

GENERAL REFEREE REPORTS

Committee on Microbiology and Extraneous Materials

Food Microbiology, Non-Dairy

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Collaborative Studies

VIDAS Listeria monocytogenes II (LMO2) Immunoassay for the Detection of Listeria monocytogenes in Foods

Study Directors, Karen M. Silbernagel, Ronald L. Johnson, and Deborah A. McIntyre (*see* 29, 15, and 29, respectively). The VIDAS *Listeria monocytogenes* II (LMO2) test (bioMerieux, Inc., Hazelwood, MO) is an automated enzyme-linked fluorescent immunoassay for the specific detection of *L. monocytogenes* in selected foods. The objective of this collaborative study was to compare the performance of the VIDAS LMO2 immunoassay (alternative method) with that of standard culture methods [AOAC Official Method **993.12** (1), U.S. Department of Agriculture/Food Safety and Inspection Service's (USDA/FSIS) *Microbiology Laboratory Guidebook* (2), and the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM; 3)] for the specific detection of *L. monocytogenes* in selected foods. Samples are enriched in order to increase the number of target analyte organisms to the detectable limit of the assay (approximately 10^5 CFU/mL). All presumptively positive results must be confirmed by the appropriate reference culture method (1–3). It is intended that the assay be applicable as a presumptive test for the presence of *L. monocytogenes* in dairy products, vegetables, seafood, raw meats and poultry, and processed meats and poultry.

A collaborative study was conducted in which 5 food types (Brie, vanilla ice cream, frozen green beans, frozen raw tilapia fish, and cooked roast beef) were analyzed. A total of 26 laboratories participated. In this study, 1404 test portions were analyzed, of which 1152 were used in the statistical analysis. There were 448 positive test portions by the alternative method and 457 test portions positive by the reference culture methods. A Chi square analysis of each of the 5 food types, at each of the 3 inoculation levels, was performed. The resulting Chi square value of 0.36 indicated that there was no overall significant difference between the alternative method and the reference culture method at the 5% level of significance.

This alternative method has been approved as a First Action method.

VIDAS Listeria (LIS) Immunoassay for the Detection of Listeria in Foods Using Demi-Fraser and Fraser Enrichment Broths

Study Directors, Karen M. Silbernagel, Ronald L. Johnson, and Deborah A. McIntyre (*see* 29, 15, and 29, respectively). The VIDAS LIS test (bioMerieux, Inc.) is an automated enzyme-linked fluorescent immunoassay for the detection of *Listeria* spp. organisms in selected foods. The objective of this collaborative study was to compare the performance of the VIDAS LIS method with that of standard culture methods (1–3) for the detection of *Listeria* spp. organisms in selected foods, using an enrichment modification of AOAC Official Method **999.06**. Moreover, the modified enrichment protocol has been implemented in order to harmonize the VIDAS LIS assay with the VIDAS LMO2 assay, above. Test portions are first enriched in Demi-Fraser broth followed by a secondary enrichment in Fraser broth without ferric ammonium citrate as opposed to the original method which involved test portion enrichment only in buffered *Listeria* enrichment broth. The 2-step enrichment was implemented to increase the number of *Listeria* spp. organisms to the detectable limit of the assay. A positive result is considered presumptive for *Listeria* spp. and must be confirmed by appropriate reference culture procedures (1–3). It is intended that the assay be applicable as a presumptive test for the presence of *Listeria* spp. organisms in dairy products, vegetables, seafood, raw meats and poultry, and processed meats and poultry.

A collaborative study was conducted in which 5 food types (Brie; vanilla ice cream; frozen green beans; frozen, raw tilapia fish; and cooked roast beef) were analyzed. A total of 15 laboratories participated. In this study, 1206 test portions were tested, of which 1170 were used in the statistical analysis. There were 433 test portions positive by the alternative method and 396 test portions positive by the reference culture methods. A Chi square analysis of each of the 5 food types, at each of 3 inocula levels, was performed. The resulting average Chi square value of 0.42 indicated that there was no overall difference between the alternative method and the reference culture methods at the 5% level of significance.

This alternative method has been approved as a First Action method.

