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Development of a sporicidal test method for *Clostridium difficile*

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SUMMARY

Background: Disinfectants with claimed activity against *Clostridium difficile* must be evaluated to ensure efficacy against the spores that comprise an environmental source of patient infection. Unfortunately there is, at present, no generally accepted method for evaluating these disinfectants. In the absence of such a method, laboratories have to adapt protocols that were not designed for products used in medical environments and consequently may use inappropriate test organisms, exposure times, and pass criteria.

Aim: To develop and evaluate a method for testing the activity of disinfectants against *C. difficile* spores using exposure times and pass criteria which are relevant to clinical application.

Methods: A Joint Working Party of the Healthcare Infection Society (HIS) and the Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infections (ARHAI) of the Department of Health in England was assembled. The Working Party adapted a previously described enzyme-based method for spore purification (the Clospore method) using an exposure time of 5 min and a 5 log₁₀ kill as a pass criterion.

Findings: Evaluation of the method by three laboratories demonstrated that the method is simple to follow and that the results are repeatable and reproducible.

Conclusion: The method described by the Working Party produces a clean suspension with a high titre of spores. It is recommended that, for a disinfectant used in the environment, the product should demonstrate a 5 log₁₀ reduction in 5 min under clean or dirty conditions to fulfil the requirements of the test.

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Introduction

For some time, concerns have been expressed about the validity of tests that measure the activity of disinfectants against *Clostridium difficile* spores.^{1–3} When *C. difficile* was highly prevalent in UK hospitals, many companies introduced products claiming to be active against spores of *C. difficile*. In the absence of a standard test against this organism, it was difficult for infection prevention and control teams to make an informed decision on the efficacy of these products and to make comparisons. Although a European Standard for sporicidal activity (EN 13704, 2002) exists, this uses *Bacillus subtilis* and *Clostridium sporogenes* rather than a clinically relevant strain such as *C. difficile* as the test organism.⁴ This European standard has also been designed for use in food hygiene, domestic, and institutional settings rather than in the healthcare environment. Consequently, the mandatory exposure time, i.e. 60 min, is too long to be representative of clinical use of disinfectants, and the conditions of the test only include a low soil (clean conditions) for pre-cleaned surfaces – a challenge for any disinfectant in the presence of high soil (dirty conditions) reflects the real world where organic material frequently contaminates inanimate surfaces. Furthermore, EN 13704 only requires a 3 log₁₀ reduction in order for a product to fulfil the requirements of the standard, whereas a 5 log₁₀ reduction is generally thought to be more appropriate for a product in clinical use. The 5 log₁₀ reduction is of particular importance for *C. difficile* as the infective dose, according to animal experiments, is extremely low.^{4,5}

Of concern is the fact that a suspension of *C. difficile* with a high titre can be difficult to prepare, and that tests using a suspension consisting predominantly of vegetative cells will give misleading results as vegetative cells are much more susceptible to chemical disinfection than spores.⁶ Consequently, a testing laboratory with poor technique might prepare test suspensions that have a low proportion of spores and could therefore erroneously report successful sporicidal activity in a product that will be of little use in a clinical environment. Validation of neutralization of residual disinfectant and possible toxicity of the neutralizer are crucial when testing chemical disinfectants. Laboratories with poor technique may also overestimate the activity of a product by inadequately neutralizing the disinfectant, resulting in an effective exposure time in excess of that stated in the standard.

In light of the above concerns, a Joint Working Party of the Department of Health (England) Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) and the Healthcare Infection Society was set up in 2011. The Task Force initially reviewed the published methods for preparation of *C. difficile* test spores and developed a reliable and reproducible method for testing disinfectant activity against this clinically important organism. The method was trialled in three different laboratories [School of Pharmacy and Pharmaceutical Sciences, Cardiff University; the Hospital Infection Research Laboratory (HIRL), Queen Elizabeth Hospital, Birmingham; and the Public Health England Laboratory (PHE), Porton Down]. Technical difficulties were identified and reasons for discrepancies between laboratories were investigated. An iterative process allowed the methodology to be improved and a final method was evaluated.

The protocol is now complete and the rationale underlying the chosen method is described as follows.

Rationale underlying the test method

Currently there is no European sporicidal disinfectant test applicable to the medical area; thus, the protocol most often adapted to test activity against *C. difficile* spores is EN 13704 (2002), which is not an ideal test for the following reasons:

- It uses *Bacillus subtilis* and *Clostridium sporogenes* spores, which are not representative of the clinical scenario. There is a need for a test that uses *C. difficile* rather than a surrogate organism.
- It requires a 3 log₁₀ spore kill to pass, which is insufficient to be relevant for a product designed for use in the medical area. The levels of *C. difficile* spores isolated from environmental surfaces, particularly during an outbreak, may be high.^{5–11}
- It uses an obligatory contact time of 60 min to determine a pass or a fail. Most situations where sporicidal activity against *C. difficile* would be required involve a liquid disinfectant applied to environmental surfaces, which is then allowed to dry naturally. This would take seconds to a few minutes.
- This test only has a simulation of clean conditions (0.3 g/L albumin in the test mixture). This provides a less stringent challenge than many of the situations in which a sporicidal disinfectant would be expected to work in healthcare where an organic load would be present.

For the above reasons the Joint Working Party developed a method for spore preparation and activity testing which is more relevant to products designed for medical use. The test proposed incorporates the following features:

- It uses a non-toxigenic strain of *C. difficile*. There is no reason to suspect that toxigenesis coincides with decreased susceptibility to disinfectants. Using a non-toxigenic strain decreases laboratory safety constraints, meaning that it will be a feasible test for a wider range of laboratories.
- It has a detailed and validated method for spore preparation incorporating growth, harvesting, purification, and verification. This protocol uses a culture method that yields increased spore numbers, and incorporates enzymatic purification and ultrasonic homogenization steps to give a high yield of spores with very low numbers of vegetative cells. This is verified by phase-contrast microscopy.
- This method gives a highly purified spore suspension with little extraneous matter that may interfere with a biocidal product's activity.
- The titre of the spore suspension allows a 5 log₁₀ reduction of spores to be demonstrated as a pass level; a target that is more relevant to patient safety than the previous 3 log₁₀ reduction.
- The exposure time used is 5 min. This represents the short-term exposure of spores to liquid disinfectant during surface disinfection in a clinical setting more realistically than the 60 min in EN 13704.
- The test has both clean (0.3 g/L albumin) and dirty (3.0 g/L albumin plus 3.0 mL/L sheep erythrocytes) conditions

included in the method to simulate that which would be found in the healthcare environment.

- The test incorporates an internal control using a standard 500 ppm available chlorine solution. This provides verification of the disinfectant susceptibility of the spore suspension.

Test method for producing a high titre of *C. difficile* spores

The methodology developed was based upon the work of Perez *et al.*, who demonstrated that the use of Clospore medium, along with an enzyme purification step, allowed the production of *C. difficile* spore suspensions at titres of up to 10^9 ($9 \log_{10}$) of $\geq 90\%$ purity (as assessed by phase-contrast microscopy).¹² *C. difficile*, NCTC 11209, was incubated anaerobically using Clospore medium, either in liquid or solid form, for 7–10 days. The organism was recovered and concentrated using several washing/centrifugation steps, prior to a purification step involving the use of an enzyme preparation and ultrasonication, to break up spore aggregates, and digest any vegetative cells. The suspension was then washed/centrifuged several more times before being heat-shocked at 70°C for 10 min to kill any remaining vegetative cells. After a final round of washing/centrifugation, the purity of the spore preparation was checked using phase-contrast microscopy, and the titre ascertained with conventional culture methods.

Test method for measuring the activity of chemical disinfectants against *C. difficile* spores

In order to verify the methodology, a multicentre trial was set up involving three laboratories (HIRL, Cardiff University and PHE). The intention was to test one disinfectant product using the spore preparation method and the disinfectant test described in Appendix 1.

