An insight into *in vitro* microbiological testing of wound dressings

KEY WORDS
- Bacteria
- Biofilm
- Infection
- Microbiology
- Swab
- Wound dressings

Medical devices such as wound dressings must undergo stringent laboratory testing during product development to satisfy safety and performance requirements of regulatory authorities. Following regulatory approval, further *in vitro* laboratory tests are commonly undertaken to compare performance characteristics of commercial products. From a microbiological perspective specifically, a variety of internationally recognised *in vitro* test methods, and also customised *in vitro* tests methods are available for investigating performance characteristics such as microbial binding, microbial barrier, antimicrobial efficacy and antibiofilm efficacy. Such test methods, together with challenges in testing antimicrobial dressings will be addressed in this paper.

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Analysis of the microbiological, biological, physical, and chemical characteristics of wound dressings is essential for two key reasons:

1 To generate data to support regulatory submissions (e.g., to BSi [UK] and the United States Food and Drug Administration [FDA]). Regulatory authorities require data to support the safety and efficacy of a new product, and most of the data are generated via *in vitro* (laboratory) tests. As examples, this may include the fluid handling, adhesion or antimicrobial characteristics of a wound dressing. Furthermore biocompatibility (toxicology) testing is essential to prove that a product is safe before use in humans. Although internationally recognised standard methods should be used where possible, customised test methods can be developed and validated to generate data to satisfy regulatory requirements.

2 To generate data to support marketing claims. Similar standard or customised test methods to those required for regulatory submissions are often used, but testing may be extended to include several similar dressing types to show differentiation in product performance. However, when comparing dressing performance, numerous factors (particularly relating to dressing construction) can influence outcomes, and consequently it is essential that specific test methods are validated for a variety of dressing types. This will be discussed in more detail later.

From a microbiological perspective specifically, regulatory authorities require data to prove that materials used in the manufacture of a dressing have an acceptably low microbial load, and that for single use products, the terminal sterilisation process has been adequate to ensure sterility of the dressing. For dressings that contain an antimicrobial agent, regulatory authorities require proof of efficacy, and data to support any specific product claims.

EXAMPLES OF COMMONLY USED MICROBIOLOGY TEST METHODS

Bacterial and viral barrier

These methods are used to demonstrate if a wound dressing can physically prevent the transmission of bacterial cells or viral particles from a heavily contaminated wound, such as a chronic wound, into the surrounding environment. From an infection control perspective this is very important because wounds often harbour infectious and antibiotic-resistant organisms that could spread within a healthcare facility. Microbe-

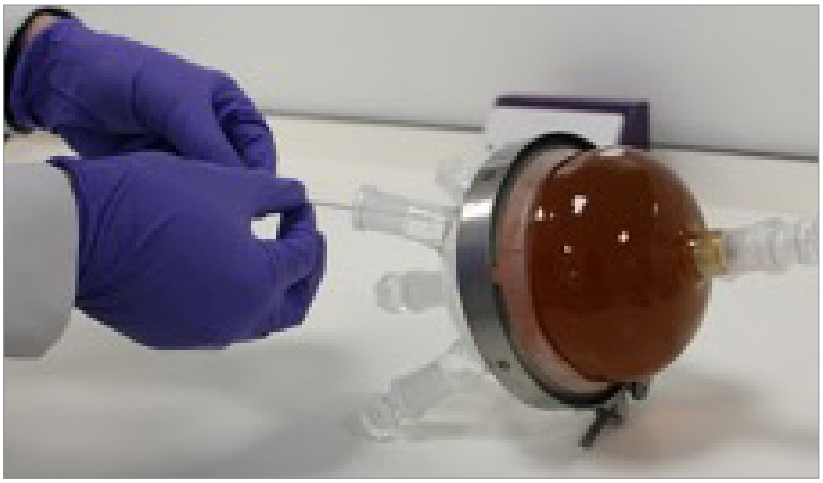

Figure 1. Bacterial barrier test model. A wound dressing is sandwiched between two sterile glass hemispheres. The wound contact surface of the dressing is challenged with a bacteria-inoculated suspension such as *Pseudomonas aeruginosa*. The outer (dry) surface of the dressing is swabbed and cultured daily to determine whether bacteria have penetrated the dressing, and if so, at what time point

