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A global disinfectant standard for cleanrooms: Presenting a harmonised approach

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Abstract

Disinfectant efficacy testing is an essential part of a facility contamination control strategy. Pharmaceutical and healthcare products facilities must know that the products they are using can achieve effective levels of microbial kill across a range of surface types.

The problem faced by microbiologists and production managers is that the various national and international standards use different methodologies. In addition, these standards and methods have not been written specifically for cleanrooms and the microbial test panels and logarithmic reduction expectations are not considered suitable.

In order to develop an international standard suitable for all pharmaceutical facility cleanrooms, Ecolab has developed the Validex™ method, which is independently assessed in this paper. This method involves using 2 cm diameter disks, to target a 3 log reduction for vegetative bacteria in 5 minutes; and a 2 log reduction for fungi and bacterial spores in 10 minutes. The key criteria and rationale for selection, along with the methodology, are presented in this paper.

1. Introduction

Following the appropriate design of cleanrooms, utilising Quality-by-Design principles, and supported by appropriate personnel training and practising aseptic techniques, maintaining environmental control in a pharmaceutical manufacturing environment is largely dependent on the facility's cleaning and disinfection programme (1). The programme requires the selection of the appropriate disinfectants, an assessment of their capability to inactivate or kill vegetative cells, plus bacterial spores and fungal spores, as applicable and their proper application and use.

A disinfectant is a chemical agent that is used to reduce the number of viable microorganisms on pharmaceutical surfaces to an acceptable level; some disinfectants are 'broad spectrum' and can kill most vegetative bacteria, others can kill bacterial and fungal spores and are generally described as sporicidal disinfectants (or 'sporicides') (2). There are also differences with the viricidal properties of disinfectants, although the inactivation of virus particles from surfaces falls outside the scope of this paper.

Disinfectants can be classified according to different properties, that include chemical type, spectrum of activity, mode of action, and method of microbial kill or inactivation (3). These different classifications are additionally important when undertaking disinfectant rotation, in order to ensure that disinfectants with different modes of action are selected for rotational purposes.

How effective a given disinfectant is in practice is dependent upon several factors such as the type of microorganism; the microbial population; the location of the microorganism and whether it is attached to a surface (and the type of surface attachment); the degree of soiling (or 'interfering substances' acting as penetrative barrier) present; the concentration and contact time of the disinfectant; the method of the disinfectant application (such as spraying, wiping, mopping, etc.); and factors such as ambient temperature and pH (4). Disinfectant effectiveness is assessed through disinfectant efficacy testing where these factors are controlled.

A problem faced by microbiologists is with the array of different (and often contradictory) national and international standards. Not only do these standards have methodological differences, they offer differing and often unrealistic acceptance criteria (particularly in relation to cleanrooms). For those working in pharmaceuticals and healthcare, a global approach applicable to these sectors is required in the absence of a suitable international standard for the cleanroom. The Life Sciences division of Ecolab has developed a new approach to meet this need (termed 'Validex™'), and the criteria and application of the methodology are discussed in this paper.

2. Why disinfectant efficacy testing matters

Qualification of a disinfectant is demonstrated through performance testing to show that the disinfectant is capable of reducing the microbial bioburden found in a pharmaceutical manufacturing area. Of the primary tests, these are divided into suspension tests (phase 1 and 2), surface tests (phase 2) and field trials (phase 3). The field trial is the final piece of the qualification jigsaw and it is essentially an assessment of environmental monitoring data; the suspension and surface tests are generally taken to be the core disinfectant efficacy tests, and both need to be assessed as satisfactory before a field trial can commerce.

Of the first two phases, the surface test is the most robust. Suspension methods evaluate the reduction of a known organism population inoculated directly into a sample of the liquid disinfectant. While data from the supplier of a disinfectant product can be used for the assessment of the suspension tests (hence there is not ordinarily a reason for the user to repeat such testing), undertaking some form of surface test is normally expected of the user by regulatory agencies (5). This is not least because it may be that the disinfectant concentration or contact time shown to be optimal for the suspension test is no longer appropriate and instead needs to be increased, especially when assessed against different types of surfaces.

As the ability to kill or inactivate microorganisms in suspension (planktonic state) is easier than microorganisms attached to surfaces (sessile state), the surface test is the one disinfectant efficacy test required by most regulatory agencies; for example, as set out by The Pharmaceutical Inspection Co-operation Scheme (PIC/S), in document PI007, where it is stated: "The effectiveness of disinfectants and the minimum contact time on different surfaces should be validated" (6).

