

## METHODS COMMITTEE REPORTS

## Committee on Antimicrobial Efficacy Testing

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**Committee Actions**

Committee M, Committee on Antimicrobial Efficacy Testing, was established in April 2007, as a "break out" from Committee H, Microbiology topic area: Efficacy Testing of Disinfectants. Several members and General Referees of Committee H were transferred to this newly established Committee.

*Committee Training Session*

The initial meeting of Committee M was held on April 18, 2007 at AOAC in Gaithersburg, MD. In attendance were Committee M members, Committee M General Referee Vipin Rastogi, Study Director Stephen Tomasino (EPA OPP), Mark Coleman, Chair Official Methods Board, and AOAC staff. The meeting was held as a training session for all Committee M members, and covered the following topics: (1) overview of the AOAC, (2) AOAC and Standards, (3) overview of EPA and AOAC contract, (4) the Official Methods review and approval process, (5) roles and responsibilities, (6) ScholarOne online review site training, (7) collaboration between AOAC, EPA and CSPA, and (7) discussion of *Clostridium sporogenes* multilaboratory study.

*OMA Chapter 6 Editorial Reviews*

An effort is underway to update and improve the methods of Chapter 6 (Disinfectants) of the *Official Methods of Analysis* (OMA) through editorial review. 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 revision. Committee M completed the review of the use-dilution methods. Review of the Tuberculocidal Activity of Disinfectants Test and the Germicidal Spray Products as Disinfectants Test are in progress.

**Summary**

Under the Federal Insecticide, Fungicide, and Rodenticide Act, the U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs has the responsibility of 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 re-occupancy. 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.

EPA is spearheading an effort to update and improve efficacy test methods for antimicrobials, particularly sporicides, to support federal Homeland Security efforts. One component of this effort involves the assessment of the AOAC Sporicidal Activity of Disinfectants Test (Method **966.04**), the method currently accepted by EPA for generating efficacy data to support the registration of sporicides. AOAC Method **966.04** is a carrier-based test that provides a qualitative measure of product efficacy against spores of *Bacillus subtilis* and *Clostridium sporogenes*. In 2005, a collaborative study resulted in the adoption of several key modifications to the Method **966.04**; however, the modifications were limited to liquid chemicals tested against *B. subtilis* on hard surfaces. Additional modifications to other components of Method **966.04**, namely for *Clostridium*, silk suture loops, and gaseous chemicals, are planned in the near future. EPA is also interested in the development and selection of quantitative test methods to augment or eventually replace Method **966.04**. In this effort, a 10 laboratory collaborative study was launched in 2006 to seek validation of a quantitative method for testing sporicides, the Three Step Method (TSM). The validation data have been collected and the final report is under preparation.

Also, a comprehensive effort is underway to update and improve the methods of Chapter 6 (Disinfectants) of the *Official Methods of Analysis* (OMA) through editorial review. The overall focus of the editorial review is to update the sources of test-specific materials, establish equivalent materials and equipment where possible, provide ranges for temperatures, pH, and time periods, clarify procedures and to reduce cross referencing. 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 revision. To date, the use-dilution methods have been editorially revised and are available through AOAC for use. Proposed revisions to Methods **961.02** and **965.12** have been submitted to AOAC Committee M. 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.

To help facilitate the EPA's initiative to improve test methods for antimicrobials products, EPA awarded AOAC 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

### *Selected Study Director Topics*

**966.04 Sporicidal 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 Website. Publication of the complete manuscript appears in the *Journal of AOAC INTERNATIONAL* (*J. AOAC Int.*; 2). In 2006, the General Referee recommended continuation of the study to expand scope of modifications to include *C. sporogenes*, suture loop carriers, and other surfaces and product formulations. Plans are underway to launch collaborative studies to further modify Method **966.04**.

The first project (Task 3 of the EPA/AOAC contract) will include modifications applicable to liquid and gaseous formulations when tested against *C. sporogenes* on hard (porcelain) and porous (silk and/or polyester) surfaces. Data from precollaborative studies on modifications to the *Clostridium* component of Method **966.04** were published in 2006 in the *J. AOAC Int.* (3).