Evaluation of VIDAS Salmonella (SLM) Immunoassay Method with Rappaport-Vassiliadis Medium for the Detection of Salmonella in Foods

Study Directors, Wendy A. McMahon and Ronald L. Johnson (*see* 4 and 15, respectively). The VIDAS *Salmonella* (SLM) assay (bioMerieux, Inc.) is an automated enzyme-linked fluorescent immunoassay for the detection of

Salmonella organisms in foods and food ingredients. This assay is an already approved Official Method (996.08). A collaborative study was conducted to obtain approval of the alternative method using the selective enrichment, tetrathionate broth, in combination with Rappaport-Vassiliadis (RV) medium in place of selenite cystine (SC) broth. The replacement of SC broth with RV medium eliminates an Environmental Protection Agency hazardous waste issue for laboratories. Eight food types (nonfat dry milk; lactic casein; dried whole egg; milk chocolate; soy flour; raw, peeled shrimp; raw, ground turkey; and raw, ground pork) were analyzed by the alternative method and the reference methods (1–3). A total of 25 laboratories participated in the study. There were 1746 portions analyzed, of which 771 were positive by the alternative method using RV medium in place of SC broth, and 775 were positive by the reference culture methods. There were no significant differences in the numbers of positive test portions obtained by the 2 methods.

This alternative method has been approved as a First Action method.

Minor Method Modification Study

Study Director, Ian Garthwaite (*see* 31). The TECRA *Salmonella* Visual Immunoassay (TECRA, Frenchs Forest, New South Wales, Australia) Final Action Method 998.09, uses RV medium as a single selective enrichment broth. A proposed modification of this method involves addition of 25 L of a new sample proprietary additive to a 1 mL aliquot of the RV medium prior to the heat-killing step. This additive allows the RV medium to be tested directly in the alternative method and eliminates the need for a post enrichment in M broth.

An in-house validation study was conducted to compare modified Method 998.09 with the reference culture method (3) for the detection of *Salmonella* in 3 food types. Two food types (milk powder and peanut butter) were each inoculated at 2 levels, whereas 3 lots of a third food type (raw poultry) were naturally contaminated. Overall, there were no significant differences in the numbers of positive test portions obtained by the modified alternative method and the reference method.

This modified alternative method has been approved as a Revised First Action method.

Recommendations

(1) **2000.06** *Detection of Salmonella in Foods, Rappaport-Vassiliadis Medium Method*: Study Director, Thomas S. Hammack, U.S. Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740-3835, Tel: 301-436-2010, Fax: 301-436-2644, E-mail: thomas.hammack@cfsan.fda.gov. This method has been adopted Final Action; it is therefore recommended that this topic be discontinued.

(2) **998.08** *Enumeration of Escherichia coli in Poultry, Meat, and Seafood Products*: Study Director, Sonya A.

Gambrel-Lenarz, 3M Microbiology Products, 3M Center, Bldg 260-6B-01, St. Paul, MN 55144-1000, Tel: 651-733-0913, Fax: 651-733-1804, E-mail: SAGambrel-Lenarz@mmm.com. Discontinue topic.

(3) **998.08** *Confirmed E. coli Counts in Poultry, Meats, and Seafoods*: Study Director, Sonya A. Gambrel-Lenarz. This method has been adopted Final Action; it is therefore recommended that this topic be discontinued.

(4) *Clostridium botulinum* Toxins A, Proteolytic B, and E, ELCA Enzyme Immunoassay: Study Director, Wendy A. McMahon, Silliker Laboratories Group, Inc., 160 Armory Dr, South Holland, IL 60473, Tel: 708-225-1435, Fax: 708-225-1536, E-mail: Wendy.mcmahon@silliker.com. A precollaborative study report has been approved, and a collaborative study is planned once the Committee approves the protocol. Continue study.

(5) **2002.08 (H56)** *Detection of Botulinum Toxins A, B, E, and F from Culture Supernatants, Amplified ELISA Procedure*: Study Directors, Joseph L. Ferreira (retired), U.S. Food and Drug Administration, 60 8th St, Atlanta, GA 30309, Tel: 404-253-2216, Fax: 404-253-1210, E-mail: jferreir@ora.fda.gov; Susan Maslanka, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, Tel: 404-639-0895, Fax: 404-639-3333, E-mail: sht5@cdc.gov; Eric Johnson, University of Wisconsin, 1925 Willow Dr, Madison, WI 53706, Tel: 608-263-6949, Fax: 608-263-1114, E-mail: eajohnso@facstaff.wisc.edu; Michael Goodnough, University of Wisconsin, 1925 Willow Dr, Madison, WI 53706, Tel: 608-263-6949, Fax: 608-263-1114, E-mail: moodnou@facstaff.wisc.edu. This method was adopted First Action in 2002. Continue study.

