



## Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores

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### Abstract

The aim of this study was to evaluate the efficacy of hydrogen peroxide vapour (HPV) against spores of *Clostridium botulinum*, for use as a method for decontaminating environments where this pathogen has been handled. Spores were dried onto stainless steel slides and exposed to HPV in a sealed glovebox enclosure, transferred to a quenching agent at timed intervals during the exposure period, before survivors were cultured and enumerated. *D*-values were calculated from graphs of log<sub>10</sub> survivors plotted against time and were found to range from 1.41 to 4.38 min. HPV was found to be effective at deactivating spores of toxigenic *Cl. botulinum*, non-toxigenic *Clostridium* spp. and *Geobacillus stearothermophilus* dried onto stainless steel surfaces. HPV could be used to decontaminate cabinets and rooms where *Cl. botulinum* has been handled. The cycle parameters should be based on studies carried out with relevant spores of this organism, rather than based on inactivation data for *G. stearothermophilus* spores, which have been used in the past as a standard biological challenge for disinfection and sterilisation procedures. HPV could provide an attractive alternative to other decontamination methods, as it was rapid, residue-free and did not give rise to the health and safety concerns associated with other gaseous decontamination systems.

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### 1. Introduction

*Clostridium botulinum* is an anaerobic, gram positive, spore-forming bacterium, which can produce an extremely potent botulinum neurotoxin (Santos-Buelga et al., 1998). The organism is classified into four groups (I to IV) based on metabolic and serological similarities (Gibbs, 2002). Within the four

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groups, it is classified into seven types (A to G), according to the antigenic structure of the toxin (Collins and East, 1998). Most cases of human botulism have been associated with types A, B, E and rarely F, which are all classed within groups I and II (Sofos et al., 1979; ICMSF, 1996). Group I (including types A, B and F) are all proteolytic, whilst Group II (including types B, E and F) are all non-proteolytic.

Three forms of human botulism have been identified. These are foodborne, wound, and infant botulism (Mims et al., 1993). The clinical disease is similar in all three forms of botulism and symptoms include blurred vision, difficulty in swallowing and speaking, muscle weakness, nausea and vomiting. Without adequate treatment, many of the patients may die within a few days of either respiratory or cardiac failure (Prescott et al., 1996). Whilst botulism is relatively rare, the severity of the disease, the high cost of treatment, and the high economic impact of outbreaks, ensures that the prevention of outbreaks remains a major aim of regulators and industry (Plowman and Peck, 2002). Safer antitoxins and vaccines are required, not only to control the naturally acquired forms of botulism, but also to meet the threat of the use of botulinum toxin as an agent of bioterrorism (Greenfield and Bronze, 2003).

In addition to research relating to the prevention and control of the disease caused by this organism, research into the use of clinically safe amounts of botulinum toxin being injected for beneficial purposes is ongoing. Benefits include reduction in pain, improved control of spasticity (Barnes, 2003) and use for cosmetic enhancements (Said et al., 2003).

With so many varied research programmes relating to this potentially fatal organism, there is a requirement for it to be handled in a microbiology containment laboratory, with specific codes of practice. *Cl. botulinum* is classified in the UK as a Hazard Group 2 pathogen (ACDP, 1990, 1995), therefore specified disinfection procedures need to be in place in the restricted area in which it is handled. Research facilities in the UK that work with this organism may choose as a local code of practice to handle it in a Containment Level (CL) 3 facility, where available. In the USA, a Biosafety Level (BSL) 3 laboratory is recommended when handling *Cl. botulinum* for activities with a high potential for

aerosol or droplet production, and those involving production quantities of toxin (US DHHS, 1999). BSL 3 and CL 3, laboratories are sealable to allow for gaseous decontamination.

