Evaluation of the effectiveness of hydrogen-peroxide-based disinfectants on biofilms formed by Gram-negative pathogens

P.K. Perumal, M.E. Wand*, J.M. Sutton, L.J. Bock

*Corresponding author. Address: Technology Development Group, Public Health England, Microbiology Services Division, Porton Down, Salisbury SP4 0JG, UK. Tel.: +44 (0) 1980 619920; fax: +44 (0) 1980 612622.

E-mail address: Matthew.Wand@phe.gov.uk (M.E. Wand).
SUMMARY

**Background:** Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-based disinfectants are widely used in a number of different healthcare settings to control bacterial colonization and contamination, and reduce the risk of cross-infection. Efficacy tests of these formulations are performed on planktonic cultures, although it is well known that biofilms are the dominant form of bacterial contamination and more difficult to eradicate.

**Aim:** To determine if the biofilms of three different Gram-negative pathogens associated with multi-drug-resistant phenotypes can be eradicated effectively using different H\textsubscript{2}O\textsubscript{2}-based disinfectants.

**Methods:** Planktonic cultures and single-species 24-h biofilms of seven strains of *Acinetobacter* spp., seven strains of *Klebsiella pneumoniae* and seven strains of *Pseudomonas aeruginosa*, including clinical isolates, were exposed to working concentrations of H\textsubscript{2}O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}-based formulations for 1 min to 24 h. Survival was monitored.

**Findings:** The levels of susceptibility of planktonic cultures to unformulated and formulated H\textsubscript{2}O\textsubscript{2} were similar in all organisms and strains tested, with minimum inhibitory concentrations ranging from 0.5 to 20 mM H\textsubscript{2}O\textsubscript{2}. However, biofilms showed up to 266-fold less sensitivity to H\textsubscript{2}O\textsubscript{2} and its formulations. The level of reduced susceptibility correlated with the strain’s propensity to form biofilm, and differed between species. The two formulations with additional acidic active ingredients performed better at short exposure times, whereas ethanol-containing products required longer exposure times to be effective.

**Conclusion:** Biofilms of a significant number of clinical isolates of multi-drug-resistant nosocomial pathogens are not susceptible to working concentrations of several H\textsubscript{2}O\textsubscript{2}-based disinfectants. This may compromise the ability to control these pathogens with such products.
Keywords:

Hydrogen peroxide
Disinfectant
Multi-drug-resistant Gram-negative organism
*Pseudomonas* spp.
*Klebsiella* spp.
*Acinetobacter* spp.
Biofilm
Healthcare-associated infection
Introduction

The emergence and spread of multi-drug-resistant (MDR) pathogens is placing an enormous burden on healthcare systems, particularly as infections caused by these organisms are becoming essentially untreatable.\(^1\) There is, therefore, a much greater need for effective infection control strategies to limit the potential for build-up of these pathogens in healthcare environments, and to minimize the spread between patients and staff. The importance of such interventions has been highlighted recently by a series of documents addressing the challenges of antimicrobial resistance.\(^2\)\(^-\)\(^4\)

Hydrogen peroxide (H\(_2\)O\(_2\)) is being used increasingly for routine and outbreak disinfection and antisepsis, both as part of liquid formulations and in vapour form to decontaminate entire rooms.\(^5\) H\(_2\)O\(_2\) has been shown to be highly effective against bacteria, spores, viruses and fungi, and its breakdown products (i.e. water and oxygen) are non-toxic.\(^6\)\(^,\)\(^7\) Many formulations available in the UK contain additional ingredients such as silver, ethanol and acids (accelerated H\(_2\)O\(_2\)) that are known to increase the efficacy of H\(_2\)O\(_2\)-based formulations.\(^6\)\(^,\)\(^8\)

Healthcare-associated infections (HCAIs) are caused by many different organisms. Three of the most relevant Gram-negative organisms are \textit{Acinetobacter baumannii}, \textit{Pseudomonas aeruginosa} and \textit{Klebsiella pneumoniae}. Gram-negative organisms are of particular concern due to the increasing presence of inherent and acquired resistance mechanisms in isolates, and the lack of development of effective antibiotics. Sources of infection with these organisms stem from contamination of environmental surfaces, hands, oral flora and endoscopes.\(^9\)\(^,\)\(^10\) A further factor influencing the likelihood of infection with these organisms is their growth on surfaces as biofilms, in which cell communities form an extracellular matrix. Cells growing in biofilms are more virulent,\(^11\)\(^,\)\(^12\) and have increased tolerance to antibiotics and disinfectants due to the protection afforded by the extracellular
matrix, phenotypic changes within the cells and other mechanisms still to be described.\textsuperscript{13} Therefore, although biofilms are the most important growth form to combat, this is not accounted for in standard efficacy testing methods for disinfectants.\textsuperscript{14,15}

