



Evaluation of hydrogen peroxide gaseous disinfection systems to decontaminate viruses

T. Pottage, C. Richardson, S. Parks, J.T. Walker*, A.M. Bennett

Biosafety Group, Novel and Dangerous Pathogens, Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, UK

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Summary This study assessed the efficacy of two commonly used gaseous disinfection systems against high concentrations of a resistant viral surrogate in the presence and absence of soiling. MS2 bacteriophage suspensions were dried on to stainless steel carriers and exposed to hydrogen peroxide vapour (HPV) and vapour hydrogen peroxide (VHP) gaseous disinfection systems. The bacteriophages were also suspended and dried in 10% and 50% of horse blood to simulate the virus being present in a spill of blood/bodily fluids in a hospital ward environment. Carriers were removed from the gaseous disinfectant at regular intervals into phosphate-buffered saline, vortexed and assayed using a standard plaque assay. The effectiveness of both the HPV and VHP systems varied with the concentration of the bacteriophage with HPV resulting in a 6 log₁₀ reduction in 10 min at the lowest viral concentration [10⁷ plaque-forming units (pfu)/carrier] and requiring 45 min at the highest concentration (10⁹ pfu/carrier). For the VHP system a 30 min exposure period was required to achieve a 6 log₁₀ reduction at the lowest concentration and 60–90 min for the highest concentration. The addition of blood to the suspension greatly reduced the effectiveness of both disinfectants. This study demonstrates that the effectiveness of gaseous disinfectants against bacteriophage is a function of the viral concentration as well as the degree of soiling. It highlights the importance of effective cleaning prior to gaseous disinfection especially where high concentration agents are suspended in body fluids to ensure effective decontamination in hospitals.

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* Corresponding author. Address: Biosafety Group, Novel and Dangerous Pathogens, Health Protection Agency Centre for Emergency Preparedness and Response, Porton Down, Salisbury SP4 0JG, UK. Tel.: +44 (0)1980 612643; fax: +44 (0)1980 612622.

E-mail address: jimmy.walker@hpa.org.uk

Introduction

Systems for vaporising hydrogen peroxide have been used in the pharmaceutical clean rooms for disinfection, microbiology laboratories to disinfect both safety cabinets and entire laboratories (to replace formaldehyde fumigation) and are currently being introduced into UK hospitals.^{1–4} Studies have shown the efficacy of hydrogen peroxide systems against a range of bacteria and viruses within hospital settings.^{3–11} However, in most of those studies the micro-organisms have been exposed after drying from sterile distilled water which may not represent their presentation in hospital settings following contamination blood, vomit or diarrhoea containing high concentrations of micro-organisms.

Hydrogen peroxide, a potent oxidant, acts against bacteria and viruses by forming HO \cdot free radicals which react with thiol groups in proteins, lipids and nucleic acids.¹² In viruses these reactions prevent the correct functioning of proteins and nucleic acids, thus inhibiting the infection and replication process. Hydrogen peroxide reacts with organic material that reduces its efficacy in the presence of organics. There are two commercial vaporised hydrogen peroxide generating systems marketed to decontaminate microbiological laboratories. One, vapour hydrogen peroxide (VHP) (Steris, Basingstoke, UK), operates as a dry system reducing the relative humidity inside the enclosure so that condensation does not form on surfaces. The other, hydrogen peroxide vapour (HPV) (Bioquell, Andover, Hants, UK), injects the hydrogen peroxide vapour into the enclosure at a level so that micro-condensation forms on the internal surfaces. Therefore, control of the environment is imperative for both systems. The VHP system is designed to generate more hydrogen peroxide per unit area than HPV as hydrogen peroxide is more reactive and corrosive in the presence of water, and so the higher the relative humidity the lower the concentration that can be used without damage occurring to materials.¹³

In this study MS2 bacteriophage, a small simple virus surrogate consisting of protein and RNA with *Escherichia coli* as its host, was used at varying concentrations to study the effectiveness of HPV and VHP. MS2 bacteriophage has been used as a surrogate for norovirus in treatment of fresh food produce, as a surrogate for non-enveloped viruses in hand-washing studies and in water disinfection to assess UV/peracetic acid, aqueous ozone and ozone/hydrogen peroxide.^{14–18}

The study investigated the efficacy of gaseous disinfectants for the decontamination of high

concentration agents suspended in culture media, blood, vomit or diarrhoea that can dry to form a protective coat around the micro-organisms. The type of spill used in this study is intended to mimic the size of a droplet which may not be detectable to the eye but may present a challenge to gaseous disinfection.¹⁹ The objective of this study was to measure the effectiveness of the gaseous disinfection against small dried spills of MS2 bacteriophage to demonstrate whether the gaseous disinfection methods can be effective by themselves or whether a pre-disinfection surface cleaning step is still required.