Fumigation with formaldehyde has traditionally been used to decontaminate safety cabinets or rooms, but it is a hazardous chemical which is a human carcinogen (Cheney and Collins, 1995; IARC/WHO, 2004) and doubts remain over its biological efficacy (Everall et al., 1982). It can also leave white paraformaldehyde residues that need to be cleaned from equipment, walls, floors and ceilings. An alternative vapour-phase decontamination method, which has been used in more recent times, is hydrogen peroxide vapour (HPV). This breaks down to insignificant amounts of oxygen and water, hence there are effectively no residues and the system is environmentally friendly, as well as having the best safety profile of the gaseous decontamination methods available (McDonnell et al., 2002). HPV is effective against a wide range of vegetative bacteria and fungi (McDonnell et al., 2002; French et al., 2004), spores of *Bacillus* spp. (Kokubo et al., 1998) and exotic animal viruses (Heckert et al., 1997). However, no published data could be found regarding the efficacy of HPV against *Cl. botulinum*.

HPV is already used widely for the decontamination of laboratory and medical equipment, hospital wards, pharmaceutical manufacturing facilities and animal houses (Klapes and Vesley, 1990; Johnson et al., 1992; Jahnke and Lauth, 1997; McDonnell and Russell, 1999; Krause et al., 2001; French et al., 2004) and could potentially be used to decontaminate safety cabinets or rooms where *Cl. botulinum* has been handled. Here we report experiments to determine whether this currently available method would be effective against *Cl. botulinum* spores and could be applied to this new application.

## 2. Materials and methods

### 2.1. Organisms used

Spore crops (stored frozen at  $-85^{\circ}\text{C}$ ) of the following strains were used: Toxigenic proteolytic Type A *Cl. botulinum*, 62A (Colworth microbiology culture collection, CMCC, 3265) from National

Canners Association; Toxigenic non-proteolytic Type E *Cl. botulinum*, Beluga (CMCC 3379) isolated from Alaskan Muktuk; Non-toxigenic, non-proteolytic *Clostridium* spp., CMCC 3676 (Virginia Polytechnic Institute, VPI, code 2093-1), CMCC 3677 (VPI 14041) and CMCC 3678 (Centres for Disease Control and Prevention 4672U1) obtained from Professor Mike Peck (Institute of Food Research, Norwich, UK).

No toxin is produced by strains 3676, 3677 and 3678, although based on the genetic characteristics and phenotype, strain 3676 closely resembles type B, E, F non-proteolytic *Cl. botulinum* toxigenic strains (Campbell et al., 1993). These three strains will be referred to in this paper as *Clostridium* spp.

## 2.2. Spore crop production

The toxigenic, proteolytic spores, 62A, were prepared according to the protocol of Gaze and Brown (1998) and spores of the toxigenic non-proteolytic strain, Beluga, were prepared using the method described by Peck et al. (1992).

Several protocols were evaluated for sporulation of the non-toxigenic strains, but the following method gave the highest yield. Spores were produced in a two-phase medium. The lower solid phase contained Robertson's cooked meat medium (Oxoid, Basingstoke, UK) with double the normal quantity of meat, giving  $150 \text{ g l}^{-1}$ ; agar (Oxoid) at a concentration of  $45 \text{ g l}^{-1}$ ; glucose (Sigma) at  $3 \text{ g l}^{-1}$ . The lower phase was dispensed in 300-ml volumes into 500-ml Duran bottles and tilted to make an agar slant. The upper liquid phase was 60 ml of de-oxygenated, sterile distilled water. The medium was inoculated with 10 ml of actively growing culture, which had been synchronised at  $37 \text{ }^\circ\text{C}$  in botulinum enrichment medium, BEM (Hobbs et al., 1982). For each strain, after 4–7 days incubation at  $30 \text{ }^\circ\text{C}$ , spores from the liquid phase of seven bottles were harvested, pasteurised in 5-ml volumes, then washed five times in ice-cold water by centrifugation at  $5 \text{ }^\circ\text{C}$ ,  $15,000 \times g$  for 10 min (Hettich Rotina centrifuge 48R, Tutlingen, Germany). After the final wash, spores were resuspended in 0.85% saline solution.