The aim of this study was to investigate the efficacy of $\text{H}_2\text{O}_2$-based disinfectants commonly used in clinical/National Health Service hospital settings against biofilms of clinically important nosocomial pathogens.

\textbf{Methods}

\textbf{Bacterial strains and culture conditions}

\textit{Acinetobacter} spp. and \textit{K. pneumoniae} strains used in this study have been described previously.\textsuperscript{11,16} The \textit{P. aeruginosa} strains used were described strains (PA01 and NCTC 13359),\textsuperscript{17} serially collected UK cystic fibrosis isolates (GH56, GH12, GH97 and GH100) and a UK neonatal outbreak strain (372261). These strains carry a variety of drug resistance mechanisms (TEM, NDM-1, \textit{aphA} and \textit{qnrS2}), and were chosen to provide information on inter- and intraspecies differences. All strains were grown in tryptic soy broth (TSB) with aeration or on tryptic soy agar (TSA) plates at 37 °C unless otherwise stated.

\textbf{Determination of minimum inhibitory concentrations, minimum bactericidal concentrations and minimum biofilm eradication concentrations}

The minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and minimum biofilm eradication concentrations (MBECs) of various disinfectants against Gram-negative strains were determined using methods described previously.\textsuperscript{11} In order to determine MBECs, overnight cultures were diluted to an optical density at 600 nm (OD\textsubscript{600}) of 0.1, 200 µL was pipetted into 96-well plates and biofilms were allowed to form at 37 °C overnight on lids with pegs (Thermo Scientific Nunc Immunoassay Transferable Solid
Phases; Thermo Fisher Scientific, Waltham, MA, USA). The lids were placed in 96-well plates containing 200 µL of a range of concentrations of each disinfectant, tested at room temperature and removed to plates containing 200 µL TSB. The MBEC was determined as the lowest concentration at which no growth was seen after 24 h in TSB. To reflect the fact that antisepsis or disinfection is unlikely to be performed for 24 h, more realistic exposure times of 1, 5, 15 and 30 min were also tested. Orbital shaking at 1200 revolutions/min in a Titramax 1000 (Heidolph Instruments, Schwabach, Germany) during exposure was also tested to emulate physical shearing of the biofilm. MICs were determined by adding 100 µL of an overnight culture at OD$_{600}$ 0.01 to 100 µL of a range of concentrations of each disinfectant, and growth was monitored after 24 h at 37 °C. The MIC was defined as the lowest concentration at which there was no growth. For MBC determination, a sterile 96-well plate replicator (Sigma-Aldrich, St Louis, MO, USA) was used to transfer 10 µL of culture from each well of the MIC plate, after exposure to a range of concentrations of each disinfectant for 24 h, on to a TSA culture plate. Plates were incubated for 24 h at 37 °C, and the lowest concentration without bacterial growth was defined as the MBC. H$_2$O$_2$ formulations used in this study are described in Table I.

<insert Table I near here>

**Analysis of biofilm formation**

The ability of all strains to form biofilms was tested using a modification of the Calgary biofilm method, as described previously. Biofilm formation was measured at an absorbance of 570 nm ($A_{570}$) using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany), and the average of three independent experiments was scored relative to the absorbance value ($A_{570}$ $\geq$ 0.4 = +++; 0.3 = ++; 0.2 = +; $\leq$ 0.1 = +/-).
**Statistical methods**

Excel 2007 (Microsoft Corp., Redmond, WA, USA) and PRISM 6 (GraphPad Software, Inc., La Jolla, CA, USA) were used for data analysis. Student’s *t*-test was used to calculate *P*-values. All data were taken from at least three independent experiments.

