

## Keywords

*Clostridium difficile* Ribotype 027*Bacillus subtilis*

Sporicidal

Surface disinfectants

Instrument disinfectants

Efficacy tests

Chemo resistance

Stefanie Büttgen<sup>1\*</sup>, Jürgen Gebel<sup>1</sup>, Britt Hornei<sup>1,2</sup>, Steffen Engelhart<sup>1</sup>, Orlando N.J. Koch<sup>1</sup>, Martin Exner<sup>1</sup><sup>1</sup> Institute of Hygiene and Public Health, University of Bonn, Bonn, Germany<sup>2</sup> Department of Pediatric Hematology and Oncology, Children's Hospital Medical Center, University of Bonn, Germany

# Comparison of chemo resistance of *Clostridium difficile* ribotype 027 spores and *Bacillus subtilis* spores against disinfectants

## Summary

**Background:** This study is the continuation of previous investigations on the efficacy of different disinfectants against *C. difficile* spores of ribotype 027. It compares the resistances of spores derived from *C. difficile* ribotype 027 and *B. subtilis* ATCC 6633 to chemical disinfectants.

**Methods:** The comparative investigations were performed using the quantitative suspension test with a high organic load based on the DGHM methods. A total of three instrument disinfectants and four surface disinfectants were tested which had been proven sporicidal in the previous study.

**Results:** In all comparative tests, *B. subtilis* spores consistently showed a considerably higher resistance to all disinfectants than *C. difficile* spores.

**Conclusions:** Therefore, disinfectants which exhibit sporicidal efficacy against *B. subtilis* can be assumed to also reliably inactivate the clinically relevant spores of *C. difficile*. According to the principle of minimization and in view of the difficulties using sporicidal disinfectants in clinical routines, tests against the more sensitive *C. difficile* ribotype 027 are still strongly recommended in order to be able to minimize the use concentrations of sporicidal disinfectants without reducing their effectiveness.

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## Introduction

The increasing incidence of *Clostridium difficile*-associated disease (CDAD) and the spread of a special variant of *Clostridium difficile*, known as *C. difficile* ribotype 027, has given rise to widespread concern, within Germany too [1,2]. Since November 2007 mandatory reporting has therefore been in place for severe courses of *C. difficile* infections pursuant to Section

6(1) Clause 5a of the German Protection against Infection Act (IfSG) [2]. In addition to rational and restrictive use of antibiotics and formulation of isolation strategies in the event of an outbreak, the implementation of well-formulated hygiene (infection-control) policies are among the most important preventive measures taken in the hospital setting. The paramount importance of hygiene measures cannot be underestimated, in particular in respect of prevention of nosocomial (hospital-acquired) infections, in view of the spores' high level of environmental resistance and ability to spread in the environment [3,4,5,6,7]. The use of effective sporicidal disinfectants is therefore urgently recommended in hospitals in the event of an outbreak of *C. difficile* infection.

Because of the paucity of data available on the sporicidal efficacy of disinfectants and the fact that, to date, there is no independent quality control system for sporicidal disinfectants, a previous study was carried out to test the declared sporicidal efficacy of disinfectants against *C. difficile* ribotype 027 under worst-case conditions (high organic load).

This present study now compared those disinfectants whose sporicidal efficacy had been demonstrated (against *C. difficile* ribotype 027 spores) in the previous study [8] for efficacy against *B. subtilis* (ATCC 6633) spores. The aim of the study was to gain insights into the chemoresistance of two spore-forming bacteria, *B. subtilis* and *C. difficile* ribotype 027. Another aim was to elucidate whether, while in line with the principle of minimisation, lower concentrations of disinfectants could be used for inactivation of *C. difficile* spores, in particular in a clinical setting.

### \*Corresponding author

#### Dipl.-Biol. Stefanie Büttgen

Institute of Hygiene and Public Health  
University of Bonn  
Sigmund-Freud-Str. 25  
53105 Bonn, Germany

Email: stefanie.buettgen@ukb.uni-bonn.de

In this article the term chemoresistance is employed to denote the level of resistance exhibited by test organisms to a disinfectant and, as such, it is subject to different evaluation criteria from those applicable to antibiotic resistance.

