Antimicrobial testing of healthcare and industrial products

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For the industrial microbiologist the testing landscape comprises a plethora of standardised testing methods. Often these are to be followed rigorously according to regulatory guidelines or rules. Sometimes the product or application may require some modification of a standard method to achieve a more realistic test result. Very occasionally, a method must be developed from scratch, based upon scientific principles and, where possible, utilising approaches used in either published papers or standards. Industrial testing is usually undertaken for one of two reasons, namely development work for new products or to satisfy regulatory requirements in order to market the product. This discussion will emphasise healthcare products, other than antibiotics, but similar principles will apply to other product categories also, albeit often to a lesser degree of testing.

Fundamentally an antimicrobial product will exert an inhibitory (static) or killing (cidal) effect on the bacterium, mould, spore, virus or parasite under study. This effect can be a very complex dynamic between the concentration of the active antimicrobial agent, length of contact time, temperature during contact time, presence of soil and the intrinsic resistance of the microorganism. Very few antimicrobials are effective against all classes of microorganism. More detailed reviews are available[1,2].

In looking to demonstrate an antimicrobial effect, there are a number of approaches that have been well documented and widely used. These range from demonstrating a zone of inhibition of growth in much the same way as an antibiotic susceptibility test is conducted[3], through qualitative and quantitative suspension tests to hard surface carrier tests where organisms are dried onto carriers before being immersed in product and then examined for growth or no growth. All of these may be described as *in vitro* or laboratory-based studies. Such studies usually represent the initial and secondary phases of development testing. There may also be some *ex vivo* studies done where the product is destined for use on skin. Such studies might use, for example, pig skin in an attempt to mimic the properties of human skin for application of product and monitoring an antimicrobial effect. Animal (*in vivo*) studies are more often used to examine the toxicology of an ingredient or product rather than for efficacy studies. Where products are intended for use on humans, such as antimicrobial hand washes or hand rubs, there is also the need for in-use studies, usually with volunteers. Now we'll look in some more detail at these tests by way of examples.

The zone of inhibition (ZOI) test employs a Petri plate containing agar and a supportive growth medium. To this an inoculum is added, either by further adding a top layer of agar with organisms present or by simply flooding the agar with organisms in suspension and then removing the surplus liquid and drying the remainder. It is important that the agar surface be dry to avoid the spreading of growth through any surface liquid which could then invalidate the test. After the plate is prepared the test substance is applied. This can be done by cutting a hole in the agar which is then filled with the test substance, applying the test substance to a sterile paper disc which is placed on top of the agar, or if the test substance is incorporated into an article the article may be placed directly onto the agar[4]. In the latter case, a top agar method is often used. After incubation any antimicrobial effects will be seen as ZOI around the applied test substance/article (Figure 1). These can be measured to gain a quantitative result but great care should be exercised in interpreting the results due to inherent limitations of the method. Some of these can be controlled such as the depth of agar, inoculum concentration, pre-incubation time and incubation time. Others may not, such as trying to compare aqueous with non-aqueous formulations because the basic principle relies on each test substance migrating through the agar at a similar rate. Nevertheless, the test can be used with good results to look for standardised antimicrobial effect in a finished product such as honey, or looking for synergistic properties of a number of aqueous product additives.

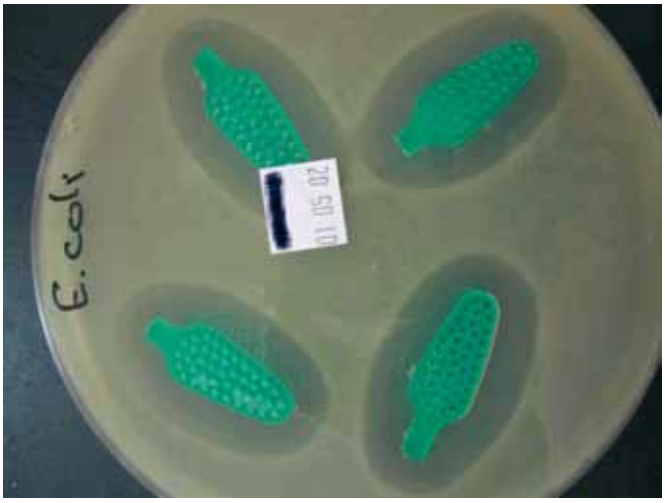

Figure 1. Zones of Inhibition of *E. coli* growth around a proposed antibacterial toothbrush head.