**Results**

MICs at 24 h (MIC\textsubscript{24}) for H\textsubscript{2}O\textsubscript{2} and formulations thereof were determined (Table II). All MIC\textsubscript{24} values are expressed as mM concentration of H\textsubscript{2}O\textsubscript{2} (as defined by the manufacturer) to allow for comparison between disinfectants. MBCs are not shown but were the same as MIC\textsubscript{24} values throughout the study. Results for unformulated H\textsubscript{2}O\textsubscript{2} showed consistent MIC\textsubscript{24} values across all strains of the three test organisms from 7 to 14 mM H\textsubscript{2}O\textsubscript{2}. Nearly all formulations showed similar distributions of MIC\textsubscript{24}, ranging from 9 to 20 mM H\textsubscript{2}O\textsubscript{2} (except for PA01 treated with Hard Surface Disinfectant 3 (HSD3) which had an MIC\textsubscript{24} of 36 mM). Only Hard Surface Disinfectant 1 (HSD1), which has an additional active ingredient of 70% ethanol, was more effective, with concentrations as low as 0.5–2 mM H\textsubscript{2}O\textsubscript{2} required to inhibit growth of all strains.

However, most Gram-negative MDR organisms in hospitals are more likely to grow as biofilms on surfaces. Therefore, disinfectants are likely to be used against bacterial biofilms. The biofilm-forming ability of the different strains was tested over 24 h (Table II). This showed marked differences between the organisms and strains. All *P. aeruginosa* isolates except GH97 and GH100 were excellent at biofilm formation (++++), whereas *K.*
pneumoniae strains showed a lower propensity to form biofilms (++ to +/-). The biofilm-forming ability of the Acinetobacter spp. strains varied (+++ to +/-) under the test conditions. The efficacy of the disinfectants was tested on single-species 24-h biofilm cultures by exposing them to various concentrations of disinfectants for 24 h. As expected, the change in sensitivity between planktonic and biofilm cultures varied significantly (P<0.0001) at both species and strain level (0–266-fold less sensitive in biofilms), with results for unformulated H₂O₂ demonstrating good correlation between the biofilm-forming ability of a test strain and reduced susceptibility (MBEC). Hence, *K. pneumoniae* strains with poor biofilm-forming ability had MBECs of 7–115 mM, *P. aeruginosa* strains with good biofilm-forming ability had MBECs of 3676 mM, and *Acinetobacter* spp. strains with the best biofilm-forming ability had MBECs of 921–3676 mM. In several cases, the MBEC of H₂O₂ was higher than that of the formulated products. A number of biofilm cultures showed bacterial survival after exposure to ≥50% working concentration of different formulations, most notably for strains of *P. aeruginosa* [five of seven strains with HSD3 and Mouthwash/Antiseptic 2 (MW/A2); seven of seven strains with Mouthwash/Antiseptic 1 (MW/A1)] and *Acinetobacter* spp. (three of seven strains with disinfectant HSD3; six of seven strains with MW/A2; seven of seven strains with MW/A1). Only a single strain–disinfectant combination of *K. pneumoniae* (NCTC13439 with MW/A1) showed an MBEC at 50% working concentration. Strains showed markedly higher susceptibility to formulations that included acids [accelerated H₂O₂ containing furoic acid in Endoscope Reprocessing (ER) and peracetic acid in Hard Surface Disinfectant 2 (HSD2)], and this reduced the MBEC range between strains of all three organisms (18–294 mM for ER; 19–74 mM for HSD2). Formulations that included ethanol showed counterintuitive results, with 15/21 strains showing MBEC ≥50% working concentration for MW/A2 compared with only one of 21 strains for HSD1, despite the former product containing higher concentrations of H₂O₂ (440 mM vs 37 mM) and ethanol (96% vs
70%). At 100% recommended working concentration, *Acinetobacter* spp. and *P. aeruginosa* biofilms were not eradicated by two of six products, with both MW/A1 and MW/A2 being ineffective. Shaking the biofilms during exposure to the disinfectants, to imitate physical shearing of the biofilm, had no significant effect on MBECs for any length of exposure (*P* ≥ 0.02, data not shown).

