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Impact of standard test protocols on sporicidal efficacy

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SUMMARY

Background: There has been an increase in the availability of commercial sporicidal formulations. Any comparison of sporicidal data from the literature is hampered by the number of different standard tests available and the use of diverse test conditions including bacterial strains and endospore preparation.

Aim: To evaluate the effect of sporicidal standard tests on the apparent activity of eight biocides against *Clostridium difficile* and *Bacillus subtilis*.

Methods: The activity of eight biocidal formulations including two oxidizing agents, two aldehydes, three didecyldimethylammonium chloride (DDAC) and amine formulations, and sodium hypochlorite were evaluated using four standard sporicidal tests (BS EN 14347, BS EN13704, ASTM E2197-11, and AOAC MB-15-03) against *B. subtilis* (ACTC 19659) and *C. difficile* (NCTC 11209) spores.

Findings: *C. difficile* spores were more susceptible to the sporicides than were *B. subtilis* spores, regardless of the method used. There were differences in sporicidal activity between methods at 5 min but not at 60 min exposure. DDAC and amine-based products were not sporicidal when neutralized appropriately. Neutralization validation was confirmed for these biocides using the reporting format described in the BS EN standard tests, although the raw data appear to indicate that neutralization failed.

Conclusion: The different methods, whether based on suspension or carrier tests, produced similar sporicidal inactivation data. This study suggests that detailed neutralization validation data should be reported to ensure that neutralization of active spores is effective. Failure to do so may lead to erroneous sporicidal claims.

Introduction

Bacterial endospores are far less susceptible to biocidal products than their vegetative counterparts.[1–3] Sporicide is the term used to define biocidal products that can destroy spores, although the term sporistatic has also been used.[1,2,4] The mechanisms leading to a sporistatic or sporicidal effect have recently been reviewed.[5] The structure of the endospores explains their resistance to biocidal products, notably the presence of spore coats, small acid-soluble proteins, a highly compressed spore membrane, and low water content.[3] To measure the efficacy of sporicides against specific bacterial endospores, several standard sporicidal tests are available.[4] In Europe, there are not yet specific test protocols to measure the efficacy of sporicides against *Clostridium difficile*, although in the UK recently Fraise and colleagues proposed a suspension test against this pathogen.[6] The use of different standard protocols against different spore formers and different bacterial strains make the comparison of sporicidal activity of

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Table I
Standard test protocols used in this study

Test method	Nature of test	Organic load: bovine serum albumin	Surface material
BS EN 14347[12]	Suspension test	N/A	N/A
BS EN 13704[13]	Suspension test	0.30%	N/A
ASTM E2197-11[11]	Hard surface test	0.30%	Stainless steel
AOAC MB-15-03[14]	Hard surface test	0.30%	Porcelain

N/A, not applicable.

biocidal products difficult.[7,8] Test parameters such as concentration of biocide, contact time, spore strain, concentration of spores, spore preparation and purification, and organic load often differs between studies. The neutralization of the biocide/biocidal products is also important to determine their sporicidal effect, but is not always effective, potentially leading to inappropriate product claims.[4,5,9] Empirically only a small number of biocides — principally oxidizing and alkylating agents — have been shown to be sporicidal.[1–3,7]

This study aimed to compare the activity of several biocides/biocidal products against *Bacillus subtilis* (the standard strain in EN tests) and *C. difficile* using various standard test protocols widely used in Europe and the USA.