Further, with the FDA 2004 aseptic processing guidance: "The suitability, efficacy, and limitations of disinfecting agents and procedures should be assessed. The effectiveness of these disinfectants and procedures should be measured by their ability to ensure that potential contaminants are adequately removed from surfaces." (7)

A similar reference to the surface test appears in the 2020 draft of EU GMP Annex 1, in relation to the method of application: "The disinfection process should be validated. Validation studies should demonstrate the suitability and effectiveness of disinfectants in the specific manner in which they are used and should support the in-use expiry periods of prepared solutions." (8)

With the surface test, representative manufacturing surface samples are inoculated with a selection of microbial challenge organisms and allowed to dry. A disinfectant is then applied to the inoculated surfaces and exposed for a predetermined contact time, after which the surviving organisms are recovered using a qualified disinfectant-neutralizing broth and test method (surface rinse, contact plate, or swab). The number of challenge organisms recovered from the test samples (exposed to a disinfectant) are compared to the number of challenge organisms recovered from the corresponding control sample (a test article not exposed to a disinfectant) to determine the ability of the disinfectant to reduce the microbial bioburden.

Successful completion of the validation qualifies the disinfectant evaluated for use. The disinfectant efficacy validation should provide documented evidence that the disinfectant demonstrates bactericidal, fungicidal, and/or sporicidal activity necessary to control microbial contamination in the facility. An important consideration is with the types of surfaces to be selected and the appropriate panel of test microorganisms.

In surface tests undertaken by end users, regulators are not expecting the same results as per the supplier registration of disinfectants. In most countries, in order to sell any labelled bactericidal, fungicidal or sporicidal products, there is a legal requirement to demonstrate that the disinfectants are effective. The supplier must provide data that demonstrates efficacy through testing, and, following evaluation, registration occurs via an appropriate authority (for example, within the European Union this is under the Biocidal Product Regulation; and in the U.S. it is with notification to the U.S. Environmental Protection Agency). These registration requirements are typically against a standard panel of organisms, with the requirement for the disinfectant product to achieve very high logarithmic reductions of the microbial challenges and with the microorganisms typically being in suspension. Where surface testing is undertaken as part of the registration process, this is typically against one surface (such as stainless steel). Therefore, the submitted registration data does not always represent the efficacy profile for products as they are used in practice and in addition there are often variables in practice between testing laboratories. Hence the supporting testing will not give the cleanroom user confidence that the disinfectant is suitable for use within the pharmaceutical environment. Therefore, user-driven multi-surface testing, more reflective of operational conditions, is required.

However, in setting out to perform surface testing, it stands that there is no 'universal' approach to disinfectant efficacy testing. There are differences in the approaches between North America and Europe, and with the guidance issued by professional bodies. This array of approaches creates confusion for the end user and also leads to unnecessary levels of testing and sometimes unrealistic acceptance criteria being applied. Some of these differences are drawn out below.

3. Global differences in efficacy tests

Neither EU GMP nor U.S. FDA guidance make any specific reference to disinfectant efficacy test standards; however, as indicated above, both major international regulators mention the importance of the pharmaceutical manufacturer evaluating the efficacy of the disinfectants used. The aim of disinfectant efficacy validation is to provide documented evidence that the disinfectant demonstrates bactericidal, fungicidal, and/or sporicidal activity necessary to control microbial contamination in the facility.

Whilst there is no regulatory obligation to apply a specific method for disinfectant efficacy validation, there are standards available to guide the microbiologist through this process, these can include standards from the CEN (European Committee for Standardization); AOAC (Association of Official Analytical Chemists International) or ASTM (American Society for Testing Materials) standards (North America); or from other national standards (such as the Australian TGA); or draw on guidance from a professional body or from non-mandatory compendia, such as USP 40-NF35 Chapter <1072> "Disinfectants and Antiseptics" (9). These standards and guidelines are contradictory and, as this paper contends, not totally suitable for evaluating disinfectants to be used within the pharmaceutical cleanroom.

The disinfectant testing standards of greatest relevance to pharmaceutical facilities are:

- U.S. ASTM E2197-17 Standard Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals (10)
- U.S. ASTM E2614-15 Standard Guide for Evaluation of Cleanroom Disinfectants (11)
- Europe EN 1276: 2019 and EN 1650: 2019 Suspension-based test methods for evaluating bactericidal and fungicidal activity of disinfectants (12, 13)
- Europe EN 13697: 2015+A1:2019 Surface-based test method for determining bactericidal and fungicidal activity of disinfectants (14)
- Europe EN 13704: 2018 Suspension-based test method for evaluating sporicidal activity of disinfectants (15)
- Europe EN 16615: 2015 Surface-based test method for determining bactericidal and fungicidal activity of disinfectants with mechanical action (wiping) (16)

