Antimicrobial activity of silver-containing dressings on wound microorganisms using an *in vitro* biofilm model

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Abstract

Antimicrobial dressings such as those containing silver are now being used widely to control wound bioburden, and tests to demonstrate their efficacy predominantly involve *in vitro* models using free-living or planktonic bacteria. In this present study a wide range of antibiotic-sensitive and resistant bacteria were tested in their quasi-sessile state using a standard agar assay and a second method used a poloxamer gel (true biofilm state – poloxamer encourages microorganisms to exhibit a more clinically relevant biofilm phenotype) technique. The antimicrobial activity of two silver dressings, a silver-containing Hydrofiber® (SCH) dressing and a nanocrystalline silver-containing dressing (NCS), were evaluated on a variety of microorganisms, using a zone-of-inhibition (ZOI) test. When grown on agar (presenting a quasi-sessile state of each organism), the antibiotic-susceptible microorganisms were generally more susceptible to the SCH dressing compared with the NCS. ZOIs associated with SCH dressing ranged between 5·7 and 17·5 mm; those for the NCS against the same group of organisms ranged between 1·9 and 8·6 mm. When grown on poloxamer gel, (presenting the biofilm state of each organism) the same group of microorganisms were less susceptible to both dressings. The SCH dressing was most effective against strains of *Pseudomonas aeruginosa*, *Candida albicans* and *Staphylococcus aureus* (ZOI range: 2·6–6 mm); the NCS was most effective against strains of *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Escherichia coli* (i.e. ZOI range: 1–2·8 mm). Similarly to the antibiotic-susceptible microorganisms, nine of ten antibiotic-resistant bacterial strains when grown on agar were more susceptible to the SCH dressing compared with the NCS. Although the microorganisms tested were universally less susceptible to the silver dressings when in their biofilm state, in the majority of cases, the SCH dressing demonstrated greater biofilm-inhibiting activity than the NCS.

Key words: Biofilms • Poloxamer • Silver dressings • Wound care

Key Points

- wounds are susceptible to microbial contamination from both exogenous and endogenous sources, and it is likely that such organisms are involved in the formation of biofilms in wounds

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INTRODUCTION

Wounds are susceptible to microbial contamination from both exogenous and endogenous sources, and it is likely that such organisms are involved in the formation of biofilms in wounds (1). Early contaminants of wound tissue are most likely to be skin flora (e.g. *Staphylococcus epidermidis*) that adhere to the wound (2), proliferate, synthesise extracellular polymeric substances and form a biofilm.

With the emergence and escalation of bacterial resistance, particularly to antibiotics (3) and associated increased health care costs, topical antiseptics are being used more extensively to control wound bioburden (1,4,5). *In vitro* techniques used to evaluate topical antimicrobial dressings are varied. One method is based on the zone of inhibition (ZOI), which measures bacterial clearance around a sample of dressing placed on an inoculated agar plate (6). However, it is now appreciated that culturing bacteria from viable tissue for use in the ZOI test alters a sessile pathogen into a lab-adapted planktonic counterpart (7). Evidence presented by Christensen *et al.* (8) and Freeman *et al.* (9) have shown that non biofilm bacteria are able to grow as colony-forming units on agar, suggesting that agar is not an ideal substratum for biofilm susceptibility testing of antimicrobials as it induces only a quasi-sessile bacterial state. The quasi-sessile state implies that the bacteria do express characteristic biofilm inducing genes and do not have the same phenotypic adaptations which lead to a true sessile and biofilm state and as such show an increased vulnerability to antimicrobials when compared to bacteria in a true biofilm state.

In this present study, a wide range of antibiotic-sensitive and resistant bacteria and a yeast were tested using a standard agar assay (Kirby–Bauer-type disc diffusion method (10), and a second method used a poloxamer technique to encourage the same strains of microorganisms to exhibit a more clinically relevant biofilm phenotype. Poloxamer gel cultures mimic many of the properties of biofilm-grown bacteria and therefore provides a reproducible method for testing the antimicrobial efficacy of biocides against biofilm bacteria (11). Poloxamer hydrogels have been used to study biofilms of *Streptococcus mutans* in plaque (12), to study homoserine lactones and biocide efficacy in biofilms (13) and coaggregation in bacteria (14). Evidence of biofilm growth in the poloxamer model has also been confirmed using confocal laser microscopy (15). Data to date indicates therefore that poloxamer induces bacteria to grow in a biofilm, suggesting this to be an ideal surface to induce biofilm phenotypes.

