

METHODS COMMITTEE REPORTS

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Study Director Report

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Summary

The U.S. Environmental Protection Agency (EPA) has statutory authority under the Federal Insecticide, Fungicide, and Rodenticide Act for regulating antimicrobial products used to control pathogenic microorganisms on inanimate surfaces. The EPA's regulations specify that product performance (efficacy) data must be submitted to support the registration of antimicrobial products, including sporicides, bearing claims to control microorganisms that pose a threat to human health. In addition, Homeland Security Presidential Directive 10 directs the EPA to take the federal lead for developing specific standards, protocols, and capabilities to address the risks of contamination following a biological weapons attack and developing strategies, guidelines, and plans for decontamination of persons, equipment, and facilities. EPA has taken action to address this directive and significantly improve the nation's ability to treat contaminated sites and to allow for safe reoccupancy. Developing proven standard methods for evaluating and testing the effectiveness of antimicrobial products, such as those used to decontaminate facilities contaminated in 2001 with spores of *Bacillus anthracis* (anthrax), is critical for protecting public health. To help facilitate EPA's initiatives to improve and develop test methods for antimicrobials products, EPA awarded AOAC INTERNATIONAL a multiyear contract in 2007. AOAC will provide services to assist EPA with single and multilaboratory validation trials, namely the procedural, technical, analytical, and statistical peer-review support services for acceptance of study design protocols and associated data, and the publication of validated methods for determining disinfectant efficacy, particularly for bioterrorism agents. The EPA is actively seeking input from the user/stakeholder community such as the Consumer Specialty Products Association (CSPA) in this effort. Roundtable discussions at AOAC Annual Meetings have been initiated by EPA to engage the stakeholder community and to seek comment on the proposed revisions.

Selected Study Director Topics

966.04 Sporocidal Activity of Disinfectants.—A collaborative study to evaluate several proposed modifications to the *Bacillus* component of the method was completed in 2006. Modified Method **966.04** (Method II), applicable for testing of liquid disinfectants against spores of *B. subtilis* on hard surfaces, was approved as a Revised First Action method (1) and is available on the AOAC *Official Methods of Analysis* (OMA) Website. Publication of the complete manuscript appears in the *J. AOAC Int.* (2). In 2006, the General Referee recommended continuation of the study to expand scope of modifications to include *Clostridium sporogenes*, suture loop carriers, and other surfaces and product formulations.

A collaborative study designed to evaluate modifications applicable to liquid and gaseous formulations when tested against *C. sporogenes* on hard (porcelain) surfaces was initiated in 2008. Egg meat medium, the culture medium for *C. sporogenes* currently specified in Method **966.04**, is no longer commercially available and finding a suitable replacement is critical. In addition, the use of a nonstandardized extract of raw soil as an amendment to egg meat medium, as stipulated in the method, may result in a highly variable spore suspension. Cooked meat medium, commercially available through Becton Dickinson (Franklin Lakes, NJ) was selected as a replacement due to its broad use for the culture and maintenance of clostridia and similarity to egg meat medium (i.e., content, sold as pellets). Manganese sulfate, shown to be a suitable replacement for soil extract in the *Bacillus* collaborative study (2), was evaluated for *Clostridium* in an effort to harmonize the sporulation protocols for both organisms. Eight laboratories participated in the study. The data have been collected and the study report is expected to be submitted to the Methods Committee on Antimicrobial Efficacy Testing by January 2009. Data from precollaborative studies on modifications to the *Clostridium* component of Method **966.04** were published in 2006 (3). The development and acceptance of a First Action alternate Method **966.04**, which includes the proposed modifications, is the goal of this project.

In a related project, EPA is generating in-house data to support modifications to the *B. subtilis* × suture loop combination. The proposed modifications will be applicable to liquid formulations when tested against spores of *B. subtilis* on a porous surface (silk and/or polyester loops) and will be consistent with previously approved modifications to the method for porcelain carriers. Comparative evaluations of current and modified procedures are being used to determine equivalency of spore loading, HCl resistance, and efficacy. The single-laboratory data will be used to design multilaboratory precollaborative studies. Test chemicals used in the efficacy component of the study are sodium hypochlorite (bleach), glutaraldehyde, and a combination of peracetic acid and hydrogen peroxide. The precollaborative studies are targeted for early 2009.