Each laboratory tested a 500 ppm available chlorine solution prepared from sodium dichloroisocyanurate (NaDCC) tablets. The concentration of available chlorine was determined prior to testing using a standard pharmacopoeial method.¹³ The test was carried out under clean conditions only, i.e. in the presence of 0.3 g/L bovine serum albumin. Contact times of 1, 5, and 60 min were tested to allow for low and high reductions to be detected. Each laboratory carried out the test in triplicate on each of two separate days. Each test was carried out using two separate batches of spore suspension. Further tests were also carried out to determine the shelf life of the spore suspension; i.e. to what extent the spore titre decreased over time.

The European Standard methods for disinfectant testing involve the following: 8 mL of NaDCC (500 ppm final concentration) mixed with 1 mL of organic load (0.03% w/v albumin; final concentration) and 1 mL of spore suspension. After pre-defined contact times 1 mL was removed and added to a previously validated neutralizing solution. The number of surviving spores was enumerated and the \log_{10} reduction calculated. The organic load used was as described in other standards used for assessing disinfectants for the medical area.

The results from these laboratories were analysed as per ASTM E691-12, to establish accuracy, repeatability and

reproducibility.¹⁴ These tests were also used as the basis for inclusion of an internal control for all tests with *C. difficile* spores.

Results

The summary of the results obtained is displayed in Table I and Figure 1. The greatest variability was seen at 5 min; this may represent a critical time, where small variations in the time taken to remove the spores to the recovery medium translate to quite large differences in the amount of viable spores remaining.

Figure 2 shows the *h*-statistic for the study, which represents the between-laboratory consistency found in the results. Figure 3 shows the *k*-statistic for the study, which represents the within-laboratory consistency of the data. In general, the *k* and *h* values did not differ significantly at the 0.5% level. Using these statistics, the 95% repeatability (*r*) and reproducibility (*R*) limits were calculated (Table II). The 95% repeatability (*r*) limit represents the value below which the absolute difference between two individual test results obtained within the same laboratory, by the same operator, may be expected to occur with a probability of ~ 0.95 . Likewise, the 95% reproducibility (*R*) limit represents the value below which the absolute difference between two individual test results obtained within different laboratories, by different operators, may be expected to occur with a probability of ~ 0.95 .

Conclusions and recommendations

The Clospore method for the preparation of spore suspensions was found to be appropriate, although the quality of the spore suspension should be verified using phase-contrast microscopy. The use of the Clospore method repeatedly generated a high titre of spores to be used in the suspension test. Testing three contact times was shown to be important, since some variability resulting in decreased repeatability and reproducibility in results was observed at a 5 min contact time. As only a small number of laboratories have taken part in this study, the precision statistics should be interpreted with care; they are a general guide to what may be expected. Nevertheless the

Table I

Summary of \log_{10} reduction in *Clostridium difficile* spores following exposure to sodium dichloroisocyanurate at 500 ppm

Laboratory	Log ₁₀ reduction at time					
	Batch 1			Batch 2		
	1 min	5 min	60 min	1 min	5 min	60 min
Laboratory 1						
Mean	0.64	1.19	6.34	0.38	0.87	5.98
SD	0.18	0.20	0.15	0.05	0.18	0.19
Laboratory 2						
Mean	0.55	3.07	6.21	0.50	2.06	6.78
SD	0.10	0.58	0.79	0.05	0.11	0.34
Laboratory 3						
Mean	0.73	1.51	5.65	0.69	1.41	4.56
SD	0.35	0.51	0.14	0.20	0.55	0.07
Overall mean	0.63	1.91	6.00	0.52	1.46	5.71
Overall SD	0.24	0.92	0.46	0.18	0.59	0.89

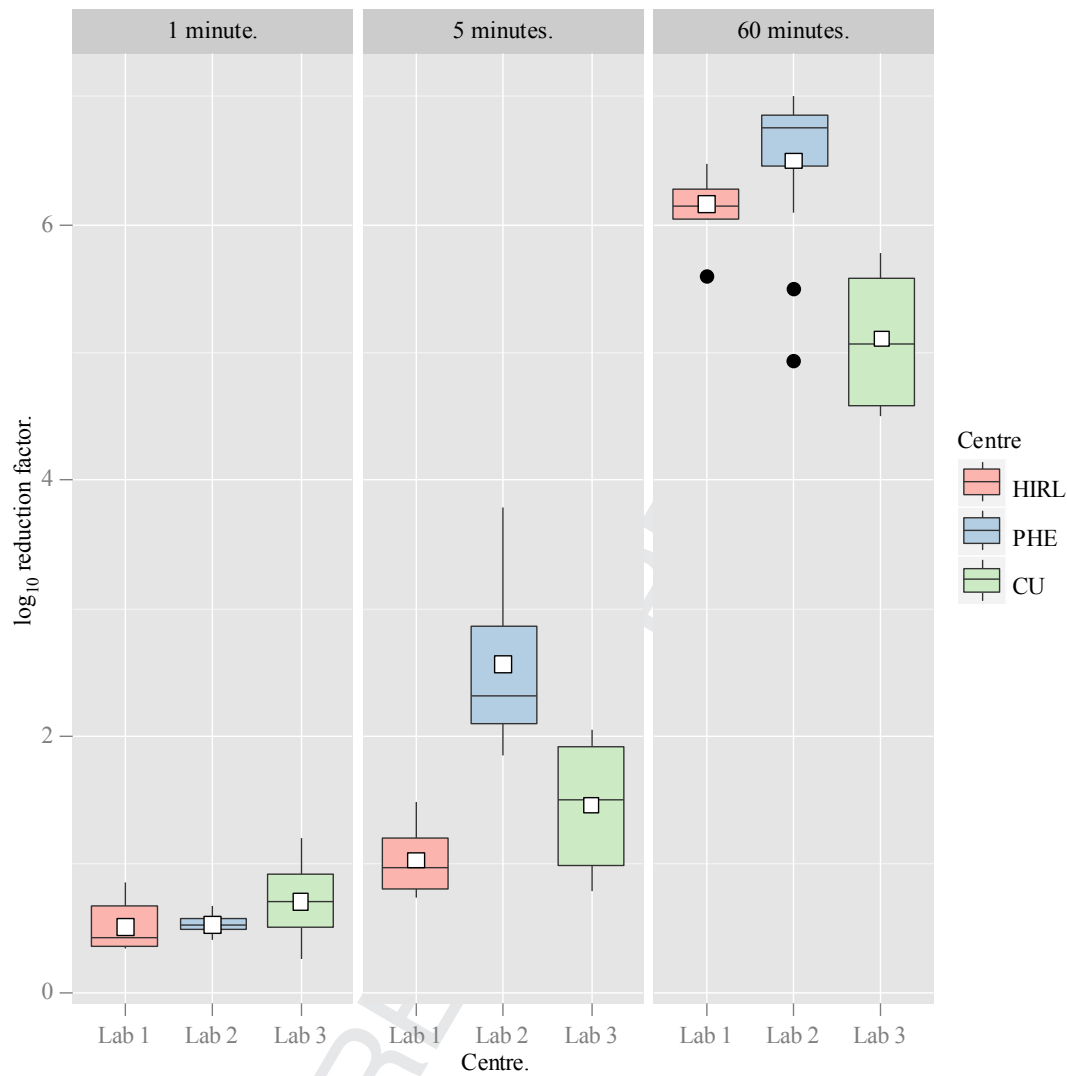


Figure 1. Summary of inter-laboratory results of 500 ppm chlorine-releasing agent against *Clostridium difficile*. HIRL, Hospital Infection Research Laboratory; PHE, Public Health England; CU, Cardiff University.

results in Table II suggest that the method proposed gives an acceptable level of repeatability and reproducibility.

