In vitro diffusion bed, 3-day repeat challenge ‘capacity’ test for antimicrobial wound dressings

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ABSTRACT
The aim of this study was to develop an in vitro wound infection model that allows the comparison of the bacterial kill rate of antimicrobial wound dressings over the course of 3 days, with renewed microbial challenges each day, under realistic wound-like conditions. A test bed model of a moderately exuding wound was constructed from a hydrogel containing releasable foetal calf serum (FCS), and cellulose discs dosed with test microbes (Methicillin-resistant Staphylococcus aureus and Pseudomonas aeruginosa) suspended in 50% FCS applied at the interface between the test dressing and the hydrogel test bed. Freshly prepared discs were used to challenge the same dressing over a 23-hour period for a course of 3 days. Different test dressings produced differing kill rates, allowing quantitative comparison of both their immediate activity and their capacity to continue working over 3 days, within a fluid-donating system similar to the situation in vivo. The reported method has significant advantages over established test procedures since it enables the researcher to assess the antimicrobial capacity of wound dressings to continue working under conditions that match those encountered in wounds. These key conditions are those that would be expected to impede the action of the dressing and protect the infecting organisms.

Key words: 3-day capacity test ● Antimicrobial wound dressings ● Diffusion test bed ● Pseudomonas aeruginosa ● Staphylococcus aureus (MRSA)

INTRODUCTION
A number of in vitro techniques have been described for testing the efficacy of antimicrobial wound dressings. In the agar diffusion methods (1,2), samples of test dressings are placed onto lawns of indicator bacteria, grown on appropriate media. The active agents within the dressings diffuse into the agar lawn, producing a zone of inhibition around the test dressings. The results from these methods depend on the speed of diffusion of the active agent from the dressing, set against the growth rate or vigour of the target species growing in the lawn, and are highly dependent on the particular physicochemical environment. Furthermore, the measurements obtained (zone diameter) have little comparative meaning for different dressings or different targets. It is not surprising that the agar diffusion/zone-of-inhibition method does not correlate to time–kill kinetic assays (3) and can produce variable results, often at odds with those observed in vivo for potentially effective wound dressings (4).

Key Points
• the purpose of this study was to build on the previous work on agar test beds and cellulose discs by adding further levels of realistic challenge, through the additional feature of controlled fluid flux
• conventional in vitro testing of antimicrobial wound dressings does not usually take into account fluid flow dynamics or the capacity to continue killing beyond 24 hours on repeat challenge

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subsequent determination of time–kill kinetics have also been described (5). These techniques are much favoured since they allow accurate determination of kill rates, which is the key comparator for different formulations, target species and physicochemical conditions. However, these methods are inappropriate for highly absorbent gel dressings that rely on oxygen diffusion and the integrity of the in-built biochemical mechanisms for generating active killing agents in situ. For these, as well as for conventional release dressings, an alternative testing method has been proposed (6). This method, described as an in vitro static diffusion method, used cellulose discs as a matrix in which to hold defined indicator species in close but indirect contact with the dressing under test. Organisms held in the discs were exposed to the antimicrobial conditions of the dressings for defined periods of time and under controlled physicochemical conditions. The organisms were readily recovered from the disc for the quantification of survivors, enabling the construction of time–kill curves and the derivation of kill rates, thus quantifying the strength of any antimicrobial effects. The method was shown to be reproducible and was applicable to a wide range of target species (including anaerobes and yeasts) and against a wide range of wound dressings and conditions (6).

The purpose of this study was to build on the previous work on agar test beds and cellulose discs, by adding further levels of realistic challenge, through the additional feature of controlled fluid flux. The fluid contained high levels of dissolved proteins and other organic substances, essentially similar to those seen in typical moderately exuding wounds. These are important factors for, in reality, antimicrobial dressings have to work under adverse conditions, caused by the presence of wound fluid (mainly serum). Wound fluid constantly perfuses into the wound bed and migrates into the dressing, so neutralising and/or diluting the effects of the antimicrobial agents. In addition, many dressings are claimed to act as ‘slow release’ or ‘controlled release’ systems, suggesting that their activity against microbes could be sustained for many days. An additional aim of this study was therefore to apply a new method (consistent with the principles of Kelsey and Sykes (7) for testing the capacity of disinfectants) to the rigorous assessment of the antimicrobial activity of wound dressings. Hence, this study includes an analysis of the capability of dressings to cope with repeated daily challenge with fresh preparations of target microbes contained in a protective matrix, over 3 days. The method was used to test four different types of wound dressing against two species of target microbes (Pseudomonas aeruginosa and Staphylococcus aureus). Results were analysed with regard to the sustainability of killing and the immediate kill rates of target cells.