Differences between standards include the panels of recommended test organisms. Sometimes this is with different types of organisms or sometimes with different strains of the same organism (for example, the European standards and the U.S. standards use different strains of Escherichia coli: ATCC 10536 and ATCC 11229 respectively). Even with the strain and culture collection source, this may impact on the test outcome where the point between passing or failing the test is marginal. Another variable between standards is with the surface coupon sizes (which range dimensionally from 5cm2 down to 1 cm2, a factor that affects the neutralisation test step); furthermore with coupons, there are variances with the permitted inoculum volumes (from 50 µL down to 10 µL). Another difference is with the test recovery methods for microbial enumeration (such as swab method, surface rinse method, or contact plate method). Standards also vary as to whether interfering substances are present (and then which type of substance and what quantity). The use of interfering substances is a factor relating to cleaning, in the context of any soil remaining on the surface to be disinfected. It is worth noting that the disinfectant efficacy standards that include use of an interfering substance are designed and used across an array of industries. For the cleanroom environment it is recommended that cleaning to remove soiling is performed prior to application of disinfectant, for this reason end user validation is commonly only performed under clean conditions.

Yet another difference is whether physical/mechanical action is permitted ('wiping') in terms of the application of the disinfectant. Consideration should be made to the target starting microbial challenge level (starting inoculum) and the subsequent logarithmic reduction (level of 'kill') that need to be achieved in order to pass the test (such as reductions of 5, 4 or 3 logs, at logarithm to base 10 with each 'log' obtained representing a 10-fold reduction in microbial numbers). With these latter two factors, a 3 log reduction, for example, may have a very different meaning depending on the starting point. Consider: a 3 log reduction from 107 cells to 104 viable cells; this would constitute a relatively large reduction in the microbial cell count. However, a 3 log reduction from 104 cells to 101 cells would, in contrast, constitute a much smaller reduction in the cell count. Hence, defining the cell count reduction can therefore be very important. A point developed later is that the selected log reduction should be reflective of the typical and maximal challenges seen within the cleanroom.

In addition to the standard panel of prepared cultures, organisms isolated from the facility should also be tested, as per current regulatory expectations. Care must be undertaken in the selection so that organisms isolated from the areas of intended use are representative of cleanroom microbiota across different cleanroom grades and types of room use. Prior to being included in the validation, it is good practice to assess the viability and stability of the environmental isolate cultures demonstrated for a suitable length of time (17).

In reviewing these standards, it is important to note that most have not been written for the pharmaceutical sector specifically and they are instead multi-industrial (including industries where high levels of microbial contamination would be expected – from farms to fracking). The lack of a universal approach and the different nuances of the tests leads to user, as well as regulatory, confusion and often the consequential outcome of over-specifying the acceptance

criteria, selecting inappropriate microorganisms, using unrepresentative surfaces and so on. Many users will thus fail to obtain satisfactory disinfectant data, including rejecting disinfectants that are potentially suitable for the cleanroom environment.

It is for these reasons that a new approach is required for pharmaceuticals. One such approach, which this author has evaluated and supports, has been set-out and detailed by Ecolab Life Sciences. This approach is called ValidexTM.

4. Validex methodology and acceptance criteria

Given the aforementioned considerations, a global disinfectant efficacy strategy suitable for the pharmaceutical industry needs to be based on as series of important factors that are most applicable to cleanrooms. These factors need to form part of the disinfectant efficacy test, and they include:

1. The microorganisms used should reflect those commonly recovered from pharmaceutical facilities (including cleanrooms of different grades and applications, such as considering what is found within changing rooms compared with wash-bays).
2. The target logarithmic reductions to be achieved by the disinfectant should reflect the levels of contamination typically seen within pharmaceutical facilities, with reference to the cleanroom grade.
3. The surfaces challenged with microorganisms should be representative of the materials found in pharmaceutical cleanrooms (with additional consideration of the surface finish).
4. Contact times assigned to the disinfectant should reflect operational conditions (that is, contact times should not be unrealistically long and where the disinfectant remains wet).
5. The degree of soiling (surface cleanliness), should be reflective of cleanroom conditions.

Each of these factors is discussed below.

The factors can also help to inform about selection of a disinfectant. A disinfectant used in the pharmaceutical sector should possess a broad spectrum of activity. This refers to the ability of the disinfectant to kill different types of microorganisms. Furthermore, the disinfectant must be rapid in action with an ideal contact time of less than ten minutes. The contact time is the time taken for the disinfectant to bind to the microorganism, traverse the cell wall and membrane and to reach its specific target site (18).

5. Validex criteria and the rationale for inclusion

In relation to the key points about the Validex method, outlined above, the following section expands upon these and outlines the rationale for the scope of each item.

