

Susceptibility of *Clostridium sporogenes* Spores to Selected Reference Substances and Disinfectants

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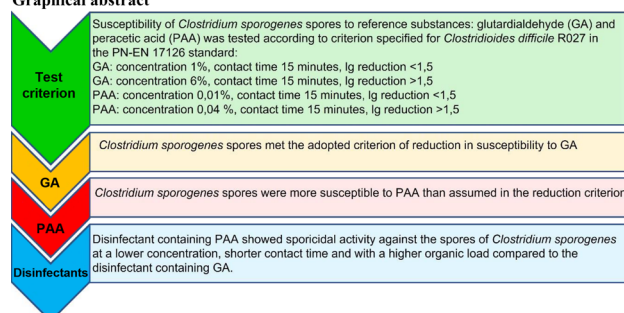
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Abstract

Research on the susceptibility of the spores of anaerobic bacteria such as *Clostridium sporogenes* or *Clostridioides difficile* is vital for assessing the sporicidal activity of disinfectants. The diverse susceptibility of anaerobic bacteria spores may lead to different disinfection parameters being determined by laboratories that prepare spore suspensions to test sporicidal effectiveness. The tests were performed using the suspension method according to PN-EN 13704:2018-09. In order to assess the susceptibility of the *C. sporogenes* spores, the criterion established for the *C. difficile* ribotype 027 spores was used in accordance with PN-EN 17126:2019-01. The susceptibility of the *C. sporogenes* spores to glutardialdehyde corresponded to the susceptibility ranges established for the *C. difficile* ribotype 027 spores. The *C. sporogenes* spore suspension was susceptible to low concentrations of peracetic acid (0.01%). A disinfectant containing peracetic acid as the active substance showed high sporicidal activity at a low concentration (1%), a short contact time (15 minutes), and a high organic load (3.0 g/l bovine albumin + 3.0 ml/l sheep erythrocytes), as compared to a disinfectant with glutardialdehyde, which was sporicidal at a higher concentration (2.5%), at a longer contact time

Graphical abstract



(60 minutes) and lower organic conditions (3.0 g/l bovine albumin). There is a need to define the minimum susceptibility criteria for the *C. sporogenes* spores to the reference substances most often found in disinfectants with sporicidal activity. Excessive susceptibility of the *C. sporogenes* spores to reference substances may result in low-performance parameters of disinfection products with sporicidal activity and lead to ineffective disinfection in practice.

Key words: glutardialdehyde, peracetic acid, susceptibility of spores, *Clostridium sporogenes*

Introduction

Spores of anaerobic bacteria have recently been the subject of studies based on a standardized methodology for the effectiveness of biocidal disinfectants (Humphreys 2011; PN-EN 13704:2018-09; PN-EN 17126:2019-01). In the medical area, sporicidal range against the *Clostridioides difficile* R027 spores has been included in the standard since 2019 (PN-EN 17126:2019-01). Usually, the sporicidal activity of disinfectants is determined using spore suspensions of aerobic bacteria such as *Bacillus subtilis* or *Bacillus cereus*.

The determination of sporicidal activity against anaerobic bacteria spores is included in PN-EN 13704:2018-09 as an additional scope defined for specific applications of disinfection products, mainly in the food industry. The test organism used in this type of research is the *Clostridium sporogenes* strain (PN-EN 13704:2018-09). It is an anaerobic bacterium widely found in healthy people's water, soil, wastewater, and feces. It takes part in the putrefactive processes; therefore, its presence in food products is highly undesirable (McSharry et al. 2021). Due to its similarity to *Clostridium botulinum* regarding the production of highly heat-resistant

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spores, the *C. sporogenes* spores have been used to assess spore survival in the canning production technology (Wang et al. 2017).

Although rare is *C. sporogenes* considered a pathogenic organism, it can cause secondary connective tissue infections or bacteremia with respiratory symptoms in immunocompetent individuals (Abusnina et al. 2019; Vecchio et al. 2020). *C. sporogenes* is often selected for testing the sporicidal activity of disinfectants due to the ease of producing large number of spores in a short culture time. PN-EN 13704:2018-09 specifies the required susceptibility of aerobic bacterial spores to reference substances such as glutardialdehyde and peracetic acid. However, it does not determine the criteria of susceptibility for the *C. sporogenes* spores due to the lack of available data (PN-EN 13704:2018-09). The susceptibility of bacterial spores is a significant feature in testing the disinfection effectiveness of sporicidal products. Disinfection effectiveness will depend on the susceptibility of spores to a given active substance (Leggett et al. 2012). The variable susceptibility of spore suspensions produced in various laboratories may lead to different disinfection parameters (concentration, contact time). It may also be the reason for the activity or inactivity of a disinfectant in terms of the parameters set by the manufacturer (Humphreys 2011). Laboratories producing spore suspensions for testing disinfectants should test their susceptibility to reference substances and meet the susceptibility range criteria for such suspensions. This approach makes it possible to compare the results of studies on the sporicidal activity of disinfectants. So far, the susceptibility criteria of anaerobic bacteria spores have been specified in the medical area in PN-EN 17126:2019-01 against spores originating from the *C. difficile* R027 strain (PN-EN 17126:2019-01).

