Disinfectant testing for veterinary and agricultural applications: A review

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Abstract
Disinfectants for veterinary and livestock use, plus skin antiseptics, are critical elements for the control of infectious agents, including zoonotic and antimicrobial-resistant micro-organisms, in managed animal species. Such agents impact animal welfare, economic performance and human health. Testing of disinfectants is needed for safety, efficacy and quality control. The present review examines the principal types of test (carrier, suspension, surface and field) that have been developed or attempted, plus the features inherent in the respective tests, particularly with respect to variability. Elements of testing that have to be controlled, or which can be manipulated, are discussed in the context of real-world scenarios and anticipated applications. Current national and international testing regimes are considered, with an emphasis on the UK, continental Europe and North America, and with further detail provided in the Supporting Information. Challenges to disinfectant efficacy include: the nature of the biological targets (bacteria, fungi, yeasts, spores, viruses and prions), the need for economical and safe working concentrations, the physical and chemical nature of contaminated surfaces, constraints on contact times and temperatures, the presence of organic soil and other barrier or neutralising substances (including biofilms), and thoroughness of pre-cleaning and disinfectant application. The principal challenges with veterinary disinfectant testing are the control of test variability, and relating test results to likely performance in variable field conditions. Despite some ambitions to develop standardised field tests for disinfectants, aside from skin antiseptic trials the myriad problems such tests pose with respect to cost, reproducibility and generalisability remain intractable.

KEYWORDS
bacterial pathogens, livestock, poultry, veterinary medicine, viral pathogens

Impacts
- The testing of disinfectants and antiseptics for veterinary and agricultural sectors is essential to establish effective usage conditions for general hygiene and to control the transmission of animal and zoonotic pathogens, plus antimicrobial-resistant bacteria, on farms, personnel, and in the food chain.
• Many real-world factors modulate disinfectant action, and a variety of basic and application-focused tests are needed to address these.
• There has been some progress in development of international testing standards, but standardised field tests currently remain unachievable.

1 | INTRODUCTION

1.1 | Biocides: definitions, use in veterinary settings and the need for testing

The control of micro-organisms affecting production and companion animals, and colonising their environments, is dependent on effective use of biocides. Such organisms include pathogens and antimicrobial-resistant organisms that can be transmitted to humans, either via the food chain or directly, such as Salmonella, Campylobacter and methicillin/meticillin-resistant Staphylococcus aureus. The present review addresses the principles and challenges of disinfectant testing and examines contemporary testing regimes for biocide efficacy in veterinary, farming and related settings.

Disinfectants are applied to inanimate surfaces to rapidly kill or inactivate micro-organisms and sometimes spores. Antiseptics have similar activity but are used on living tissues, and are considered here insofar as they relate to skin hygiene. Disinfectants generally disrupt membrane function and/or interfere with nucleic acids or cytoplasmic components of biological targets, and typically involve multiple target sites (Maillard, 2002; McDonnell & Russell, 1999; Morente et al., 2013).

Beyond basic testing of the biocidal activity of disinfectant agents, appropriate ‘in-use’ working concentrations and exposure times need to be established, for general and special applications and for operator and environmental safety considerations. In principle, test results should be repeatable within the same laboratory and reproducible in different laboratories. They should also yield results (pass/fail or quantitative values) that relate to anticipated applications.

1.2 | Factors affecting disinfectant efficacy in the field

Important factors influencing the action of livestock and veterinary disinfectants are summarised in Figure 1. Factors of particular

![Diagram of disinfectant efficacy](image)
relevance to laboratory testing include: temperature, disinfectant concentration and exposure time, organic soil, biofilm, effects of drying, and qualities of surfaces and of diluting water.

Organic soil is almost universal in livestock environments and such material is strongly associated with attenuation of disinfectant efficacy (Brouillard-Delattre et al., 1994; Kirk et al., 2003; Luyckx et al., 2017; Steinmann et al., 1995; Wales et al., 2006), but it has markedly differing effects between microbicidal agents (Bessemens, 1998). Biofilms comprise extracellular polysaccharide and protein matrices constructed by mono- or multi-species bacterial and/or yeast communities on surfaces and at fluid/gas interfaces. Biofilm matrix presents a diffusion barrier and a neutralising environment for some biocides. This protective effect varies with: substrate surface, age of biofilm, hydration, fluid shear conditions during biofilm formation, and the mix of microbe species in the biofilm (Akinbobola et al., 2017; Steinmann et al., 1995; Wales et al., 2006), but it has marked resistance to disinfectants, ease of manipulation and culture, diversity, and variation in disinfectant concentration according to local surface features or evaporation. This allows some additional examination of field-type conditions. Surfaces may be inert (such as steel, glass or concrete) or biological, which includes skin or substitutes thereof (important for hand hygiene) or a complex matrix in the case of biofilm. Biofilm is potentially of great significance in infrequently-cleaned and disinfected environments such as animal housing.