proof dressings can therefore be of great value in preventing the spread of healthcare-related infections. *Figure 1* shows a test method set-up for investigating bacterial barrier properties of wound dressings. Briefly, a dressing is sandwiched between two glass hemispheres, and the wound contact side is challenged with a fluid culture of a pathogenic bacterium, e.g., *Pseudomonas aeruginosa*, or methicillin-resistant *Staphylococcus aureus* (MRSA). The outer (dry) surface of the dressing is then swabbed daily via the glass port holes to determine whether any bacterial cells have penetrated the dressing. A viral barrier method uses a similar principle, but a bacterium-infecting virus is used as a non-pathogenic surrogate for human blood borne viruses such as hepatitis B and HIV that may be transmitted via wound fluids.

Antimicrobial barrier

In addition to the physical barrier methods, antimicrobial dressings may provide a microbial barrier because of their antimicrobial agent rather than their physical ability. This test is suitable for porous/fibrous dressings, such as alginate, gelling fibre dressings, and involves placing a dressing onto an agar plate followed by inoculation of the top surface of the dressing with a fluid culture of

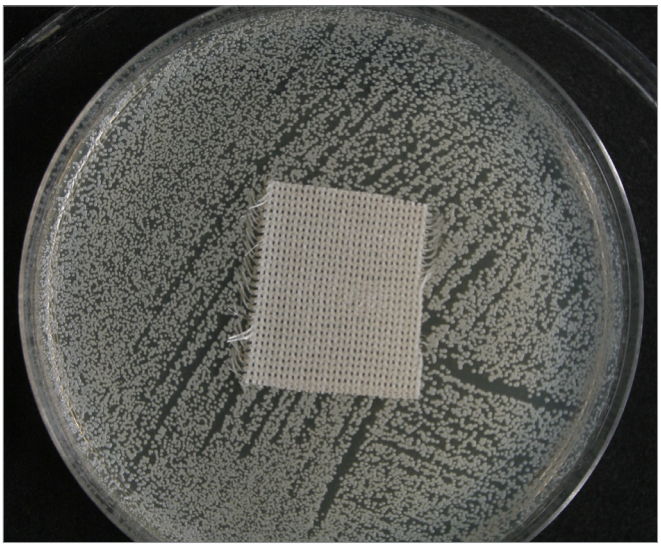

Figure 2. Zone of inhibition test with a non-antimicrobial dressing. A bacterial suspension (*Staphylococcus aureus*) is inoculated across the surface of an agar plate. A sample of a non-antimicrobial dressing (sterile gauze, negative control) is then applied to the surface of the agar plate. Following incubation, growth of bacterial colonies across the agar plate in observed, including growth around the dressing sample, indicating that the dressing has not inhibited the bacterium in the form of a zone of inhibition.

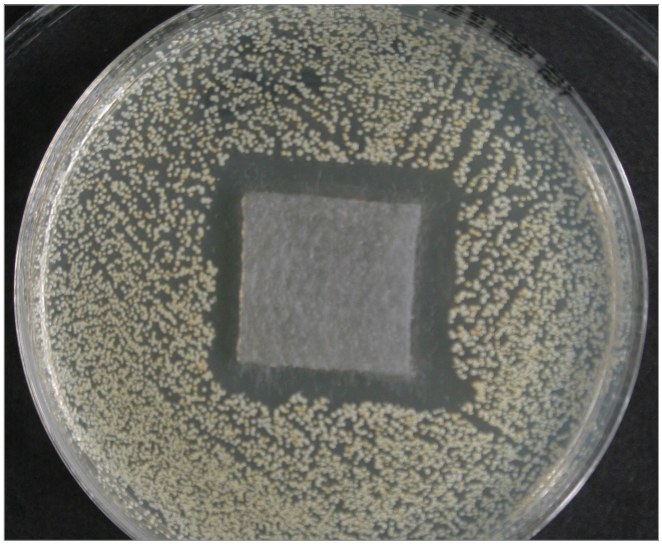

Figure 3. Zone of inhibition test with an antimicrobial dressing. A bacterial suspension (*Staphylococcus aureus*) is inoculated across the surface of an agar plate. A sample of an antimicrobial dressing is then applied to the surface of the agar plate. Following incubation, growth of bacterial colonies across the agar plate in observed, except for an area of no growth (zone of inhibition) around the dressing where the antimicrobial agent has leached-out and prevented bacterial growth.