The study aimed to determine the susceptibility of the *C. sporogenes* spores to selected reference substances, i.e., glutardialdehyde and peracetic acid, and to evaluate the usefulness of these data for the study of disinfectants containing the aforementioned active substances.

Experimental

Materials and Methods

Materials and bacterial suspension. *C. sporogenes* ATCC® 3584™ was used to produce a spore suspension with a density of 1.5×10^6 CFU/ml up to 5.0×10^6 CFU/ml. The strain used differed from the strains proposed for assessing the sporicidal activity of the products under additional conditions (*C. sporogenes* ATCC® 19404™, CIP 79.3). It was used because the standard allows using other strains when tested under additional conditions

if they can be shown to lead to the same results. In this case, the correct number of spores required by the standard was obtained. The *C. sporogenes* ATCC® 3584™ spore suspension met the quality requirements for spore suspensions.

Reference substances were as follows: 1) glutardialdehyde (GA) – 25% aqueous solution (Sigma-Aldrich, USA). A 1.25% solution and a 7.5% solution were prepared from a 25% glutardialdehyde solution. Glutardialdehyde concentrations in the spore susceptibility test were 1% and 6%, respectively; 2) peracetic acid (PAA) – a solution containing approx. 35% of peracetic acid in acetic acid, stabilized (Acros Organics, Belgium). A 5% solution was prepared from a 35% peracetic acid solution, in which the concentration of peracetic acid was determined by titration with a sodium thiosulfate solution (PN-EN 17126:2019-01). After determining the peracetic acid content in the stock solution, 0.05% and 0.0125% solutions were prepared. Peracetic acid concentrations in the spore susceptibility test were 0.04% and 0.01%.

Disinfectants were as follows: 1) composition of disinfectant A (Phagocide D, Christeys, France): 2.5% glutardialdehyde, ready to use. Disinfectant A is intended for instruments disinfection in clean conditions after prior cleaning them; 2) composition of disinfectant B (Neoform Active, Dr. Weigert, Germany): 1% product solution contains 0.15% peracetic acid, product for dilution. Disinfectant B is intended for cleaning and disinfecting the surface of medical devices and other equipment. Disinfectant A was tested as the ready-to-use product without dilution before the test, while a 1% solution of disinfectant B was prepared in sterile hard water with pH 7.0 ± 0.2 . Sterile hard water was prepared according to PN-EN 13704:2018-09.

Disinfectant A, as a ready-to-use product, was tested at a concentration of 80%. Disinfectant B solution was prepared at a concentration 1.25 times higher due to the dilution of the disinfectant in the study.

Preparation of *C. sporogenes* ATCC® 3584™ spore suspension. *C. sporogenes* ATCC® 3584™ was cultured in a deoxygenated liquid medium with tryptone (Tryptone Broth – TB) for 48 hours at 37°C under anaerobic conditions to obtain a 10^7 CFU/ml bacterial suspension to inoculate a tryptone agar medium (Tryptone Agar – TA). The TA medium was previously deoxygenated and then inoculated with 2–3 ml of the 48-hour *C. sporogenes* suspension. The excess inoculum was discarded. The culture in the TA medium was carried out in three 160 ml Roux bottles for 10 days at 37°C under anaerobic conditions. After three days, the start of sporulation was verified under the microscope. After 10 days, the surface of the substrate was rinsed with sterile water to obtain a spore suspension. The resulting suspension was purified by washing four times

and centrifuging at $4,000\times g$ for 20 minutes. The suspensions were subsequently heated for 10 minutes in a water bath at 75°C. A series of dilutions in water was prepared, and the suspensions were plated on MTSA (Meat Glucose Yeast Agar) to determine their density (dilutions with 10^{-4} and 10^{-5}). Incubation lasted 48 hours at 37°C in anaerobic conditions. The spore suspension was stored in a sterile tube with glass balls in the water at 2–8°C for four weeks to mature the spores (PN-EN 13704:2018-09). Before each examination, the spore suspension was viewed under an optical microscope ($400\times$ magnification) to assess its quality (the absence of vegetative and germinative forms) and counted in the Thoma cell counting chamber.