Based on the results of this study, the Clospore method is recommended for spore preparation. It is also recommended that an internal control comprising 500 ppm NaDCC (or other equivalent chlorine-releasing agent) under clean conditions be included in all testing of *C. difficile* spores. For a spore suspension to be regarded as a sufficiently stringent challenge, the internal control should not produce a $>1 \log_{10}$ reduction after 1 min. It is expected that the internal control would produce a $\geq 5 \log_{10}$ reduction after 60 min. Failure to do so may represent excessive debris/clumping within the spore suspension.

This study has demonstrated that the Clospore method produces a clean suspension with a sufficient number of spores that can be used to demonstrate a $5 \log_{10}$ reduction. The Joint Working Party recommends that, for a disinfectant used in the environment, the product should demonstrate a $5 \log_{10}$ reduction in 5 min under clean or dirty conditions to fulfil the requirements of the test. This requirement is in line with the EN test for bactericidal efficacy. Five minutes is a more practicable time for disinfectants used for this purpose.

Conflict of interest statement

The views expressed in this publication are those of the authors and not necessarily those of Public Health England or any other Government Agency.

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The Joint Working Party received funding to cover travel and other expenses from the Healthcare Infection Society.

Appendix 1. Test methodology for assessing the efficacy of chemical disinfectants used in the medical area against spores of *Clostridium difficile*

Requirements

The product shall demonstrate $\geq 5 \log_{10}$ reduction in 5 min, when tested under simulated clean conditions (0.3 g/L bovine albumin solution) or dirty conditions (3 g/L bovine albumin solution, plus 3 mL/L washed sheep erythrocytes). For instrument

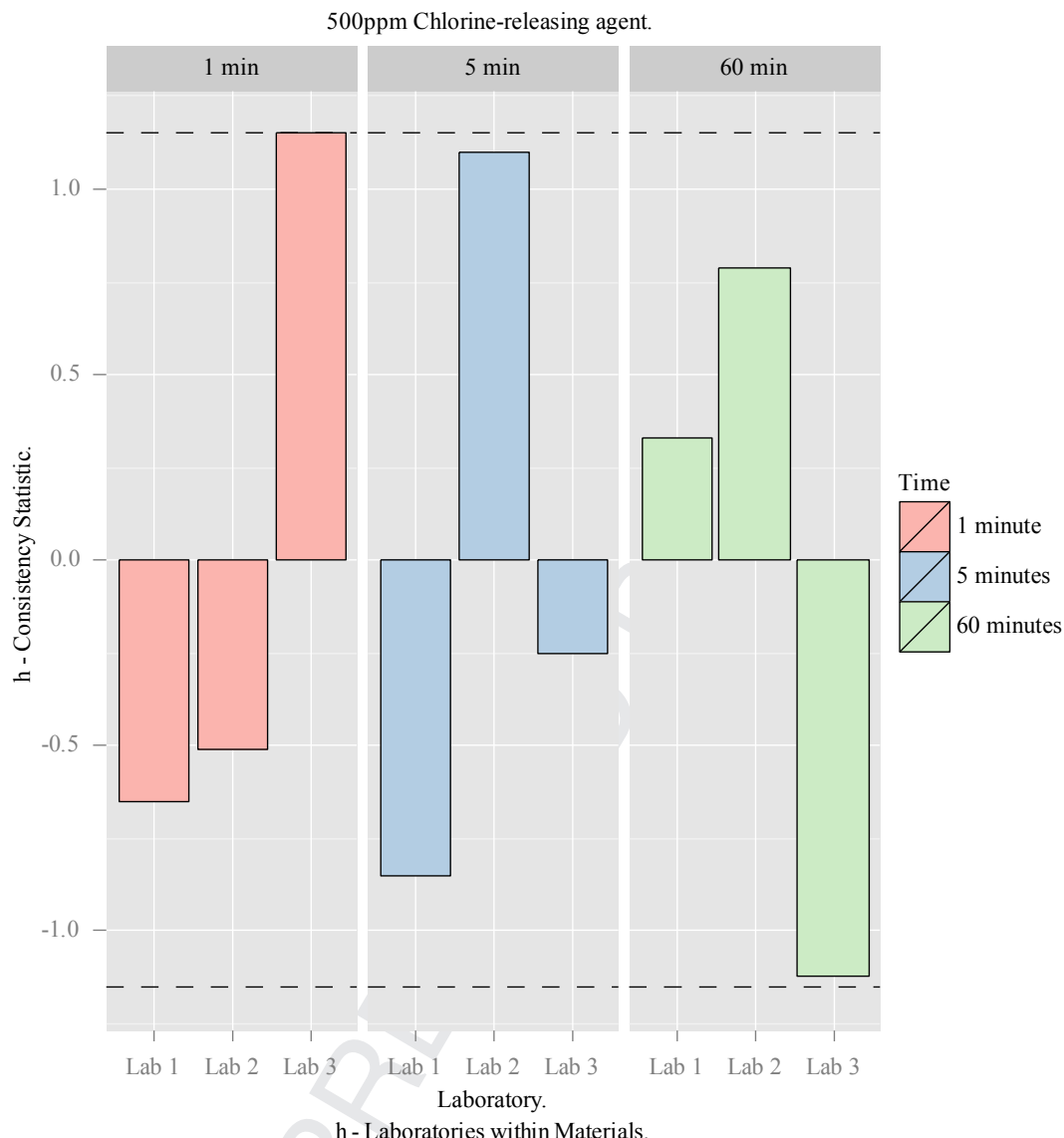


Figure 2. *h*-Statistic for the study, representing the between-laboratory consistency found in the results.

disinfectants, where recommended by the manufacturer to be used at higher temperatures, the product needs to demonstrate the 5 log₁₀ reduction only at the recommended temperature.

Test conditions

Instrument disinfection or surface disinfection.

Test organism: *Clostridium difficile* NCTC 11209.

Test temperature and time

At the lowest recommended temperature, range 20–60°C, contact time:

- (a) obligatory 5 min;
- (b) additional according to the manufacturer's recommendation, but no longer than 5 min.

Interfering substance(s)

Clean conditions: 0.3 g/L bovine albumin solution.

Dirty conditions: 3.0 g/L bovine albumin solution plus 3.0 mL/L washed sheep erythrocytes.

The obligatory contact times for surface disinfectants are chosen on the basis of expected practical application of the product.

Test method

Principle

A sample of the product as delivered and/or diluted with hard water is added to a test suspension of spores in a solution containing an interfering substance. The mixture is maintained at the temperature for the contact time specified. At the end of this contact time, an aliquot is taken and immediately neutralized or suppressed by a validated method. The method of choice is dilution–neutralization. If a suitable neutralizer cannot be found, membrane filtration may be used. The numbers of surviving spores in each sample are determined and the reduction is calculated.