All incubation stages were carried out in an anaerobic environment using Genboxes with anaerobic generators (Biomerieux, Basingstoke, UK). All

de-gassing was carried out in Genbags with anaerobic generators (Biomerieux), following steaming of the pre-autoclaved, sterile media.

## 2.3. Counts of clostridia spore crops following drying on stainless steel slides

Spore crops were vortexed for 5 min prior to use (to disrupt any clumps present). A small aliquot (10  $\mu\text{l}$ ) of each spore suspension was spread onto the upper surface of sterile square stainless steel slides of  $12 \times 12 \text{ mm}$ , and left to dry (for approximately 90 min). Once dry, each slide was placed into 5 ml of catalase quench [100  $\mu\text{l}$  of 1% filter sterilised catalase (Sigma C-9322) per 10 ml of 0.1% peptone diluent (Biomerieux)]. After 0 and 4 h in the quenching agent, slides were vortexed for 2 min, to release the spores into suspension for counting (the 0-h samples were placed into quenching agent and vortexed immediately). Suspensions were serially diluted in 9 ml of 0.1% peptone diluent and plated onto Anaerobic Blood Agar, ABA (Biomerieux) using the 0.1-ml spread plate technique. Plates were incubated anaerobically at  $30 \text{ }^\circ\text{C}$  and counted after 72 h.

## 2.4. Assessing the inactivation of clostridia spores on stainless steel by HPV

Spores were inoculated onto stainless steel slides and dried, as described above. Slides were placed in an open petri-dish (inoculated surface facing upwards) on the base of a  $0.4\text{-m}^3$  glovebox enclosure, next to plastic universal tubes containing 5 ml of catalase quench (used to ensure that any residual hydrogen peroxide, absorbed into the suspension during the transfer of slides, did not continue to act on the spores during the decontamination cycle). The glovebox was sealed, with the HPV generator (Clarus™ L, BIO-QUELL (UK), Hampshire) connected to one of the glove ports, whilst manipulations were carried out by working through the glove attached to the other port. An external aeration unit, to accelerate the catalytic decomposition of HPV after the cycle, was also connected to the glovebox via a transfer hatch. Slides were placed into tubes of quench using forceps at timed intervals during the decontamination cycle. A control tube containing an inoculated slide in quench was left uncovered in the enclosure to verify the

Table 1  
Counts of clostridia spore crops following drying on stainless steel slides (calculated as weighted means for duplicate slides)

Strain	Log <sub>10</sub> count per slide after 0 h in quench	Log <sub>10</sub> count per slide after 4 h in quench	Log <sub>10</sub> difference
62A <i>Cl. Botulinum</i> (toxigenic, proteolytic)	6.26	6.18	-0.08
Beluga <i>Cl. Botulinum</i> (toxigenic, non-proteolytic)	7.38	7.49	+0.11
3676 <i>Clostridium</i> sp. (non-toxigenic, non-proteolytic)	6.00	5.98	-0.02
3677 <i>Clostridium</i> sp. (non-toxigenic, non-proteolytic)	6.15	6.11	-0.04
3678 <i>Clostridium</i> sp. (non-toxigenic, non-proteolytic)	6.36	5.28	-1.08

activity of the catalase quench. During the decontamination cycle, HPV concentrations were monitored by an internal sensor in the Clarus™ L. The ambient temperature in the laboratory was approximately 23 °C (internal cabinet temperature was not measured in these experiments, but in a typical cabinet HPV cycle, the temperature rises by approximately 5 °C).

Following the decontamination cycle, an aeration cycle was run and the enclosure was only opened once the HPV levels were <1 ppm. This was measured by the internal sensor in the Clarus™ L and verified by a hand-held HPV sensor (Portasens II, ATI, Ashton-

under-Lyne, UK). The glove was then detached from the cabinet and samples were taken out through the open glove port. Each slide was vortexed for 2 min, to release the surviving spores into suspension. Suspensions were serially diluted and plated onto ABA as described above.