Testing the spore susceptibility to reference substances and the disinfectant sporicidal activity against *C. sporogenes*. The spore susceptibility and the sporicidal activity of disinfectants were tested in accordance with PN-EN 13704:2018-09. This document determines the sporicidal activity of disinfectants by the suspension method in the food, industrial, domestic, and institutional areas. The reference substance/disinfecting product at an appropriate concentration is combined with a spore suspension with a density of 1.5×10^6 CFU/ml to 5.0×10^6 CFU/ml and sterile distilled water/an interfering substance. Sterile distilled water was used instead of an interfering substance for susceptibility testing to reference substances. The sporicidal activity of disinfectants A was evaluated in clean (0.3 g/l bovine albumin) and dirty conditions (3.0 g/l bovine albumin). For disinfectant B, the dirty conditions consisted of an organic load containing 3.0 g/l of bovine albumin and erythrocytes from sterile sheep blood (3.0 ml/l) because this product was intended to clean and disinfect medical device surfaces. Disinfectant A was tested under conditions of a lower organic load because it is intended only for the disinfection of clean instruments, i.e., cleaned before disinfection. When testing the spore susceptibility to reference substances, the contact time was 15 minutes. However, disinfectant A was tested with contact times of 15 and 60 minutes, while disinfectant B with a contact time of 15 minutes. 1 ml of water/interfering substance was mixed with 1 ml of the spore suspension for two minutes, and after this time, 8 ml of reference substances or disinfectants was added. After the contact time, 1 ml of the mixture was transferred into a tube with 1 ml of water and 8 ml of neutralizer. The reference substance/disinfectants were neutralized with a suitable neutralizer for 5 minutes. The composition of the neutralizers is provided in Table I.

The mixture was inoculated on Meat Glucose Yeast Agar (MTSA) at a volume of 1 ml in duplicate and incubated for 48 hours at 37°C under anaerobic conditions. Validation of the selected experimental condi-

Table I
The composition of neutralizers used in the tests.

Disinfectants/ reference substances	Composition
A) Glutardialdehyde	polysorbate 80 – 30 g/l saponin – 30 g/l histidine – 1 g/l in diluent
B) Peracetic acid	polysorbate 80 – 10 g/l sodium thiosulfate – 5 g/l lecithin – 3 g/l in diluent

tions (A), neutralizer toxicity (B), and dilution-neutralization (C) were performed according to the procedures of PN-EN 13704:2018-09 for each test. The susceptibility of spores to reference substances and sporicidal activity of the tested disinfectants were expressed as a reduction in decimal logarithm (lgR). Due to the lack of susceptibility criteria for *C. sporogenes* spores to reference substances in PN-EN 13704:2018-09, the susceptibility of the spores was determined according to the criteria established for *C. difficile* R027 spores in accordance with PN-EN 17126:2019-01. The reduction required should be ≥ 3 on a log decimal scale (PN-EN 13704:2018-09). The results came from six independent experiments performed in one repetition following the PN-EN 13704:2018-09 method. The reproducibility of the PN-EN 13704:2018-09 method was ± 0.09 in decimal logarithm.

Results

The susceptibility of *C. sporogenes* spores to reference substances. The susceptibility of *C. sporogenes* spores to reference substances – glutardialdehyde and peracetic acid – was tested. The results are shown in Table II. The susceptibility of *C. sporogenes* ATCC® 3584™ spores to glutardialdehyde was within the accepted susceptibility limits for the *C. difficile* R027 spores at concentrations of 1% and 6% and with a contact time of 15 minutes. The reduction of $\lg R < 1.82$ on a decimal logarithmic scale showed that spores were not susceptible to the concentration of 1% during the contact time of 15 minutes, while at the concentration of 6% at the same contact time, the reduction was higher ($\lg R$ 2.11) and the number of viable spores was lower than in conditions described above. The reduction value below < 1.82 on a decimal logarithmic scale observed for glutardialdehyde was due to the number of spores entered into the study and meant that at the concentration of 1%, the growth of *C. sporogenes* ATCC® 3584™ spores was observed within an unquantifiable range.

C. sporogenes ATCC® 3584™ spores were susceptible to peracetic acid at a concentration of 0.01% for 15 minutes contact time and did not meet the criteria

Table II
The susceptibility of *Clostridium sporogenes* ATCC 3584 spores to glutardialdehyde and peracetic acid under the parameters defined for *Clostridioides difficile* R027 spores (results from two independent experiments, the reproducibility of PN-EN 13704: 2018-09 method was ± 0.09).

