Testing Surface Disinfectants

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Testing surface disinfectants: quantitative, semi-quantitative, qualitative, and alternative methods

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[*Key words*: dried surface test, biofilm test, efficacy measure, log reduction, most probable number, P/N LR formula]

The microorganisms against which a disinfectant is tested can be either planktonic microbes suspended in a liquid or a collection of surface-associated microbes. Various suspension test methods have been thoroughly evaluated and are widely used, for example, to screen chemicals for antimicrobial activity and to assess the efficacy of drinking water disinfectants or recreational water disinfectants (AOAC method 965.13, 2000; Bloomfield and Looney, 1992). Methods for testing a disinfectant against surface-associated microbes typically use easily manipulated carriers; e.g., glass disks. A variety of methods are used to place and hold microbes on the carriers. This article focuses on tests using surface-associated microbes because it is anticipated that most new standardized disinfectant tests will be surface tests. However, the discussion can be applied to suspension tests if the suspension test beakers or tubes are considered to be carriers.

In brief, a surface disinfectant test is conducted as follows. Some of the microbe-bearing carriers are treated with the disinfectant and others serve as untreated carriers. At the end of the designated contact time, the disinfectant is neutralized to stop its activity. Untreated carriers usually receive the same manipulations and neutralization as the treated carriers, except that an inactive treatment, such as dilution water, is applied instead of the disinfectant.

An effective disinfectant is formulated to kill most of the microbes on the treated carrier. When an effective disinfectant is tested, the treated carriers should hold few viable microbes, relative to the untreated carriers. Therefore, disinfectant efficacy is quantified by comparing a measure of the viable microbes on the treated carriers to the measure on the untreated carriers.

Dried surface and biofilm tests

Most tests devised for surface disinfectants are either dried surface tests or biofilm tests.

- **Dried surface test** – In some methods, a sample of planktonic microbes is pipetted onto the carrier (e.g., AOAC method 2008.05, 2008). Alternatively, the carrier is immersed in a suspension of planktonic microbes for a short period of time (e.g., 15 minutes) under static conditions, allowing the microorganisms to adhere to the carrier (e.g., AOAC Method 955.15,

2009). In either case, the inoculated carrier surface is dried and the microbes usually are held on the surface within a dried organic film.

- **Biofilm test** –The carrier is inoculated by placing it in a microbe-abundant aqueous environment, typically for 24 h or longer. The formation of the biofilm is initiated when planktonic organisms leave the suspended state in the aqueous medium, adhere to the surface, replicate, and populate the surface. Biofilm microorganisms live in a self-organized, cooperative, sessile community of microorganisms, typically embedded in a matrix of extracellular polymeric substances (**EPS**, known as slime in the vernacular). The biofilm growth protocol controls relevant biological and physicochemical factors, such as the hydrodynamics and the chemistry of the aqueous medium (e.g., ASTM method E2562-07, 2007; Goeres et al., 2009). Prior to application of the disinfectant, the carriers may be dried or hydrated (Buckingham-Meyer et al., 2007). Biofilm carriers simulate surface contamination over time in a specific environment, which may be moist, hydrated, or intermittently hydrated.

Categories of disinfectant test methods and associated measures of viable microbes

Disinfectant tests can be classified according to the techniques used to quantify the viable microbes. Such a classification is helpful because knowledge of the quantification process is required to analyze the results of a test . We have found that all standardized disinfection tests can be partitioned into four categories –quantitative tests, semi-quantitative tests, qualitative tests, or alternative tests. A similar classification system has been used for describing the methods used in food microbiology (Corry, 2007).

- **Quantitative test –** Individual viable microbes on each carrier are enumerated. The microbes are harvested from the carrier surface into suspension, with a separate suspension for each carrier. (At present, there are no standard methods for *in situ* enumeration.) Most quantitative tests rely on viable plate count techniques (e.g., spread plate, pour plate, or drop plate) to enumerate the harvested, suspended, viable microbes. In a viable plate count, a small sample volume of the suspension is spread out on a nutrient agar plate so that the individual microbes are spaced apart. When the plate is incubated, each viable microbe repeatedly divides and eventually grows into a visible colony. Each colony represents a colony forming unit (**cfu**), which is presumably due to a single viable microbe. The cfu count is scaled up according to the volume plated to arrive at the **density** of viable microbes, for which the units are usually "cfu per cm^2 of carrier surface area" or "cfu per carrier." The typical quantitative test utilizes between 3 and 10 treated carriers and between 3 and 10 untreated carriers. Disinfectant efficacy is measured by the log reduction (**LR**), found by subtracting the mean of $\log_{10}$-transformed densities for the treated carriers from the mean of $\log_{10}$-transformed densities for the untreated carriers (Zelver et al., 2001). If the disinfectant kills none of the microbes, the expected LR is zero. If the disinfectant is effective, the expected LR is positive.