**MATERIALS AND METHODS**

**Growth and maintenance of micro-organisms**

Methicillin-resistant *Staphylococcus aureus* (methicillin-resistant Llewellyn strain SMH 22115) (MRSA) and *P. aeruginosa* (PAO 1161) were maintained from frozen stocks on nutrient agar (Oxoid Ltd, Basingstoke, UK). Broth cultures of *S. aureus* and *P. aeruginosa* were grown in 1% tryptone–0.5% yeast extract (TYE) (Oxoid Ltd).

**Experimental wound dressings**

The wound dressings tested were Iodozyme (Insense, Bedford, UK), Silvercel hydro-alginate (Johnson & Johnson, Skipton, UK), Contreet (Coloplast Ltd, Peterborough, UK) and Acticoat (Smith & Nephew Medical Ltd, Hull, UK). All wound dressings were supplied in their standard packaging and were cut into square sections (25 × 25 mm) immediately before use.

**Discs and disc inoculation**

Discs were sterilised and inoculated as described by Thorn et al. (6). For 3-day experiments, discs were prepared from fresh broth cultures at the start of each day. Cultures (10 ml) were centrifuged (PK121R multi-speed centrifuge; ALC Ltd, Cologno Monzese, Italy) at 8000 g for 5 minutes. Cells were harvested and resuspended in 1 ml of TYE + foetal calf serum (FCS; Biosera, Ringmer, UK) (50%, v/v). The FCS was heat inactivated (57°C for 30 minutes) to remove complement activity before use. Following inoculation by immersion in TYE/FCS cell suspension, discs were shown to contain approximately the same numbers of viable cells at about 1 × 10⁷ cfu per disc.
Hydrogel test bed preparation

Hydrogel test beds were prepared in the following manner: the monomer, sulpho-propylacrylate powder (potassium salt; Aldrich code 251631), was dissolved in de-ionised water to a concentration of 10% (w/v). Once this reagent was thoroughly dissolved, a quantity of 10× concentrated phosphate-buffered saline stock solution (PBSa; Sigma D1408, Poole, Dorset, UK) was added to give a final 1× concentration. Cross-linker was added [PEG-700 diacrylate (Aldrich 455008)] at a concentration of 0.95% (w/v). Finally, photoinitiator was added (1-hydroxycyclohexyl phenyl ketone; Aldrich 405612) to a final concentration of 0.01% (w/v). Once all reagents were completely mixed, aliquots of the pre gel mix were poured into 10-cm² square petri dishes to give 25 g per dish, and these were irradiated under ultraviolet light at a power of 100 mW/cm² at the gel surface for approximately 25 seconds. The gels formed were firm to the touch and not sticky.

The resulting hydrogel formulation was tested for its fluid transfer characteristics. This was performed by placing a 2-cm² patch of an iodozyme wound dressing onto the surface of the test bed, and at various time intervals, the patch was removed and the weight increase measured using a microbalance. A test bed of 1% (w/v) agar with 1× concentration PBSa was also tested for comparison.

Test method

Hydrogel test beds were pre-soaked with 3 ml of a 50% (v/v) solution of heat-inactivated FCS in PBSa (pH 7.2). Plates were incubated for 1 hour to let the FCS solution soak into the surface of the hydrogel. Excess fluid remaining after this period was aseptically removed by suction pipette. Thus, the test microbes were steeped in serum within the discs, and further serum was brought into contact from the reservoir within the model wound hydrogel test bed polymer. Cut square sections (25 × 25 mm) of test wound dressings (n = 16 for each type) were distributed evenly onto the surface of the test bed plates (five per plate; 16 plates). Sample positions were determined by reference to a grid matrix (intersection of rows and columns), and the sequence of samples was determined according to Latin square principles. Each replicate test plate contained one cut square section of each test dressing and one uncovered control. The test plates loaded with dressing samples were immediately challenged by introducing the microbe-laden test discs (one per square) placed between the test bed hydrogel layer and the test dressing squares, the components being manipulated with sterile forceps. The test bed assay plates (with discs and cut dressings) were incubated at 32°C for the duration of the experiments.