Validation of the Quantitative Three Step Method for Sporocides.—In 2008, the Three Step Method (TSM), a quantitative procedure for determining the efficacy of liquid sporicides, was granted First Action status. Based on the data, the TSM successfully met the statistical parameters for validation for quantitative test methods. The TSM was responsive to the change in efficacy of the chemical treatments and was highly repeatable. The scope of the TSM validation included testing liquid formulations against spores of *B. subtilis* (a surrogate for virulent strains of *B. anthracis*) on a hard, nonporous surface. Method **966.04** (Method II) was used as the reference method. The TSM uses 5 × 5 × 1 mm glass coupons to deliver spores into the sporicidal agent (400 μL) contained in 1.5 mL microcentrifuge tubes, 3 coupons per chemical treatment. Following exposure to the test chemical and neutralization, spores are removed from the carriers in 3 fractions by loosely washing (fraction A), sonication (fraction B), and prolonged agitation and spore germination (fraction C). Liquid from each fraction is plated on recovery medium for viable spore enumeration. Control counts (water control) are compared to the treated counts and the level of efficacy is determined by calculating the Log₁₀ reduction (LR) of spores; LR = log₁₀ (mean spores/control carrier) – log₁₀ (mean spores/treated carrier). The method was adopted *Official Methods* **2008.05** (4). The outcome of the collaborative study has been published (5). In the near future, the Study Director will seek to expand the scope of the TSM protocol (i.e., make minor modifications) to include additional coupon materials to represent porous surfaces relevant to buildings and environmental surfaces (e.g., wood, ceiling tile, concrete). Data (e.g., carrier counts, recovery) on porous coupons were presented to the Methods Committee on Antimicrobial Efficacy Testing during the 2008 Annual Meeting. In addition, the Study Director is interested in expanding the use of the TSM beyond sporeforming bacteria to include vegetative bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Preliminary data on this topic were also presented to the Methods Committee on Antimicrobial Efficacy Testing during the 2008 Annual Meeting.

Editorial Revisions to OMA Chapter 6 (Disinfectants).—The Use-Dilution methods (Methods **955.14**, **955.15**, **964.02**), the Tuberculocidal Activity of Disinfectants test (Method **965.12**), and the Germicidal Spray Products as Disinfectants test (Method **961.02**) have been prioritized for editorial review. Editorial revisions to the Use-Dilution methods and the Tuberculocidal Activity of Disinfectants test have been submitted and approved. In 2008, proposed editorial changes to the Germicidal Spray Products as Disinfectants test were submitted to the Methods Committee on Antimicrobial Efficacy Testing. The changes were discussed during the 2008 Roundtable and included input previously provided by the CSPA.

Procedural Modifications to Disinfectant Test Methods.—EPA has generated data to support procedural modifications to the Use-Dilution methods and the

Confirmative in vitro Test for Determining Tuberculocidal Activity (Method **965.12** II).

The AOAC Use-Dilution methods, **955.14** (*Salmonella enterica*), **955.15** (*Staphylococcus aureus*), and **964.02** (*Pseudomonas aeruginosa*), are used to measure the efficacy of hospital disinfectants on hard inanimate surfaces. The methods do not provide procedures to assess log density of the test microbe on inoculated penicylinders (carrier counts). Without a clear, standardized methodology for measuring and monitoring of carrier counts, the associated efficacy data may not be as reliable and repeatable. A report (*J. AOAC Int.* manuscript) was submitted to the Methods Committee on Antimicrobial Efficacy Testing that provides a standardized procedure to address this issue, and based on carrier count data collected by 4 laboratories over an 8 year period, minimum and maximum log density values were proposed to qualify the test results. In addition, a presentation was made by the Study Director to the Methods Committee on Antimicrobial Efficacy Testing during the 2008 Annual Meeting outlining the proposed modifications. Carrier count data were collected concurrently with performing 242 Use-Dilution tests. The tests were conducted on products bearing claims against *P. aeruginosa* and *S. aureus* with and without an organic soil load (OSL) added to the inoculum (depending on the specific product label claim). Six carriers were assayed per test for a total of 1452 carriers. All 242 mean log densities were between 6.0 and 7.5 [geometric means between 1.0×10^6 and 3.2×10^7 colony-forming units (CFU/carrier)]. Across microbes and OSL treatments, the mean log density (\pm SEM) was 6.7 (\pm 0.07) per carrier (a geometric mean of 5.39×10^6 CFU/carrier). The mean log density across 6 carriers per test showed good repeatability (0.29) and reproducibility (0.32). A minimum mean log density of 6.0 and a maximum of 7.5 (geometric mean of 3.2×10^7 CFU/carrier) were proposed as validity requirements for *S. aureus* and *P. aeruginosa*. This range provides for the potential inherent variability that may be experienced across a wide range of laboratories and the slight effect due to the addition of an OSL. A follow-up report is planned to present data to support the carrier count procedure and carrier counts for *S. enterica*.

Middlebrook 7H9 (M7H9) agar is the medium specified in Method **965.12** II for maintaining stock cultures of *Mycobacterium bovis* BCG. EPA also uses M7H9 agar plates for inoculum enumeration. Premade M7H9 is not commercially available and therefore, preparation requires valuable time and resources; however, Middlebrook 7H11 (M7H11) agar is available commercially and its use as an alternate growth medium is under investigation. Based on comparative plate counts and colony morphology, the media are comparable; thus, M7H11 is an adequate alternate to M7H9. An official modification to Method **965.12** II will be pursued with AOAC to allow the use of M7H11 for maintaining stock cultures and for plating inoculum.