Representative microorganisms

Disinfectant standards include a range of recommended microorganisms, not all of which are of relevance to a typical pharmaceutical facility; in addition, there are organisms found in a greater predominance within the pharmaceutical setting which are excluded from the standards.
It is recommended that the test panel of organisms is populated with microorganisms commonly found in pharmaceutical facilities (19), to be assessed against the common types of surfaces. To construct an appropriate panel a review of the facility cleanroom microbiota should be undertaken. In undertaking a review, the microbiologist should ensure that cleanrooms of different grades and uses are assessed. The microorganisms recovered from a Grade B cleanroom within the aseptic core will be different from those recovered from a dirty equipment wash-room, for example. Notably there will be variations between different facilities within the same region, as well as regional and national differences with the common types of organisms isolated; such variations will be more abundant in lower grade cleanrooms where controls around personnel gowning are less strict.

Nonetheless, there are several types of organisms that either have direct association with the human microbiota of the skin or which are representative examples of areas where water is used or in terms of material transfer (where the risk of environmental cross-contamination exists). Examples of representative organisms, and which are detailed in the Validex approach as USP recommendations are listed below, this is not an exhaustive list as new and differing environmental isolates may be recovered within the end users' facility:

- Staphylococcus aureus
- Staphylococcus epidermidis
- Corynebacterium jeikeium
- Micrococcus luteus
- Escherichia coli
- Pseudomonas aeruginosa
- Burkholderia cepacia
- Bacillus subtilis
- Bacillus cereus
- Candida albicans
- Penicillium chrysogenum
- Aspergillus brasiliensis

The above organisms differ on the basis of being either Gram-positive or Gram-negative, in the case of bacteria (and hence have different cell wall compositions and thickness, including propensity to absorb an external chemical, which may react differently when they come into contact with different chemicals); between being bacteria or fungi; and with the ability to form spores or not. Further, these organisms are reflective of what is carried on operators (S. aureus, S. epidermidis, and M. luteus), linked with poor hygiene (E. coli), associated with water systems (potentially Ps. aeruginosa or B. cepacia), linked to equipment transfer (B. subtilis or B.cereus), or representative of common fungi - of both yeast-like and filamentous morphologies (C. albicans, P. chrysogenum, and A. brasiliensis). Such organisms should be drawn from a recognised culture collection. Where organisms can form spores (with the above, in the case of B. subtilis and the filamentous fungi) and the disinfectant to be evaluated carries a sporidical claim then it needs to be verified that the spores of the organism are being subject to testing rather than vegetative cells. In addition, efficacy studies need to be supported from organisms isolated from the manufacturing environment, especially where such organisms are different to the above list (as indicated above). For example, a more common fungus might be a species of Cladosporium (which has a strong link with the as-built environment and poorly operating HVAC systems).

General factors to consider when selecting additional organisms and potential sources of environmental isolates include:

- Ensuring that all process cleanrooms are reviewed (from Grade A to Grade D)
- Review data from water systems, especially where water is drawn in order to dilute disinfectants, or for areas where equipment is processed
- Assess data around airlocks, to gain an insight into organisms potentially arising from equipment transfer

- Assess data from changing rooms, for indicators of human skin residential or transient organisms
- Ensure that the review covers a sufficiently long period of time to account for variables such as seasonal variation

There are no absolute numbers for the inclusion of environmental isolates given that selection will depend on the available trends and how similar or dissimilar the organisms characteristic of the environment are, when they are compared to the recommended type cultures already included in the study. In practice, between 2 and 4 environmental isolates is common.

In addition to pinpointing common organisms, it may be appropriate to include other organisms which are not frequently recovered but which have been recovered from critical areas (such as Grade A) and which, from a literature review, may be theoretically more difficult to kill. Such reviews should be conducted periodically (for example, annually) drawing on microbial identification data. Following such reviews, additional disinfectant efficacy studies can be conducted if the microbial profile shows significant shift towards a particular organism of concern.

Different organisms will display different susceptibility profiles to different types of disinfectants. While organisms do not appear to develop resistance to a disinfectant or to low environmental concentrations of disinfectant, some organisms are inherently more resistant to a given disinfectant than others. Practical application of the disinfectant by the end user can affect how well a disinfectant will work, such as issues with concentration, contact time and the method of application. This is especially so with organisms which are inherently more resistant.