Field tests typically have been reported in livestock and human hospital environments. Their value potentially lies in comparative evaluation of differing protocols and agents in similar environments, and in identifying scenarios (environment, surface or equipment) that pose particularly stiff challenges. Furthermore, such tests may identify common pitfalls and practical limits to what can be achieved with certain approaches (Decun et al., 2009; Roesler et al., 2007). However, many factors militate against standardisation in agricultural settings. These include: local water quality, prevailing temperature and relative humidity, varied materials and configurations (structural, flooring and equipment), damaged surfaces, the practicability of comprehensive cleaning, presence of biofilms, the density of bacterial communities, prevailing bacteria and/or viruses, plus drainage and airflow affecting pooling and drying (Burbarelli et al., 2015; Connor et al., 2017; Otter et al., 2015), and they are markedly less susceptible than suspended cells (Cabeça et al., 2012; Harding et al., 2014).

In field situations, gross organic soil, biofilm and post-cleaning residual hydrophobic organic material on surfaces can protect persistent resident pathogens despite regular cleaning and disinfection (C&D), for example in the case of Salmonella in hatcheries. The selection and application of pre-disinfection cleaner can substantially affect disinfectant performance in the face of organic soil and/or biofilm. Cleaning agents (usually detergents) may also show substantial microbicidal activity in their own right (Hancox et al., 2013) and may variously interfere or synergise with the action of a subsequently-applied disinfectant (Gosling et al., 2017).

A post-disinfection drying downtime of one or more days may add substantially to the observed microbicidal effect of C&D procedures, at least for materials where effective drying occurs (Connor et al., 2017; Hancox et al., 2013; Martelli et al., 2017). Furthermore, drying between applications of cleaner and of disinfectant will allow porous surfaces to absorb more disinfectant in the latter stage (Böhm, 1998).

1.3 | Types of disinfectant tests

Approaches to disinfectant testing have been reviewed by Reybrouck (1998). Biological targets are usually defined bacteria, yeasts, spores, viruses, or (latterly) prions, or sometimes mixtures of these. The principal types of test (carrier, suspension, capacity, surface and field) are summarised in Table 1.

Simple minimum inhibitory or minimum bactericidal concentration (MIC or MBC) multi-well suspension tests can be used to screen for activity (Bloomfield et al., 1994; Roesler et al., 2007) and to avoid approving an in-use concentration that is below the disinfectant’s MIC, which can happen with short, ‘clean’ tests with low nutrient availability and minimal organism adaptation time (DVG, 2017a). Performance can be further assessed using suspension tests, typically at anticipated ‘in-use’ concentrations, with and without interfering substances added to the solution to mimic organic soil.

Surface tests introduce additional variables, for example: surface characteristics, attachment and changes in viability of target organisms before disinfection, and variation in disinfectant concentration according to local surface features or evaporation. This allows some additional examination of field-type conditions. Surfaces may be inert (such as steel, glass or concrete) or biological, which includes skin or substitutes thereof (important for hand hygiene) or a complex matrix in the case of biofilm. Biofilm is potentially of great significance in infrequently-cleaned and disinfected environments such as animal housing.

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2 | KEY FACTORS IN THE DESIGN AND CONDUCT OF TESTS

2.1 | Choice of biological target

2.1.1 | Bacteria, yeasts and fungi

Principal considerations have included: operator safety, intrinsic resistance to disinfectants, ease of manipulation and culture, diversity, and relevance to disinfectant application environments. Hospital infections and human disease potential have heavily influenced such choices, with Escherichia coli and Pseudomonas aeruginosa (Gram-negative), Staphylococcus aureus and Enterococcus spp. (Gram-positive) often being employed (Bloomfield et al., 1994; Cabeça et al., 2012). For some specific applications, other organisms may
TABLE 1 Outline of the principal types of disinfectant test

| Test type                  | Features & process                                                                 | Outcome(s) measured                                      | Comments                                                                                       |
|----------------------------|-----------------------------------------------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Carrier                    | Physical ‘carrier’ object (e.g. metal cylinder, wire loop) coated with biological target, immersed in excess disinfectant then cultured, with or without neutralisation | Presence/absence of growth from carrier. Semi-quantitative results may be obtained from culture of multiple carriers | Oldest type of test. Example references Lück and McDonald (1983) and Gangi et al. (1997)          |
| Suspension                 | Aqueous suspension of planktonic bacteria or other target, mixed with disinfectant. Culture (typically semi-quantitative or quantitative) after neutralisation | Presence/absence of growth or reduction of viable counts compared with controls | Inherently repeatable given uniform association between target and disinfectant, plus tight control of concentrations |
| Capacity                   | Target organisms in suspension added incrementally to disinfectant solutions at or near ‘in-use’ concentrations. Aliquots of test mix taken after each addition for neutralisation and culture | Reduction in viable count compared with amount of target/organic soil added | Addresses the repeated use of solutions in hospitals for cleaning and disinfection, e.g. by mopping (Kelsey & Sykes, 1969) |
| Surface (or ‘surface carrier’) | Target attached (typically dried-on, can be biofilmed or within organic soil matrix) to a surface of defined chemical/biological and physical features (e.g. steel, wood, rubber), and covered with a modest volume of disinfectant. After neutralisation, target recovered, usually by sonication and/or scraping, for quantitative culture or other detection of viability system | Typically, reduction of viable counts compared with controls | Combination of many variables including dynamic elements (diffusion, evaporation) means this test type can be regarded as a test of disinfection process, not simply activity (Reybrouck, 2007). More susceptible to issues with repeatability than suspension tests |
| Field                      | Attempts to evaluate efficacy in real-world environments with protocols that follow recommended or observed practice. Wide range of possible scenarios. Also testing of field-contaminated materials in laboratory (Furuta & Yoshizawa, 1997; Huneau-Salaün et al., 2010). Targets for recovery may be pathogens (e.g. Salmonella, Staphylococcus aureus), or endogenous or applied indicator organisms (e.g. Enterobacteriaceae, Serratia marcescens). Sampling by swabs, rinses, impression plates, pieces of material, etc. | Often comparative culture of treated and untreated (control) areas. May use molecular biological tools to detect viable target, particularly for viruses. Reduction against control or to a predefined surface density are typical measures | Generally, standardisation not currently achievable due to the number of variables and resources needed for repetitive trials (Holah et al., 1998). Tight control of variables, even if possible, would limit the generalisability of results in agricultural settings. Hand sanitiser tests may be an exception (Cremieux et al., 1989) |