Reference substance	Concentration [%]	Contact time [min]	The reduction required [lg]	The reduction obtained [lg]
Glutardialdehyde	1.0	15	< 1.5	< 1.82
Glutardialdehyde	6.0	15	≥ 1.5	2.11
Peracetic acid	0.01	15	< 1.5	2.54
Peracetic acid	0.04	15	≥ 1.5	> 3.38

for *C. difficile* R027 spores. The reduction was higher than 1.5 on a decimal logarithmic scale (lgR 2.54). However, at a concentration of 0.04% during the contact time of 15 minutes, they showed susceptibility to peracetic acid under the criterion established for the *C. difficile* R027 spores.

The above results were obtained in experimental conditions that meet the basic limits set in the PN-EN 13704:2018-09 standard. The number of spores in the mixture for the spore’s susceptibility test to the reference substances ranged from 5.34 to 5.53 on a decimal logarithmic scale. The experimental conditions (control A) and the toxicity of the neutralizer (control B) did not affect *C. sporogenes* spores. Therefore, the dilution-neutralization method has been validated (method validation C) (Table SI).

The sporicidal activity of the disinfectants. Disinfectant A achieved the required spore reduction and thus sporicidal activity in both clean and dirty conditions at the concentration of 80% with a contact time of 60 minutes. However, it was not active against *C. sporo-*

genes spores under clean conditions, at a concentration of 80%, and during the contact time of 15 minutes (Table III).

The sporicidal activity of disinfectant B was determined in clean and dirty conditions, except that the dirty conditions contained 3.0 ml/l sheep erythrocytes and 3.0 g/l bovine albumin. This modification characterizes the organic load conditions for determining medical areas’ disinfection parameters. The additional interfering substance did not reduce the activity of the tested product. It showed sporicidal activity at a concentration of 1% with a contact time of 15 minutes in clean and dirty conditions (Table IV).

The above results were obtained in experimental conditions that meet the basic limits set in the PN-EN 13704:2018-09 standard. The sporicidal activity of disinfectant A was assessed with the spore suspensions at the log densities from 5.34 to 5.52. For disinfectant B, the log density of the spore suspension was 5.46. The experimental conditions (control A) and toxicity of the neutralizer (control B), and the dilution-neutralization method

Table III
Sporicidal activity of the glutaraldehyde-based disinfectant A. Composition: 2.5% glutardialdehyde (results from three independent experiments, the reproducibility of PN-EN 13704: 2018-09 method was ± 0.09).

Disinfectant	Concentration [%]	Contact time [min]	Organic load conditions	The reduction required [lg]	The reduction obtained [lg]
A	80	15	clean*	≥ 3	< 1.84
A	80	60	clean	≥ 3	> 3.19
A	80	60	dirty**	≥ 3	3.24

* – 0.3 g/l bovine albumin solution, ** – 3.0 g/l bovine albumin solution

Table IV
Sporicidal activity of peracetic acid-based disinfectant B. Composition: 1% product solution contains 0.15% peracetic acid (results from 1 experiment, the reproducibility of PN-EN 13704: 2018-09 method was ± 0.09).

Disinfectant	Concentration [%]	Contact time [min]	Organic load conditions	The reduction required [lg]	The reduction obtained [lg]
B	1	15	clean*	≥ 3	> 3.31
B	1	15	dirty**	≥ 3	> 3.31

* – 0.3 g/l bovine albumin solution, ** – 3.0 g/l bovine albumin solution plus 3.0 ml/l erythrocytes

(validation method C) did not affect *C. sporogenes* spores when testing both products (Tables SII and SIII).

Disinfectant B showed higher sporicidal activity at the lower disinfection parameters (concentration, contact time) and higher organic load than disinfectant A. The sporicidal activity of the disinfectants A and B towards *C. sporogenes* spores confirmed their manufacturers' disinfection parameters (concentration, contact time).

Discussion

The susceptibility of *C. sporogenes* spores to glutardialdehyde and peracetic acid was evaluated using the PN-EN 17126:2019-01 for *C. difficile* R027 spores. It indicated that *C. sporogenes* spores show similar susceptibility to glutardialdehyde as *C. difficile* R027 spores did, as they met the criteria adopted for this test organism. However, they are very susceptible to peracetic acid. Treatment with 0.01% peracetic acid reduced spore numbers to the level required for disinfectants of sporicidal activity (≥ 3 on a decimal logarithmic scale). The higher sporicidal activity was demonstrated for the product based on peracetic acid than the product containing 2.5% glutardialdehyde. According to Rutala et al. (1993a) *C. sporogenes* spores were less susceptible to disinfectants containing glutardialdehyde than the spores of *C. difficile*.