Appropriate microbial challenges

With challenge inocula, as discussed above, the challenge (starting inoculum) needs to be sufficiently high in order that an accurate cell reduction can be measured. In addition, starting inocula should be sufficiently high in order to compensate for in-test dilutions and to account for some loss of viability during drying. Furthermore, since the standard disinfection test methodology requires population reduction to be assessed using serial dilution; in order to avoid inaccuracies stemming from low organism populations, the use of higher starting challenges not only demonstrates a high-kill level it avoids issues that can arise from low population plate counts in terms of consistent recovery.

It is also important that the test inoculum is achievable and recoverable using a positive control produced using water, and prepared in a way that is robust enough so that the cell numbers can be recovered in triplicate. With bacteria, 1.5 to 5.0 x 107 CFU/mL is recommended; for yeast-like fungi and filamentous fungi, 1.5 to 5.0 x 106 CFU/mL is recommended; and for bacterial spores, 1.5 to 5.0 x 106 CFU/mL is recommended, within the Validex™ guidance. These levels of starting inoculum are lower than those stated in the disinfectant efficacy standards, however, as previously mentioned, the standards are designed for a wide array of industries and the parameters detailed in Validex™, established through scientifically robust development work (Ecolab Report TRB-2017-032-01), are designed specifically for the cleanroom environment where, by design, there is a much lower level of bioburden present.

With target levels of microbial kill (log reduction), the criteria should be reflective of the regulatory recommended permitted maximum levels of microorganisms in the cleanroom and then tracking the level of kill through an appropriate logarithmic reduction. With the log reduction, the highest microbial surface level permitted in an EU GMP Grade D cleanroom, for instance, is 100 CFU/cm2. Under this requirement, there is little value with seeking a 6-log reduction from a disinfectant. In this circumstance, a two or three log reduction is more than adequate.

A further reason for setting realistic challenges is because test organisms, whether type cultures or those isolated from the plant, will be grown as healthy laboratory cultures and challenged whilst in the logarithmic phase of growth. Such organisms are typically more resistant than organisms within the cleanroom environment, which are often not growing and subject to external stress factors (20).

Hence, it is important to set appropriate and realistic acceptance criteria so that products are not being unnecessarily or overly challenged, based on the fact that disinfectants are being used in a cleanroom environment. On this basis, recommended challenges under the Ecolab Validex™ proposal are:

Table 1: Target log10 reduction values

Disinfectant	**Acceptance Criteria (log_{10})**
Vegetative cells	>Log 3 (or 99.9% reduction)
Bacterial and fungal spores	>Log 2 (or 99% reduction)

The above acceptance criteria (as presented in Table 1) are equivalent to those recommended in USP <1072>, reflecting the realistic microbial challenges within cleanrooms. Therefore, where contamination is at or above permitted Grade D levels (which would be reflective of a poorly controlled facility), the user would have test data to support the appropriate level of contamination reduction required to bring the facility well within the expected level of microbial control.

Representative surfaces

Prior to initiating disinfectant efficacy validation, a comprehensive survey of the materials comprising the room surfaces (floors, walls, windows) and equipment (such as stainless steel, acrylic, polyvinyl chloride and so on) present in the facility and which could potentially be exposed to the disinfectant, needs to be conducted. Where there are many different surfaces, a bracketing strategy can be adopted. Factors such as porosity and the effects of prolonged exposure to the disinfectant should be considered when identifying which surfaces are “worst case” (21).

In terms of selecting common surfaces, the Ecolab Validex™ approach was to construct a matrix to enable cleanroom managers and microbiologists to input different surface types in order to select appropriate surfaces for testing. This matrix draws on the following ‘worst case’ factors:

- Hydrophobicity
- Surface roughness
- Potential for chemical interaction at surface
- Prevalence
- Contamination risk (such as with horizontal surfaces are a greater risk than vertical; plus, those surfaces that are frequently touched)
- Proximity to product, areas where critical activities are performed

From this assessment of material properties and composition, common surface types may include:

- Vinyl flooring
- Epoxy coated flooring
- Glass
- High density polyethylene (HDPE) plastic bags
- Stainless steel (grade 304)
- Powder coated steel
- Plastic polycarbonate
- Plastic vinyl curtains
- Trespa® (high pressure laminate as used as a laboratory bench material),
- Polyurethane coated walls
- Polymeric flooring
- Plexiglass
- Methyl methacrylate (MMA) / Acrylic flooring
- Polycarbonate
- Gloves

The surface finish quality for the coupons used in disinfectant efficacy studies typically resembles that of a newly fabricated material. It is recognised that facilities will have materials that are older and where material becomes damaged, abrasions can provide reservoirs for microorganisms or easier opportunities for surface attachment. However, creating surface abrasions and roughness is not a variable that can be controlled and hence it cannot be introduced into the test method. In addition, where surfaces become particularly damaged in cleanrooms, the surfaces should be repaired or replaced.