be chosen for relevance or safety (Table 2). Details of commonly-employed yeasts and fungi are also in Table 2.

There can be marked variation in the susceptibility of bacterial species, and of strains within a species, to disinfectants under test conditions (Sander et al., 2002). Furthermore, the modulating effects of other test variables on disinfectant activity can vary markedly according to the bacterial target. Examples include the interaction observed between target, water hardness and activity of quaternary ammonium compounds (QAC) in one study (Bessems, 1998), and the effect of organic soil on susceptibility of Salmonella Typhimurium or S. aureus to disinfectants (Stringfellow et al., 2009).

For field trials, organisms monitored as markers for disinfection efficacy should ideally be sufficiently abundant to allow a reduction of several log10 units to be detected. Suitable options in livestock environments include enterococci (Gram-positive), Enterobacteriaceae and total aerobic counts (Luyckx et al., 2017).

### 2.1.2 Viruses

Test viruses ideally should be readily propagated to high titres, be safe to handle, and be robust in the face of disinfectants and (for surface tests) of drying (Krug et al., 2011; Rabenau et al., 2014). There is much variation in these qualities, with non-enveloped viruses generally being more robust to adverse environmental and chemical conditions, but loss of activity upon drying is still commonly substantial (Krug et al., 2011; Schürmann & Eggers, 1983). The complexity of propagation, recovery and enumeration of viable virus adds to the challenge of reproducibility (Rabenau et al., 2014). Some viruses that have been selected as suitable for test procedures are listed by ASTM International and the Organisation for Economic Co-operation and Development (OECD) (ASTM, 2011; OECD, 2013).
Preparation of the biological target

The physiological and physical state of the biological target will affect test performance as will any associated material, for example culture medium or biofilm. As previously mentioned, stock strains also appear to alter in susceptibility over time (Bloomfield et al., 1994; van Klingeren, 1995). For suspension tests with bacteria, the use of stationary-phase cultures sedimented and re-suspended in defined media provides targets in reproducible numbers and in a state of limiting nutrition and physiological stress, such as is likely to be encountered in real-world applications.

Organisms dried on to surfaces may show increased resistance to disinfectant action, but they also spontaneously lose viability over a matter of hours, necessitating tight control over the timing of preparation and test procedures (Pines et al., 2013). In addition, the bacterial species and density of inoculum will affect surface distribution patterns and consistency of results, and mechanical spreading tends to reduce the viability of the inoculum (Bloomfield et al., 1994).

Qualities of diluting water

Water hardness (dissolved calcium and magnesium minerals) can have a substantial effect on interactions between certain combinations of bacteria and biocide (Bessens, 1998), and standardised testing usually specifies deionised/distilled water (EPA, 2014), or water of a defined hardness (APHA, 2016; Bloomfield et al., 1993; CEN, n.d.). Water supplies in the field can have effects on disinfectant efficacy that are either understood, such as the effect of pH on free chlorine (Holah, 1995), or which may be unpredictable and not correlated with usually-measured physical and chemical properties (Davison et al., 1996; Wales et al., 2013).

Surface qualities

Surface variables include: roughness, porosity, chemical nature and qualities such as hydrophobicity. Such factors need to be controlled, but they do allow testing to address certain real-world scenarios,
for example polyethylene drinking water systems or concrete, metal and wood structural material in livestock accommodation (Hancock et al., 2013). Highly standardised surface tests tend to use readily-available and non-reactive materials, typically stainless steel or glass, with a defined surface roughness (van Klingeran et al., 1998; Pines et al., 2013).

Results of laboratory studies (Marin et al., 2009; Yilmaz & Kaleta, 2003) and field trials (Connor et al., 2017; Luyckx et al., 2015; Martelli et al., 2017) emphasise the tendency for rough and damaged surfaces to reduce the efficacy of disinfectants. In addition, porous surfaces present a large surface area and possibly enhanced attachment opportunities for target organisms. They too are associated with reduced bactericidal and virucidal effect of disinfectants (Gamal et al., 2014; Jang et al., 2014; Lyutskanov et al., 2010).