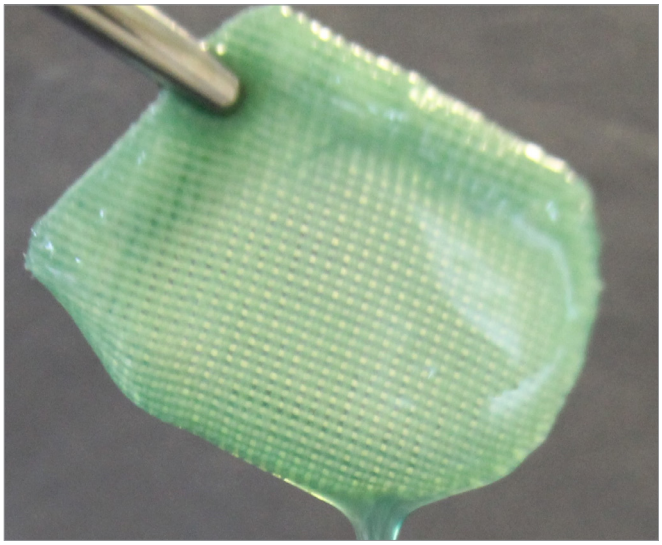

Figure 4. *Pseudomonas aeruginosa* biofilm slime. A piece of sterile gauze was immersed in a suspension of *Pseudomonas aeruginosa*. After 48 hour incubation, *Pseudomonas aeruginosa* cells have attached to the gauze and produced a thick, slimy biofilm. The green coloration is caused by a pigment produced by *Pseudomonas aeruginosa*.

bacteria. The dressing is incubated for 24 hours after which the dressing is removed, and the agar plate re-incubated for a further 24 hours to observe growth of any bacterial colonies that would confirm penetration.

Zone of inhibition (for antimicrobial dressings)

This is a relatively simple test that measures the migration (leaching) of an antimicrobial agent from a test material and enables a wide variety of bacterial species to be tested for their susceptibility to antimicrobial agents (antiseptics such as iodine and silver, and antibiotics). A small volume of a fluid bacterial culture (e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*) is evenly spread across an agar surface. A small piece of an antimicrobial dressing, e.g. 1cm^2 or 2cm^2, is then placed on the surface of the agar plate. After 24–48 hours incubation, bacterial colonies will have grown on the agar plate, but if it is susceptible to the antimicrobial dressing, a zone of inhibition around the dressing will be evident where leaching of the antimicrobial agent has occurred (*Figures 2–3* show examples of no zone, and zone of inhibition). This zone can be measured to enable comparison of the sensitivity of different organisms to the antimicrobial dressing (Jones et al, 2004).

Sustained antimicrobial activity and speed of action

Important claims for antimicrobial dressings include their ability to maintain activity throughout the wear time of a dressing, and to initiate antimicrobial activity rapidly once a dressing has been applied to a moist wound. The basis for a relevant *in vitro* test is the AATCC 100 method which is a quantitative internationally recognised test for assessing the efficacy of antimicrobial textile materials. It has now been adapted for testing wound dressings and can be used to measure both the length of time that the antimicrobial agent works against microorganisms in a simulated wound fluid (i.e., sustained activity), and the speed of action of the antimicrobial dressing. This method involves growing a bacterial species in a fluid culture medium that contains organic matter, such as foetal calf serum, to simulate wound conditions more closely (Bowler et al, 2004). Since organic matter can neutralise some antimicrobial agents, its inclusion creates a more stringent and realistic test for an antimicrobial dressing. Following the immersion of a small piece of dressing into the inoculated simulated wound fluid, small volumes are withdrawn at numerous time points, e.g., 30 minutes, 4 hours, 24 hours, 48 hours, 72 hours, to count bacterial cells and determine both the speed of action of the antimicrobial dressing, as well as its ability to sustain activity over a prolonged period of time (e.g., for the maximum duration that a dressing may be applied to a wound). Regulatory authorities such as the US FDA request that a wound dressing can show a 10,000 times reduction in microbial challenge population (compared with the starting population at time zero), against Gram-positive and Gram-negative bacteria (at least three relevant wound microorganisms of each), yeasts and fungi.