Probably the most widely used technique in antimicrobial testing is the suspension test. There are numerous standards published by several standards bodies which can be consulted[5-7]. Essentially the product or test substance is dispensed in a liquid form and the microorganism inoculum is added and mixed with it. Then after appropriate contact times, samples are taken and checked for growth or no growth or serially diluted and quantified. A variation is the minimum inhibitory concentration (MIC) test where the test substance is diluted in a microbial growth medium. Then after inoculation, the media are examined directly for growth or no growth to establish the lowest concentration of test substance that was able to prevent the test organism growth. This can be further developed to sub-culture the medium with inhibition of growth to a fresh medium with neutralisers present. In this way, further information can be gleaned to establish whether the test substance has a bactericidal effect. Thus the minimum lethal concentration (MLC) can be determined.

One aspect of antimicrobial testing that cannot be overemphasised is the need for suitable neutralisers of the test substance[8,9]. Examples of neutralisers are Tween 80, lecithin and sodium thiosulphate. These need to be established by performing validation studies. Such studies comprise spiking the test substance/neutraliser mixture with low numbers of organisms and then attempting to recover them in a similar manner to which would be used in the test recovery. Without such controls in place for the experimental protocols there can be no assurance that the results for a given contact time are correct because of the possibility that antimicrobial activity persists after the sampling event.

Three examples of suspension tests will be discussed to describe the variety and utility of the approach. The preservative efficacy test (PET) is a functional test for the effectiveness of preservatives in multi-use formulations[10]. These may be therapeutic products or consumer goods. There is a variety of test protocols to conduct this evaluation but essentially all follow the same principle. The product under test is inoculated with the organism/s of interest. For pharmacopoeial testing it is normal to inoculate a variety of organisms as single inocula. However, for personal care products it is also common for a mixture of organisms to be used. In some protocols there are one or more re-challenges with organisms. Once the product has been inoculated, samples are collected at different time intervals which may be as little as a few hours to as long as 28 days. Samples are then serially diluted and plated to quantify any survivors and results evaluated against the interpretive guidelines.

A useful method for evaluating the bactericidal or fungicidal properties of a formulated product is the Time-Kill study[11]. In a way, this is similar to the PET in that it uses the product inoculated directly with organisms as a starting point. Depending on the proposed label claims and use of the product, it is sampled at appropriate time intervals and tested for survivors. This information is then used to report the kinetics of the organism's death, which is normally reported as a logarithmic reduction or percentage kill relative to the inoculum.

Another variety of this kind of method is represented by the "TGA test" for evaluating disinfectants[12]. It is a modification of the "capacity" test originally developed by Kelsey and Sykes in the UK[13]. Actually, the TGA test comprises four different testing protocols, namely options A through D for different types of use. Option A and B are for hospital-grade disinfectants tested in "clean" or "dirty" conditions, option C is for household or commercial-grade disinfectants and option D is for antiseptics.

Table 1. Summary of TGA disinfectant test options requirements.