Test bed sampling

Immediate samples (for time 0), and at various later sampling times, were taken by lifting the wound dressing squares to remove the test or control discs (using sterile forceps). Each sample disc was added to a 10-ml volume of sterile PBSa containing 0.05% (w/v) Na2S2O3 as I2 and Ag+ neutraliser and vortex mixed for 1 minute to release surviving cells in suspension. Neat suspensions were serially diluted in a 1:10 dilution series down to 10⁻² in PBSa. Viable counts were then performed of neat and diluted suspensions using a spiral plater (Don Whitley Scientific, Shipley, UK) onto a nutrient agar recovery medium (Oxoid CM3) to determine the numbers of survivors at different time points. Recovery plates were incubated at 37°C aerobically for 24 hours, and the number of survivors determined as cfu per sample (i.e. cfu per cm² since the discs have a surface area of 1 cm²).

One test bed system of 16-assay plates per test organism was started and challenged on day 1 and incubated, and the samples were removed at various time points. After the last 23 hour, disc samples had been extracted the same test bed was rechallenged at 24 hours with freshly prepared discs (day 2) and reincubated, and the samples were removed at various times. After the last 23 hour, disc samples from day 2 were removed, the test bed system was again challenged with fresh discs (day 3), which were again incubated and sampled up to 23 hours.

Analysis of results

For each test species (MRSA and P. aeruginosa) and each condition (four test dressings and one control), plots were made of log numbers of survivors over reaction time. Kill rates were determined by linear regression, enabling direct comparison of the results over time for the tests and controls for each target species. Graph construction, statistical analyses, and
modelling were conducted with the use of GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). An analysis of covariance was used to ascertain whether there was a significant difference between the rates. A $P$ value of $<0.05$ was regarded as statistically significant.

RESULTS

The rate and extent of fluid donation from the hydrogel polymer test bed to the Iodozyme wound dressing, from the moment when the two were brought into contact with each other, was found to be comparable with that predicted for a moderately exuding wound [this is extrapolated from a rate of $0.5g/cm^2$/day, as determined by Thomas et al. (8)], shown in Figure 1. The 1% w/v agar gel [used in a previous study (6)] donated fluid at a much higher rate, with large, uncontrolled quantities of fluid being absorbed into the dressing in the first few hours.

The kill curves obtained for *P. aeruginosa* (Figure 2) show that on day 1, the Acticoat dressing had the highest kill rate (Table 1), followed by the Iodozyme dressing. The measured rates for Silvercel and Contreet were low in comparison. By day 2, the Iodozyme had by far the highest kill rate of the four dressings ($P<0.002$). The kill rate for Acticoat was significantly reduced ($P<0.0001$) to less than 25% the value obtained on day 1. The kill rate for Silvercel was also lower ($P<0.0001$), while that for Contreet increased significantly ($P<0.001$) to just over twice the rate recorded on day 1 (Table 1), making its efficacy on day 2 close to that of Acticoat. Kill rates on day 3 were not significantly different from those measured on day 2 for each respective dressing. On day 1, the samples taken at 23 hours indicate that survival or regrowth of *P. aeruginosa* occurred with Acticoat. On day 2, the 23-hour data indicated that the Silvercel dressing had a killing effect, although low over the first 8 hours had continued giving a $>2$-log reduction by 23 hours. The other silver dressings (but not Iodozyme) also allowed significant microbial survival/regrowth before 23 hours. The other silver dressings (but not Iodozyme) also allowed significant microbial survival/regrowth by 23 hours on day 2. A similar pattern was observed on day 3; Silvercel had a kill rate that was statistically indistinguishable from the controls over the first 8 hours, nevertheless continued to slowly reduce the numbers of disc survivors so that a $>2$-log drop was observed by 23 hours. On day 3, as on day 2, the other silver dressings allowed some degree of microbial survival or regrowth by 23 hours, which, in this case, was also observed for the Iodozyme dressing.

In the MRSA experiments, the dressings could be ranked in a similar order to that observed in the experiments with *P. aeruginosa*, although all the kill rates were lower. Again, the Acticoat dressing showed the highest kill rate on day 1, with Iodozyme giving a lower but still significant kill rate (Figure 3; Table 1).

**Figure 1.** Fluid donation/absorption into an Iodozyme wound dressing from either a hydrogel polymer or a 1% w/v agar test bed compared with a theoretical wound exudation curve extrapolated from a previous study (8). Predicted wound exudation from theoretical model (●), 1% w/v agar test bed (■) and hydrogel polymer test bed (▲) are shown.