Methods

Bacterial strains

Two spore-producing bacteria were used in all testing procedures: *Clostridium difficile* (NCTC 11209) and *Bacillus subtilis* (ACTC 19659). Both bacteria are relevant to standard disinfectant testing procedures. Vegetative bacterial cells for both strains were stored on protect beads (Fisher Scientific, Loughborough, UK) at −80°C (±1°C). Liquid spore stock cultures of *C. difficile* were cultured using the Clospore method.[10] This liquid medium was chosen as it enables the production of large concentrations of purified *C. difficile* spores.[6,10] *Bacillus subtilis* liquid spore cultures were prepared in accordance with the ASTM method E2197-11.[11] Spore suspensions were washed, resuspended in phosphate-buffered saline (Fisher Scientific) and stored at 4°C for one month before use. Regular enumeration and sterility checks were performed to ensure spore stock purity. Total spore count was measured using a haemocytometer. The percentage of germinating spores was estimated by comparing total count and viable spore count (after germination) for each bacterium. The percentage of germinating spores was 88.06% for *C. difficile* and 83.49% for *B. subtilis*. A viable count was performed prior to each test. The average counts of viable spore stock for *B. subtilis* and *C. difficile* were 7.02 ± 0.59 and 7.39 ± 019 $\log_{10}$ respectively.

Formulations, biocides, and neutralization

Eight formulated biocides were tested for their sporicidal activity at 5 and 60 min with four different standard test procedures (Table I). All disinfectants were supplied by Anios (Lille, France) except for sodium hypochlorite, which was purchased from Fisher Scientific. The disinfectants were as follows: glutaraldehyde (GTA; tested at 2% v/v; pH 6.0); *ortho*-phthalaldehyde (OPA; tested at 0.55 and 0.65% v/v; pH 7.0); didecyldimethylammonium chloride (DDAC; labelled 191501; tested at 1%; pH 6.0); bis(aminopropyl)laurylamine (labelled 191502; tested at 1% w/v; pH 11.5); a combination of DDAC (1% w/v) and bis(aminopropyl)laurylamine (1% w/v) (labelled 191503; pH 11.0); two oxidizers: ANIOXY-TWIN (tested at 1200 ppm) and ANIOSEPT ACTIV (tested at 2% v/v); sodium hypochlorite (NaOCl; tested at 5000 ppm; pH 7.8). ANIOSEPT ACTIV was made 2 h before use. All tests were performed at 20°C in clean conditions (Table I).

The aldehydes, oxidizers, and sodium hypochlorite were neutralized after 5 and 60 min contact time with a solution composed of 5 g/L sodium thiosulphate, 30 g/L Tween 80, 30 g/L saponin, 1 g/L L-histidine, and 3 g/L azolectin (Fisher Scientific). This universal neutralizer was initially used to quench the activity of DDAC and amines when the BS EN14347 protocol was used. Filtration neutralization according to BS EN 13704 was subsequently used for all test protocols.[12,13]

Neutralization toxicity and efficacy to quench each biocide were confirmed with the aldehyde, oxidizing agents, and sodium hypochlorite. The failure of chemical neutralization to quench the activity of DDAC and amines was further investigated whereby both chemical neutralization and neutralization by filtration were compared following exposure to biocides at three concentrations (0.5, 1, and 2% v/v).

Modification to standardized testing procedures

Four sporicidal test protocols were used in this study; the BS EN 14347, the BS EN 13704, the ASTM E2197–11, and the AOAC MB-15-03.[11–14] Due to the nature of this study standardized test methods were modified somewhat to ensure test consistency. We were interested in studying the effect of the test procedures themselves on sporicidal activity and not the effect of the spore preparation and viable count enumeration protocols. With this in mind, all testing procedures followed enumeration with the pour plating method in accordance with BS EN 14347.[12] Apart from the preparation of spore stock and enumeration of viable count following exposure, the test procedures described in the standard were strictly followed.

Reproducibility

Unless otherwise mentioned, tests were carried out in triplicate on three separate occasions. The data analysed were normally distributed (Shapiro–Wilk test; $P > 0.05$); hence *t*-test analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and post-hoc Tukey tests were conducted to analyse the results using SPSS® software where appropriate.

Results

For the purpose of this study, a biocide formulation was deemed to be sporicidal if it achieved >4 $\log_{10}$ reduction in spore number. We found that GTA was not sporicidal even after 60 min exposure (Figures 1 and 2). The other aldehyde, OPA, also failed to achieve 4 $\log_{10}$ reduction in *B. subtilis* spores even after 60 min contact (Figure 2b), but it was sporicidal against *C. difficile* spores after 60 min exposure (Figure 1b). The DDAC and amine formulations tested were not sporicidal when