Skin is a complex surface type, with studies in this area commonly comparing contamination and recovery techniques on volunteers’ hands (Cremieux et al., 1989; Macinga et al., 2011). One ASTM International test (ASTM E2897) employs prepared and sterilised pig skin as the surface material, whereas an artificial skin substitute is used elsewhere (prEN 17422, Table S3).

2.5 | Mode of disinfectant application

In standardised surface tests a measured volume of disinfectant is placed on a surface, or applied by using a prescribed wiping or spraying pattern (Bloomfield et al., 1993; EPA, 2014), whereas standard spray-disinfection procedures are usually employed in field tests (Connor et al., 2017; Fankem et al., 2009). With small disinfectant volumes evaporative effects during the tests may be of significance, and larger volumes can enhance the observed biocidal effects (van Klingeran et al., 1998). Therefore appropriate and consistent volumes need to be employed, and in standardised conditions of temperature and humidity.

There have been recent developments of airborne disinfection systems, using either aerosols (fogging or ionised ‘dry mist’ systems) or disinfectant vapour, commonly intended for disinfection of complex environments such as hospital patient rooms and animal houses (Barbut et al., 2009; Luyckx et al., 2017; Schneider, 2013). Laboratory testing has employed carrier surfaces, for example contaminated stainless steel discs, placed in varied locations within test rooms (Fu et al., 2012; Piskin et al., 2011), and standard test protocols are emerging (ALG, n.d.; CEN, n.d.). Attempts to model real-world scenarios have suggested that the effects of location, dose, virus and virucide on outcomes can be substantial and unpredictable (Montazeri et al., 2017).

2.6 | Disinfectant exposure: concentration, time and temperature

The theoretically predictable relationships between killing/inactivation and: exposure time, disinfectant concentration and temperature become complex outside of tightly-controlled suspension tests (van Klingeran, 1995; Mazzola et al., 2009), and even these show marked differences in observed parameters between differing combinations of agent and target (Bessems, 1998). Furthermore, variability in the results of disinfectant tests is likely to be higher when the concentration of active disinfectant experienced by the target is at or near ‘borderline’ biocidal conditions (van Klingeran et al., 1998).

Whilst ‘in use’ concentrations should be well within the highly-effective killing range, the addition of ‘real-world’ factors to a test (for example, a porous surface, organic soil or biofilm) may consequently reveal much less efficacy, or more variability, than anticipated (Furuta & Yoshizawa, 1997). Microbicidal classes vary substantially in their dilution coefficients. Thus, the activity of phenolic and cresol-based agents reduces rapidly with decreasing concentration (high dilution coefficient), whereas QAC have a low dilution coefficient and therefore they may show a wider effective range of concentrations, performing better in tests that tend towards ‘borderline’ conditions for other microbicide classes (Gosling et al., 2017; Russell et al., 1979).

Exposure time is a factor for which testing regimes take their cues from real-world applications, and between five and 30 min have been popular intervals in experimental and standardised tests. Five minutes has been considered realistic for water-based non-foaming agents on non-porous vertical surfaces (Holah, 1995), although there may not be good field data to substantiate this. Where interfering organic material is present, extended exposure times appear not to be associated with greater biocidal effect (Berchieri & Barrow, 1996), whereas increased disinfectant concentration can overcome such a limit (Furuta & Yoshizawa, 1997; Gosling et al., 2016).

Very short contact times, such as those encountered in agricultural and veterinary practice when using boot dips, are generally not covered in standard tests and they pose challenges for laboratory testing. Nevertheless this information may be highly relevant for day-to-day disinfectant use on farms where high numbers of organisms are present, for example with Campylobacter on poultry farms.

Ambient temperature may correlate well with effect, even for surface and field tests (Böhm, 1998), but field temperatures (particularly in colder climates) may depart markedly from those usually employed in the laboratory, making specific testing at such temperatures potentially more informative and reliable than extrapolating from ‘standard’ temperature results (Dee et al., 2005).

2.7 | Neutralisation of disinfectant

Neutralising disinfectant action provides a defined end to biocidal activity, i.e. standardisation of single or multiple sampling points. Testing of neutraliser methods should be performed using the relevant combination of target and microbicidal(s) (Mackinnon, 1974). Additional benefits, of protecting the detection system (usually bacterial or cell culture) and allowing damaged yet viable targets to recover and be detected, have prompted recommendations that control procedures should include testing neutralising agents for inhibitory effects upon the viability of disinfectant-exposed targets.
and the systems used to detect them (MacKinnon, 1974; Russell et al., 1979).

Dilution is a simple neutralisation method, either using conventional dilution or membrane filtration plus washing (Russell et al., 1979). Some disinfectants that associate strongly with bacterial cell walls (QAC, phenolic agents), or which have low dilution coefficients (QAC) may resist this mode of neutralisation (MacKinnon, 1974; Roesler et al., 2007). Neutralisation by addition of chemical agents, is a well-established alternative, with agents having been established empirically for the most part. They include: non-ionic surfactants, lecithin, thiourea compounds, various culture media, histidine or histamine (for formaldehyde), catalase (for hydrogen peroxide), and pH-modifying agents for organic acids (ASTM, 2013a; Carrique-Mas et al., 2007; MacKinnon, 1974; Neuberger, 1944; Russell et al., 1979). Chemical neutralisers have been used in field investigations (Luyckx et al., 2017; Lyutskanov et al., 2010), where a ‘disinfect plus dry downtime’ cycle may be appropriate before applying neutraliser.