### Key Points
- the rate and extent of fluid donation from the hydrogel polymer test bed was found to be comparable with that predicted for a moderately exuding wound
- the order of efficacy of the four wound dressings tested against *Pseudomonas aeruginosa* and MRSA, as determined by kill rates ($k$), was Iodozyme $>$ Acticoat $>$ Contreet $>$ Silvercel $>$
- regrowth occurred to varying extents under all wound dressings tested, possibly due to either resistant variants or localised biofilm formation within the cellulose disc
Both of the other kill rates (Contreet and Silvercel) were low and not significantly different from those of the controls. By days 2 and 3 the Iodozyme dressing had by far the highest kill rate, while none of the silver dressings produced kill rates significantly different from those of the respective controls. Microbial survival or regrowth was apparent at 23 hours on days 1, 2 and 3 for all silver containing dressings. This was not observed with Iodozyme.

**DISCUSSION**

The purpose of this study was to build on the in vitro static diffusion method, based on cellulose discs as the microbe carrier matrix (Thorn et al. (6), to provide a wound bed model featuring the most important factors capable of inhibiting the action of most antimicrobial agents – high levels of serum proteins and a realistic flux of protein-loaded fluid from the test bed into the dressing. This was achieved by developing a wound bed model constructed
from a fluid-donating hydrogel polymer that could donate serum to the absorbent test dressings at about the same rate as a typical, moderately exuding wound. Although the fluid uptake after 24-hour contact time was less than the theoretically predicted value derived from a straight line extrapolation of the ‘standard’ uptake rate, the observed slowing of uptake after 4 hours is believed to reflect the situation under compression, in which the build-up of hydrostatic pressure actually slows the rate of exudation. A further degree of reality arose from the way the microbes were suspended in FCS within a porous cellulose matrix, placed between the serum-donating hydrogel test bed and the absorbent test dressing. For antimicrobial wound dressings, this arrangement is especially challenging (and realistic), as it results in the microbes becoming bathed in protective serum proteins. The net flux of serum from the hydrogel polymer test bed into the dressing also creates a gradual flow against which the antimicrobial agents must be able to diffuse before they can have their effect.

The static diffusion bed kinetic kill model described by Thorn et al. (6) was characterised by a slow initial response to the application of iodine-generating dressings. The assay used a 2-hour equilibrium period, wherein the dressing was kept in contact with agar gel containing 99% water, before the placement of the target microbe-loaded discs. During this time, the test hydrogels became very swollen, as they did their job of rapidly absorbing the water originally carried in the agar gel. This aspect of the model was not representative of the situation occurring in real wounds, as the large degree of swelling resulted from an uncontrolled absorption of excess fluid from the test bed. Two immediate problems arose from this, the first of which was that the rapid ingress of unlimited quantities of liquid opposed the diffusion of antimicrobial iodine in the direction of the disc matrix. The second was that the test dressing squares became excessively swollen very rapidly and to an extent never observed when similar dressings were placed on wounds. This impeded the biochemical functioning of the dressing (designed to drive a steady delivery of iodine and oxygen) and caused the material to curl and lift away from the agar surface, requiring weights to hold it in place. By replacing the agar gel in the assay bed with the specially formulated hydrogel polymer, these problems were obviated. In fact, the water release properties of this material were designed to be comparable with the fluid release rates of a typical, moderately exuding leg ulcer. The swelling of the test squares when in contact with the new test bed was close to that observed for this kind of dressing when in use on real wounds (venous leg ulcers).

In addition to the main modification of replacing the agar test bed with the new polymeric hydrogel, further minor modifications were made to the assay. An ideal antimicrobial assay for wound dressings would be one that exposes the dressing to the same (or very similar) physicochemical conditions that occur in a real infected wound. Typically, this would include a temperature between ambient and 37°C, a pH close to that of blood or tissues and a chemical environment characterised by high concentrations of nutrients and serum. For these reasons, the 3-day assays described in this study used a temperature of 32°C (9) and pH 7.2 (10) and incorporated TYE-FCS in the disc microenvironment, backed up by an effectively unlimited supply of FCS in the assay test bed. As a consequence of these modifications, the response of the assay system was more discriminating and, arguably, more realistic. The resultant kill rates for *P. aeruginosa* and MRSA allowed consistent comparisons to be