Methods

Micro-organisms

E. coli NCIMB 9481 host strain was inoculated onto Trypase Soya Broth agar (TSBA, bioMérieux, Marcy l'Etoile, France) plates and incubated at $37 \pm 2^\circ\text{C}$ (19–20 h). A 10 μL loop of the *E. coli* was used to inoculate 60 mL sterile Trypase Soya Broth (TSB), mixed thoroughly [(120 rpm) for 150 min] and incubated at $37 \pm 2^\circ\text{C}$. The MS2 suspension was then prepared by inoculating 4×10^{11} plaque-forming units (pfu) into 60 mL TSB and the suspension aerated by shaking at $37 \pm 2^\circ\text{C}$ (3 h) and centrifuged at 2000 g (2×20 min) to remove the cell debris, and the supernatant used neat, 1:10 and 1:100 dilution in sterile distilled water. Suspensions of bacteriophage in defibrinated 10% and 50% horse blood (TCS Biosciences, Claydon, Bucks, UK) to simulate a challenge soil, such as human blood or bodily fluids, were also prepared.

Preparation of coupons

Stainless steel coupons (1 cm in diameter, 316 grade, M-Tech Diagnostics, Warrington, Cheshire, UK) were cleaned using Decon 90 (Decon 90, Brighton, Sussex, UK), then autoclaved at 126°C for 11 min, followed by storage in a drying oven (50°C for 2 h). Aliquots (10 μL) of the selected suspension were pipetted on to the centre of the coupon, then dried (37°C for 1 h) before being stored at room temperature prior to use and used within 24 h of production. Bacteriophage recovery from coupons was measured by comparing the recovery of bacteriophage from the coupons after drying to the bacteriophage loading calculated from the bacteriophage suspension for all the drying formulations used.

Exposure chamber

A Class III safety cabinet (Health Protection Agency, Porton Down, UK) of internal diameter (0.87 m^3) was used as the exposure chamber.

Collection and assay of samples

At each time point, three biological indicator coupons were removed from the exposure plate using sterile forceps and were placed into separate 25 mL universals containing 5 mL of phosphate-buffered saline (PBS).

After the exposure period the Class III cabinet was vented, the universals were vortexed for 1 min (or until all microbial residue present on the coupon had been resuspended) and the samples assayed following serial dilution.

MS2 bacteriophage assay

A fresh TSBA plate was inoculated with *E. coli* NCIMB 9481 from a stock plate previously stored at $4 \pm 2^\circ\text{C}$. This plate was incubated at $37 \pm 2^\circ\text{C}$ for 19–20 h. The *E. coli* 9481 was subcultured by transferring a $10\text{ }\mu\text{L}$ loopful from the plate to 10 mL sterile nutrient broth in a glass universal bottle, which, after mixing, was incubated at $37 \pm 2^\circ\text{C}$ for 260 min. Meanwhile, stoppered bottles containing 3 mL volumes of soft bacteriophage agar (1 L consists of 4 g Agar, 25 g nutrient broth and 1 L of sterile water) were heated for at least 120 min at $90\text{--}100^\circ\text{C}$ and then stored at $60 \pm 2^\circ\text{C}$ until required. The bottles were then cooled to 45°C . The serial dilutions of the MS-2 suspension in PBS ($100\text{ }\mu\text{L}$) were added to the soft agar followed immediately by three drops of the *E. coli* 9481 suspension using a 50D ($20\text{ }\mu\text{L}$ per drop) Pasteur pipette. After mixing, the soft agar was poured immediately on TSBA plates (in duplicate) that were then incubated at $37 \pm 2^\circ\text{C}$ overnight. The clear plaques were counted and the total phage recovery was calculated.

Operation of the Bioquell Clarus L HPV generator

The Bioquell Clarus L HPV generator (Bioquell) was connected to the Class III cabinet using its hydrogen peroxide supply hose, return hose, pressure monitor tube and hydrogen peroxide sensor. Each fumigation experiment run with the HPV generator used 40 mL of 30% hydrogen peroxide (Sigma, Poole, Dorset, UK). A cycle had been created for the fumigation of the Class III cabinet by Bioquell

with a 10 min conditioning phase, a 60 min gas dwell and 90 min aeration phase. The experiment was timed from the beginning of the gassing phase.