## Materials and Methods

### Test organisms

The test organisms used were spores of *C. difficile* ribotype 027 (E.J. Kuiper, Leiden University Medical Centre, Netherlands) and spores of *B. subtilis* laboratory strain ATCC 6633 (DSMZ, Braunschweig, Germany).

### Spore enrichment

Spore enrichment for *C. difficile* and *B. subtilis* spores was based on a method developed and evaluated by the Department of Hygiene and Public Health at Bonn University [9,10].

### Spore enrichment: *C. difficile*

*C. difficile* spore enrichment was performed in a meat broth that had been inoculated in each case with a colony of a pure culture of *C. difficile* and incubated under anaerobic conditions for 24 hours at 37 °C. Columbia agar plates (Becton Dickinson GmbH, Heidelberg, Germany), which had been reduced in advance for 24 hours, were each inoculated with aliquots of 0.1 ml of the meat broth culture. The inoculated agar plates were incubated under anaerobic conditions for seven days at 37 °C and for 14 days at room temperature while protecting them against the light.

The spores were recovered by rinsing off the nutrient medium with 0.067 M phosphate buffer. Following centrifugation for 10 min at 3000 g, three further washing steps were carried out as well as resuspension in 0.067 M phosphate buffer for subsequent storage at 4 °C. This was followed by heat inactivation in a water bath for 10 min at 80 °C to inactivate any remaining vegetative cells. Those viable spores capable of germination were identified after cooling down the spore suspension using the plate-casting method with GS-BHI agar to which 10 µg/ml lysozyme had been added (50,000 units/mg) (Serva, Heidelberg, Germany). This was followed by incubation for 48 hours at 37 °C under anaerobic conditions. The mean spore

suspension concentration was around 10<sup>6</sup> spores /ml [9].

### Spore enrichment: *B. subtilis*

*B. subtilis* spore enrichment was performed in accordance with the laboratory standard method SOP Bonn in a liquid medium and is based on a method developed by Schaeffer [11]. Enrichment was carried out in a small bioreactor developed at the Department of Hygiene and Public Health [12]. This bioreactor was filled with a litre of the sporulation medium and inoculated with five colonies of the second *B. subtilis* subculture, followed by incubation at 37 °C and a shaking frequency of around 120 rpm. As soon as there was evidence of turbidity of the medium (after around 4–5 hours), the medium was subjected to sterile aeration (pump capacity: 150 l/h) and incubated, under aerobic conditions, for a further 72 h at 37 °C.

Before spore recovery and the various washing steps, the sporulation medium underwent ultrasonic treatment in an ultrasonic basin for 2 min at 10 °C. Centrifugation for 15 min at 5000 g (5 °C–10 °C) was carried out using 100 ml aliquots of the sporulation medium. The bacterial sediments were collected and washed three times again using around 25 ml sterile distilled water. After the last wash step, the sediment was collected in 70 ml sterile distilled water. To inactivate any remaining vegetative cells, heat inactivation was performed at 80 °C for 10 min. After allowing the spore suspension to cool down, those spores capable of germination were determined using trypticase soybean agar (TSA). Experience shows that for *B. subtilis* a spore yield of between 10<sup>8</sup> and 10<sup>9</sup> spores/ml can be expected. The spore suspension was stored at 4 °C.

### Disinfectants

A total of seven different disinfectants, based on different active substances that had been declared as having sporicidal activity by the manufacturers, were investigated for efficacy against spores of *B. subtilis* ATCC 6633. Of the seven disinfectant products, three had been approved as instrument disinfectants and four as surface disinfectants. In a previous study it had been possible to confirm that these seven disinfectants were effective against *C. difficile* ribotype 027 in the presence of a high organic challenge [8].

### Active substance combinations

For the instruments, products based on a peroxide compound and aldehydes + aldehyde-releasing agents were chosen. For the surface disinfectants, products based on aldehydes, quaternary ammonium compounds, chlorine compounds and peroxide compounds were tested. The substances glutaraldehyde (Merck, Darmstadt, Germany), 1% - 15 min, and peracetic acid (Sigma-Aldrich, Munich, Germany), 0.1% - 15 min, which had been tested within the framework of European multicentre trials by CEN TC 216, were used in parallel as standard or reference substances.