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### Key Points

- In addition to the main modification of replacing the agar test bed with the new polymeric hydrogel, further minor modifications were made to the assay.
- As a consequence of these modifications, the response of the assay system was more discriminating and, arguably, more realistic.
Comparison of the efficacy of one type of dressing with another or one type of dressing against a range of different target species is best expressed in terms of a common measuring unit, \( k \), the kill rate or cells made between various antimicrobial dressings, bringing out differences in performance that would have been hidden in less challenging models. Within each study set, there were kill curves at each end of the performance scale, with some showing maximal kill rates, some intermediate and some negligible. The differences exposed by this technique went still further, in that apparently similar silver dressings could be seen to differ in the pattern of microbial kill within a 23-hour test period. For example, some could achieve modest but distinct kill rates through the first part of a 23-hour period, only to apparently wane in the later stages sufficiently to allow regrowth by the next morning. Others under identical conditions had a slower initial kill rate, but which was consistently maintained through the full 23 hours, thereby achieving a similar end result. This would not have been evident in other test formats.

Comparison of efficacy of one type of dressing with another or one type of dressing against a range of different target species is best expressed in terms of a common measuring unit, \( k \), the kill rate of cells. To reliably measure the kill rate requires that viable count of survivors are made at different times,

\[ \text{Day 1} \]
\[ \text{Day 2} \]
\[ \text{Day 3} \]

Figure 3. Kill curves obtained for four types of antimicrobial wound dressings tested against MRSA over a 3-day period. (○), Untreated control; (▲), Acticoat dressing; (▼), Contreet dressing; (■) Silvercel dressing and (●), Iodozyme dressing, and the dashed line shows the minimum level of detection.
preferably over at least three or more log reduction cycles for an active dressing and having sufficient points (five or more) to determine the regression of slope of kill. These features were all incorporated into this study, which showed that the lodozyme dressing maintained a strong microbial killing effect over the whole 3-days of the capacity test and was significantly more effective against 

P. aeruginosa

and MRSA than the three silver-based wound dressings tested. The immediate killing effect on day 1 of Acticoat silver dressing was not maintained when challenged with fresh target discs on days 2 and 3 against 

P. aeruginosa

and not distinguishable from those of the controls on days 2 and 3 against MRSA. However, the 23-hour data on day 2 with MRSA did show a >2-log-fold drop, showing that the dressing did continue to work slowly. The Silvercel dressing appeared to exert a slow but continued killing effect on 

P. aeruginosa

on days 2 and 3, as evidenced by the numbers of survivors recovered at 23 hours. In contrast, Acticoat and Contreet dressings produced an immediate kill of two to three log cycles over the first 4 hours, but at 23 hours, on days 2 and 3 survival and regrowth were apparent. It is difficult to explain these phenomena when the immediate kill rate on day 2 suggests (by extrapolation) that all target cells should be killed by 8–10 hours, yet survivors were clearly present at 23 hours. To further complicate the interpretation, the dressings that had permitted survivors and regrowth still possessed substantial killing potential as clearly showed by the immediate killing of a new challenge observed on day 3. This effect will be studied further to determine if it can be explained in terms of resistant variants or, perhaps, by the formation of small areas of biofilms in pockets of the cellulose disc that were relatively inaccessible to the antimicrobial molecules.

The challenge of 10^7 organisms per day for 3 days would be unrealistic in a real wound unless it was grossly reinfected. If the immediate activity of the dressing reduced the initial number by four or five log fold, then only a few survivors might be expected on days 2 and 3. However, the point of rechallenge was not to emulate every detail of a typical wound condition but to maximise the work that the dressing must do, so that its remaining antimicrobial capacity can be gauged.

Overall, this refined version of the in vitro static diffusion method has been shown to expose key differences in the bacterial killing capacity of various antimicrobial dressings, not normally discernible in normal in vitro test systems. This discriminating ability depends on the incorporation of key challenging features that resemble important factors encountered in exuding wounds, especially an influx of protein and nutrient-rich fluid into the dressings from a controlled fluid release wound bed model. The system made it possible to evaluate the antimicrobial performance of the dressings when the microbial challenge was prolonged for 3 days. Under these rigorous conditions similar to those of wounds, it was perhaps surprising that a newly developed, controlled release iodine dressing (based on de novo synthesis of iodine driven by an enzyme) was by far the most effective antimicrobial product.

REFERENCES