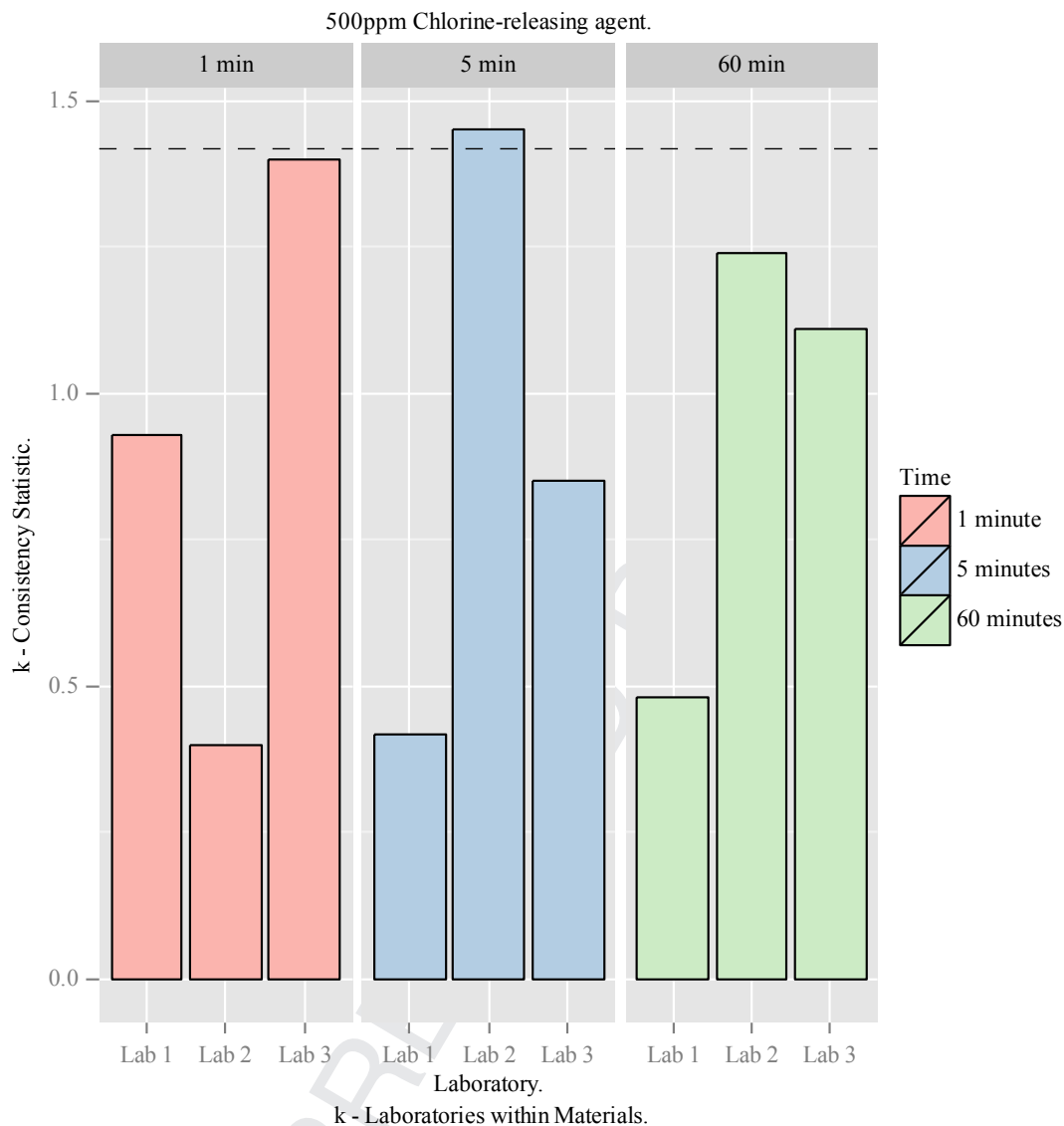


Figure 3. k-Statistic for the study, representing the within-laboratory consistency of the data.

Materials and reagents

Test organism: *C. difficile* NCTC 11209.

The required incubation temperature and conditions for this test organism are $36 \pm 1^\circ\text{C}$ in anaerobic conditions for five days.

Culture media and reagents

General. All weights of chemical substances given in this SOP refer to the anhydrous salts. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

Table II

Reproducibility and repeatability limits for the inter-laboratory study

	1 min	5 min	60 min
Reproducibility limits (<i>R</i>)	0.55	2.44	2.36
Repeatability limits (<i>r</i>)	0.55	1.68	1.47

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organism.

Note 1: To improve reproducibility, it is recommended that commercially available dehydrated material be used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

Note 2: For each culture medium and reagent, a time limitation for use should be fixed.

Water. The water shall be demineralized water (ultrapure water) or freshly glass-distilled water. Water for injections may be used.

Sterilize in the autoclave. If the water is used for preparation of culture media and subsequently sterilized, initial sterilization is not necessary.

The NCTC and ATCC numbers are the collection numbers of strains supplied by these culture collections. This information

is given for the convenience of users and does not constitute an endorsement of the product named.

Preparation of *Clostridium difficile* spore suspension from liquid medium (Clospore). This is based on J. Perez and S.A. Sattar' standard operating procedure (University of Ottawa, Canada; 20 May 2008).

Materials. All available from Sigma–Aldrich unless otherwise stated. The list is supplied for the convenience of users; equivalent products may be available from alternative suppliers.

- *Clostridium difficile* NCTC 11209
- Columbia Broth (QueLab Laboratories, Montreal, QB, Cat. No. QB-39-1106; 500 g)
- *C. difficile*-specific agar plates
CLO (bioMérieux Cat. No 43431) or
CDMN agar (Oxoid Cat. No: SR0173 added to CM0601 and SR0050)
- Special Peptone Mix (SPM) (Oxoid Laboratories, Cat. No. LP0072)
- Brain Heart Infusion (BHI) (Oxoid Laboratories, Cat. No. CM0225)
- Yeast Extract Powder (HiMedia, Cat. No. 61001-636) or Difco (Becton–Dickinson), USA
- Lysozyme (Egg white) – Activity 20100 units/mg. (Pharmacia LKB Biotechnology AB, Uppsala, Sweden)
If unavailable, Lysozyme (Sigma Cat No. L6876), activity 40,000 units; note: use half as much, as is twice as active.
- Trypsin (Sigma Cat: T0134, Lot: 24H0069 from Porcine Pancreas.15000 BAEE units/mg.) If unavailable: Trypsin (Sigma cat. no, T0303)
- Na₂HPO₄
- NaH₂PO₄
- KH₂PO₄
- K₂CO₃
- CaCl₂·2H₂O
- (NH₄)₂SO₄
- MgSO₄
- Taurocholic acid sodium salt hydrate (Sigma, Cat. T4009-259, Batch No. 048K1437)
- L-Cysteine
- 0.1 M sodium phosphate buffer pH 7.0
- Sterile water (either double-distilled water (ddH₂O), or sterile distilled water (sdH₂O) suffices)
- Sterile plastic transfer pipettes (Pasteur pipettes)
- 15 mL sterile polypropylene tubes (Falcon tubes)
- 50 mL sterile polypropylene tubes (Falcon tubes)
- 1 L polycarbonate or high-density polypropylene culture flasks or Duran flasks.
- 200–1000 µL pipette and suitable sterile tips
- 20–100 µL pipette and suitable sterile tips
- 1–20 µL pipette and suitable sterile tips
- 37°C incubator with anaerobic conditions:
Anaerobic Chamber (ACh) with internal incubator at 37 ± 2°C.
Or anaerobic jars capable of being incubated at 37 ± 2°C, with anaerobic sachets and indicators (Oxoid Cat. Nos. AN035A and BR0055B)
- Centrifuge with capacity for larger volumes and capable of maximum 10,000 g;

e.g. Beckman Centrifuge Avanti J-25 with rotors JA-25-50 and JLA 16-250;
Heraeus Biofuge Primo R with Sorvall 6 × 50 mL 75007588 rotor;

Eppendorf 5702 with 4 × 50 mL A-4-38 rotor.

- Appropriate centrifuge tubes, e.g. 50 mL Falcon tubes
- Sonicating water bath (frequency 40 kHz ± 2 kHz)
- Water bath capable of 70°C

Preparation of media and reagents. Note: All agar/broths used to cultivate *C. difficile* must be pre-reduced (oxygen removed) before growth. This is defined as incubation of media in anaerobic conditions for at least 17 h; all lidded containers of broth/agar must have loosened lids to allow reduction to occur. All growth of *C. difficile* must take place at 37 ± 1°C with anaerobic conditions.

Columbia Broth (CB). Prepare 500 mL of Columbia Broth as per the manufacturer's instructions (35 g/L from QueLab Brand). Keep at 4°C ± 2°C in cold room or refrigerator.