### Quantitative suspension test

Quantitative suspension tests were conducted in accordance with the standard methods of the German Society for Hygiene and Microbiology (DGHM) 2001 – Determination of the bactericidal and fungicidal efficacy in the quantitative suspension test (Chapter 9) [13].

The mean baseline concentration of spores capable of germination was 2.54 × 10<sup>6</sup> cfu/ml. The mean baseline concentrations of the *B. subtilis* spores was 1.34 × 10<sup>6</sup> cfu/ml. All tests were performed using a reproducible methodology; the figures show the mean reduction values in the form of bar graphs. A maximum deviation of 0.5 log<sub>10</sub> units was a precondition for calculation of the mean values.

### Challenge

All microbiological / hygiene tests were conducted using a high organic challenge i.e. with the addition of 0.3 % albumin and 0.3 % sheep erythrocytes. The reason for using this high challenge was to obtain proof that the products could be safely used in the hospital setting.

## Results

### Reference substances: Comparison of spores of *C. difficile* ribotype 027 and *B. subtilis*

Figure 1 illustrates the efficacy of the two standard (reference) substances glutaraldehyde (pH 6.59), 1% - 15 min, and peracetic acid (pH 3.47), 0.1% - 15 min, in the quantitative suspension test, using a high organic challenge against *C. difficile* ribotype 027 spores (dark-blue bars) and *B. subtilis* (light-blue bars). From the

figure can be seen that *B. subtilis* spores exhibited higher resistance to the reference substances than did the *C. difficile* spores of the special ribotype 027 variant. Noteworthy is the markedly higher stability of *B. subtilis* spores to glutaraldehyde.

### Efficacy testing of instrument disinfectants

Currently, a reduction by 3 log<sub>10</sub> levels is required at European level in CEN TC 216 as confirmation of the efficacy of sporicidal products [14]. Figure 2 illustrates a comparison of the efficacy of three different instrument disinfectants investigated in quantitative suspension tests against spores of *C. difficile* ribotype 027 and *B. subtilis*. Under the given test conditions using a high organic challenge one product (Sekusept aktiv) was able to meet the requirement that spores be reduced by at least three log levels. This was the case for both test organisms. In the previous studies, adequate efficacy under worst-case conditions was also demonstrated against *C. difficile* for the products Pera Safe (Antec International) and Gigasept FF new (Schülke & Mayr GmbH) [8]. However, for the concentration-time ratios used, and in the presence of a high organic load, these products did not prove to be sufficiently effective against *B. subtilis* spores.

### Active substance concentrations tested: instrument disinfectants

The product Sekusept aktiv (2% – 30 min), which is based on peroxide compounds and manufactured by Ecolab GmbH & Co., was used with an absolute active substance concentration of 10,000 mg/L and Pera Safe (1.62% – 10 min), manufactured by Antec International, was used with an active substance concentration of 2,600 mg/L. Gigasept FF new (6% – 6 h) manufactured by Schülke & Mayr GmbH is a product based on aldehydes and aldehyde-releasing agents and, at the specified concentration, it contains 7,140 mg/l aldehydes and 1,920 mg/l aldehyde-releasing agents.

### Surface disinfectants: comparison of efficacy

A total of four surface disinfectants whose efficacy against *C. difficile* ribotype 027 spores had been demonstrated in a previous study [8] were investigated in the quantitative suspension test for efficacy against *B. subtilis* spores. Figure 3 high-

lights the fluctuating chemoresistance of the two test organisms against the four different surface disinfectants (Pursept FD (Merz GmbH), Actichlor Plus, Acticlens and Incidin active (Ecolab GmbH & Co)). Under the prevailing test conditions, none of the surface disinfectants tested were able to reduce *B. subtilis* spores by the required 3 log levels. However, all the surface disinfectants were able to adequately inactivate spores of *C. difficile* ribotype 027 in the quantitative suspension test, while using a high organic challenge (reduction > 3 log levels).