In this study, we have utilised the biofilm-inducing properties of poloxamer to provide a more relevant method to test the effectiveness of antimicrobial dressings on biofilm microorganisms.

METHODS AND MATERIALS

Media and reagents

Mueller–Hinton broth (MHB) and Mueller–Hinton agar (MHA; Laboratory M, Bury, UK) were used throughout. Poloxamer F127 was obtained from Univar (Essex, UK) and all other chemicals were purchased from BDH (Poole, UK), Bio Rad (Hemel Hempstead, UK) or Sigma (Poole, UK).

Test materials

The antimicrobial dressings used in this study were a silver-containing Hydrofiber® (SCH) dressing (ConvaTec™, Princeton, NJ, USA) and a nanocrystalline silver-containing dressing (NCS) (Smith and Nephew™, London, UK). The NCS dressing consists of a rayon/polyester non woven inner core laminated between two layers of silver-coated high-density polyethylene mesh. The layers are held together with ultrasound welds. The SCH dressing comprises of sodium carboxymethylcellulose Hydrofiber® and ionic silver. A Hydrofiber® dressing without silver was used as a control.

Microorganisms

A wide variety of aerobic bacteria (including antibiotic-resistant strains) and *Candida albicans* known to be associated with wound colonisation and infection were included in the study. These included reference isolates: *Staphylococcus aureus* (National Collection of Industrial and Marine Bacteria [NCIMB] 9518), *Pseudomonas aeruginosa* (NCIMB 8626), *Escherichia coli* (NCIMB 8545), *C. albicans* (National Collection of Pathogenic Fungi [NCPF] 3179); clinical isolates: *Enterococcus faecalis* (clinical isolate 141), *Klebsiella pneumoniae* (clinical isolate 033); and antibiotic-resistant bacteria: *P. aeruginosa* (NCIMB 8506), vancomycin-resistant enterococcus (VRE) (National Collection of Type Cultures [NCTC] 12201), VRE (clinical isolate 1), VRE (clinical isolate 2), methicillin-resistant *S. aureus* (MRSA) (NCTC 12232), MRSA (Cardiff clinical isolate 1), MRSA (clinical isolate 026), MRSA (clinical isolate 10371), MRSA (clinical isolate 10442) and *Serratia marcescens* (clinical isolate 1173146). An overnight culture of each microbial isolate was prepared in maximal recovery diluent (MRD; Laboratory M) at a concentration of approximately 1×10^5 colony-forming units/mL. A 1-ml volume of each organism suspension

Key Points

- with the emergence and escalation of bacterial resistance, particularly to antibiotics, and associated health care costs, topical antiseptics are being used more extensively to control wound bioburden
- in this study, a wide range of aerobic bacteria were tested using a standard agar assay and a second method used a poloxamer technique to encourage the same strains of microorganisms to exhibit a more clinically relevant biofilm phenotype

Key Points

- on agar, the antibiotic-susceptible microorganisms were generally more susceptible to the SCH dressing compared to NSD
- the SCH dressing was more effective against strains of *P. aeruginosa*, *C. albicans* and *S. aureus*
- NSD was most effective against strains of *K. pneumoniae*, *E. faecalis* and *E. coli*

was inoculated onto the surface of MHA and poloxamer gel plates in duplicate, which were then gently agitated to allow full coverage of the inoculum on the agar or gel surface, and then allowed to dry.

Poloxamer hydrogels (biofilm phenotype induction)

Poloxamer F127 hydrogels are diblock co-polymers of polyoxyethylene and polyoxypropylene that demonstrate thermoreversible gelation properties. At temperatures below 15°C, poloxamer is liquid and fully miscible with water but changes to a firm gel at temperatures in excess of 15°C. In this study, poloxamer hydrogels were prepared by mixing MHB with poloxamer F127 to make a final concentration of 30% poloxamer (w/v) (11). The solutions were chilled overnight to allow the poloxamer to go into solution. Prior to use, the poloxamer solution was autoclaved at 121°C for 15 minutes and then stored at 4°C. The solutions were mixed gently after incubation. Twenty-five-millilitre volumes of the poloxamer solutions were added to Petri dishes and then incubated at 35°C (±3°C) to allow the poloxamer to solidify.