Surface size

In developing the coupon (a regular sized piece of each material for the testing), it is necessary that the size of the test coupon is standardised to ensure consistency of testing for replicate tests on surfaces of the same material types and to facilitate comparisons between different surfaces. Experimental data indicates that a better recovery is generally obtained from a smaller sized coupon (Ecolab Report TRB-2017-032-01).

The disinfectant efficacy test uses samples of the surface materials discussed above. These need to be of a defined size. With selecting the size of the samples (or 'coupons'), the disinfection standards differ. From one perspective, if the same sample inoculum and disinfectant is applied to a surface, the size of the surface should not be an influencing factor. However, in developing the Validex method is was shown there are differences between test outcomes in relation to the volumes applied and the surface size. Experimental data, using a consistent volume and using disks made from stainless steel of 2 cm and 5 cm sizes, showed that optimal log reductions were obtained through the use of 2 cm diameter sized disks. The finding applied to 10 µl and 50 µl volumes. Therefore, this size of test surface is recommended. Based on the 2 cm surface size and considering the above discussion about optimal recovery, the Validex approach recommends 50 µl for organism inoculation and 100 µl for disinfectant application to the surface (Ecolab Report TRB-2017-032-01).

Contact times

There is little value in evaluating disinfectants targeted to kill vegetative microorganisms with contact times for longer than 5 or 10 minutes. One reason is practical; a busy pharmaceutical facility will not be able to wait for 60 minutes to use a cleanroom or item of equipment every time a disinfectant is applied. Another reason is scientific; given the rapid air changes found in many cleanrooms and clean air devices (especially at EU GMP Grade A where there can be fast air movement, higher temperatures and low humidity), treated surfaces will dry quickly and no surface will remain 'wet' for a prolonged period, triggering the need for repeated application. Therefore, relatively short contact times should be targeted from the outset of selecting a disinfectant.

The 5 to 10-minute target contact times is applicable to kill or to inactive vegetative cells. It is recognised that longer contact times are expected for sporicidal disinfectants (15 to 20 minutes or longer). However, these extended contact times can be more easily accommodated, given that most rotational regimes use sporicides less often than standard disinfectants. To represent practical conditions, a 5 minute contact time should be targeted for vegetative bacteria and a 10 minute contact time targeted for fungi and bacterial spores. For some disinfectants, these times may need to be increased. In terms of the data relating to different surface materials, the surface that requires the longest contact time in order to kill the target organism should be the time that is used or applied to all surfaces. Having different contact times for use within the facility will undoubtedly lead to human error; therefore, a single contact time should be used for consistency.

Temperature

The temperature at which a disinfectant is applied can alter the efficacy of the disinfectant. Disinfectants which are sensitive to temperatures (or to be used at temperatures other than at ambient) can be assessed through the use of a temperature coefficient, or Q10 (which relates the increase in activity to a 10°C rise in temperature, or corresponding decreases in activity) (22). Given that most cleanrooms operate at ambient and temperatures in cleanrooms tend not to fluctuate widely given HVAC controls), the Validex™ approach uses 18-25oC as standard. Where disinfectants are used in cold rooms (typically 2-8oC), separate evaluations may be required and the outcomes are likely to vary between disinfectants manufactured from different ingredients. The outcome could be an increase in concentration or with the contact time (23).

Disinfectant dilution

Disinfectants that are not supplied ready-to-use are presented as concentrates, which require dilution in order to create the in-use concentration.

Disinfectant standards default to the use of 'water-of-standard-hardness' (essential potable water, with hardness linked to the amount of dissolved calcium and magnesium in the water) as the diluent (within European disinfectant norms this is water containing (300 mg kg −1 $CaCO_3$) (24). Within pharmaceutical environments, water of pharmaceutical grade is required (that is water which has been through a purification process, which not only chemically treats the water, but which also reduces the bioburden to a low level). The Validex™ approach uses water for injection (WFI) as this is reflective of in-use conditions found in pharmaceutical facilities.

Disinfectant neutraliser selection

An appropriate neutraliser is required as part of the disinfectant efficacy validation method. Neutralisation testing proves that the assay method used adequately neutralises any antimicrobial agent in the product that could interfere with testing. The neutraliser needs to be effective against the chemical formulation of the disinfectant and non-toxic to the test organisms. The Validex™ approach recommends suitable neutralisers for different types of disinfectants, as set out in Table 2.