Microbial binding capacity

Some wound dressings can absorb, and trap wound microorganisms within or on the wound contact surface of the dressing by physical mechanisms (e.g., hydrophobic interaction, or gelling fibre trapping technology). This is considered to be beneficial

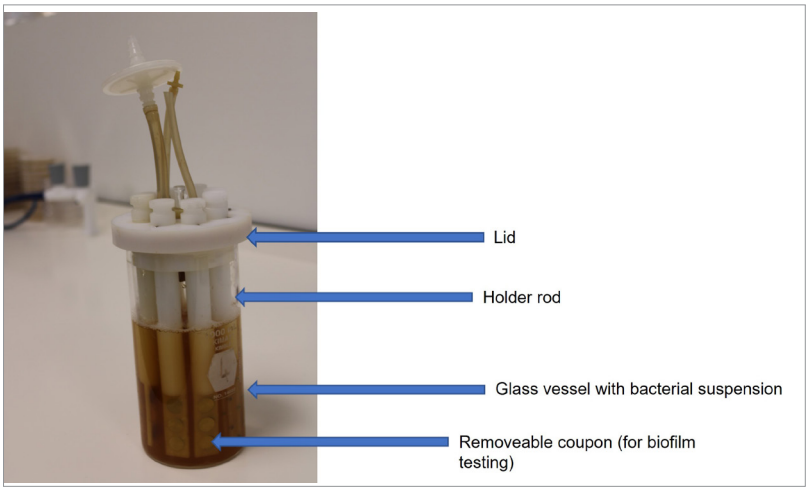

Figure 5. CDC reactor biofilm model. This reactor consists of eight polypropylene coupon holder rods suspended from a lid. Each rod holds three small coupons, and there are 24 coupons in total. The lid and holder rods are placed within a glass vessel into which a bacterial suspension is circulated and stirred. A uniform and consistent biofilm forms on the coupons over 24–48 hours, and the coupons can then be removed and washed prior to direct application of wound dressings to test for their antibiofilm properties.

in reducing wound microbial load without killing microorganisms. Although no standardised method exists for measuring microbial binding capacity, customised methods have been described (Bowler et al 1999; Rippon et al, 2019). Non-antimicrobial, microbial binding dressings can be assessed against both planktonic and biofilm-encased bacteria utilising appropriate customised and validated methods (Rippon et al, 2018).

In vitro biofilm test methods

The above examples of *in vitro* microbiological test methods are used commonly to meet both regulatory and commercial requirements. All such test methods have historically involved the use of 'planktonic' microbial cultures, meaning that the tests are performed against the free-living/swimming and susceptible forms of bacteria. However, in recent years it has become evident that bacteria found in hard-to-heal chronic wounds exist predominantly in the 'biofilm' form which provides much greater protection to bacteria against antimicrobial agents (e.g., antibiotics and antiseptics). A visual indication of biofilm growing on sterile gauze is shown in *Figure 4*). Biofilm is now known to prevent wound healing (Metcalf et al, 2013). Consequently, testing antimicrobial dressings against biofilm bacteria is the most stringent and relevant challenge, and is most representative of a chronic wound situation.

A variety of *in vitro* test methods now exist for investigating antibiofilm properties of antimicrobial agents, and methods have now been adapted for testing wound dressings (Suleman et al, 2020). Two of the most widely used biofilm models are the CDC (Centres for Disease Control and Prevention) biofilm reactor model (*Figure 5*), and the Drip Flow reactor model. These models are increasingly used in wound care and have been developed and validated to differentiate antibiofilm performance of dressings (Suleman et al, 2020). Adaptations of these methods typically involve the growth of biofilm on an inert, reproducible surface onto which wound dressing samples can be applied. Addressing factors such as substrate selection and simulated wound fluid flow rate enhances their applicability to chronic wound modelling. Additionally, validated customised *in vitro* test methods have also been used to test the antibiofilm properties of some wound dressings (Parsons et al, 2018).

CHALLENGES IN TESTING ANTIMICROBIAL DRESSINGS

Although standard, internationally recognised test methods can be used for testing some wound dressings (e.g., BS EN ISO 20645 for zone of inhibition testing of antimicrobial fabrics, and an FDA adapted version of AATCC Test Method 100 for testing antimicrobial wound dressings), such methods need to be modified to reflect wound conditions more accurately. Any adaptation to a standard method means that re-validation is required to ensure that the method will continue to produce reproducible and robust results. *In vitro* testing of wound dressings is further complicated by the fact that dressings are extremely variable in their type and construction, such as hydrocolloids, gelling fibre dressings, alginates, hydrogels, foams, and consequently it is very difficult to develop a test method that is suitable for all dressing types.

