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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

Public Health England, Microbiology Services Division, Porton Down, Salisbury, UK

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SUMMARY

Background: Hydrogen peroxide (H_2O_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_2O_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_2O_2 and H_2O_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_2O_2 were similar in all organisms and strains tested, with minimum inhibitory concentrations ranging from 0.5 to 20 mM H_2O_2. However, biofilms showed up to 266-fold less sensitivity to H_2O_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_2O_2-based disinfectants. This may compromise the ability to control these pathogens with such products.

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

* 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).

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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_2O_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_2O_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_2O_2) that are known to increase the efficacy of H_2O_2-based formulations.[6,8]

Healthcare-associated infections (HCAIs) are caused by many different organisms. Three of the most relevant Gram-negative organisms are *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *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.[13] Therefore, although biofilms are the most important growth form to combat, this is not accounted for in standard efficacy testing methods for disinfectants.[14,15]

The aim of this study was to investigate the efficacy of H_2O_2-based disinfectants commonly used in clinical/National Health Service hospital settings against biofilms of clinically important nosocomial pathogens.

Methods

Bacterial strains and culture conditions

Acinetobacter spp. and *K. pneumoniae* strains used in this study have been described previously.[11,16] The *P. aeruginosa* strains used were described strains (PA01 and NCTC 13359),[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, *aphA and 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.

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.[11] In order to determine MBECs, overnight cultures were diluted to an optical density at 600 nm (OD_{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_2O_2 formulations used in this study are described in Table I.

Analysis of biofilm formation

The ability of all strains to form biofilms was tested using a modification of the Calgary biofilm method,[18] as described previously.[11] 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

Table I
Compositions of formulations used in this study

Formulation	mM H_2O_2 in 100% working concentration	Added active ingredient in 100% working concentration
Hard Surface Disinfectant 3	2206	0.075% w/v Silver
Mouthwash/Antiseptic 2	1324	None[a]
Endoscope Reprocessing	588	<2.5% w/v 2-Furoic acid
Mouthwash/Antiseptic 1	441	96% v/v Ethanol
Hard Surface Disinfectant 2	294	0.08% w/v Peracetic acid
Hard Surface Disinfectant 1	37	70% v/v Ethanol

[a] Contains phosphoric acid, which is not listed as an active ingredient.