**Shorter exposures of biofilms to H\textsubscript{2}O\textsubscript{2} formulations**

When biofilms were exposed to unformulated H\textsubscript{2}O\textsubscript{2}, all organisms were affected to different degrees within the first 5 min, with little change in the next 25 min and little variation between strains of the same organism. *K. pneumoniae* strains were affected by much lower concentrations of H\textsubscript{2}O\textsubscript{2} (average MBEC 1882 mM) than either *Acinetobacter* spp. or *P. aeruginosa* strains (average 4704 and 2998 mM, respectively) (Figure 1a,b). Whereas both *K. pneumoniae* and *Acinetobacter* spp. biofilms were further affected by continued exposure, *P. aeruginosa* strains showed no increase in sensitivity to H\textsubscript{2}O\textsubscript{2} after 24 h of exposure (Figure 1a,b). In many cases, as with unformulated H\textsubscript{2}O\textsubscript{2}, disinfectants had little or no effect on biofilms within the first 1 min of exposure, although the majority of biofilm eradication was achieved within the following 4 min. There was only limited additional effect with extended incubation over the next 25 min (Figure 1c–h). There were exceptions, such as HSD2, which continued to show a reduction in biofilm over 30 min for all organisms. MW/A1 was not effective against biofilm growth of any strains of *A. baumannii* or *P. aeruginosa* within 30 min of exposure to 100% working concentration (Figure 1d,h). HSD1 was only effective against *K. pneumoniae* strains and the two worst biofilm-forming strains of *P. aeruginosa* within that time, although this was the most effective disinfectant over 24 h of exposure. Of the remaining disinfectant formulations, only HSD2 was effective at 100% working concentration against the majority of *P. aeruginosa* and *Acinetobacter* spp. strains, and HSD3
and ER were effective at 100% working concentration against the majority of *A. baumannii* strains at 30 min (Figure 1c,g). In contrast, all formulations except HSD1 and MW/A1 were effective at less than 50% working concentration against most *K. pneumoniae* strains after 30 min (Figure 1e). For nearly all disinfectants, as with unformulated H$_2$O$_2$, there was a further significant drop in MBEC between 30 min and 24 h in all organisms including *P. aeruginosa* ($P<0.0001$). Disinfectants that showed an effect within the first 30 min showed continued biofilm reduction over 24 h (HSD2, HSD3 and ER), whereas most disinfectants that were ineffective within 30 min (MW/A1 and MW/A2) showed very little or no activity towards *P. aeruginosa* or *Acinetobacter* spp., even after 24 h (Figure 1c,g).

<insert Figure 1 near here>

**Discussion**

Due to increasing levels of antibiotic resistance, disinfection and decontamination of healthcare settings are becoming essential factors in combating the spread of HCAI MDR infections. A disinfectant frequently used in healthcare settings due to its low toxicity is H$_2$O$_2$.\textsuperscript{5} This study set out to investigate the effectiveness of H$_2$O$_2$-based disinfectants against HCAI MDR Gram-negative biofilms. The study focused on three Gram-negative pathogens from the ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Enterobacter* spp., *Escherichia coli*) group of pathogens,\textsuperscript{19} which are of increasing concern for healthcare professionals. A range of commonly used H$_2$O$_2$-based disinfectants, covering applications from mouthwash, antisepsis, hard surface disinfection and endoscope reprocessing, with and without additional active ingredients such as peracetic acid and silver, were tested. All formulations were available for purchase in the National Health Service during the study period (January–September 2013).
As no European standard exists to determine efficacy of a biocide against biofilms of different Gram-negative organisms, methods were selected that would enable comparison between different applications and formulations of the disinfectants against various organisms. Although current standards looking at planktonic cultures concentrate on defined levels of log-reduction, eradication should be the final aim in biofilm cultures due to rapid regrowth, likelihood of recurring infections, higher virulence, and location of biofilms in invasive devices such as catheters and endoscopes. Single-species biofilms formed over 24 h present a standardized challenge to disinfectants, but are likely to be easier to eradicate than ‘real-life’ biofilms due to the lack of patient fluids, the short growth time, and the lack of masking and protective effects that are often found in multi-species biofilms. Failure to eradicate even these simple biofilms should, therefore, provide cause for concern for the more complex biofilms commonly encountered in healthcare settings. Although the study did not simulate mechanical cleaning, as would be used for some hard surface disinfectants, there was no evidence of increased biofilm removal with agitation (results not shown).

Use of the Calgary method for biofilm formation and MBEC measurement at relevant time points allows high throughput and simple comparison between formulations and organisms to define relative efficacy of removal. It also allows comparison of effectiveness of the disinfectant on a variety of strains, allowing distinction between its effect on fast-growing and biofilm-forming strains compared with weaker strains. This makes the method a possible starting point for the development of a much-needed standard for testing biocide efficacy against biofilms, and investigation into the mechanisms of H₂O₂ tolerance.