***Clostridium difficile* sporulation medium (Clospore) preparation (500 mL).** It is recommended that this medium be prepared by dissolving the reagents in the same order in which they are described. To a 500 mL Duran bottle add 300 mL of distilled water and then:

SPM	5.0 g
KH ₂ PO ₄	1.30 g
(NH ₄) ₂ SO ₄	0.30 g
CaCl ₂ ·2H ₂ O	0.04 g
Yeast Extract Powder	5.0 g
K ₂ CO ₃	1.74 g
MgSO ₄	0.06 g
ddH ₂ O	up to 500 mL

To prepare Clospore solid medium, add 15 g/L agar to the above formulation.

The pH before autoclaving should be 7.9 ± 0.2. If not, adjust it with KOH. Once sterilized the pH should increase up to 9.0 ± 0.2. Autoclave the flasks using the liquid cycle for 45 min at 121°C (retention time is 20 min).

Wait for 2 or 3 h until the temperature drops to ~50–60°C, aliquot 25 mL Clospore into 6 × 50 mL sterile falcon tubes, or in 500 mL batches to give higher yields of spores, and pre-reduce.

After 17 h of pre-reduction and before inoculation, the pH should drop to 8.2 ± 0.3. After sterilization, the pH can be 8.8–8.9 and after 17 h reduction it should drop to 8.7 or less.

Semisolid medium for spore recovery (BHIS). This can be used for spore enumeration. Alternatively, *C. difficile* specific agar plates can be used.

BHI	37.0 g
Yeast extract	5.0 g
L-Cysteine	1.0 g
Na taurocholate	1.0 g
Bacteriological agar	15 g
Complete with ddH ₂ O to:	1000 mL

Heat and boil for 1 min, sterilize on liquid cycle, 45 min at 121°C. Lysozyme 10 mg in 10 mL of ddH₂O, added by filtration (0.2 µm sterile membrane).

0.1 M Na phosphate buffer pH 7.0. Weigh 8.1934 g of anhydrous Na₂HPO₄ and 5.8374 g of NaH₂PO₄·H₂O. Dissolve in ~800 mL of ddH₂O. Adjust pH to 7.00 with 1 M NaOH or 1 M HCl and fill to 1000 mL.

Enzymatic solution. Weigh the necessary quantity to prepare a solution of 20 µg of lysozyme and 15 µg of trypsin per mg of wet weight spore and dissolve it in 25 mL of 0.1 M sodium phosphate buffer, pH 7.00 (scale amount of enzyme used up/down in case of use of enzyme with different activity). The spore wet weight depends on the harvest's yield. Sterilize the solution through a maximum pore size of 0.45 µm sterile Millipore filter at the time you add this solution directly to the centrifuge tube with the spore suspension to be treated.

Procedure

Note: The liquid culture method is preferable; however, the solid culture method can be used if access to a centrifuge with suitable capacity for harvesting (>200 mL total rotor capacity) is unavailable.

Preparation of liquid culture

Inoculum preparation:

- From the stock spore suspension kept at 4 ± 2°C (or the original suspension kept at –80°C) take a loop and spread it on a reduced CDMN or CLO plate. Incubate the plate for 48 h at 37 ± 1°C under anaerobic conditions or until the colonies are 2–4 mm in diameter
- At the same time, keep in anaerobic conditions 15 mL sterile Falcon tubes containing 5 mL of CB for the pre-inoculum preparation, and the same number of 50 mL Falcon tubes with 20 mL of CB as number of culture flasks you have to inoculate, for the inoculum preparation. For example, 4 × 500 mL (or 4 × 25 mL) of Clospore broth require 4 × 20 mL inoculum CB, which require 4 × 5 mL pre-inoculum CB.
- Select a colony and resuspend it in a 15 mL sterile plastic tube containing 5 mL of pre-reduced CB.
- Incubate for 24–48 h under anaerobic conditions. Inoculate 50 µL into each of 50 mL plastic tubes containing 20 mL of pre-reduced CB.
- Incubate them for 24–48 h in the anaerobic chamber.

Flask inoculation:

- Take the pre-reduced Clospore broth out of anaerobic conditions and tighten their lids.
- Inoculate each Falcon tube of 25 mL Clospore broth with 1 mL of inoculum as fast as you can, without any agitation or introduction of air bubbles. For larger volumes of broth, add all 20 mL inoculated CB to 500 mL Clospore broth, in a similar, steady fashion.
- Return the inoculated flasks to anaerobic conditions without any agitation, first loosening the lids. Incubate the flasks without any agitation for 7–10 days at 37 ± 1°C.
- At the end of incubation, assess the number and viability of spores to calculate the yield of the process.
- For quality control purposes, here one can inoculate a Lysogeny Broth (LB) or Trypticase Soy Agar (TSA) plate

(500 µL) and incubate aerobically overnight to check for absence of growth.

Harvesting liquid culture:

- Centrifuge the sporulated culture at 10,000 g (or as high as your rotor allows up to this speed) for 10 min at room temperature. Discard the supernatant in a waste container and repeat the process until the total volume of the culture is centrifuged. Pool the sediment in one centrifuge tube and wash the pellet up to three times with 25 mL of sterile water.
- Resuspend the pellet in 10 mL of sterile water and transfer the suspension into a previously weighed 50 mL centrifuge tube. Rinse the centrifuge tube several times and complete the volume to 20 mL. Centrifuge at 10,000 g for 10 min and discard the supernatant.
- Weigh the tube with the pellet and keep it at 4 ± 2°C until the next step. Calculate the obtained wet weight of the spores. This wet weight should be used to prepare the enzymatic solution.

Clospore plate method

Inoculum preparation:

- From the stock spore suspension kept in the cool room (or the original suspension kept at –80°C), take a loop and spread it on a reduced CDMN/CLO plate. Incubate the plate for 48 h at 37 ± 2°C in anaerobic conditions or until the colonies are 2–4 mm in diameter.
- At the same time, keep in anaerobic conditions 4 × 15 mL sterile Falcon tubes containing 5 mL of CB for the pre-inoculum preparation and 40 Clospore plates.
- Select a colony and resuspend it in a 15 mL sterile plastic tube containing 5 mL of at least 24 h pre-reduced CB.
- Incubate for 16 h in anaerobic conditions.
- Inoculate 40 Clospore plates (90 mm) with 500 µL, spread evenly over the plate with a sterile spreader and allow drying at room temperature in a laminar air flow cabinet.
- For quality control purposes, here one can inoculate an LB/TSA plate (500 µL) and incubate aerobically overnight to check for absence of growth.
- Incubate anaerobically for 7–10 days at 37 ± 1°C. At the end of incubation, assess the number and viability of spores to calculate the yield of the process.

Harvesting for solid culture:

- Wash each plate by adding 2 mL sterile water to a plate, scraping the surface with a sterile spreader and pipetting the liquid into a sterile and previously weighed 50 mL centrifuge tube.
- Centrifuge at 10,000 g for 15 min at 4 ± 2°C.
- Pour off supernatant and wash up to three times in 30 mL sterile water, discarding the supernatant.
- Weigh the tube and the pellet and keep it in the cold room until the next step. Calculate the obtained wet weight of the spores. This wet weight should be used to prepare the enzymatic solution.

Purification of the spore suspension (both liquid and plate harvest):

- Resuspend the pellet (no more than 1 g of wet weight) in 10 mL of 0.1 M sodium phosphate buffer, pH 7.0 and mix thoroughly in a vortex.