EFFICACY OF SILVER DRESSINGS AGAINST POLOXAMER BIOFILMS

Dressing preparation and evaluation

Circular samples (2-cm diameter) of dressings were aseptically cut from a non SCH dressing (control), a SCH dressing and an NCS. With the use of sterile forceps, one circle of each dressing type was placed onto each inoculated MHA and poloxamer gel plate and pressed down gently to ensure close contact. A non SCH dressing and an SCH dressing were hydrated with 0·5 mL MRD to more closely mimic hydration in wound conditions. An NCS was hydrated with 0·5 mL sterile distilled water (as per the manufacturers' instructions: the dressing requires hydration with sterile water before applying the dressing to the wound). Plates inoculated with bacteria were incubated at 35°C ± 3°C for 24 hours, and those inoculated with yeasts were incubated at 25°C ± 2°C for 48 hours.

After 24-hour incubation, all plates were observed and the ZOIs around each sample on both the MHA and poloxamer gel plates were measured. All tests were carried out in duplicate.

Disodium ethylenediaminetetraacetic acid (EDTA, Fisher, Loughborough, UK) was used as a positive control (16–18) and saline as a negative control. For both the positive and negative controls, two sterile glass rings (diameter 15 mm) were placed aseptically onto each Petri dish containing poloxamer gel. A 400-μL aliquot of 4% (w/v) disodium EDTA. pH 4·0, was added to one ring and 400 μL of 0·85% (w/v) saline (Oxoid) added to the other. The dishes were incubated at 35°C ± 3°C for 24 ±1 hour and then examined for signs of ZOIs of growth, which were measured using digital calipers (Mitutoyo, Singapore, Malaysia).

Statistical analysis

Data were analysed by the Mann–Whitney *U*-test.

RESULTS

Figures 1a–1d illustrate the antimicrobial efficacy data for the SCH dressing and the NCS dressing against a variety of aerobic bacteria (including antibiotic-resistant strains).

On agar, the antibiotic-susceptible microorganisms were generally more susceptible to the SCH dressing compared with the NCS (i.e. in 6/6 cases, Figure 1a). ZOIs associated with SCH dressing ranged between 5·7 and 17·5 mm; those for the NCS against the same group of organisms ranged between 1·9 and 8·6 mm. All data were shown to be statistically significant ($P < 0·05$). When presented in their biofilm form (poloxamer gel), the same group of microorganisms were less susceptible to both dressings (Figure 1b); the SCH dressing was most effective ($P < 0·05$) against strains of *P. aeruginosa*, *C. albicans* and *S. aureus* (ZOI range: 2·6–6 mm), and the NCS was most effective ($P < 0·05$) against strains of *K. pneumoniae*, *E. faecalis* and *E. coli* (ZOI range: 1–2·8 mm).

Similarly to the antibiotic-susceptible microorganisms, nine of ten antibiotic-resistant bacterial strains presented on agar were more susceptible ($P < 0·05$) to the SCH dressing compared with the NCS (Figure 1c) (ZOIs ranged between 5·1 and 17·9 mm for the SCH dressing and between 4·1 and 8·3 mm for the NCD). In their biofilm (poloxamer gel) form (Figure 1d), antibiotic-resistant strains of bacteria showed reduced susceptibility to both dressings, but in nine of ten cases, the SCH dressing proved to be the more effective ($P < 0·05$) dressing, with equivalent activity being