### Active substance concentrations tested: surface disinfectants

Pursept FD (Merz GmbH) was tested with an active substance concentration of 11,700 mg/l aldehydes + 6,000 mg/l quaternary ammonium compounds (7.5% – 8 h) and 23,400 mg/l aldehydes + 12,000 mg/l quaternary ammonium compounds (15% – 4 h). The chlorine-based product Actichlor Plus (Ecolab GmbH & Co) when used at a concentration of 4.1% contains an absolute active substance concentra-

tion of 10,000 mg/l. Based on information from the manufacturer, the peroxide products Incidin active and Acticlens (Ecolab GmbH & Co) were used at an active substance concentration of >1,000 mg/l.

## Discussion

The present study compared the chemoresistance of *B. subtilis* and *C. difficile* spores. For these comparative tests spores of the hypervirulent *C. difficile* isolate ribotype 027 from a Dutch hospital and a well-studied laboratory strain of *B. subtilis* (ATCC 6633) were used. The wild type variant of *C. difficile* was selected because in a previous comparative test it had proved to be markedly more resistant than the laboratory strain of *C. difficile* (ATCC 9689) and, furthermore, it is more representative of everyday practice. The laboratory strain of *B. subtilis* (ATCC 6633) is used in European test standards as a reference standard for sporicidal efficacy.

The aim of this study was to demonstrate that disinfectants that are effective

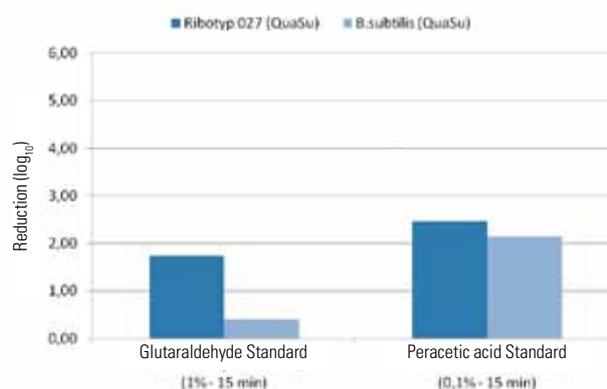


Figure 1: Comparison of reduction of *C. difficile* ribotype 027 (dark-blue bars) and *B. subtilis* spores (light-blue bars) using peracetic acid and glutaraldehyde (reference substances) in the quantitative suspension test (QuaSu) with a high organic challenge.

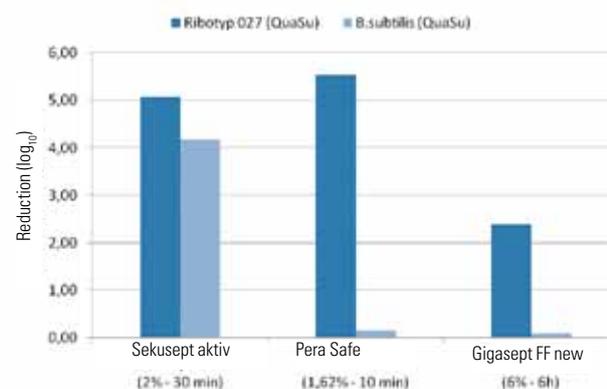


Figure 2: Efficacy testing of three sporicidal instrument disinfectants against *C. difficile* ribotype 027 (dark-blue bars) and *B. subtilis* spores (light-blue bars). The tests were conducted using a high organic challenge in the quantitative suspension test (QuaSu).

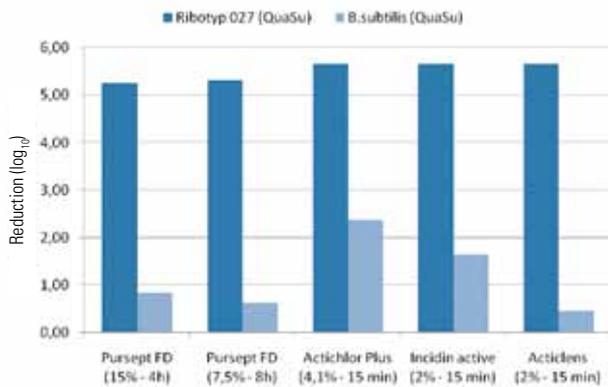


Figure 3: Efficacy testing of four surface disinfectants against *C. difficile* ribotype 027 spores (dark-blue bars) and *B. subtilis* spores (light-blue bars) using a high organic challenge in the quantitative suspension test (QuaSu).

against *B. subtilis* spores are also able to reliably inactivate those *C. difficile* spores that are of relevance in a clinical setting. Two important other issues were investigated within the framework of the present study:

1. Are the *B. subtilis* spores more resistant than the *C. difficile* ribotype 027 variant which is currently of epidemiological significance?
2. Is it possible, in line with principle of minimisation, to reduce the concentrations of disinfectants, in particular in the hospital setting?