Option	Product class	Organisms used	Soil type	Number of challenges	Inoculum density
A	Hospital-grade – clean conditions	*P. aeruginosa* *P. vulgaris* *E. coli* *S. aureus*	HW [a]	2	$2x10^8 – 2x10^9$
B	Hospital-grade – dirty conditions	*P. aeruginosa* *P. vulgaris* *E. coli* *S. aureus*	HW [a] yeast	2	$2x10^8 – 2x10^9$
C	Household/ commercial	*E. coli* *S. aureus*	HW [a] nutrient broth	1	$2x10^8 – 2x10^9$
D	Antiseptic [b]	*P. aeruginosa* *P. vulgaris* *E. coli* *S. aureus*	HW [a] serum	1	$2x10^6 – 2x10^7$

[a] hard water; [b] excluding those for intact skin only

The various test options are summarised in Table 1. Once the product is made up to its use-dilution, the inoculum with soil is added and mixed. After eight minutes' contact, 20μl is transferred to five sub-culture tubes with nutrient broth and neutraliser. These tubes are then incubated and examined for growth or no growth. Providing at least three tubes show no growth, the test is said to have passed. This procedure is repeated on three separate occasions to demonstrate consistency of results. The test is thus a semi-quantitative method because, although specified numbers of organisms are used in setting up the test, the results are based purely on growth or no growth of the organism. It will be noticed that this test uses a very restricted variety of organisms, originally chosen to reflect 'hospital' environment organisms. The test forms part of the minimum performance requirements for hard surface disinfectants described in Therapeutic Goods Order 54[14]. This document provides a framework of performance tests to be met for the various categories for disinfectants; some others will be covered below.

In developing evidence of efficacy for a disinfectant product, it makes sense to do at least some of the tests using hard surfaces rather than in suspension tests. This has long been the approach in the USA and when Australia formalised its disinfectant regulatory testing regimen it incorporated hard surface carrier testing as a cornerstone of the evaluation process[14]. Unlike the suspension tests where organisms are inoculated directly into the product in suspension, the carrier tests attempt to be a little more realistic in the challenge mode by drying organisms onto test surfaces before using these to challenge the product. This is not to simulate a biofilm-type environment where organisms can become highly resistant to disinfection, but rather to replicate the transient attachment of organisms to surfaces that need to be disinfected. Under the American system described in a series of AOAC[7] standards, the carriers may be glass, stainless steel or porcelain depending on the organism under evaluation. Typically 60 replicates are used and after exposure to the disinfectant under the proposed use conditions, the carriers are transferred to a growth medium with neutraliser and incubated before again checking for growth or no growth. The methods are labour-intensive and laborious. They are also known to be prone to errors[15,16] that are a cause for concern in regulatory mandated testing and have led to the development of modified methods in an attempt to remedy the situation[17]. Here much smaller test surfaces (coupons) are used and the testing is completed in micro-centrifuge tubes to facilitate ease of processing. If adopted by regulatory authorities, it will greatly simplify the carrier testing methodology.

One thing most regulatory standards have in common is the use of mandatory standards that specify particular test strains of organisms. If sponsors of products wish to make specific claims for organisms not included in the standardised testing procedures then they are required to demonstrate efficacy with the specific organism. For example, most regulatory standards would include *Staphylococcus aureus* in the test organism test panel. However, if it was desired to extend the claim to include MRSA then this specific variety of *S. aureus* would need to be tested. When it comes to viruses this can pose problems as it is not always feasible to test the particular virus for which a claim is desired. In this case it is usual to use surrogate viruses instead of the actual virus. An example is the use of feline calicivirus for disinfectant studies instead of enteric norovirus, which is presently not culturable[18]. Similarly a panel of surrogate organisms have recently been proposed to evaluate disinfectants as general biocides[19] Thus the use of surrogates can enable a more complete description of a disinfectant's range of activity.

Finally, in conducting these laboratory studies it is now expected that work will be conducted under conditions of Good Laboratory Practice (GLP) as outlined in the international standard ISO 17025[20]. This approach encourages standardisation of procedures and the reproducibility required for a legislative framework for controlling the marketing of antimicrobial products in the healthcare industry. Hopefully, this short introduction will give the reader some insight to the world of regulatory and development testing of industrial products and, in particular, healthcare products.

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Biography

Dr Paul Priscott is Managing Director of AMS Laboratories, a therapeutic products testing laboratory based in Sydney and is also National Convenor of the Cosmetics & Pharmaceuticals Special Interest Group (CAPSIG) of the Australian Society for Microbiology.