Table 2: Recommended neutralisers against different disinfectants

Disinfectant type	**Recommended neutraliser**
Quaternary ammonium compounds, amphoterics, amines	Lecithin, saponin, polysorbate-80, sodium dodecyl sulphate
Oxidising compounds e.g. chlorine, hydrogen peroxide, peracetic acid	Sodium thiosulphate, polysorbate-80, lecithin. Catalase is recommended for hydrogen peroxide products
Alcohols	Lecithin, saponin, polysorbate-80

Culture media

There is a range of different culture media and incubation times and temperatures available to the microbiologist (25). The culture media selected and the parameters to trigger growth should be suitable for the recovery of specific organisms. The Validex approach makes recommendations in terms of culture media:

- Tryptone soya broth / agar - for vegetative bacteria.
- Glucose yeast extract agar - for bacterial spores.
- Malt extract agar / broth - for fungi (yeast and filamentous fungi).

Sabouraud dextrose agar was evaluated for yeast as an alternative to malt extract agar. As part of organism preparation, sporulation agar / broth are recommended for the preparation of spore forming organisms.

The recommended incubation temperatures and times are, as per Table 3:

Table 3: Incubation temperature and time parameters

Organism	**Incubation temperature**	**Incubation time**
Bacteria (type cultures)	36°C (±2.5°C)	48 hours (± 4 hours)
Bacteria (environmental isolates)	32°C (±2.5°C)	48 hours (± 4 hours)
Yeast-like fungi	30°C (±2.5°C)	48 hours (± 4 hours)
Filamentous fungi	30°C (±2.5°C)	3 – 5 days
Bacterial and fungal spores	30°C (±2.5°C)	3 days

Soiling

Disinfectant standards outline challenges with soiling present (where bovine serum albumin [BSA] is often used to represent 'soil'). The application of additional soil (to create so-termed 'dirty' conditions) should not be necessary when evaluating disinfectants in a pharmaceutical facility since disinfectants should be being applied to clean surfaces.

In adopting the 'clean' condition, a small level of soiling is still incorporated given that cleaning efficiency is not straightforward to evaluate down to the level of complete elimination of soil from a surface. The standard soil is bovine serum albumin, at a concentration of 0.03% (this is part of the Validex™ approach, and it is in keeping with the existing standards). The addition of a small amount of proteinaceous material also assists with the survival of some Gram-negative organisms where viability would otherwise be lost during the drying of the organism onto the test surface, prior to the application of the disinfectant. The validation of the drying conditions, based on an assessment of cell or spore population and viability, is an important pre-step prior to commencing the disinfectant study.

6. Validex™ Methodology for the pharmaceutical products sector

Based on the above criteria, and supporting rationale, the Validex™ method was designed and subject to robust comparative testing. The method requires the use of surface disks of 2 cm diameter, with the range of surfaces to be tested drawn from a matrix. Testing is undertaken under 'clean' conditions, with tests performed in triplicate in order to demonstrate reproducibility. The Validex™ test panel of microorganisms includes:

- *Staphylococcus aureus* ATCC 6538
- *Escherichia coli* ATCC 10536
- *Pseudomonas aeruginosa* ATCC 15442
- *Bacillus subtilis* ATCC 6633
- *Candida albicans* ATCC 10231
- *Penicillium chrysogenum* ATCC 11709
- *Aspergillus brasiliensis* ATCC 16404
- *Staphylococcus epidermidis* ATCC 12228
- *Micrococcus luteus* ATCC 49732
- *Burkholderia cepacian* ATCC 25416
- *Penicillium chrysogenum* ATCC 11709

The organisms selected should be traceable to an approved culture collection (American Type Culture Collection strains are referenced above). The strains recommended are in keeping with pharmacopeia test strains for method suitability testing in relation to bioburden and sterility test assessments. In relation to the recommended organism list, environmental isolates based on cleanroom microbiota trending can be added (28).

In terms of suitable contact times, reflective of practical considerations within the cleanroom, the target should be 5 minutes for vegetative bacteria and 10 minutes for fungi and spore forming bacteria. With log reductions for these microbial challenges, based on the typical (and maximally permitted) levels of contamination found in a facility, a 3-log reduction of vegetative bacteria and a 2-log reduction of fungi and bacterial spores will be sufficient in order to maintain facility microbial control. These criteria are assessed under a typical 'ambient' temperature range of 18-25oC.

With log reductions, these are reported to two decimal places. Replicate tests need to be within 1-log difference of each other to be valid. A further factor affecting test validity is with the control samples, which must demonstrate the appropriate challenge level. The final assessment for whether or not a disinfectant has passed the test, for a specific surface and against a challenge organism, is subject to the formula set out in the European disinfectant standards, comparing the difference between the number of survivors from the treated surface against the challenge inocula (the control organisms).