- Add the 20 mL freshly prepared enzymatic solution and gently mix.
- Sonicate the suspension on ice for 60 s, then cool on ice for 30 s, and repeat this cycle once. Incubate the suspension for 30 min at 45°C in a water bath. Repeat the sonication–water bath step once.
- Centrifuge the suspension at 10,000 g for 10 min and wash the pellet up to three times with sterile water, resuspending in 30 mL of sterile water.
- Heat for 10 min at 70°C, and then submerge the tube in ice for 5 min.
- Centrifuge the suspension at 10,000 g for 10 min and resuspend the sediment in a final volume of 10 mL of sterile water. Transfer it to a sterile plastic tube and keep the suspension in the cold room at $4 \pm 2^\circ\text{C}$.
- Count the spore number and the survivors (see below).

Quality control

Sonication:

- As *C. difficile* spores tend to aggregate into clumps over time, immediately prior to testing, sonication must be performed.
- As repeated sonication over time can lead to loss of spore viability, sonication must only be performed on one small aliquot of spores at a time; enough to perform testing.
- Sonication prior to testing should be performed in a thin-walled glass vessel for 5 min.

No. of cells/mL = (average of counted cells/0.0005834) $\times 10^3$ (for slides)

No. of cells/mL = (average of counted cells/0.003821) $\times 10^3$ (for the Neubauer chamber)

Storage of spores:

- Storage of spores at $4 \pm 2^\circ\text{C}$ must be performed for a minimum of two weeks, to allow for spore maturation. Immature spores may be more susceptible to disinfectants and give inaccurate results.
- Spore preparations must be discarded after 12 months of production, due to loss of viability.
- Prior to testing, the preparation should be assessed for concentration and percentage of spores.

Assessing spore concentration (cfu/mL). Note: Concentration of spores/mL can be found by multiplying the percentage spores seen by microscopy by the cfu/mL concentration.

- Perform a 1-in-10 serial dilution in sterile water of the spore solution, and assay by plating out duplicate 100 μL volumes on pre-reduced *C. difficile* specific agar. Colony-forming units (cfu) can be counted on duplicate plates.
- Cfu/mL can be calculated by:

$$\frac{\text{cfu}}{\text{mL}} = \frac{\text{average of cfu on plates} \times 1000}{\text{volume plated} \times \text{dilution factor}}$$

Alternatively, the Miles–Misra or other suitable validated methods can be employed (Miles AA, Misra JO. The estimation

of the bactericidal power of the blood. *J Hyg* 1938;38: 732–749).

Assessing percentage spores

Phase-contrast microscopy with imaging software:

- Add 10 μL of a well-vortexed solution to the surface of a glass slide and cover with a 22×22 mm cover slide or using an improved Neubauer chamber. It may be necessary to serially dilute (10^{-1} – 10^{-2}) the suspension, depending on the yield.
- Take random pictures (8–10) in a phase-contrast and epifluorescent microscope: Axioskop 2 Plus, Carl Zeiss with Lens Plan-Neofluar, Camera Axiocam MRm, Monochrome Carl Zeiss and MR Grab software 1.0.0.4. by using the $\times 40$ objective lens. Process images with the Software Image-Pro Plus version 5.1 to count the total spore number as highly refractile (phase-bright) particles present in the sample.
- The factor used for the estimation of the cell concentration in the microscope slides is calculated considering the picture area and height for a volume of 10 μL . The theoretical factor is amended by comparing the spore and cell concentration in stock cultures with those obtained with an improved Neubauer chamber used as standard. The lower sensitivity for the slide method is $1.2 \times 10^6/\text{mL}$ and for the Neubauer chamber $2 \times 10^5/\text{mL}$ which corresponds to one cell or spore average per picture (field).
- The final formulae to estimate the cell or spore numbers are the following:

Phase contrast microscopy by eye:

- Add 10 μL of a well-vortexed spore preparation to the surface of a glass slide and cover with a 22×22 mm cover slide or using an improved Neubauer chamber. It may be necessary to serially dilute (10^{-1} – 10^{-2}) the suspension, depending on the yield.
- View under phase contrast and estimate the percentage of highly refractile, (phase-bright) spores that are present, compared to debris or dark vegetative cells.

Spore staining method:

- If phase contrast microscopy equipment is unavailable, perform a Schaeffer–Fulton spore stain of 10 μL of final suspension on a glass slide and estimate percentage of dark green spores compared to debris or red vegetative cells, visible under light microscopy.

Increasing spore yield.

- Increasing the volume of liquid or the number of plates inoculated can give a higher spore yield.
- Decreasing the number of washes during the purification process can give a higher yield, as spores can be lost from pellet upon discarding of supernatant. However, too few

washes may lead to a decrease in quality, so no fewer than four washes in total are recommended.

Disinfectant test method

Materials

Standard hard water

Stock solution A:

1.98 g	MgCl ₂ (anhydrous)
4.62 g	CaCl ₂ (anhydrous)
100 mL	Distilled water

1. Weigh out the MgCl₂ and CaCl₂ and transfer to a 100 mL volumetric flask.
2. Using a clean measuring cylinder, measure 100 mL of distilled water and add ~50 mL to the chemicals. Gently rotate the flask until the dry chemicals have dissolved.
3. Gradually add the rest of the measured volume of distilled water to the solution to make up to 100 mL.
4. Secure the lid on the bottle and shake thoroughly to mix the ingredients.
5. Sterilize by autoclaving at 121°C for 15 min.

Label appropriately. This may be stored at 4 ± 2°C for up to one month prior to use.

Stock solution B:

3.5 g	NaHCO ₃
100 mL	Distilled water

1. Weigh out the NaHCO₃ and transfer to a 100 mL volumetric flask.
2. Follow steps 2–4 as described above in the preparation of solution A.
3. Sterilize using a sterile Millipore unit with a maximum pore size of 0.45 µm.
4. At the end of the sterilizing procedure, transfer the filtered media into a sterile 250 mL container. This may be stored at 4 ± 2°C for up to one month prior to use.

Requirements for preparation of working solution of hard water

The following volumes are required to produce 100 mL hard water:

0.6 mL	Solution A
0.8 mL	Solution B
98.6 mL	Pre-poured bottle of sterile distilled water

1. Aseptically transfer 0.6 mL of solution A to the bottle of sterile distilled water.
2. Transfer 0.8 mL of solution B to the same bottle of sterile water.
3. Before immediate use, shake the bottle well in order to mix the water and solutions.
4. Use on day of preparation.
5. Dispose of any unused hard water after use.

Diluent

1.0 g	Tryptone
8.5 g	NaCl
1 L	Distilled water

1. Weigh out the tryptone and NaCl and transfer to a 1 L volumetric flask.
2. Add 1 L distilled water.
3. Sterilize by autoclaving at 121°C for 15 min.

Interfering substance

General. The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 10 times its final concentration in the test.

The ionic composition (e.g. pH, calcium and/or magnesium hardness) and chemical composition (e.g. mineral substances, protein, carbohydrates, lipids and detergents) shall be defined.

Note: The term 'interfering substance' is used even if it contains more than one substance.

Clean conditions (bovine albumin solution: low concentration)

Dissolve 0.30 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 mL of diluent.

Sterilize by membrane filtration, store at 4 ± 2°C and use within one month.

The final concentration of the bovine albumin in the test procedure shall be 0.3 g/L.

Dirty conditions (mixture of bovine albumin solutions: high concentration with sheep erythrocytes)

Dissolve 3.00 g of bovine albumin fraction V (suitable for microbiological purposes) in 97 mL of diluent.

Sterilize by membrane filtration.

Prepare at least 8.0 mL fresh sterile defibrinated sheep blood. Centrifuge the erythrocytes at 800 g for 10 min. After discarding the supernatant, resuspend erythrocytes in diluent. Repeat this procedure at least three times, until the supernatant is colourless.

Resuspend 3 mL of the packed sheep erythrocytes in the 97 mL of sterilized bovine albumin solution (see above). To avoid later contamination this mixture should be split in portions, and kept in separate containers for a maximum of seven days at 4 ± 2°C.