Testing of Towelettes.—Standardizing test methodology for measuring the performance of antimicrobials formulated as towelette-based products is another key priority. During a

2008 Roundtable discussion, the Study Director and colleagues discussed the need to review current methods or pursue the development of a new method for resolving this issue. EPA's Microbiology Laboratory's Standard Operating Procedure on testing towelettes was presented as an example of a method currently in use. Further discussion with the Methods Committee on Antimicrobial Efficacy Testing and stakeholders will be necessary to address key methodology issues such as the type and size of carriers, number of towelettes per carrier, wiping pattern, measuring surface wetness, and incubation/recovery of used towelettes.

Call for Methods.—AOAC, under contract with the U.S. EPA, conducted a call for methods in an effort to verify/validate technology to augment and/or replace standard plate counts for use in quantitative methodology such as Method **2008.05** (TSM). The attributes of interest included (1) easy to use; (2) more efficient than standard plating techniques with regard to the amount of time spent by the analyst performing the plating, as well as the resources required; (3) commercially available; (4) enumeration medium contains a generic substrate to support the growth of *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *S. enterica*; (5) enumeration medium has a quick turnaround time; (6) technology for reading results is automated and commercially available (the capacity to enumerate colonies using the test kit can be separate from the recovery system); and (7) enumeration technology provides the ability to store images of the enumerated medium and its associated data. Several companies submitted product information to AOAC; the protocol for down-selecting technologies is under development and may include the use of the Methods Committee on Antimicrobial Efficacy Testing or the formation of an expert review panel to assist in the evaluation.

Future Initiatives.—During the 2008 Annual Meeting, several new priorities and topic areas were proposed by the Study Director. In addition to the towelette test, the new initiatives include (1) the development of chemical reference standards for use in collaborative studies and proficiency tests for antimicrobial methods; (2) the need for specific method performance criteria for antimicrobial tests; (3) standardized methodology for testing virucidal products; and (4) development, standardization, and validation of methods for biofilm. It is anticipated that each topic area will be further developed prior to the 2009 Annual Meeting.

The Study Director seeks continued support from AOAC, the Methods Committee on Antimicrobial Efficacy Testing, and the stakeholder community in the arena of antimicrobial test method development and revision.

Committee Actions

The Methods Committee on Antimicrobial Efficacy Testing, established in April 2007, continues to evaluate improvements, interpretations, and additions related to Chapter 6 (Disinfectants) of the OMA.

OMA Chapter 6 Editorial Reviews

An effort is underway to update and improve the methods of Chapter 6 (Disinfectants) of the OMA through editorial review. Editorial changes to the method, "Tuberculocidal Activity of Disinfectants" (**965.12**) were approved by the committee. Review of the Germicidal Spray Products as Disinfectants Test is in progress.

Work Items

(1) *Carrier Counts in the Use-Dilution Method.*—The EPA requested that the Methods Committee on Antimicrobial Efficacy Testing consider a proposal to add additional features to the AOAC Use-Dilution methods (**955.15** and **964.02**) designed to enhance the Use-Dilution methods through standardization of microbial populations on dried inoculated carriers.

Recommendations

- (1) Continue study.
- (2) Evaluate intralaboratory and interlaboratory variability on carrier counts.
- (3) Understand impact of carrier counts on experimental outcome.
- (2) *Inquiry into the Addition of a Pellicle Attenuation Step into the Use-Dilution Method.*—The Efficacy Working Group of the CSPA has inquired about a proposal to increase the reproducibility of the AOAC Use-Dilution methods (**955.15** and **964.02**) for *Pseudomonas aeruginosa*. This modification proposes dilution of the broth culture to be used for testing 1:50 in nutrient broth to solubilize any remaining pellicle

fragment and determination of carrier counts following a 30–60 min soak in subculture broth.

Recommendations

- (1) Continue study.
- (2) Examine scientific evidence supporting the change.
- (3) *Proposed Modification to the Clostridium sporogenes Portion of the AOAC Sporicidal Activity Test (966.04).*—A collaborative study is underway to evaluate procedural modifications, including the establishment of a commercially available culture media, as suggested by EPA to aid in the conduct and reproducibility of testing *C. sporogenes* with the AOAC Sporicidal Activity Test.

Recommendations

- (1) Continue study.
- (2) Review data generated during collaborative investigation.

References

- (1) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **966.04** (Method II)
- (2) Tomasino, S.F., & Hamilton, M.A. (2006) *J. AOAC Int.* **89**, 1373–1397
- (3) Tomasino, S.F., & Samilot-Friere, L.C. (2007) *J. AOAC Int.* **90**, 825–833
- (4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2008.05**
- (5) Tomasino, S.F., Pines, R.M., Cottrill, M.P., & Hamilton, M.A. (2008) *J. AOAC Int.* **91**, 833–852