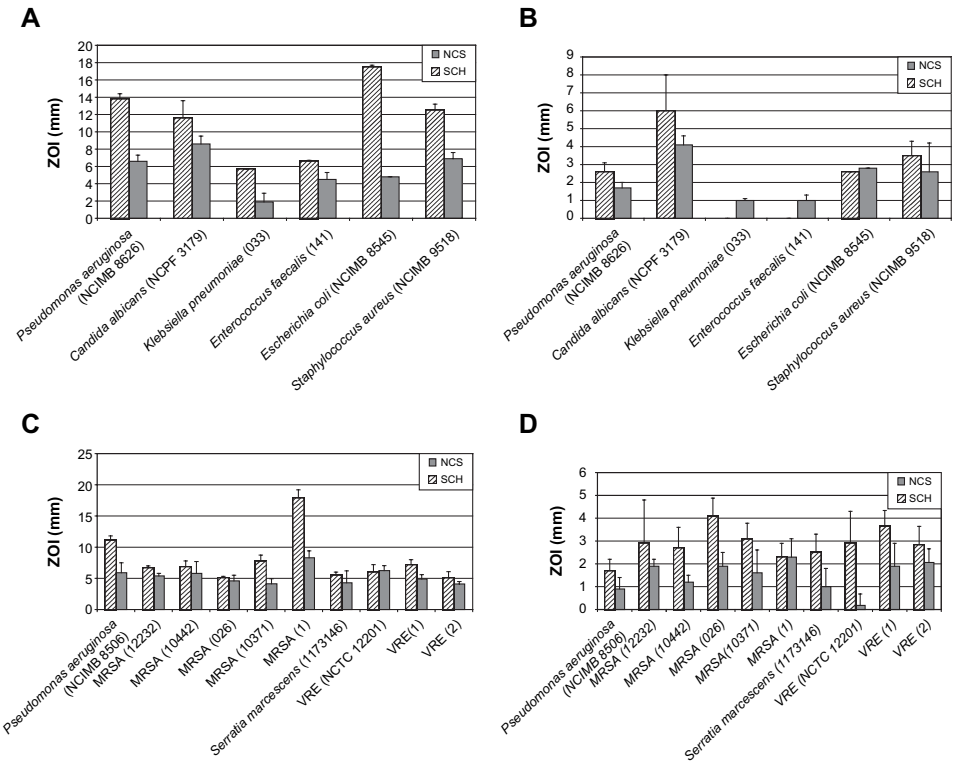

Figure 1. (a) Zone of inhibition (ZOI) induced by silver-containing dressings against bacteria and yeast grown on Mueller–Hinton agar (MHA). Results are expressed as means ± standard error. (b) ZOI induced by silver-containing dressings against bacteria and yeast grown on poloxamer gel. Results are expressed as means ± standard error. (c) ZOI induced by silver-containing dressing against multiple resistant bacteria grown on MHA. Results are expressed as means ± standard error. (d) ZOI induced by silver-containing dressings against multiple resistant bacteria on poloxamer gel. Results are expressed as means ± standard error. SCH, silver-containing Hydrofiber® dressing; NCS, nanocrystalline silver-containing dressing; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococcus.

demonstrated against one clinical strain of MRSA (ZOI ranged between 1·7 and 4·1 mm for the SCH dressing and between 0·9 and 2·3 mm for the NCS).

The positive control produced a zone of clearing against *P. aeruginosa* and *S. aureus* biofilm. Both silver dressings produced a zone of clearing around both MRSA and *P. aeruginosa* when grown on agar (Figures 2a, 2c) and poloxamer gel (Figures 2b, 2d), although these were small, particularly for the NCS dressing.

DISCUSSION

Using two silver-containing dressings, the SCH dressing and the NCS, activity against biofilm microorganisms has been demonstrated, although differences in the level of activity was observed. In 15 of the 16 microorganisms tested on agar, the SCH dressing produced greater ZOIs compared with the NCS. Although the NCS dressing contains approximately ten times more silver (w/w of dressing) than the SCH dressing, this study showed that the concentration of silver did not correlate with the level of activity, and this has been reported elsewhere (19). Ionic silver (the active form of silver, Ag^+) exerts antimicrobial activity at very low concentrations (20), and *in vitro* studies have shown that silver can be effective at concentrations well below the previously reported minimum inhibitory concentrations values (19).