For evaluation, the Validex™ method was performed across three laboratories and additionally in one laboratory using three different laboratory technicians to examine the same disinfectant, surface, and microorganism sets. To assess reproducibility and repeatability between laboratories, standard scores (also referred to as z-scores) were calculated to assess variability. A z-score represents the number standard deviations from the mean and can be placed on a normal distribution curve. Hence, the z-score indicates how far above or below average a score is from the mean (29). Through the laboratory comparison, based on a normal distribution of 97.72%, the z-score range was between -2.0 and +2.0, which is indicative of a low level of variation (a score of above 3.0 is deemed unsatisfactory).

7. Discussion

The selection of a disinfectant for use in a cleanroom is a complex decision for there is no single disinfectant that is effective against all microorganisms. The microbiologist must tread along a path divided between scientific properties, practical application, and safety considerations. On making the selection, disinfectants should be evaluated under simulated-use conditions, integrating the net effects of disinfectant preparation, concentration, application and contact time with different challenge organisms and surface properties.

For disinfectant evaluation, there is no standard method outlining step-by-step instructions of how disinfection qualifications studies should be conducted. Furthermore, the global disinfectant standards as they are currently constituted are not wholly suitable for cleanroom disinfection evaluations. The Ecolab Validex™ approach, which has been independently assessed in this paper, offers a more realistic means to perform an evaluation of disinfectants, to the level where facility control can be demonstrated.

Based on the criteria outlined in this paper, validation studies should prove that disinfectants:

- Are effective against the types organisms encountered in cleanrooms
- Can reduce microbial populations down to a suitably low level, based on the maximal levels typically recovered from cleanroom surfaces
- Are compatible with and effective against microorganisms found on the predominant surfaces used in the cleanroom
- Do not leave residues on cleanroom surfaces that would interfere with organism recovery from contact plates

These important considerations need to be captured within a disinfectant test protocol. With each of the key aspects, the rationale for each must be explained and documented. Following execution of the protocol a report must be generated, containing any methodological deviations and out-of-specification or invalid test results. The report must conclude as to the disinfectant efficacy test outcome in relation to the acceptance criteria.

For disinfectants which pass the test and are then deemed suitable for use within the cleanroom, cleanroom procedures must reflect the practices adopted during the qualification, such as disinfectant concentration, contact time and method of application to surfaces. The adoption of a disinfectant for the cleanroom requires a follow-up assessment based on a field trial (sometimes known as a phase III trial) which includes an evaluation of microbial counts and species recovered.

In terms of future actions, a future ValidexTM trial may involve the use of mechanical action. The method of application for most disinfectants to a surface is either by spraying and wiping or mopping (activities that are commonly described as involving 'mechanical action'). This is because there are advantages with this, since wiping can increase the efficacy of disinfection as well as physically removing particulates, soiling materials (and residues), and microorganisms (microorganisms that are detached from surfaces are easier to kill) (11). The activity of wiping additionally ensures a controlled delivery of the disinfectant onto the surface. Therefore, the inclusion of mechanical action is an important part of disinfectant efficacy evaluation.

In summary, in relation to the Validex™ approach the following standard test criteria are appropriate for the cleanroom setting. The key factors that shape the Validex™ approach are described in Table 4.

Table 4: Main Validex testing factors and acceptance criteria

Factor	**Test criteria**
Starting inoculum	1.5 to 5.0 x 10^7 bacteria, 1.5 to 5.0 x 10^6 yeast, bacterial and fungal spores
Surface size	2 cm (coupon diameter)
Test temperature	18-25^{o}C
Contact time	5 mins vegetative bacteria, 10 mins yeast, bacterial and fungal spores. (Tolerance ± 10 seconds)
Testing cycle	Triplicate testing (any retests to be performed twice in triplicate)
Acceptance criteria	3 log vegetative bacteria, 2 log yeast, bacterial and fungal spores

The Validex™ standard guides the user to select microorganisms that are representative of the manufacturing plant; to use populations at levels based on actual environmental monitoring data; and to ensure challenges are conducted using representative surface materials. Through this the Validex™ standard can help to nudge the pharmaceutical sector towards the execution of better targeted and more meaningful disinfectant efficacy studies.

With the Ecolab Validex™ approach setting out appropriate and reproducible criteria for the evaluation of disinfectants for use in facility cleanrooms, it would be useful for pharmaceutical manufacturers and health organisations, together with manufacturers of disinfectants, to press for this type of approach to become the de facto standard. This can happen through users developing protocols along the lines recommended, having disinfectant suppliers recommending the revised criteria, and, most importantly, engaging with regulators to present the scientific arguments behind the approach and seeking global acceptance.

Through such measures it should be possible to construct a global standard for disinfectant efficacy testing which is applicable to the pharmaceutical sector.,

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