The final concentration of bovine albumin and sheep erythrocytes in the test procedure shall be 3 g/L and 3 mL/L respectively.

Clean and dirty conditions for the modified method for ready-to-use products

Follow in general the procedures for preparation, but prepare the interfering substance in fivefold higher concentrations:

- (a) Clean conditions: dissolve 1.50 g bovine albumin (instead of 0.3 g) in 100 mL of diluent.
- (b) Dirty conditions: dissolve 15.0 g bovine albumin (instead of 3.0 g) in 85 mL of diluent (instead of 97 mL).

Prepare at least 40 mL (instead of 8.0 mL) sheep blood. Resuspend 15 mL (instead of 3.0 mL) of the packed sheep erythrocytes in 85 mL of sterilized bovine albumin solution (see above).

Sterile defibrinated sheep blood

The sterile defibrinated sheep blood can be acquired from a commercial supplier or prepared according to EN 14820.

Working culture of test organism

Use spore suspension prepared as previously described.

Validation of spore stock suspension

Check the viability and the susceptibility of each spore batch after at least two weeks' storage at $4 \pm 2^\circ\text{C}$. Each test should include 500 ppm av. Cl_2 at 20°C as a validation control.

With *Clostridium difficile* NCTC 11209 (under clean conditions only):

500 ppm av. Cl_2 solution should achieve a \log_{10} reduction of <1 in 1 min;

500 ppm av. Cl_2 solution should achieve a \log_{10} reduction of ≥ 5 in 60 min.

After validation, store the spore stock suspension at $4 \pm 2^\circ\text{C}$ and use within one year.

Test suspension ('N')

Adjust the number of spores in the suspension to 1.5×10^7 to 5×10^7 cfu/mL using distilled water. Assay the solution by a validated method [see 'Assessing spore concentration (cfu/mL)' section above]

Validation suspension ('N_v')

To prepare the validation suspension (N_v), dilute the test suspension with diluent to obtain 3.0×10^2 cfu/mL to 1.6×10^3 cfu/mL [about one-fourth ($1 + 3$) of the 10^{-3} dilution]. Mix.

Note: In the case of the modified method, the procedure is the same resulting in 3.0×10^3 cfu/mL to 1.6×10^4 cfu/mL.

Assay the suspension as previously described.

Maintain and use the validation suspension (N_v) in the same way as the test suspension.

Incubation and counting of the test and the validation suspensions

(a) Incubate the plates anaerobically for 72–120 h. Discard any plates that are not countable for any reason.

(b) Note: For each plate the exact number of colonies and determine the V_c values (see below).

(c) Calculate the numbers of cfu/mL in the test suspension 'N' and in the validation suspension 'N_v' using the methods given below.

Product test solutions

As it is diluted to 80% during the test and the method validation, the concentration of a product test solution shall be 1.25 times the desired test concentration.

Product test solutions shall be prepared in hard water. The product as received may be used as the test solution; in this case the highest tested concentration is 80%. If ready-to-use

products, i.e. products that are not diluted when applied, do not demonstrate the required reduction, the modified test procedure may be used in order to test a higher product concentration (97%).

Dilutions of ready-to-use products shall be prepared in water instead of hard water. The modified method cannot be used for handwash products, since 62.5% represents the highest accepted concentration (50%), multiplied by 1.25.

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogeneous preparation, stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculant (for example, through the addition of the interfering substance), it shall be recorded in the test report.

Note: Counting micro-organisms embedded in a precipitate or flocculant is difficult and unreliable.

Procedure for assessing the sporicidal activity of the product

General

Experimental conditions (obligatory and additional). Besides the obligatory temperature, contact time, interfering substance, and test organisms, additional experimental conditions (including test organisms) may be selected according to the practical use considered for the product:

(a) Temperature ($^\circ\text{C}$):

The obligatory temperature to be tested is 20°C .

The allowed deviation for each chosen temperature is $\pm 2^\circ\text{C}$.

(b) Contact time, t (in min):

The obligatory contact time to be tested is 1 min for a hand rub/wash, 5 min for a surface disinfectant, and 5 min for an instrument disinfectant.

The allowed deviation for each chosen contact time is ± 10 s, except for ≤ 1 min where it is 5 s.

(c) Interfering substances:

The obligatory interfering substance to be tested is 0.30 g/L bovine albumin under clean conditions, or a mixture of 3 mL/L sheep erythrocytes and 3.0 g/L bovine albumin under dirty conditions, according to practical applications. Additional interfering substances may be tested according to specific fields of application.

(d) Test organism:

Clostridium difficile, NCTC 11209

Additional spore-forming test organisms may be tested but details on the validation of the spore suspension must be provided.

Choice of test method

The method of choice is the dilution–neutralization method. To determine a suitable neutralizer, carry out the validation of the dilution–neutralization method, using a

neutralizer chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer.

Note: In special circumstances, it may be necessary to use a 1-in-100 dilution, or a filtration method for neutralization and recovery.

General instructions for validation and control procedures

The neutralization and/or removal of the sporicidal and/or sporstatic activity of the product shall be controlled and validated – only for the highest product test concentration – for each test organism and for each experimental condition (interfering substance and temperature). These procedures (experimental condition control, neutralizer control, and method validation) shall be performed with the same neutralizer used in the test.

Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions, test suspension, validation suspension, diluent, hard water, and interfering substance) to the test temperature.

Dilution–neutralization method

General. The test and the control and validation procedures should be carried out at the same time.

Test N_a : determination of sporicidal efficacy. The procedure for determining sporicidal efficacy is as follows:

(a) Pipette 1.0 mL of the interfering substance into a tube. Add 1.0 mL of the test suspension, N . Start the stopwatch immediately, mix, and allow the tube to equilibrate at the chosen test temperature for $2 \text{ min} \pm 10 \text{ s}$.

At the end of this time, add 8.0 mL of the product test solution. Restart the stopwatch at the beginning of the addition. Mix, and place the tube at the required temperature for the chosen contact time t . Just before the end of t , mix.

(b) At the end of t , take a 1.0 mL sample of the test mixture N_a and transfer into a tube containing 8.0 mL neutralizer and 1.0 mL water. Mix and place at the required temperature. After a neutralization time of $5 \text{ min} \pm 10 \text{ s}$, mix and immediately take a sample of 1.0 mL of the neutralized test mixture N_a (containing neutralizer, product test solution, interfering substance and test suspension) in duplicate and inoculate using a surface plate technique.

Additionally transfer 1 mL of this mixture into a tube containing 9 mL of diluent to obtain 10^{-1} dilution of N_a , repeat this procedure to obtain 10^{-2} and 10^{-3} dilutions of N_a . Take samples of 1.0 mL from each dilution tube in duplicate and inoculate using the surface plating technique.

(c) Perform procedures (a) and (b), using any other product test solutions at the same time.

(d) Perform procedures (a) to (c), applying any additional experimental conditions.

Experimental conditions control A: validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions. To validate the selected experimental conditions and/or to verify the absence of any lethal effect in the test conditions, the procedure is as follows:

(a) Pipette 1.0 mL of the interfering substance used in the test into a tube. Add 1.0 mL of the validation suspension, N_v . Start the stopwatch immediately, mix, and allow the tube to equilibrate at the chosen test temperature for $2 \text{ min} \pm 10 \text{ s}$.

At the end of this time, add 8.0 mL of hard water [In the case of ready-to-use products (except handwash products), distilled water is used instead of hard water.] Restart the stopwatch at the beginning of the addition. Mix and place the tube at the required temperature for t . Just before the end of t , mix again.

(b) At the end of t , take a sample of 1.0 mL of this mixture A in duplicate and inoculate using the spread plate technique.