### 2.5. Use of *Geobacillus stearothermophilus* (formerly known as *Bacillus stearothermophilus*) as biological indicators

*G. stearothermophilus* spores, dried onto stainless steel discs of approximately 10 mm diameter, were supplied (by Apex Laboratories, USA) sealed in Tyvek pouches, each giving a 6-log<sub>10</sub> inoculum. At various contact times during the sterilisation cycle, these were placed into 10-ml volumes of Tryptone Soya Broth, TSB (Biomérieux) with added catalase (approximately 100 µl of 1% filter sterilised catalase to each 10 ml of TSB). The samples were examined for turbidity over 7-day incubation at 55 °C.

## 3. Results

### 3.1. Counts of clostridia spore crops following drying on stainless steel slides

Table 1 shows the log<sub>10</sub> counts of the spore crops after drying on stainless steel slides. It can be seen that in four of the five strains there was <0.25 log<sub>10</sub> difference between those which had been left in

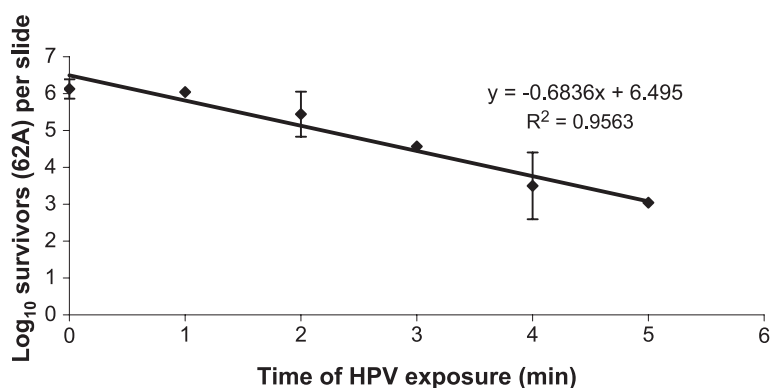


Fig. 1. Inactivation of *Cl. botulinum* 62A spores using HPV (error bars: time 0 shows the S.D. for four slides and times 2 and 4 show the S.D. of three slides, over two separate experiments).

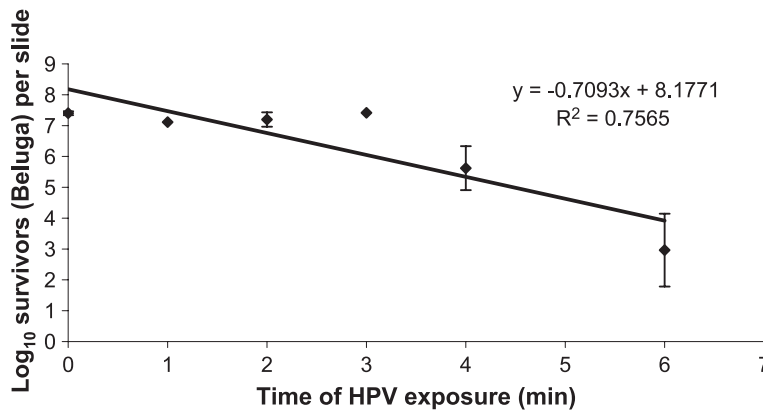


Fig. 2. Inactivation of *Cl. botulinum* Beluga spores using HPV (error bars: time 0 shows the S.D. for four slides and times 2, 4 and 6 show the S.D. of three slides, over two separate experiments).

quenching agent for 4 h prior to enumeration, compared to those which were enumerated immediately after placing in the quenching agent. The apparent increase in count over 4 h for the Beluga strain is very small (0.11 log<sub>10</sub> increase) which is likely to be due to plating error. The European Standard (Anon, 2003) for enumeration of microorganisms using a colony count technique (obtained using the same method, operator, laboratory and equipment within a short period of time) states that the difference in replicates of microbial count should not be greater than 0.25 log<sub>10</sub> microorganisms ml<sup>-1</sup>.