Egg meat medium (EMM), 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 EMM, as stipulated in the method, may result in a highly variable spore suspension. The primary focus of the precollaborative study was to find replacements for EMM and soil extract. A carrier count procedure, the establishment of target carrier counts (spores/carrier), and a neutralization confirmation procedure were also evaluated. The precollaborative study was limited to liquid products tested against *Clostridium* on a hard surface carrier (porcelain penicylinder). Cooked meat medium, commercially available through Becton Dickinson, was selected 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.

In the precollaborative study, spore suspensions of *C. sporogenes* were generated using EMM with soil extract (EMM/SE), cooked meat medium with soil extract (CMM/SE), and cooked meat medium with 5 µg/mL manganese sulfate (CMM/MnSO<sub>4</sub>). The titer of the spore suspension, carrier counts, resistance to hydrochloric acid (HCl), and efficacy against 3 liquid sporicidal agents were used to evaluate the potential of CMM and MnSO<sub>4</sub> as replacements. Based on the titer of spore suspensions, the

carrier counts, the HCl resistance pattern, and the efficacy studies, CMM/MnSO<sub>4</sub> was shown to be a suitable culture and sporulation medium and is recommended as a candidate for the replacement for EMM/SE. The CMM/MnSO<sub>4</sub> method is simple, very similar to the current method, reproducible, and eliminates the use of the highly variable garden soil extract. The development and acceptance of a Revised First Action alternate Method 966.04 is the goal of the project. Test conditions (e.g., contact times, product concentrations) are currently being refined by Study Director. The collaborative study protocol is expected to be submitted to Committee M by the end of 2007.

In a closely related project (Task 1 of the EPA/AOAC contract), additional modifications to Method 966.04 applicable to liquid formulations when tested against *B. subtilis* on a porous surface (silk and polyester carriers) will be pursued. The proposed modifications will be consistent with previous modifications involving porcelain carriers. The collaborative study protocol is expected to be submitted to Committee M by the end of 2007. The development and acceptance of a First Action alternate Method 966.04, which includes the proposed modifications, is the goal of this project.

*Validation of the Quantitative Three Step Method (TSM) for Sporicides*—In late 2006, an interlaboratory collaborative study was launched to seek validation of a quantitative method, the TSM. The purpose of the study was to evaluate the TSM according to procedures outlined in the AOAC Official Methods Program for method validation. Ten laboratories, representing both the federal and private laboratories, participated in the study. The approved

validation protocol was limited to the evaluation of the TSM for testing liquid chemicals against spores of *B. subtilis*, a surrogate for *B. anthracis*, on a representative hard nonporous surface (glass). The test chemicals used in the study represent three different classes of active ingredients: sodium hypochlorite (NaOCl), glutaraldehyde, and a combination of peracetic acid and hydrogen peroxide (PA/HP). The method's performance for porous materials (e.g., wood, concrete) and gaseous formulations will require additional collaborative studies.

Although the details of the method have been published (4), the TSM is considered a new method for the purpose of the validation study with a limited amount of historical use in the regulatory arena. 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 Log<sub>10</sub> reduction (LR) of spores; LR = log<sub>10</sub> (mean spores/control carrier) – log<sub>10</sub> (mean spores/treated carrier).