### 2.8 Recovery and detection, with or without enumeration, of viable biological target

Laboratory test results may be expressed as pass/fail against a reduction target or as a (semi-) quantitative reduction, against appropriate controls. For surface-mounted targets, physical removal via methods such as scraping, agitation with glass beads and/or sonication, is usually needed for enumeration by culture. Any variability in outcomes associated with these removal methods remains unquantified. Methods for enumeration in situ of surface-adhered viable bacteria rely on microscopy or spectrophotometry (Günter et al., 2017; Köse & Yapar, 2017; Smith & Hunter, 2008; Yu et al., 1993). These techniques have not been formally compared with culture techniques in equivalent settings, and as yet they have not been adopted for standardised testing.

In the field, agar contact plates are convenient and of suitable sensitivity for aerobic counts after disinfection, but sampling for specific organisms (such as *E. coli* or enterococci) benefits from a more sensitive technique such as wiping with neutraliser-moistened swabs (Luyckx et al., 2015). For the assessment of hand sanitisers, recovery has been performed by sampling defined areas or by complete immersion of the test hand in neutraliser/recovery medium, a technique that recovers a large proportion of bacteria or viruses (Cremieux et al., 1989; Macinga et al., 2011; Steinmann et al., 1995), and which has been adopted in many standards as the glove or ‘glove juice’ method (ASTM, 2013b, 2017a).

Assessing the activity of disinfectants against bacterial or fungal spores has been reviewed recently by Leggett et al. (2016). Complications arise in distinguishing between lethal and static effects, between activity against spores versus against derived vegetative cells, and in recognising sub-lethal damage that can be bypassed by treatment with lysozyme or, plausibly, following ingestion by an animal. It is evident that there are significant knowledge gaps in this area.

For virus recovery, cell culture remains the principal system, but inoculation of susceptible species with recovered material has also been used to determine if an infective dose remains after disinfection (Baker et al., 2018). For prions, detecting residual infectivity has potentially become simpler with the development of in vitro cell culture and biochemical assay techniques that correlate with activity in whole-animal studies (Wagenführ & Beekes, 2012).

### 2.9 Organic soil and biofilm

Typically, serum, serum albumin or yeast are used as interfering organic material in tests, in pursuit of standardisation and reproducibility (APHA, 2016; Bloomfield et al., 1993; Pines et al., 2013). A mix of high and low molecular weight polypeptides (bovine serum albumin plus tryptone or yeast extract) plus mucous material (bovine mucus) has also been used (ASTM, 2017b; OECD, 2013). Some studies focussed on field conditions have trialled more complex materials, including dairy fat/protein composites, faeces and chicken litter (Brouillaud-Delattre et al., 1994; McLaren et al., 2011; Stringfellow et al., 2009); standardisation of such materials is challenging but some, such as skimmed milk, can yield reproducible results.

Tests incorporating biofilm are not currently part of routine assessments, but some test standards have been developed. Laboratory systems for growing biofilms are diverse, as are the type of growth conditions that they allow and control (Buckingham-Meyer et al., 2007; Ceri et al., 1999).

### 2.10 Cleaning and drying

There is currently a lack of verified methods for assessing cleaning before disinfection. Bacterial spores can be used as markers if the disinfection step in a laboratory test is not expected to be sporicidal (Brouillaud-Delattre et al., 1994). As discussed in the introduction, a drying stage may contribute significantly to the microbicidal effect of C&D. Therefore, depending on anticipated conditions of disinfectant use, tests that include drying steps after application of cleaning and/or biocidal agents, may more closely indicate the efficacy of disinfection procedures, compared with simpler tests that aim to quantify the effect of the disinfection agents alone.

### 2.11 Special applications

Beyond the disinfection of environmental surfaces in livestock accommodation and veterinary clinical environments, special applications include hand and skin sanitisation (discussed elsewhere) and chemical sterilisation (to include sporicidal activity) of equipment such as endoscopes. C&D of animal transporters is one further special application of particular relevance. Challenges for transporters
include: a major risk for the spread of pathogenic or antimicrobial-resistant organisms between groups of animals, a number of surfaces with varying texture and finish, use in commercial situations demanding a fast turnaround, and C&D sometimes in very low ambient temperatures (Baker et al., 2018; Böhm, 1998; Dee et al., 2005). Given their particular circumstances, their relatively small size and the limited range of materials and structures encountered, this is one area where cost-effective and informative field tests might realistically be developed.

2.12 | Consistency and interpretation of tests

Repeatability and reproducibility are maximised in tests where there are a few, inherently-controllable variables and where the measured outcome is not very sensitive to changes in those variables. For example, a test of stationary-phase planktonic bacteria, with or without well-defined uniformly distributed organic matter (such as horse serum or yeast extract), fully-mixed with disinfectant and with a readily-achievable pass/fail criterion (Bloomfield et al., 1993). If undissolved ‘soil’ is introduced, for example faeces or chicken litter, then liquid/solid interfaces are introduced, plus sometimes other organisms, and outcomes are more variable (Berchieri & Barrow, 1996; McLaren et al., 2011; Stringfellow et al., 2009).

