be robust. Thus, a suitable test method to evaluate efficacy of chemical disinfectants against *C. difficile* spores under simulated practical conditions is presented, including a reliable protocol for preparing *C. difficile* spores.

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Conflict of interest statement
None declared.

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