The level of quality assurance was consistent with EPA Good Laboratory Practice Standards (FIFRA 40 CFR Part 160). Test method training and study protocol familiarization were provided to each laboratory via teleconference in

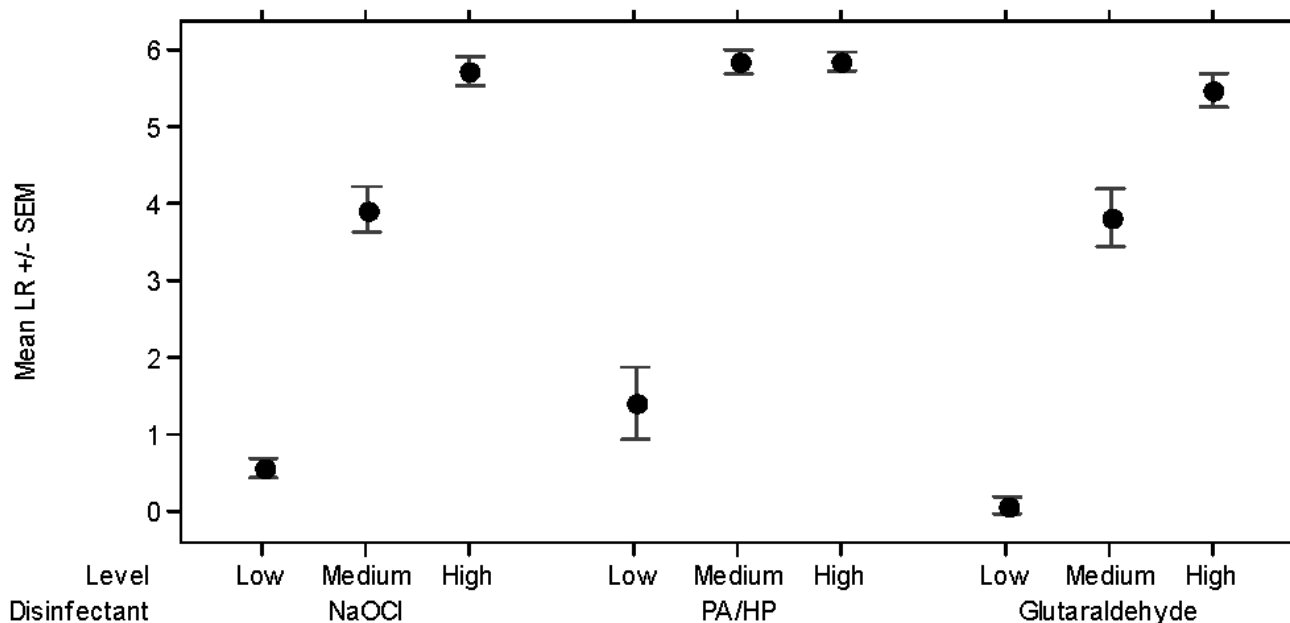


Figure 1. Preliminary TSM validation data. Mean LR values with  $\pm$  SEM (standard error of mean) error bars for the 9 treatments based on 24 TSM tests, 3 tests in each of 8 laboratories. The SEM takes account of both interlaboratory and intralaboratory variation.

advance of launching the study. Each laboratory designated a technician team to conduct the study. Each laboratory was asked to practice the test methodologies in advance of the study to gain proficiency. The Study Director provided the method protocols, standardized data sheets, media preparation sheets, test chemicals, and selected media and reagents. Test chemicals and the Material Safety Data Sheets were provided to each collaborator by the Study Director. A single production lot of each test chemical was used in the study.

The study design called for each of the 10 laboratories to test 3 chemicals at 3 projected levels of efficacy for each (i.e., high, medium, and low efficacy) using the TSM, one test chemical at 3 levels per day. Three replications (days) were required. The performance of the TSM was assessed by generating and comparing control counts and LR data, assessing the degree and sources of variability associated with the data, both within and between laboratories, and to evaluate responsiveness. AOAC Method **966.04** (Method II) was used as the reference method. For the purpose of this study, only the *Bacillus* and hard surface (porcelain penicylinders) components of Method **966.04** were conducted.