For surface tests, the interface and small disinfectant volume introduce variables not present in suspension tests, and there is not a consistent or predictable relationship between performance in a suspension and a surface test (van Klingereren, 1995). Nonetheless, the repeatability of a highly-standardised surface test can in practice be similar to a suspension test, but in both cases variation in the biological target inoculum can be a substantial constraint on both reproducibility and repeatability (Bloomfield et al., 1994). In general, results become less repeatable or reproducible as more variables are introduced into a test, and the more a test incorporates ‘borderline’ conditions that will demonstrate substantial changes in outcome when conditions alter (Maretrå et al., 2009).

The need to consider what counts as non-significant variation in test outcomes is illustrated by intra- and inter-laboratory trials of microbicidal effect (ME, i.e. reduction in viable bacterial counts). Two studies of repeatability and/or reproducibility (the latter using three laboratories) for stainless steel surface tests reported that non-significant variation in ME exceeded 1.6 log_{10} units (Bloomfield et al., 1994; van Klingereren, 1995). Similarly, in a seven-laboratory ring trial of steel surface tests using local stocks of defined target bacteria strains, the range of ME values for a given test (same agent, concentration and target) generally exceeded two (and often four) log_{10} units (van Klingereren et al., 1998).

Surface tests are a versatile tool for assessing disinfection processes in highly standardised model scenarios, but there is no ready translation of results to outcomes in the field. In farm situations, target end-points of routine C&D are either undefined or empirical, for example up to one colony-forming unit of aerobic bacteria per square centimetre (Tamási, 1995), and it is not clear whether present testing methodology allows a pass/fail criterion to be set that has a predictable relationship with the likelihood of pathogen elimination or a particular end-point in the field. There are also factors in the field which can be intractable even with optimised C&D technique. For veterinary and agricultural applications these include: substantial residual soiling of certain materials and structures (for example, belts conveying eggs or droppings), short persistence time on non-porous vertical surfaces or the undersides of equipment/fittings, and low ambient temperatures (Böhm, 1998; Huneau-Salaün et al., 2010; Mueller-Doblies et al., 2010; Tamási, 1995).

3 | REGULATORY AND TESTING REGIMES FOR DISINFECTANT APPROVAL

Requirements for tests to be consistent (using few and highly-controllable variables) and to give a firm indication of performance in the field are to some extent irreconcilable. Using current methodologies the various aims of disinfectant testing require that more than one type of test be performed, and current testing schemes recognise this. There follows a summary of notable examples for which details are readily available.

3.1 | International testing authorities

The principal standards bodies that develop disinfectant tests and have international stances are: AOAC International, ASTM International, the European Committee for Standardization (CEN), and the OECD. The International Organization for Standardization (ISO) provides a classification framework (ISO, n.d.). AOAC and ASTM standards have been adopted as the basis for many of the USA-approved tests, as discussed below. The USA and OECD used international ring trials of candidate methods to develop a draft guidance document on quantitative methods for testing microbes used on hard non-porous surfaces (OECD, 2013; Table S5).

3.2 | European Union

A technical committee (TC 216) of the CEN was established in 1989 to develop a unified European disinfectant testing regime for food hygiene, medicine, veterinary practice and agriculture (Bloomfield et al., 1993). The testing structure adopted includes basic (Phase 1) quantitative suspension tests to establish biocidal activity and to evaluate any neutralisers (A3P, 2017; CEN, n.d.). Phase 2 tests, subdivided into suspension (Step 1) and surface or carrier (Step 2) subgroups, then establish performance at or around manufacturers’ recommended concentrations, with low and high amounts of added organic soil (CEN, n.d.; van Klingereren et al., 1998). Within Phase 2, variations in soil type and load, target organisms, concentrations, time, temperature and (where applicable) surface material can be tested according to anticipated (and consequently licensed)
uses (A3P, 2017). The most relevant Phase 1 and 2 tests, published or in development, are outlined in Tables S1 and S3. An ambition to develop ‘Phase 3’ field performance tests has encountered severe problems with standardisation (Bessem, 1998; van Klingeren et al., 1998), as discussed earlier. The European Union Biocidal Product Regulations (BPR) 528/2012 governs safety and efficacy approval for biocide active substances and compound products, and the CEN/TC 216 testing scheme provides a route to compliance with the BPR efficacy requirements for member states. However, other tests are acceptable for BPR compliance, such as variants used within the multistage licensing regime of Germany.