  There are other methods available for enumerating viable microbes, including colony counts on membrane filters and microscopic direct counts after exposing the microbes to a fluorescent viability stain. New technologies may well lead to the routine use of automated microbe counting or colony counting instruments. If *in situ* viable microbe counts become feasible, then the harvesting and dilution series steps can be eliminated.

- **Semi-quantitative test –** Instead of cfu enumerations, positive/negative (**P/N**) outcomes are observed for either the treated carriers or all the carriers. In theory, the outcome is "positive" if the suspension tube or the carrier contains at least one viable microbe and the outcome is "negative" if there were no viable microbes, which would happen if, for example, all microbes were killed by the disinfectant treatment. From the P/N observations, the log density of viable

microbes is calculated using the most probable number (**MPN**) method (Garthright and Blodgett, 2003). The MPN method is based on a binomial likelihood model for the P/N responses. The calculation of the MPN requires a computer program or access to tables, except for the one-dilution MPN which is a simple formula. The log-transformed MPN is used in place of the mean log density in LR calculations. The mathematical equations and formulas will not be presented here; they are available in the references.

The efficacy result of a semi-quantitative test is generally less precise than the result of a quantitative test. Precise measurement systems, such as the hydrophobic grid-membrane filter method that relies on MPN calculations (Tsuji and Bussey, 1986), are called semi-quantitative here, even though many practitioners would consider the precision sufficient to justify the quantitative designation.

The viable microbes can be measured differently on the untreated carriers than on the treated carriers, leading to three types of semi-quantitative tests, which we call type SQ_1, type SQ_2, and type SQ_3. For each type, efficacy is measured by the LR, but the way that LR is calculated differs among the types.

- Type SQ_1 semi-quantitative test – The viable microbe density is enumerated on the untreated carriers exactly as for a quantitative test, but the microbes are not harvested from the treated carriers. Instead, a positive/negative (P/N) result is observed for each treated carrier as a whole. The most-used method for determining the P/N result is to place the carrier in a tube containing sterile nutrient broth and incubate for an appropriate period. If the tube becomes turbid due to microbial growth, the result is a positive; if the tube remains clear indicating that there was no microbial growth, then the result is a negative. The type SQ_1 test is used when it is impossible to harvest the microbes from treated carriers (e.g., the disinfectant may fix the microbes to the carrier), when the real-life application dictates that the disinfectant treatment should achieve complete kill, or when the P/N result is cost-effective compared to the viable plate count. Type SQ_1 tests typically use between 3 and 10 untreated carriers and many (60 or more) treated carriers (AOAC Method 966.04, 2006; AOAC Method 991.47, 2006). The LR value is the log density for treated carriers subtracted from the mean log density for untreated carriers, where the log density for treated carriers is estimated by taking the $\log_{10}$ transformation of a modified, one-dilution MPN density estimate (e.g., the P/N formula for LR, equation 1 in Tomasino and Hamilton 2006).

- Type SQ_2 semi-quantitative test – Microbes are harvested into suspension from each untreated and treated carrier, and a dilution series is formed for each suspension. However, instead of conventional plate counts, multiple suspension tubes (or wells) are created for each dilution, a P/N outcome is observed for each tube, and the density of viable microbes for each carrier is estimated using the MPN method. A type SQ_2 test might be used when the microbes cannot be counted, as happens when testing some virus types for which direct enumeration of viable virus particles is impossible, although P/N results can be determined. An example is the EPA dried surface test against hepatitis B virus (EPA, 2008). It uses 2 untreated carriers and 2 treated carriers for each of 2 lots of the disinfectant; the P/N outcome is observed for each of 4 microtiter wells at each dilution in the dilution series for each carrier. To calculate LR, the log densities of both the untreated and treated carriers are the $\log_{10}$ transformation of the corresponding MPN estimates.