### 3.2. Inactivation of the clostridia spores on stainless steel slides by HPV

Figs. 1 and 2 show inactivation graphs of the toxigenic *Cl. botulinum* proteolytic and non-proteo-

lytic strains, respectively. No viable microorganisms were recovered after 6- and 7-min exposure for strains 62A and Beluga, respectively (the limit of the test is <50 cfu per steel slide). Data points without error bars show counts of survivors from single slides. Replicates were conducted at times 0, 2, 4 and 6 min, for the toxigenic strains and the error bars represent the standard deviations. Fig. 3 shows inactivation graphs for the three non-toxicogenic, non-proteolytic clostridia strains. The *D*-value (time in min for a 1 log<sub>10</sub> reduction) for each strain has been calculated from each graph. These range from 1.41 to 4.38 min and are shown in Table 2. Comparative *D*-values should be interpreted with caution, because of the different methods used to produce proteolytic compared to non-proteolytic strains, which could have affected relative resistance to HPV. The concentration of HPV, measured by the sensor at a single point in the

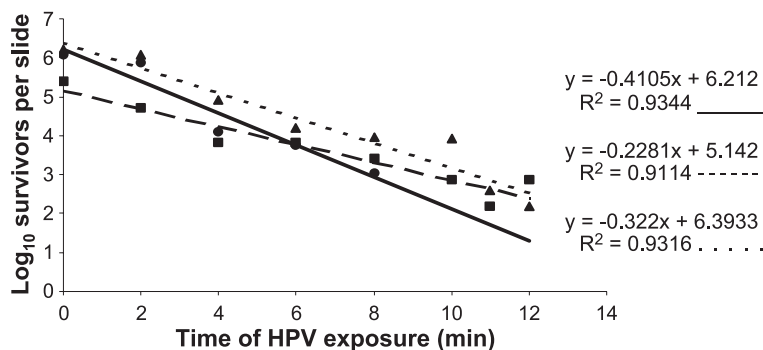


Fig. 3. Inactivation of clostridia spores, no replicates, (—●—), strain 3676; (---■---), strain 3677; (---▲---), strain 3678. Equations listed in this order to the right of the figure.

Table 2  
D-values for the disinfection of the five clostridia strains

Strain	D-value (min) at 355 ppm HPV
62A <i>Cl. Botulinum</i> (toxigenic, proteolytic)	1.46
Beluga <i>Cl. Botulinum</i> (toxigenic, non-proteolytic)	1.41
3676 <i>Clostridium</i> sp. (non-toxigenic, non-proteolytic)	2.44
3677 <i>Clostridium</i> sp. (non-toxigenic, non-proteolytic)	4.38
3678 <i>Clostridium</i> sp. (non-toxigenic, non-proteolytic)	3.11

enclosure, was found to be >100 ppm when read at 2 min into the gassing cycle, >300 ppm at 4 min and reaching an average of 355 ppm after 12 min.

### 3.3. Inactivation of *G. stearothermophilus* biological indicators by HPV

Table 3 shows that survivors of *G. stearothermophilus* could be detected after 4-min exposure to HPV, but that none were recovered after an exposure time of 6 min. In this study, spores of *G. stearothermophilus* were generally found to be more sensitive to HPV than spores of *Clostridium* spp.

## 4. Discussion

The results suggest that if clostridia spores of 62A, Beluga, 3676 or 3677 were left in the quenching agent for 4 h before plating, this would not have an adverse effect on the counts (shown in Table 1). This was tested because it was not known how quickly the HPV concentration would decrease during the aeration cycle to a level which was safe enough (<1 ppm) for the operator to remove the quenched samples from the enclosure. Table 1 shows that the counts of 3678 appeared to decrease by just over 1 log<sub>10</sub> following the 4 h in quench. In reality, samples were removed from the glovebox and plated out within 2 to 3 h of the end of the gassing cycle. Fig. 3 shows that in a subsequent experiment, 6.23 log<sub>10</sub> were recovered from the untreated slide of 3678 in quench (plated out within 3 h of the end of the gassing cycle). Thus, the time that the slides were left in quenching agent

before plating seems unlikely to have an adverse effect on the counts.