### 3.2.1 Germany

The German Veterinary Society (Deutsche Veterinärmedizinische Gelleschaft; DVG) examines and approves products for animal husbandry, the food sector, veterinary practice and animal shelters (DVG, n.d.). A particular feature of the German approach is the use of independent expert reviewers approved by the Committee for disinfection that supervises the process for each product. Reviewers oversee testing by laboratories (which are required to participate regularly in ring trials) and provide reports. Four principles are advanced as the basis of DVG product testing (DVG, 2019a):

- **Independence**, via the use of approved experts
- **Repeatability**, via two independent experts and at least two independent test repetitions
- **Validity**, via further review by the Committee
- **Relevance**, with reference to anticipated applications and to managing the risk of selecting for reduced susceptibility by establishing MIC values for all tested organisms

Guidelines for disinfectant testing are published by DVG (2019b), and these vary by sector. In general, an initial testing report is required, including results from EN-compliant quantitative suspension and surface tests (DVG, 2015). This is reviewed by the Committee, who then specify further tests (EN-compliant where available) in a laboratory and reported by an expert who are both independent of the initial submission (DVG, 2019a). The second set of tests use the least-susceptible target organisms identified in the initial submission, and determine the conditions of use in the approval listing. DVG guidelines that are outside of EN specifications include the incorporation of a reference antimicrobial substance (selected according to the chemical nature of the dominant component in a disinfectant product) as an internal control for variation in the susceptibility of target organisms (DVG, 2017c).

**Livestock sector**

Requirements for bactericide testing and approval within the German livestock sector are outlined in the Supporting Information (Table S11 and Appendix S1), and summarised on the DVG website (DVG, 2018a). Appendix S2 in the Supporting Information gives a model testing regime for approval of a livestock bactericide (DVG, 2017b, 2018b).

Virucidal activity is assessed using defined strains of enveloped (vaccinia and Newcastle Disease) and non-enveloped (ECBO, Reo) viruses, with cell monolayer detection systems (DVG, 2018a; 2019b; Table S7). Anti-protozoan cyst and roundworm ovicidal activities, if required, are tested using Cryptosporidium parvum oocysts and Ascaris suum eggs, respectively (DVG, 2019b; Table S7).

**Veterinary applications**

Testing requirements are similar to the livestock regime and use smooth non-porous surface tests (DVG, 2015). For veterinary practice and animal shelters, preventive bactericidal disinfection can be approved on the basis of two reports following testing to standards published by the Association for Applied Hygiene (Verbund für Angewandte). There are also DVG-specified tests, closely-following relevant CEN tests in the case of bacteria and fungi. Approval for ‘special’ disinfection applications (fungi, parvovirus and *Giardia*) requires expert reports according to current DVG test guidelines (DVG, 2019b; Table S7).

### 3.3 United Kingdom

Currently the UK adheres to the BPR, and the Department for Environment, Food and Rural Affairs (Defra) Disinfectant Approvals Scheme tests only disinfectants that are BPR-compliant, although there is substantial latitude for the type of efficacy test that may support such compliance. The Defra scheme aims solely to ensure disinfectant efficacy for the prevention and control of outbreaks of notifiable animal and zoonotic disease. Defra approval follows standardised suspension tests under five Disease Orders (Table 3 and Table S2). The General Orders Scheme covers notifiable diseases and infectious agents not listed under a specific Order, for example Salmonella spp. All five tests are semi-quantitative disinfection suspension tests incorporating organic soil (APHA, 2016; Defra, 2014). The Tuberculosis Orders and General Orders tests follow the British Standards BS 6734:2004 and its antecedent BS 6743:1986, respectively. The Defra tests, uniquely among other current and relevant British Standards, are not currently adapted from CEN standards.

### 3.4 United States of America

The regime for testing and licensing disinfectants for public health purposes in compliance with efficacy and safety requirements of the Federal Insecticide, Fungicide, and Rodenticide Act and the Federal Food, Drug, and Cosmetic Act is administered by the United States Environmental Protection Agency (EPA; EPA, 2013). The reference tests for licensing under the EPA closely follow those developed and published by AOAC International or ASTM International (EPA, 2018b), or are based upon OECD procedures. Standard
TABLE 3 Overview of tests specified by the Diseases of Animals (Approved Disinfectants; England) Order 2007

| Order | Test organism | Other details |
|-------|---------------|---------------|
| Diseases of Poultry Order and Avian Influenza and Influenza of Avian Origin in Mammals Order (DoP and AL&IAOM) | Newcastle disease virus strain Herts 33 | WHO hard water diluent, 2.5% baker’s yeast soil, 4°C, 30 min contact time, performance standard is 4 log₁₀ unit reduction in egg infective doses |
| | Swine vesicular disease virus (SVDV) | Swine vesicular disease virus UK (British field strain) G 27/72 | WHO hard water diluent, 4°C, 30 min contact time, performance standard is 4 log₁₀ unit reduction |
| | Foot-and-mouth disease virus (FMDV) | Foot- and-mouth disease virus O1 BFS (British field strain) 1860/UK/67 | As SVDV test, plus 1% foetal bovine serum soil |
| | Tuberculosis disease (TB) | | Follows British Standard BS 6734:2004, 4°C, 2.5% yeast soil, 60 min contact time, in-use dilution, mixed 1:1 with inoculum. Performance standard is 4 log₁₀ unit reduction |
| General Orders (GO) | Salmonella Enteritidis S9574/07 | Based upon former British Standard BS 6734:1986; 4°C, 2.5% yeast soil, 30 min contact time, in-use dilution, mixed 1:1 with inoculum. Performance standard is 5 log₁₀ unit reduction |

Operating Procedures (SOPs) for all bacterial and yeast EPA tests are available on the EPA website (EPA, 2014). Summary details of most tests are in Tables S4–S6.