In their biofilm form, antibiotic-sensitive bacteria and *C. albicans* generally showed reduced susceptibility to both silver-containing dressings. While the SCH dressing continued to demonstrate greater efficacy against five of the nine strains tested, the NCS dressing performed more favourably against strains of *K. pneumoniae*, *E. cloacae*, *E. faecalis* and *E. coli*. With respect to antibiotic-resistant bacteria, the majority of which were MRSA and VRE strains, the SCH dressing performed more effectively than the NCS in nine out of ten cases (one strain of VRE being an exception)

Key Points

- using two silver containing dressings, and the NSD, activity against biofilm microorganisms has been demonstrated, although differences in the level of activity were observed
- ionic silver (the active form of silver) exerts antimicrobial activity at very low concentrations
- the ZOI test to evaluate the efficacy of antimicrobial wound dressings is open to debate, as indicated by a number of authors
- this is due to the fact that ionic silver may interact with the test medium and therefore affect its diffusion

Key Points

- from this study, it is evident that factors other than ionic silver alone play a role in the antimicrobial efficacy of silver-containing dressings
- although microorganisms presented in their biofilm form are invariably less susceptible to ionic silver, the combined unique physical and antimicrobial properties associated with the SCH dressing induced an effect that is greater than that induced by the NSD
- in a wound environment, dressing selection should be carefully considered to ensure that the antimicrobial efficacy of a dressing is maximised *in vivo* where biofilms reside

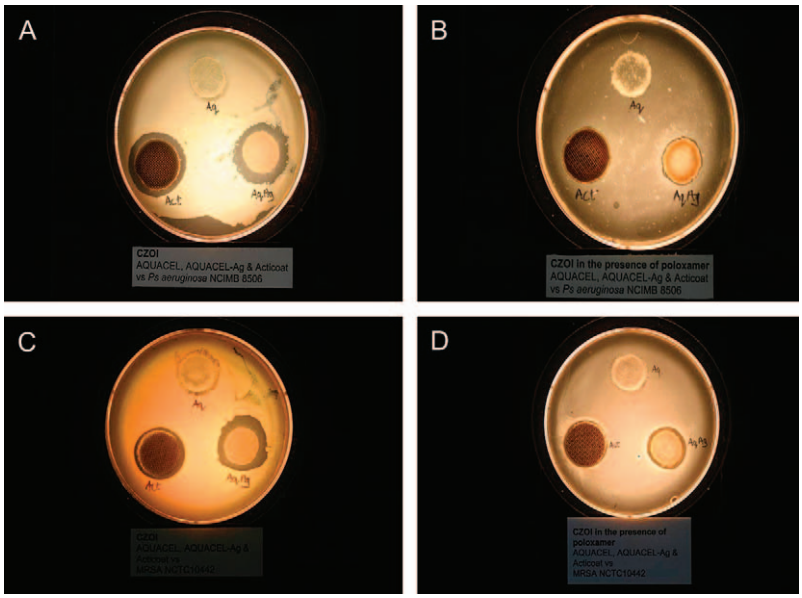

Figure 2. (a) Zone of inhibition (ZOI) against *Pseudomonas aeruginosa* (NCIMB 8506) on Mueller–Hinton agar (MHA). (b) ZOI of *P. aeruginosa* (NCIMB 8506) on poloxamer gel. (c) ZOI against methicillin-resistant *Staphylococcus aureus* (NCTC 10442) on MHA. (d) ZOI of MRSA (NCTC 10442) on poloxamer gel Act (Acticoat™) is the nanocrystalline silver-containing dressing, Aq (Aquacel®) is a non silver-containing Hydrofiber® dressing, and AqAg (Aquacel® Ag) is a silver-containing Hydrofiber® dressing).

for the quasi-biofilm forms, and in nine out of ten cases when the same bacteria were presented in their biofilm form. However, ZOIs were again generally smaller than for their quasi-biofilm counterparts for both dressings.

As a test method to evaluate the efficacy of ionic silver on sessile (biofilm) bacteria, it is evident from this research that, ionic silver is less effective on poloxamer-grown biofilm than on agar-grown quasi-sessile bacteria. Biofilms are well known to be recalcitrant to antimicrobials and have been documented in both infected and non-infected chronic wounds (21), and therefore it is most likely that bacteria within biofilms needs to be controlled in order to facilitate wound progression.

It is recognised that *in vitro* data cannot be directly extrapolated to clinical outcomes. Nevertheless, *in vitro* models can be beneficial to enhance our understanding of how topical antimicrobial agents and wound dressings may facilitate wound management and every effort must be taken to ensure that *in vitro* models are both as stringent and realistic as possible, as was the case in this study.

Although the microorganisms tested in this *in vitro* model were universally less susceptable to the silver dressings when in their biofilm state, in the majority of cases, the SCH dressing demonstrated greater biofilm inhibiting activity than the NCS dressing.

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