In the interest of hospital hygiene, the use of disinfectants endowed with sporicidal efficacy is urgently required to prevent the spread of infection via surfaces close to the patient as well as the spread of *C. difficile* subtypes that pose a risk of infection. There are ample data attesting to the persistence and spread of Clostridia in hospitals [15,16,17,18,19], and these underline the importance of strict implementation of hygiene measures for prevention and control of CDAD outbreaks.

However, the difficulty is in finding disinfectants endowed with an adequate level of sporicidal efficacy [20] for the clinical setting since at present there is no approved list of sporicidal products for prophylactic disinfection. The manufacturer bears responsibility for any declaration of a product as “sporicidal” and such declarations are often based on tests conducted in accordance with the European Basic Sporicidal Efficacy Test (EN 14347) [21], which is performed without the use of an organic load. Often, the user cannot judge whether, or not, the test product’s spectrum of action covers an organic

challenge. Not least for that reason, independent testing and listing are urgently recommended. One problem faced by the manufacturer or by an independent listing committee derives from the fact that there is still no quantitative suspension test or practice-oriented germ carrier test available at European level to test sporicidal efficacy in the human medicine setting [22].

Within the framework of this present study, as well as during a previous study, it has been demonstrated that the quantitative suspension tests could be modified to permit sporicidal efficacy testing[8], also when using a high organic challenge. It was necessary to greatly prolong the exposure times used in the quantitative suspension tests, compared with the usual test times of a maximum of 60 min, to in some cases 8 h.

Clarification of these issues were given prime consideration in the present study. As regards the chemoresistance, it has been possible to confirm that the *B. subtilis* spores are more resistant than those of the *C. difficile* ribotype 027 variant which is currently of epidemiological significance. It can be assumed that the spore-forming test organism *B. subtilis* – as has often been postulated – serves to cover all *C. difficile* variants [23].

In all the instrument and surface disinfectants tested, *B. subtilis* spores proved to be markedly more resistant than the spores of *C. difficile* ribotype 027. *B. subtilis* spores were also markedly more resistant against the reference substance glutaraldehyde. In the case of peracetic acid, less pronounced differences were observed between the anaerobic spore-forming

bacterium *C. difficile* and the aerobic spore-forming bacterium *B. subtilis*.

In line with the principle of minimisation, and in the patients’ interest, efforts should be made to try to reduce the concentrations of sporicidal disinfectants. Patients often have problems tolerating sporicidal disinfectants despite adequate ventilation. For that reason it is urgently recommended that the sporicidal efficacy of disinfectants be tested against *C. difficile* ribotype, which is of clinical relevance and relatively resistant, because in all probability this would permit the use of much lower concentrations of many sporicidal disinfectants. The marked discrepancy in the chemoresistance profiles between *B. subtilis* and *C. difficile* spores highlights the need for testing sporicidal efficacy of disinfectants against *C. difficile*, in particular in the hospital setting.

## Conclusion

The tests conducted to date confirm the higher resistance of *B. subtilis* spores to chemical disinfectants, also compared to the *C. difficile* ribotype 027 variant which is currently of epidemiological significance. It can therefore be assumed that disinfectants that are effective against *B. subtilis* spores are also able to reliably inactivate those *C. difficile* spores that are of relevance in a clinical setting. In line with the principle of minimisation, there is an urgent need to test the sporicidal efficacy of disinfectants against resistant *C. difficile* isolates. In particular in the clinical setting, it is advisable that a distinction be made between *C. difficile* and *B. subtilis* in order to identify those concentrations of sporicidal disinfectants that can be well tolerated by patients. Independent testing and listing of sporicidal disinfectants, analogous to that of bactericidal test methods [13, 24], are urgently needed because of the rising incidence of *C. difficile*-associated disease [13,25].

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## Conflict of Interest

The authors declare that there is no conflict of interest as understood by the International Committee of Medical Journal Editors.

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