(6) **2002.10 (H5)** *ISO vs AOACI Reference Culture Methods for the Detection of Motile and Non-Motile Salmonella in Selected Foods*: Study Director, Philip T. Feldsine, BioControl Systems, Inc., 12822 SE 32nd St, Bellevue, WA 98005-4318, Tel: 425-603-1123, Fax: 425-603-0070, E-mail: ptf@biocontrolsys.com. This study represented a cooperative effort between AOACI and the Central European Commission to develop performance data for ISO method 6579 (4). The ISO method was adopted First Action in 2002. Continue study.

(7) **2002.07 (H11)** *SimPlate Total Plate Count Color Indicator (TPC-CI) for the Enumeration of Total Aerobic Microorganisms in Foods*: Study Director, Philip T. Feldsine. This method was adopted First Action in 2002. The field experience with this method has been positive; therefore, the Study Director recommends that this method be adopted Final Action. The co-General Referees concur. Continue study.

(8) **996.09** *Visual Immunoprecipitate Assay for the Analysis of Ground Beef for Escherichia coli O157:H7*: Study Director, Philip T. Feldsine. This method was originally validated for the detection of *E. coli* O157:H7 in selected foods (dairy foods, meats, poultry products, fruits, nut meats, seafood, pasta, and liquid eggs) using an 18–28 h enrichment protocol. This method was adopted First Action in 1996 and Final Action in 1999. A method modification was validated to revise the enrichment protocol for raw and cooked beef

products only to allow for an 8 h enrichment. This method modification was adopted Revised First Action in 2002. Because the performance of this method modification for the analysis of raw and cooked beef products in the field has been satisfactory, the Study Director recommends that this method modification be adopted Final Action. The co-General Referees concur. Continue study.

(9) **997.03** *Visual Immunoprecipitate Assay for the Detection of Listeria monocytogenes and Related Listeria spp. in Selected Foods*: Study Director, Philip T. Feldsine. This method was originally validated for the detection of *L. monocytogenes* and related *Listeria* species in selected foods (dairy foods, red meats, pork, poultry and poultry products, seafood, fruits, vegetables, nut meats, pasta, chocolate, eggs, and bone meal). This method was adopted First Action in 1997 and Final Action in 1999. The method was subsequently validated for environmental surfaces and was adopted Revised First Action in 2001. Because the performance of this method for the analysis of environmental surfaces has been satisfactory, the Study Director recommends that this revised First Action method be adopted Final Action. The co-General Referees concur. Continue study.

(10) **2002.11** *SimPlate Yeast and Mold Color Indicator (Y&M-CI) Method for Enumeration of Yeasts and Molds in Foods*: Study Director, Philip T. Feldsine. This method was adopted First Action in 2002. Because the field experience with this method has been positive, the Study Director recommends that the method be adopted Final Action. The co-General Referees concur. Continue study.

(11) **996.10 MOD 9/21/00** *Assurance Enzyme Immunoassay for the Detection of Escherichia coli O157:H7 in Ground Beef*: Study Director, Philip T. Feldsine. This method was originally adopted First Action in 1996 and Final Action in 1999 for the detection of *E. coli* O157:H7 in selected foods (dairy foods, meats, poultry products, fruits, nut meats, seafood, pasta, and liquid eggs), using an 18–28 h enrichment protocol. A method modification was validated to revise the enrichment protocol for raw and cooked beef products only to allow for an 8 h enrichment. This method modification was adopted Revised First Action in 2002. Because the performance of this method modification for the analysis of raw and cooked beef products in the field has been satisfactory, the Study Director recommends that this method modification be adopted Final Action. The co-General Referees concur. Continue study.

(12) **H68** *SimPlate CEC Quantitative Method for Total Coliforms and Escherichia coli in Foods*: Study Director, Philip T. Feldsine. A precollaborative study manuscript has been submitted for review, and a collaborative study is nearing completion. Continue study.

(13) **996.14** *Assurance Polyclonal Enzyme Immunoassay for the Detection of Listeria monocytogenes and Related Listeria Species in Selected Foods*: Study Director, Philip T. Feldsine. This method was adopted Final Action in 2001 for the analysis of selected foods. This method was subsequently adopted Revised First Action in 2001 to include the analysis

of environmental surfaces. The co-General Referees recommend that this method be adopted Final Action for the analysis of environmental surfaces. Continue study.