- Type SQ_3 semi-quantitative test – Each treated carrier provides a whole-carrier P/N response as for a type SQ_1 test and for each untreated carrier, the microbes are harvested, the suspension is serially diluted, P/N outcomes are observed, and the MPN estimate of density for the untreated carrier is calculated as for a type SQ_2 test. To calculate LR, the log densities of both the untreated and treated carriers are the $\log_{10}$ transformation of the corresponding MPN estimates. This may be the method of choice when working with anaerobic bacteria, viruses, or other problematic microbes.

- **Qualitative tests** –A P/N outcome is observed for each treated carrier, exactly as for a type SQ_1 test; however, there are no untreated carriers. The test results are conveyed by two integers, the number of treated carriers and the number that were negative. The typical qualitative test utilizes many (60 or more) treated carriers. For most applications, the disinfectant is judged to be efficacious if, and only if, almost all the treated carriers are negative (e.g., 59 or 60 negative carriers among 60 total carriers). This test provides only soft (qualitative) data because it possesses no internal check to see whether there is a suitable number of viable microbes on the carriers; e.g., for a dried surface test, to check that most microbes were not killed by the drying step prior to application of the disinfectant treatment.

- **Alternative tests** – For either the treated carriers or the untreated carriers, use an alternative, possibly indirect, measure of viable microbe density, in units that are defined in the test method protocol. Alternative techniques for measuring viable microbes are receiving intense study, particularly techniques based on advances in fundamental microbiology and molecular biology. One can anticipate that fast, automated, inexpensive techniques for measuring viable microbes eventually will become standardized methods.

References

AOAC International (2000) Official Method 965.13: *Disinfectants (water) for swimming pools.* AOAC International, Gaithersburg, MD.

AOAC International (2006) AOAC Official Method 966.04, *Sporicidal activity of disinfectants.* AOAC International, Gaithersburg, MD.

AOAC International (2006) AOAC Official Method 991.47, *Hard surface carrier test.* AOAC International, Gaithersburg, MD.

AOAC International (2008) Official Method 2008.05: *Efficacy of liquid sporicides against spores of Bacillus subtilis on a hard nonporous surface quantitative three step method (First Action).* AOAC International, Gaithersburg, MD.

AOAC International (2009) AOAC Official Method 955.15 *Testing disinfectants against Staphylococcus aureus: Use-dilution Method*. AOAC International, Gaithersburg, MD, USA.

ASTM Standard E2562-07 (2007): *Standard test method for quantification of Pseudomonas aeruginosa biofilm grown with high shear and continuous flow using CDC biofilm reactor*. ASTM International, West Conshohocken, PA.

Bloomfield, S. F. and Looney, E. (1992) Evaluation of the repeatability and reproducibility of European suspension test methods for antimicrobial activity of disinfectants and antiseptics. *J. Applied Bacteriology* 73:87-93.

Buckingham-Meyer, K., Goeres, D.M., and Hamilton, M.A. (2007) Comparative evaluation of biofilm disinfectant efficacy tests. *J. Microbiol. Methods* 70:236-244.

Corry, J. E. L., Jarvis, B., Passmore, S., and Hedges, A. (2007) A critical review of measurement uncertainty in the enumeration of food micro-organisms. *Food Microbiology* 24:230-253.

EPA (2008) Protocols for testing the efficacy of disinfectants against Hepatitis B virus (HBV). (http://www.epa.gov/oppad001/hbv.htm)

Garthright, W. E. and Blodgett, R. J. (2003) FDA's preferred MPN methods for standard, large or unusual tests, with a spreadsheet. *Food Microbiology* 20:439-445.

Goeres, D.M., Hamilton, M. A., Beck, N. A., Buckingham-Meyer, K., Hilyard , J. D., Loetterle, L. R., Lorenz, L. A., Walker, D. K., Stewart, P. S. (2009) A method for growing a biofilm under low shear at the air–liquid interface using the drip flow biofilm reactor. *Nature Protocols*. 4:783-788.

Tomasino, S. F. and Hamilton, M. A. (2006) Modification to the AOAC sporicidal activity of disinfectants test (Method 966.04): collaborative study. *Journal of AOAC International* 89:1373-1397.

Tsuji, K. and Bussey, D. M. (1986) Automation of microbial enumeration: development of a disposable hydrophobic grid-membrane filter unit. *Applied and Environmental Microbiology* 52:857-860.

Zelver, N., Hamilton, M., Goeres, D., and Heersink, J. (2001) Development of a standardized antibiofilm test. in *Methods in Enzymology – Biofilms II*, Vol. 337, R.J. Doyle (Ed), Academic Press, New York, NY, pp 363-376.

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