The data analysis and preparation of the final report are underway. Data from 9 laboratories were submitted; however, due to deviations in the protocol, one data set was deemed invalid and was excluded from the analysis. The preliminary analysis shows that the overall mean ( $\pm$ SEM) log densities for the TSM control counts was 6.86 ( $\pm$ 0.08) and that the method is very responsive to levels of product efficacy (Figure 1). Across the 9 treatments, the mean LR ranged from 0.1 to 5.8. For the mean LR values, the repeatability standard deviation ranged from 0.17 to 0.72 and the reproducibility standard deviation ranged from 0.34 to 1.43. With the exception of 2 treatments for PA/HP, the TSM produced LR values that properly ordered the efficacy levels. However, LR values for PA/HP were consistent across laboratories. Relative to other antimicrobial test methods (5), the standard deviation values are acceptably small, thus, the method appears suitable for consideration as a validated method.

*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 were submitted and approved in 2005 (6). EPA previously submitted draft editorial revisions to Method **965.12** to Committee H and discussed the revisions with AOAC officials, Committee H members, and stakeholders during the Disinfectant Roundtable on September 19, 2006, at the AOAC Annual Meeting. In addition, revisions to Method **961.02** were submitted to the 2006 Roundtable attendees, but the details were not discussed during the session; rather, a teleconference was held on October 30, 2006 with members of the CSPA and AOAC to review the revisions.

*Procedural Modifications to Disinfectant Test Methods.*—EPA has initiated research to support procedural modifications to the use-dilution methods and the

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

The AOAC Use-Dilution methods do not provide procedures to enumerate the test microbe on stainless steel carriers (carrier counts) or guidance on the expected target populations for the test microbe. An enumeration procedure and single laboratory carrier count data were published in 2006 in the *Journal of AOAC INTERNATIONAL* (7). Carrier counts from 78 Use-Dilution tests conducted over a 6 year period were compiled and analyzed. A mean carrier count of 6.6 logs (ca  $4.0 \times 10^6$  CFU/carrier) was calculated for *Staphylococcus aureus* (Method **955.15**) and *Pseudomonas aeruginosa* (Method **964.02**). Carrier counts from 3 additional laboratories have been added to the database; the data have been analyzed and are comparable to the data presented in the initial paper. The data were generated over a 7 year period from 242 efficacy tests. The average  $\log_{10}$  count ( $\pm$ SEM) per carrier across microbes with the presence/absence of 5% organic burden was 6.73 ( $\pm$ 0.061); repeatability and reproducibility standard deviations per carrier set were 0.29 and 0.31, respectively. The data will be used to support the addition of an enumeration procedure and the establishment of a minimum target carrier count level as official modifications to the Use-Dilution methods.

Also, Middlebrook 7H9 (M7H9) agar is the medium specified in AOAC 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 AOAC Method **965.12** II will be pursued with AOAC to allow the use of M7H11 for maintaining stock cultures and for plating inoculum.

The Study Director seeks continued support from AOAC, Committee M, and the stakeholder community in the arena of antimicrobial test method development and revision.

#### *Topics Discussed at Committee Meeting*

*Review and approval of Study Director report.*—The Study Director provided an overview of the report focusing on several of the major accomplishments of the previous year. The report was approved, as written, by the Committee.

*Editorial revisions status.*—The tuberculocidal method revisions were discussed and will be completed soon. The Germicidal Spray Products Test was identified as the next revision for Committee M review.

*Discussion of three step method validation data.*—The Study Director presented the results from the Three Step Method validation including the preliminary data and the improved method and accompanying video clips. The EPA contract funded a project that imbedded demonstrational video clips into the proposed method, accessible through the OMA *Online*. Each of the 12 videos have a run time of

approximately 20s, and demonstrate a critical procedural aspect of the Three Step Method. These videos will, of course, be subject to Committee review since they are part of the method being reviewed.

Discussion on AOAC **966.04**—*Clostridium sporogenes*. There was a discussion on the status of the collaborative study to expand the procedural modifications for method **966.04** to include changes for testing *Clostridium sporogenes*.

Discussion of use-dilution method carrier count data.—The Study Director presented data on the average carrier counts achieved at 4 government laboratories for *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Review of contract and upcoming work.—An overview was given on future work as outlined in the contract signed between AOAC INTERNATIONAL and EPA in mid-July.

## References

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