Similar data on the susceptibility of *C. sporogenes* compared to the susceptibility of *C. difficile* spores was provided by Humphreys (2011). Other study found that the susceptibility of *C. difficile*, *B. subtilis*, and *C. sporogenes* spores changes with the sporicidal disinfectant applied and depends on the spore culture conditions (Perez et al. 2005). At the same time, it was observed that the dilution of disinfection products with 2% glutardialdehyde to a lower concentration did not lead to the inactivation of *C. difficile* spores at contact times commonly used in the disinfection of semi-critical devices, e.g., flexible endoscopes (Rutala et al. 1993 b). In this study, the activity of the glutardialdehyde-based product (2% glutardialdehyde) resulted in the required reduction of *C. sporogenes* spores during the 60-minute contact time, both in clean and dirty conditions, while this product was inactive during the 15-minute contact time in clean conditions. The product based on peracetic acid showed sporicidal activity at a lower concentration and at a shorter contact time, both in clean and dirty conditions. The higher activity of the disinfectant containing peracetic acid might be due to the higher *C. sporogenes* spores susceptibility to peracetic acid than the one specified PN-EN 17126:2019-01 for *C. difficile* R027 spores, and the higher content of peracetic acid in the disinfectant (0.15% peracetic acid at 1% disinfectant concentration).

Since the suspension methods for the sporicidal activity evaluation are not suitable due to practical reasons (too long contact times, testing without organic load or only under clean conditions, or lack of sporicidal tests on surfaces) (Humphreys 2011; Gemein et al. 2022), dirty load conditions were introduced following the guidelines for the medical area (3.0 g/l bovine albumin and 3.0 ml/l sheep erythrocytes) in this study. Disinfectants containing peracetic acid are sensitive to contact with organic pollutants (especially blood) that may lead to the loss of their activity (Kampf et al. 2014). However, the organic load conditions applied here did not cause a decrease in the peracetic acid concentration and a reduction in its sporicidal activity. The low reactivity of disinfectants in contact with organic contaminants/body fluids was reported as one of the primary features determining their biocidal effectiveness (Yokoyama et al. 2021).

Compared to the preparation containing glutardialdehyde, the product containing peracetic acid acted on the spores at lower concentrations and a shorter time, even in a significant organic load. Comparable results for preparations containing peracetic acid were described by Gemein et al. (2022). They demonstrated that disinfectants based on peracetic acid showed higher sporicidal activity than disinfectants containing hydrogen peroxide and glutardialdehyde, both in suspension and surface tests (Gemein et al. 2022). These characteristics of peracetic acid were used to develop disinfectants for rapid disinfection, including sporicidal activity, for example in hand disinfection. Acidification of ethanol with peracetic acid (1,200–2,000 ppm) limited the transmission of infections caused by the spores of *C. difficile* (Nerandzic et al. 2015). According to PN-EN 13704:2018-09, the strong sporicidal activity of peracetic acid was also found against *B. subtilis* and *C. difficile* R027 spores, proving that the use of disinfectants with strong disinfecting effects eliminates the differences in the susceptibility of spores and their importance in testing this type of products (Votava and Slitrová 2009).

However, the results of this study on the *C. sporogenes* spore susceptibility to peracetic acid demonstrated the need to determine the minimum parameters of spore susceptibility to a given reference substance. The sporicidal activity of disinfectants should not be determined based on too high spore susceptibility since it may lead to ineffective disinfection.

Conclusions

The susceptibility of spores to the reference active substances may determine the sporicidal activity of disinfection products. Excessive susceptibility of spore

suspensions to these substances may lead to the designation of underestimated parameters of disinfection, which in practice will not provide sporicidal activity. It is also necessary to consider the spore suspension quality (lack of vegetative and germinative forms). Otherwise, the research may not determine the bactericidal activity but the sporicidal one.

The methodological problem is also the appropriate selection of the organic load that reflects the practical conditions of using the sporicidal disinfectant. Despite these limitations, the development of methods that confirm the sporicidal activity of disinfectants is necessary due to the contamination of surfaces, instruments, and textiles by spores of anaerobic bacteria both in the medical and non-medical areas.

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Conflict of interest

The author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Supplementary materials are available on the journal's website.