Operation of the Steris VHP 1001 generator

The Steris VHP 1001 generator was connected to the Class III cabinet using the hydrogen peroxide supply and return hose. Each fumigation experiment operated with the VHP generator used hydrogen peroxide of 31% supplied in a cartridge (Amsco, USA). A cycle for use within the Class III cabinet was designed and validated by Steris' technicians, the parameters for the cycle were a 10 min dehumidification phase (20% RH), 4 min conditioning (VHP, 4 g/min), 90–180 min sterilisation (VHP, 3 g/min) and 90 min aeration with an air exchange rate of $32\text{--}34\text{ m}^3/\text{h}$. The experiment was timed from the conditioning stage.

Experiment to establish neutralising effect of PBS

Blank stainless steel coupons were exposed to an HPV fumigation, as described above for 60 min. At set time-points of the fumigation (30 and 60 min) the blank coupons (in triplicate) and control coupons (in triplicate) that were unexposed to HPV were placed into PBS (5 mL). After aeration of the cabinet the universals had $10\text{ }\mu\text{L}$ of the stock phage suspension pipetted into them and these samples were then assayed according to the method described previously.

Results

The recovery of MS2 bacteriophage from the stainless steel coupons varied from 21.7% (50% horse blood dried), 22.7% (10% horse blood dried), 48.5% and (undiluted dried), 81.3% (1:10 suspension dried) and 118.5% (1:100 suspension dried) after drying.

The HPV experiment was carried out with different starting concentration of MS2 demonstrating that the rapidity of inactivation was dependent on the initial carrier loading (Figure 1).

The lowest loading (3.3×10^7 pfu/carrier) was reduced to the detection limit within 10 min of the same time taken for the $6\log_{10}$ reduction, the medium loading (3.1×10^8 pfu/carrier) was reduced to below the detection limit within 40 min of the same time taken for the $6\log_{10}$ reduction. The highest loading (1.4×10^9 pfu/carrier) was reduced to below the detection limit

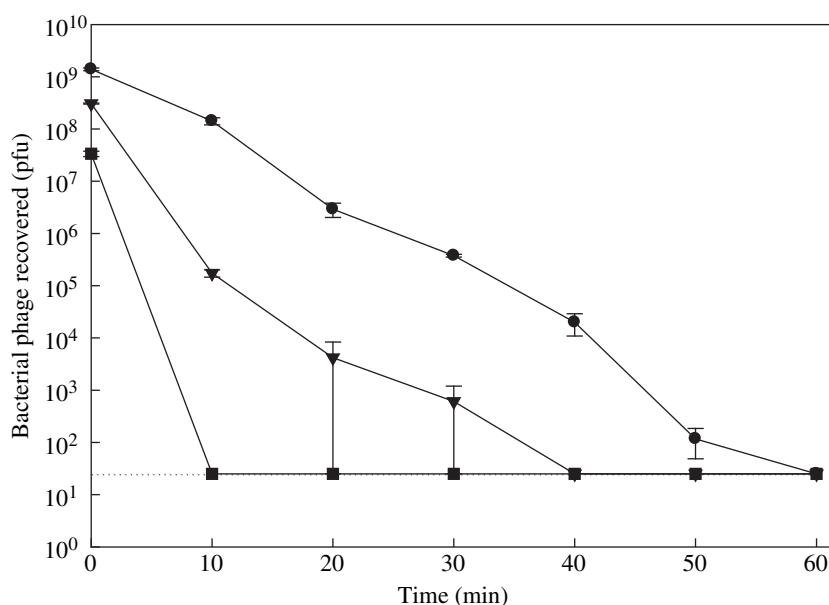


Figure 1 The effect of hydrogen peroxide vapour on the recovery of MS2 bacteriophage dried from suspensions of different titre [$\sim 10^7$ plaque-forming units (pfu)/carrier (squares), $\sim 10^8$ pfu/carrier (triangles), $\sim 10^9$ pfu/carrier (circles)].

within 60 min and took 50 min for a $7.1 \log_{10}$ reduction.