Neutralizer control B: verification of the absence of toxicity of the neutralizer

To verify the absence of toxicity of the neutralizer, the procedure is as follows:

(a) Pipette 9.0 mL of the neutralizer used in the test into a tube. Add 1.0 mL of the validation suspension (N_v). Start the stopwatch at the beginning of the addition, then mix. Transfer 1.0 mL of this mixture into a tube containing 8.0 mL of neutralizer and 1.0 mL of distilled water. Mix and place the tube at the required temperature for $5 \text{ min} \pm 10 \text{ s}$. Just before the end of this time, mix.

(b) At the end of this time, take a sample of 1.0 mL of this mixture B in duplicate and inoculate using the surface plating technique.

Method validation C: dilution–neutralization validation. To validate the dilution neutralization method, the procedure is as follows:

(a) Pipette 1.0 mL of the interfering substance used in the test into a tube. Add 1.0 mL of diluent and then, starting a stopwatch, add 8.0 mL of the product test solution only of the highest concentration used in the test. Mix and place the tube at the required temperature for t . Just before the end of t , mix again.

(b) At the end of t , transfer 1.0 mL of the mixture into a tube containing 8.0 mL of neutralizer.

Restart the stopwatch at the beginning of the addition. Mix and place the tube at the required temperature for $5 \text{ min} \pm 10 \text{ s}$ (for hand-rub and handwash products only $10 \pm 1 \text{ s}$). Add 1.0 mL of the validation suspension. Start a stopwatch at the beginning of the addition and mix. Place the tube at the required temperature for $30 \text{ min} \pm 1 \text{ min}$. Just before the end of this time, mix again. At the end of this time, take a sample of 1.0 mL of the mixture C in duplicate and inoculate using the surface plating technique.

Incubation and counting of the test mixture and the control and validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows:

- (a) Incubate the plates anaerobically for 72–120 h. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu.
- (b) Note for each plate the exact number of colonies and determine the V_c values (see below).
- (c) Calculate the numbers of cfu/mL in the test mixture N_a and in the validation mixtures A, B, and C using the method given below.

Modified dilution–neutralization method

In the following, only the modifications are described.

Test N_a .

- (a) Pipette 0.2 mL of the five-fold concentrated interfering substance into a tube. Add 0.1 mL of the 10-fold concentrated test suspension. Start the stopwatch immediately, mix, and place the tube at the chosen test temperature for $2 \text{ min} \pm 10 \text{ s}$.
- (b) At the end of this time, add 9.7 mL of the undiluted product test solution.
- (c) Restart the stopwatch at the beginning of the addition. Mix and place the tube at the required temperature for the chosen contact time t . Just before the end of t , mix again.
- (d) Follow the subsequent instructions for the unmodified method.

Experimental conditions control A: validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions.

- (a) Pipette 0.2 mL of the interfering substance used in the test into a tube. Add 0.1 mL of the 10-fold-concentrated validation suspension as described for this modified method. Start the stopwatch immediately, mix, and place the tube at the required temperature for $2 \text{ min} \pm 10 \text{ s}$.
- (b) At the end of this time, add 9.7 mL of water. Restart the stopwatch at the beginning of the addition.
- (c) Follow the subsequent instructions for the unmodified method.

Neutralizer control B: verification of the absence of toxicity of the neutralizer. Follow the procedure as described in the unmodified method.

Method validation C: dilution–neutralization validation.

- (a) Pipette 0.2 mL of the interfering substance used in the test into a tube. Add 0.1 mL of diluent. Then, starting a

stopwatch, add 9.7 mL of the product test solution. Mix and place the tube at the required temperature for t . Just before the end of t , mix again.

- (b) At the end of t , transfer 1.1 mL of the mixture into a tube containing 8.8 mL of neutralizer.

Restart the stopwatch at the beginning of the addition. Mix and place the tube at the required temperature for $5 \text{ min} \pm 10 \text{ s}$ (for hand-rub and handwash products only for $10 \pm 1 \text{ s}$). Add 0.1 mL of the 10-fold-concentrated validation suspension as described for this modified method. Start a stopwatch at the beginning of the addition and mix. Place the tube at the required temperature for $30 \text{ min} \pm 1 \text{ min}$. Just before the end of this time, mix again.

At the end of this time, take a sample of 1.0 mL of the mixture C in duplicate and inoculate using the spread plate technique.

Calculation

General

The first step in the calculation is the determination of the V_c values, the second the calculation of N , N_0 , N_a , N_v , N_{v0} , A, B, and C. The third step is the calculation of the reduction R.

Determination of V_c values

- (a) Determine the number of cfu on each plate, using only those plates where colonies are well separated.
- (b) For counting the test suspension N , the validation suspension N_v , and for all counts of the dilution–neutralization method, determine and record the V_c values according to the number of plates used per 1 mL sample.

Note: If more than one plate per 1 mL sample has been used to determine the V_c value, the counts per plate should be noted.

Calculation of N and N_0

N is the number of cells per mL in the test suspension. If two dilutions of the test suspension are evaluated, calculate the number of cfu/mL as the weighted mean count:

$$N = \frac{c}{(n_1 + n_2/10)10^{-6}}$$

where c is the sum of V_c values taken into account, n_1 is the number of V_c values taken into account in the lower dilution, i.e. 10^{-6} , n_2 is the number of V_c values taken into account in the higher dilution, i.e. 10^{-7} , and 10^{-6} is the dilution factor corresponding to the lower dilution.

Note: For the modified method, N is 10-fold higher and therefore the dilutions to be evaluated are 10-fold higher. The formula above has to be changed accordingly. N_0 is the number of cells per mL in the test mixture at time 'zero'. It is one-tenth (one-hundredth in the modified method) of the weighted mean of N due to the dilution by the addition of the product and interfering substance.

Calculation of N_a

N_a is the number of survivors per mL in the test mixture at the end of the contact time and before neutralization. It is

10-fold higher than the V_c values due to the addition of neutralizer and water.

Calculate the mean for each dilution step N_a^0 , N_a^{-1} , etc., using the following formula:

$$N_a = 10 \frac{c}{n}$$

where c is the sum of V_c values taken into account, and n is the number of V_c values taken into account.

Calculation of N_v and N_{v0}

N_v is the number of cells per mL in the validation suspension. It is 10-fold higher than the counts in terms of V_c values due to the dilution step. N_{v0} is the number of cells per mL in the mixtures A , B , and C at the beginning of the contact time (time zero). Calculate N_v and N_{v0} using the following formulae:

$$N_v = 10 \frac{c}{n}$$

$$N_{v0} = c/n$$

where c is the sum of the V_c values taken into account and n is the number of V_c values taken into account.

Calculation of A , B , and C

A , B , and C are the numbers of survivors in the experimental conditions control A , neutralizer control B , and method validation C . They correspond to mean of the V_c values of the mixtures A , B , and C . Calculate A , B , and C using the following formula:

$$A, B, C = c/n$$

where c is the sum of V_c values taken into account and n is the number of V_c values taken into account.

Expression of results

The reduction R is the logarithmic ratio of N_0 to N_a . For each product concentration and experimental condition, calculate the decimal \log_{10} reduction using the formula:

$$R = \log N_0 - \log N_a$$

Interpretation of results: conclusion

General

According to the chosen experimental conditions (obligatory or obligatory and additional) the sporicidal concentrations determined according to this standard may differ. A product can only pass the test if A , B , and C are $\geq 0.5 \times N_{v0}$.

Sporicidal activity for surface disinfection products

The product shall be deemed to have passed this method if it demonstrates in a valid test for surface disinfection products a reduction of $\geq 5 \log_{10}$ within 5 min for products used on surfaces.

The test will be considered valid if the validation test using 500 ppm av. Cl_2 solution under clean conditions only demonstrates:

<1 \log_{10} in 1 min.

$\geq 5 \log_{10}$ reduction in 60 min.

If these criteria are not met, the test is not valid.

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