The variability in wound dressing types and constructs also significantly affects how an

Table 1. Watchouts and implications in in vitro test method design and data interpretation	
Test method 'watchout'	**Implication**
Ensure that both a positive control (i.e., material known to have an antimicrobial effect) and a negative control (i.e., material known to have no antimicrobial activity) are included in a test method.	This enables microbial growth curves in the presence of wound dressings to be compared directly against a test material that is known to have potent antimicrobial activity (i.e., positive control), and a test material that has no antimicrobial activity (i.e., negative control). Ideally this would involve an antimicrobial dressing being compared directly against the same dressing without the antimicrobial agent
Putting microbial numbers into perspective	A microbial reduction from 100,000,000 (8 log) to 10,000,000 (7 log) is equivalent to a 90% reduction. Whilst this number looks impressive, in antimicrobial reduction terms this reflects a low level of activity and would be insufficient to generate an antimicrobial reduction claim that would be accepted by regulatory authorities (a 99.999% reduction, [i.e., 10,000 times / 4 log reduction] would typically be required for regulatory approval)
Complexity of an *in vitro* test method	While an *in vitro* test method should simulate a clinical situation as closely as possible, it is also essential that a test method is robust, reproducible, and repeatable, and consequently restricting test variables to a minimum is an important consideration. An example would be including multiple organisms in a test method. While this approach is more representative of a chronic wound environment, one organism may outcompete another, so without careful selection and method validation, reduction in a particular organism may have been caused by being outcompeted by another organism, rather than the antimicrobial component in a wound dressing
Statistical interpretation (Error bars not shown)	Standard deviations show the spread of the data. When average numbers are shown without standard deviations the variation in the data set is unknown thus accurate conclusions cannot be drawn

antimicrobial agent may be made available from a dressing. Using silver-containing dressings as an example, some dressings readily and uncontrollably release silver, others provide a slower, sustained delivery, and silver availability from some dressings is compromised by the construction of the dressing. For example, a silicone adhesive wound contact layer can interfere with silver availability and hence antimicrobial action (Walker et al, 2011; Cavanagh et al, 2013).

Since complex hard-to-heal wounds are invariably polymicrobial, an *in vitro* microbiology model should ideally involve multiple species. But since microorganisms often compete with each other, introducing more organisms can increase variability in results. Consequently, creating a multi-species model requires careful planning and upfront work to determine how selected organisms will co-exist.

Although *in vitro* models should reflect the clinical situation as best possible, the more complex a model is, the more variables are introduced, and this compromises the robustness of a test method. Basic *in vitro* studies are likely to produce the most reproducible data sets and thus relatively small-scale studies can generate good, robust data. At the other end of the scale, the significant variabilities associated with clinical studies (no two patients or wounds are alike) means that a large participant number is typically required to produce reproducible data about a products performance. Consequently, a balance needs to be struck between simplicity and simulating a wound environment as closely as possible.

STUDY DESIGN AND INTERPRETATION

The quality of the data output from any *in vitro* study is dependent on the study design, inclusion

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of appropriate controls, and understanding the implications of the data and relevance to the clinical situation. Examples of 'watchouts' in the performance of, and interpretation of data generated from microbiological *in vitro* test methods are described in *Table 1*.

INDEPENDENT TESTING LABORATORIES

Given the physiological complexities that exist in chronic wounds, and the variations in type and construction of wound dressings, significant challenges exist in developing representative, robust, and reproducible *in vitro* models for testing wound dressings. While standard, internationally recognised test methods exist that can be applied to wound dressings, these methods invariably require adapting (and re-validating) to mimic a wound scenario more closely. In this respect, independent, accredited, and certified testing laboratories have a significant role to play in using, developing, and validating appropriate, customised test methods, and for providing accurate and impartial data in support of both regulatory submissions and commercial requirements.

SUMMARY

A range of laboratory-based test methods can be used to demonstrate the safety and performance/efficacy of wound dressings. *In vitro* testing provides a relatively quick (compared with *in vivo* studies) and reproducible way to ascertain the performance of a product, that can be extrapolated to predict its effect on multiple wound types. It is vital to select the appropriate test method to mimic the clinical scenario of interest and to ensure that the integrity and quality of the data is maintained.

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