With VHP all the concentrations approximately 10^9 and 10^{10} pfu/carrier were not completely inactivated within the 90 min experiment (Figure 2). The highest concentration gave a $5.2 \log_{10}$ reduction after 60 min but did not achieve a $6 \log_{10}$ reduction within the time-course of the experiment. The 10^8 pfu/carrier suspension

gave a $6.4 \log_{10}$ reduction within 30 min whereas 60–90 min was required for a $6.0 \log_{10}$ reduction for the 10^9 pfu/carrier suspension.

The addition of soiling in the form of 10% and 50% horse blood to the bacteriophage suspensions resulted in a $5.8 \log_{10}$ reduction being obtained with 10% suspension and only a $2.7 \log_{10}$ reduction being achieved with 50% blood using the HPV system (Figure 3) over the 180 min exposure period.

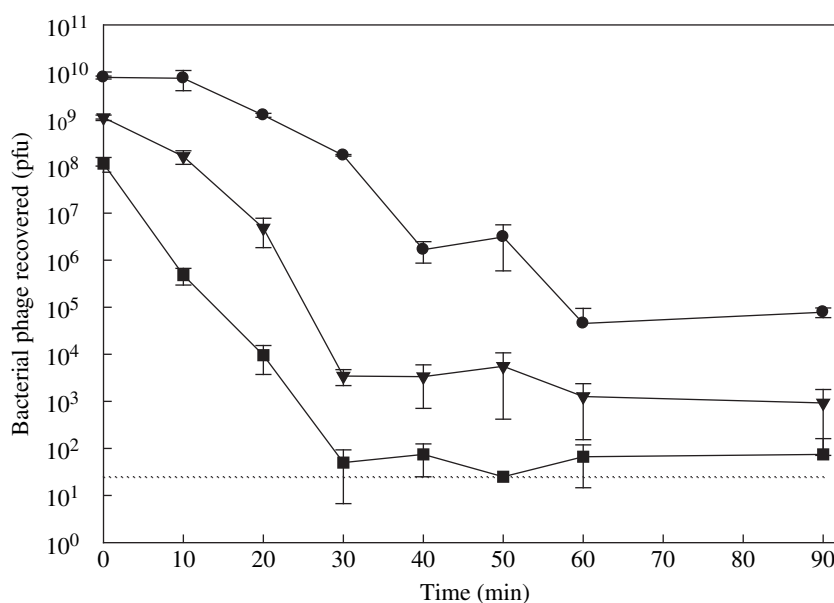


Figure 2 The effect of vaporised hydrogen peroxide on the recovery of MS2 bacteriophage dried from suspensions of different titre [$\sim 10^8$ plaque-forming units (pfu)/carrier (squares), $\sim 10^9$ pfu/carrier (triangles), $\sim 10^{10}$ pfu/carrier (circles)].

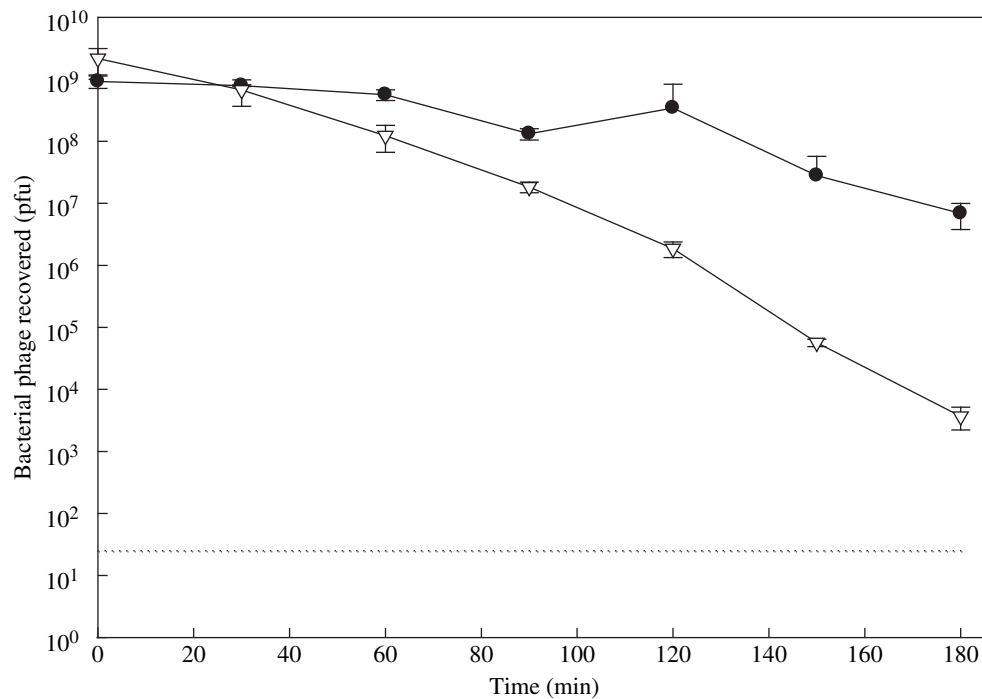


Figure 3 The effect of hydrogen peroxide vapour on the recovery of MS2 bacteriophage dried from 10% (triangles) and 50% (circles) horse blood.