In terms of planktonic growth, all strains of all three organisms tested were sensitive to similarly low concentrations of H₂O₂ and tested disinfectants. This is likely due to the mode of action of H₂O₂, which, at low concentrations, oxidizes DNA, enzymes, proteins and other cell components; this activity is very non-specific and affects a wide range of
The efficacy of H$_2$O$_2$-based disinfectants was, however, significantly reduced when the organisms were grown as biofilms. Biofilm structures consist of an extracellular matrix made up of polysaccharides, extracellular DNA, enzymes and other cell components, which are excreted by cells and form a protective layer, although the precise composition of the extracellular layer may vary considerably between organisms and strains. However, studies have shown that poor biocide penetration, oxygen limitation and metabolic inactivity, as well as destruction of the matrix, are important in reducing biofilm eradication. Protection from H$_2$O$_2$ may be due to catalases, alginate or other free radical scavengers excreted by the cells and trapped in the matrix, which break down H$_2$O$_2$ before it can affect the cells.

Biofilm-forming ability was linked to growth kinetics of the strains and to the number of viable cells, with those strains with a shorter doubling time and greater numbers of viable cells in the biofilm forming larger biofilm structures in 24 and 48 h than those with a slow growth rate (data not shown).

When exposed to disinfectants for realistic times of 1–30 min, several of these biofilms were insensitive to 100% working concentration. Different formulations showed different effects over time, which implies that there may be more than one mechanism of biofilm eradication at play: an initial mechanism that acts immediately upon exposure, such as oxidation of components of exposed cells; and a second mechanism that continues to affect the biofilm over time, perhaps related to ongoing penetration of H$_2$O$_2$ into the biofilm with continued oxidation of components of the matrix and cells. *K. pneumoniae* strains were more affected initially, and were also affected by formulations that did not contain additional active ingredients, implying that they were more sensitive to H$_2$O$_2$. There was a clear distinction between the sensitivity of biofilms of any organism to disinfectants containing H$_2$O$_2$ alone as an active ingredient and those that contained added ingredients. Unformulated H$_2$O$_2$ and...
H₂O₂-only disinfectants were much less effective against biofilms after 24 h of exposure, even at 100% working concentration, than those that contained additional active ingredients. This supports the finding that H₂O₂ alone cannot eradicate biofilms at lower concentrations. The added ingredients appear to allow access to the biofilm structure, possibly by affecting the matrix and/or its components. Disinfectants containing low concentrations of acids in addition to H₂O₂ showed a smaller difference in efficacy between planktonic and biofilm cultures of the same strains. Strong acids, including peracetic acid, are known to have a synergistic effect with H₂O₂, both by stabilizing H₂O₂ and having a biocidal effect themselves. Neither of the two disinfectants that contained ethanol were very effective for short-term exposure, but showed varying effectiveness over 24 h; counter-intuitively, the disinfectant that contained a higher concentration of both H₂O₂ and ethanol was less effective for biofilm eradication. Ethanol can have a detrimental effect on the effectiveness of H₂O₂ against planktonic cultures at low concentrations as it scavenges OH⁻ radicals, reducing the oxidizing effect of H₂O₂ on cell components. Within a biofilm, however, ethanol may serve to dehydrate the extracellular matrix and allow cell components to be oxidized more readily by H₂O₂. This may take time, explaining the delayed activity of ethanol-containing disinfectants on biofilms. Alternatively, H₂O₂ may be chemically quenched by the matrix, thereby making ethanol the sole active component against the biofilm. Overall, the implication is that H₂O₂ alone is not very active against biofilms, but requires an additional ingredient such as a strong acid or moderate concentrations of ethanol to be effective.

Biofilms are a serious consideration for healthcare disinfection with H₂O₂-based products, as several commonly used antiseptics and disinfectants are unable to eradicate simple biofilms within a realistic time frame at the recommended concentration. This, together with the fact that different formulations affect biofilms differently over time, can
have serious implications for cleaning and antiseptic regimes, and supports the call for disinfectants/antiseptics to be tested against biofilms.

**Conflict of interest statement**

None declared.

**Funding source**


**References**