(14) *Enumeration of Total Aerobic Microorganisms in Foods, SimPlate Total Plate Count Color Indicator (TPC-CI) System*: Study Director, Philip T. Feldsine. This topic is a duplicate of 7, above, and should be deleted.

(15) **MOD 999.06** *VIDAS Listeria (LIS) Immunoassay for the Detection of Listeria Species in Foods*: Study Director, Ronald L. Johnson, bioMerieux, Inc., 595 Anglum Rd, Hazelwood, MO 63042-2320, Tel: 314-506-8182, Fax: 314-731-8276, E-mail: Ron.JOHNSON@biomerieux.com. This method has been adopted as Final Action; it is therefore recommended that this topic be discontinued.

(16) **996.08** *VIDAS SLM Method for Detection of Salmonella in Foods*: Study Directors, Wendy A. McMahon and Ronald L. Johnson. This method has been adopted Final Action; it is therefore recommended that this topic be discontinued.

(17) **2001.07** *Salmonella in Selected Foods by Immuno-Concentration Salmonella (ICS) and Selective Plate (BS, HE, SMID) Procedure*: Study Directors, Wendy A. McMahon and Ronald L. Johnson. A new method for the detection of *Salmonella* organisms in foods in 48 h has been developed which uses both the VIDAS immuno-concentration (ICS) assay and a combination of 3 selective agar plates: bismuth sulfite (BS), Hektoen enteric (HE), and *Salmonella* identification (SMID). After overnight preenrichment of the test portion, immunological capture of the *Salmonella* organisms is accomplished by the VIDAS ICS assay. Following the VIDAS ICS assay, the released *Salmonella* cells are streaked to BS, HE, and SMID agars. The method was approved First Action for selected foods in 2001 and subsequently approved First Action for foods in general in 2004. It is recommended that the title of the topic be changed to “*Salmonella* in Foods by Immuno-Concentration (ICS) and Selective Plate (BS, HE, SMID) Procedure.” Continue study.

(18) **2001.08** *Salmonella in Selected Foods by Immuno-Concentration Salmonella (ICS) and Selective Plate (BS, HE, XLD) Procedure*: Study Directors, Wendy A. McMahon and Ronald L. Johnson. A new method for detection of *Salmonella* organisms in selected foods in 48 h has been developed which uses both the VIDAS ICS assay and a combination of 3 selective agar plates: BS, HE, and xylose lysine desoxycholate (XLD). After overnight preenrichment of the test portion, immunological capture of the *Salmonella* organisms is accomplished by the VIDAS ICS assay. Following the VIDAS ICS assay, the released *Salmonella* organisms are streaked to BS, HE, and XLD agars. The method was approved First Action for selected foods in 2001 and subsequently approved First Action for foods in general in 2004. It is recommended that the title of the topic be changed to “*Salmonella* in Foods by Immuno-Concentration (ICS) and Selective Plate (BS, HE, XLD) Procedure.” Continue study.

(19) **2001.09** *Salmonella* in Selected Foods by Immuno-Concentration *Salmonella* (ICS) and Enzyme-Linked Immunofluorescent Assay (ELFA): Study Directors, Wendy A. McMahon and Ronald L. Johnson. A new method for detection of *Salmonella* organisms in selected foods in a minimum of 24 h has been developed which uses both the VIDAS ICS assay and the VIDAS *Salmonella* (SLM) assay, the latter being Official Method **996.08**. After overnight preenrichment of the test portion, immunological capture of the *Salmonella* organisms is accomplished by the VIDAS ICS assay. Following the VIDAS ICS assay and release of the *Salmonella* cells, preenrichment is accomplished in a nonselective growth medium for 5–6 h (6–7 h for nonfat dry milk) followed by detection of *Salmonella* organisms in the test portion by the VIDAS SLM assay. The method was approved First Action for selected foods in 2001 and subsequently approved First Action for foods in general in 2004. It is recommended that the title of the topic be changed to “*Salmonella* in Foods by Immuno-Concentration *Salmonella* (ICS) and Enzyme-Linked Immunofluorescent Assay (ELFA).” Continue study.