With the VHP generator a 7.6 \log_{10} reduction was obtained within 120 min and reduction to the detection limit was found after 150 min with the 10% blood. With the 50% blood only a 4.2 \log_{10} reduction was obtained over the 180 min (Figure 4).

The results from the experiment to demonstrate the neutralising effect of the PBS showed that there was no significant difference in the recovery of phage from coupons which had and had not been exposed to hydrogen peroxide.

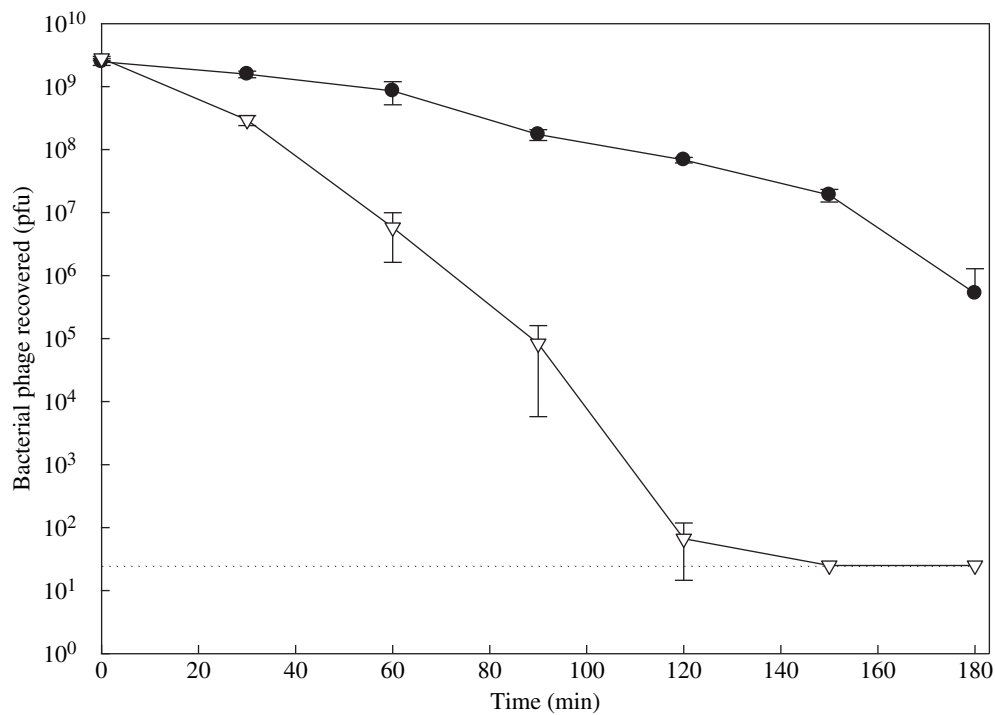


Figure 4 The effect of vaporised hydrogen peroxide on the recovery of MS2 bacteriophage dried from 10% (triangles) and 50% (circles) horse blood.

Discussion

Gaseous hydrogen peroxide has previously been used for the reduction of microbial contamination [*Serratia* sp., *Clostridium difficile*, meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and gentamicin-resistant Gram-negative rods (GNR)] in the hospital environment following cleaning.^{8,10,11} In this current study both the gaseous hydrogen peroxide disinfectants tested (HPV and VHP) were effective against MS2 bacteriophage; however, extended decontamination times were required where soiling was present. The results of gaseous disinfection studies are often expressed as time taken to give a 6 log₁₀ reduction or as decimal reduction time (D-values, 1 log₁₀ reduction). The results of this study demonstrate that the use of D-values must be interpreted with caution as few of the kill curves are linear or even sigmoidal in form. Many of the observed kill curves (especially Figure 2) in this study have an initial linear phase followed by a pronounced trailing phase. The use of a D-value taken from the 'linear' phase will greatly underestimate the time taken for complete inactivation of the dried organism. It has also been found that time taken to produce a 6 log₁₀ reduction of MS2 bacteriophage was dependent on the initial bacteriophage concentration especially in the case of HPV. These differences in efficacy can be explained in terms of the action of both HPV and VHP.