For animal health applications, EPA approval is required for any product that makes a public health claim (EPA, 2018a). Products that do not (explicitly or implicitly) make a public health claim do not generally require EPA approval on the basis of efficacy data although manufacturers are required to ensure and document that these products perform as intended. Nonetheless some such products are subject to discretionary regulation for claims against pathogens, such as foot and mouth or avian influenza A viruses, that have animal health significance and/or the potential to infect humans (EPA, 2018a, Section C/2/i/d).

EPA general guidelines (EPA, 2018a) require organic soil to be included when specifically claimed on the product’s label and/or indicated by the product’s pattern of use. The core AOAC ‘use dilution’ test for bactericidal disinfectants (AOAC tests 955.14, 955.15, 964.02; EPA designation MB-05-14; Appendix S3) is a carrier test, using stainless steel cylinders with an in-use disinfectant concentration. A quantitative non-porous surface test is also listed (MB-25). Other relevant tests are specified in current efficacy testing guidelines (EPA, 2018b), or the EPA online analytical methods (EPA, 2014). There is also a method for testing mono-species biofilm S. aureus or P. aeruginosa (MB-20, based on ASTM E2871). Virucidal testing relies on ASTM International standards for suspension and glass surface tests (ASTM 1052 and 1053). Current guidance from the EPA for foaming, fogging, gas, and vapour applications is to consult with the agency before testing, to determine the appropriate methodology (EPA, 2018b). Guidelines for anti-prion efficacy testing are also published by EPA (EPA, 2012).

Skin sanitiser antiseptics are regulated under the Food and Drug Administration (FDA; Rutala & Weber, 2008), which has recently ruled that many previously-used agents cannot be generally regarded as safe and effective by criteria including bacterial reductions in clinical simulation experiments using volunteers’ hands (FDA, 2015). It has indicated that ASTM International tests (ASTM, 2019, also Table S4) may be useful to establish efficacy claims for these products (FDA, 2017).

4 | DISCUSSION

There is no universal test for disinfectant efficacy, and current procedures may broadly be considered either as tests of disinfectant activity, where the effect is measured in suspension in an excess of microbicide, or tests of disinfection process, where dynamic and ‘real-world’ elements such as surface materials, surface adherence, small disinfectant volume and mechanical action may be included. For both test types, biological targets are selected by scientific consensus, including judgement of likely field challenge.

A prominent feature of published data is the variability observed, even within standardised tests. Beyond robust protocols and careful technique, there are precautions that may be taken in order to safeguard repeatability and reproducibility of tests. These include: the use of reference biocidal substances as internal controls, limiting the source of each biological target to a specified reference centre, defined (and minimal) passaging of targets before use, and periodic phenotypic (MIC- and MBC-type) and genotypic (potentially whole-genome-sequence based) tests to verify target stability. Ring trials amongst approved laboratories,
using a standard panel of biocides, can provide larger-scale quality control. Care also needs to be taken in deciding on pass/fail criteria. Setting a high performance threshold may require high working concentrations and exposure times, with potentially more consistent test results. However, the resulting label conditions of use may prove uneconomic, unrealistic or unsafe, and thus either not be adhered to or pose a threat to operator and environmental safety.

National testing and approval regimes still vary substantially. Within Europe, the CEN/TC 216 process has focussed on a single, structured testing regime with the flexibility to incorporate application-specific tests, including tests for surgical hand antisepsis and for fogging and misting devices. In the USA, the approach appears to have been more eclectic, selecting and adapting (where necessary) useful tests from a number of sources. This includes, importantly, tests for surface-biofilmed organisms. The approval process for German livestock products stands out in its provision for independent repeat testing and review.

The advent of biofilm surface tests is an important step, particularly for applications in environments that are habitually moist and not intensively cleaned and disinfected, such as livestock drinker systems. Hand sanitiser and antiseptic tests have to contend with variation between regions (particularly fingernails), and variation between volunteers in respect of, for example, skin and nail surface, resident microorganisms and prior hygiene routines. There are starting to be test protocols involving ex vivo animal skin or artificial skin, which may yield less variability or at least provide the opportunity to increase repetitions to demonstrate consistency.

The lack of a satisfactory ‘one-size-fits-all’ test has led authorities to consider specific variants of standardised tests for specific applications. This allows test conditions and pass/fail criteria to be set that either do not have to account for a ‘worst case’ disinfection scenario, or which address a particular biological target of concern. One such emerging concern, likely requiring higher concentrations or application rates of disinfectants, is eliminating persistent contamination of animal housing by commensal bacteria that carry multiple drug resistance.

Field evaluation of disinfection has thus far proven impossible to standardise, despite an ambition to do so articulated over 20 years ago by CEN/TC 216. Field studies of C&D have served mostly to illustrate deficiencies in conventional regimes, although sometimes improved infection control has been observed even when measures of residual microbial contamination are not significantly improved compared with controls. Unresolved issues include: what biological target organisms to measure, what surface conditions to use (field materials are often damaged or deteriorated), and what conditions of diluent source, soiling, temperature, humidity and downtime might yield results that are more useful than standardised laboratory surface tests. If ‘standardised’ field study protocols do emerge, it may be that interpretation and application of their results would need to be carefully limited to scenarios that are highly similar to the test conditions.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.