Johnston et al. (2002) discuss the importance of using an appropriate quenching agent during liquid disinfection studies. In each HPV experiment, a control tube (Time 0) containing an inoculated slide, submerged in quenching agent, was left uncovered in the enclosure during the gassing cycle. Spores of each strain were recovered from these quench controls with no reduction in numbers, so the catalase quench was effective at preventing the action of HPV on the submerged slides.

The concentration of HPV was measured at a single point in the enclosure. A slight lag on the inactivation graphs (which is most evident in Fig. 2) may be due to the time required to reach micro-condensation and hence optimum kill conditions within the chamber. It is difficult to correlate the HPV measured by the sensor, at a single point in the enclosure, with the concentration at the surface of the slides, and HPV concentration alone may not be the best measure of HPV biocidal action (Watling et al., 2002). For this reason, D-values are quoted at 355 ppm, the highest concentration reached (which will err on the side of safety when calculating cycle parameters). The D-value calculated from Fig. 2, for Beluga, should be interpreted with care, as the decrease in the number of survivors is not as linear ( $r^2=0.76$ ) as that calculated for the other strains (all have an  $r^2$  value >0.90). However, Beluga appears to be more sensitive to HPV (no survivors at, or after, 7 min) than strains 3677 or 3678 (>2 log<sub>10</sub> survivors at 12 min), so cycle parameters could be assessed using

Table 3  
Inactivation of *G. stearothermophilus* biological indicators by HPV

Time of HPV exposure	Growth after incubation in TSB+catalase
0	+
2	+
4	+
6	–
8	–
10	–
11	–
12	–
20	–
TSB-catalase sterility check	–

*D*-values of the more resistant non-proteolytic spores with more linear inactivation kinetics.

There is evidence that different methods of spore production (including sporulation temperature and sporulation medium) can influence the subsequent resistance of spores. This has been studied frequently for spores of *Bacillus* spp. and mainly for heat resistance (De Pieri and Ludlow, 1992; Palop et al., 1999a,b; Cazemier et al., 2001). In a review by Palop et al. (1999b), the authors state that spores of *Bacillus* spp. are usually more heat resistant when they are formed at higher temperatures. However, Peck et al. (1995) found that there was no significant difference in the heat resistance of non-proteolytic *Cl. botulinum* whether spores were formed at 25, 30 or 35 °C. Melly et al. (2002) studied the effect of sporicidal agents and found that spores of *Bacillus subtilis* prepared at higher temperatures were more resistant to liquid hydrogen peroxide, as well as Betadine, formaldehyde, glutaraldehyde and a superoxidised water, Sterilox. In heat resistance experiments, Mazas et al. (1995) found that the sporulation media affected *D*-values differently for each strain and Wright et al. (1995) showed that formaldehyde resistance was dependent on the sporulation media.

It is possible, therefore, that the methods of spore production used in this study affected the relative resistance of the *Cl. botulinum* strains tested to HPV. However, for the non-proteolytic strains (both toxigenic Beluga, and the non-toxigenic 3676, 3677 and 3678) the sporulation medium was Robertson's cooked meat medium (with double meat concentration) and the sporulation temperature was 30 °C. It seems reasonable that direct *D*-value comparisons can be made between these four non-proteolytic strains. The method used to produce spores of the proteolytic strain (62A) was different, as it used Anellis broth as the sporulation medium at a higher temperature of 37 °C. The method of spore production for the proteolytic strain was different to that used for the non-proteolytic strains, as the two groups have different requirements for optimum sporulation and spore yield. Therefore, it may be inappropriate to compare the *D*-value of 62A directly with those of the other strains. However, an important consideration is that when an area is to be decontaminated, the spore crops used to establish