The effectiveness of the HPV system was dependent on the initial bacteriophage concentration (Figure 1) where the time taken for a 6 log₁₀ reduction varied between 10 and 60 min. The presence of organic blood matter resulted in a slower inactivation of the bacteriophage, which was exacerbated as the organic blood concentration increased (Figure 3). These results demonstrate that log₁₀ reductions can vary with microbial loading and must be interpreted with great care. HPV is a 'wet' system that relies on micro-condensation for its antimicrobial effects. Marcos-Martin *et al.* found that biological material acted as a preferred nucleation site for the condensation of vapour during a sterilisation process indicating that the sterilisation process using HPV should be more efficacious.²⁰ But the VHP system generates more hydrogen peroxide per unit area, meaning that any potential soiling and organic material will not affect the VHP system to the same extent as the HPV system. A slight condensation layer was observed during the disinfection process and this condensation layer of hydrogen peroxide suspension may be more rapidly effective against the lower levels of bacteriophage. Nevertheless, it completely inactivated all bacteriophage

suspensions within 60 min. With less condensation it may have taken the HPV longer to dissolve/penetrate the dried bacteriophage and to act on the different layers of bacteriophage. HPV was less effective than VHP against bacteriophage in dried blood and this may have been due to insufficient condensation being able to rehydrate the dried blood and also inactivation of hydrogen peroxide by catalase present in the blood. If HPV is used for gaseous disinfection where blood or any other heavy soiling may be present it is recommended that a pre-cleaning or liquid-disinfection step is included in the decontamination procedure. All the coupons were treated in exactly the same manner prior to the decontamination processes. The control coupons were measured in the same way as the exposed coupons.

The VHP system, which produces the higher concentration of H₂O₂, rapidly reduced the levels of the dried MS2 bacteriophage on the coupons but was unsuccessful at completely inactivating them within the 90 min. This may have been due to the production of a dry vapour (a dry vapour is achieved when the concentration of sterilant in the vapour is below the dew point, therefore condensation will not form on surfaces where the vapour is held) that did not rehydrate and penetrate the dried layers of bacteriophage. Unlike the HPV system the VHP has produced a similar log₁₀ reduction for each of the bacteriophage suspensions used and the inactivation times are similar. The VHP was more effective than the HPV at inactivating the bacteriophage in blood. The VHP generates more hydrogen peroxide than the HPV system (286 g against 40 mL of a 30% suspension = 29 g) and this may have overcome the catalase in the blood to produce a more rapid inactivation than for HPV.

The recovery of MS2 bacteriophage from the metal coupons varied between 21.7% and 118.5% of that expected from the assay. The highest concentrations were recovered from the lowest dilutions of the bacteriophage and the lowest concentrations were from the neat suspension and the suspensions containing blood. When the bacteriophage was dried from a high concentration suspension, it was more likely to form aggregations that did not completely disaggregate. Therefore these results may underestimate the presence of the dried bacteriophage as aggregates will be measured as one plaque-forming unit. The high levels of survival of the bacteriophage diluted in distilled water show the inherent resistance of this bacteriophage to the drying process.

It would be expected that 10⁶ bacterial suspension of spores would demonstrate greater

resistance to hydrogen peroxide than a higher concentration of MS2 bacteriophage. However, this study has demonstrated that when dealing with a high concentration of a biological agent in a laboratory the use of an appropriate biological indicator may be more suitable, i.e. pathogenic viruses will be more resistant if encased in a heavy organic soil such as blood.

When planning a gaseous disinfection it is important to consider the possible level of contamination present and the presence of a potentially protective soiling. There is recognition that surfaces should be cleaned prior to decontamination using gaseous hydrogen peroxide decontamination because biological soiling reduced the efficacy of the technology.²¹ A number of studies have demonstrated the usefulness and controllability of gaseous hydrogen peroxide for reducing microbial contamination of *Serratia* sp., *Clostridium difficile*, MRSA, VRE and GNR in the inanimate hospital environment following cleaning.^{8,10,11} Therefore pre-cleaning of surfaces with appropriate disinfectants prior to the deployment of gaseous disinfection is recommended. Gaseous disinfection with hydrogen peroxide systems alone should not be regarded as a combined cleaning/disinfection procedure.

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Conflict of interest statement

None declared.

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