decontamination parameters should be prepared by the same methods as the spores which are regularly handled in that area. In this case, taking the most resistant spore crop tested as an example (3677), a 12 log<sub>10</sub> reduction (laboratory/cabinet contamination is unlikely to be above this level, even if a concentrated spore crop had been spilled) would be predicted to occur in 53 min at approximately 355 ppm HPV assuming that the kill dynamics remain approximately linear. When decontaminating a 250-m<sup>3</sup> laboratory suite where *Cl. botulinum* had been handled, using hydrogen peroxide vapour (Clarus R™ BIOQUELL (UK)), it was for approximately 85 min with a greater injection rate and consequently increased total hydrogen peroxide delivery. Thus there was a large safety margin built into the room decontamination process.

Comparing the *D*-values of the non-proteolytic strains used in this study, the three non-toxigenic strains were all more resistant to HPV than the toxigenic one. As non-toxigenic strains are safer to work with, they would make ideal surrogates for future HPV testing. These non-toxigenic strains would predict longer *D*-values, hence over-estimating the treatment that would be required to inactivate spores of toxigenic *Cl. botulinum*. It would be valuable to conduct further work to investigate the efficacy of HPV for deactivation of the toxins produced by *Cl. botulinum*. Although a toxin's structure is vastly different to that of a spore, there is evidence that other sporicidal agents, such as solutions of sodium hypochlorite (0.1%) or sodium hydroxide (0.1 M) readily inactivate the toxins produced by *Cl. botulinum* (US DHHS, 1999).

According to McDonnell et al. (2002) bacterial spores, particularly *G. stearothermophilus*, have been shown to be the most resistant to HPV (more than viruses, vegetative bacteria, fungi, and mycobacteria). For this reason, spores of *G. stearothermophilus* have frequently been used to represent a "worst-case" biological challenge in HPV disinfection experiments. Results presented in this paper show that spores of *G. stearothermophilus* were generally found to be more sensitive to HPV than spores of *Clostridium* spp. However, as described above, sporulation methods may have influenced results. In addition, the commercially purchased *G. stearothermophilus* biological indicators may have used a different grade of metal to

the in-house prepared clostridia-inoculated-slides and variation in the carrier material is known to have an effect on the performance of biological indicators (Shintani and Akers, 2000). Spores of the *Clostridium* spp. were resuspended and stored in a weak saline solution (which could have had a protective effect on the spores dried onto the steel slides) whereas the *G. stearothermophilus* spores were purchased dried onto the discs from a sterile distilled water solution.

Methods also differed in that the surviving *Clostridium* spp. were enumerated following vortexing (which cannot guarantee that all viable cells are dislodged for counting), whilst the *G. stearothermophilus* biological indicators were transferred directly into broth and incubated for 7 days (theoretically allowing for resuscitation and subsequent growth of a single sub-lethally damaged spore, but not allowing enumeration). However, this methodology issue adds weight to the fact that the *Clostridium* spp. spores are more resistant than the *G. stearothermophilus* spores because it was possible to detect *Clostridium* spp. at contact times when the *G. stearothermophilus* had been completely destroyed, despite the fact that the broth method for *G. stearothermophilus* is theoretically more sensitive than the enumeration technique employed for the *Clostridium* spp. It would be useful, as a future study, to check the proportion of viable spores remaining attached to the steel slides after vortexing, to establish what effect these cells might have on the enumeration results. Further work would also be useful to determine the efficacy of HPV on different surfaces, such as porous ones.

The study shows that HPV is effective for the inactivation of *Cl. botulinum* spores, under the conditions tested, and could be applied for the decontamination of areas where this organism has been handled. The cycle parameters for HPV decontamination (e.g. volume of hydrogen peroxide, injection rate and contact time) should be based on studies carried out with relevant spores of this organism (prepared by relevant methodologies) rather than based on inactivation data for *G. stearothermophilus* spores. In summary, HPV offers a safe, clean, environmentally friendly and effective method for the decontamination of areas used to handle *Cl. botulinum* such as biological safety cabinets and microbiology containment laboratories.

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