(20) **2000.13 (H8)** Eight-Hour Reveal Screening Test for the Detection of *Escherichia coli* O157:H7 in Selected Foods: Study Director, Mark A. Mozola, Neogen Corp., 620 Leshler Pl, Lansing, MI 48912, Tel: 517-372-9200, Fax: 517-372-0108, E-mail: mmozola@neogen.com. Because the performance of this method in the field has been satisfactory, the Study Director recommends that this method be adopted Final Action. The co-General Referees concur. Continue study.

(21) **2000.14** Twenty-Hour Reveal Screening Test for the Detection of *Escherichia coli* O157:H7 in Selected Foods and Environmental Swabs: Study Director, Mark Mozola. Because the performance of this method in the field has been satisfactory, the Study Director recommends that this method be adopted Final Action. The co-General Referees concur. Continue study.

(22) **H-16** Total *E. coli* [Counts] in Food Samples for Determination of 10^4 CFU/g Action Levels: Study Director, Michael A. Grant, U.S. Food and Drug Administration, 22201 23rd Dr, SE, Bothell, WA 98021-4421, Tel: 425-402-3179, Fax: 425-483-4996, E-mail: mgrant@ora.fda.gov. This topic is a duplicate of 23, below, and should be deleted.

(23) **H-16** Improved Analysis of Food Samples for Total *Escherichia coli* Populations to Determine Whether 10^4 CFU/g Action Levels Have Been Exceeded: Study Director, Michael A. Grant. Continue study.

(24) **2001.07** *Salmonella* in Selected Foods, HE, BS, SMID Procedure: Study Director, Wendy A. McMahon. This topic is a duplicate of 17, above, and should be deleted.

(25) **3M** Petrifilm Staph Express for *Staphylococcus aureus* in Meat, Seafood, and Poultry: Study Director, Wendy A. McMahon. In a collaborative study, the 3M Petrifilm Staph Express Count plate method was compared with AOAC Official Method **975.55** (1) for the enumeration of *S. aureus* in selected foods. Details of this collaborative study were provided in last year’s General Referee Report (5). Despite the

title of this topic, the method has been adopted First Action as Official Method **2003.11** for the specific and exclusive analysis of cooked, diced chicken; cured ham; smoked salmon; and pepperoni. Continue study.

(26) **996.08** *Salmonella* in Foods, VIDAS SLM Method: Study Directors, Wendy A. McMahon and Ronald L. Johnson. This method has been adopted Final Action, it is therefore recommended that this topic be discontinued. Moreover, this topic is a duplicate of 16, above.

(27) **2001.09** *Salmonella* in Selected Foods by Immuno-Concentration *Salmonella* (ICS) and ELFA: Study Director, Wendy A. McMahon. This topic is a duplicate of 19, above, and should be deleted.

(28) **2001.08** *Salmonella* in Selected Foods, HE, XLD, and BS Procedure: Study Director, Wendy A. McMahon. This topic is a duplicate of 18, above, and should be deleted.

(29) **999.06 MOD (Hg135)** Evaluation of VIDAS *Listeria* (LIS) Immunoassay: Study Directors, Karen M. Silbernagel (retired), rtech Laboratories, MS 0075, PO Box 64101, St. Paul, MN 55164-0101, Tel: 651-766-1303, Fax: 651-486-0837, E-mail: kmsilbernagel@landolakes.com and Deborah A. McIntyre, rtech Laboratories, MS 0075, PO Box 64101, St. Paul, MN 55164-0101, Tel: 651-481-2636, Fax: 651-486-0837, E-mail: DAMcIntyre@landolakes.com. This modified assay has been validated as described previously (see *Collaborative Studies*) and has been adopted First Action. Change method number from **999.06 MOD (Hg135)** to **2004.06**, and continue study.

(30) **2003.01 (H-51)** Enterobacteriaceae in Foods, Dry Rehydratable Film Method: Study Director, Deborah A. McIntyre. This method was approved First Action in 2003. Continue study.

(31) **H995.22 MOD 5-23-01** TECRA Enrichment for *Listeria* in Foods: Study Director, Ian Garthwaite, TECRA International Pty Ltd, 13 Rodborough Rd, Frenchs Forest, NSW, 2086, Australia, Tel: 61-2-8977-3000, Fax: 61-2-9453-3422, E-mail: ian.garthwaite@tecra.net. This modification of Method **995.22** uses a new and less toxic enrichment procedure for the TECRA *Listeria* Visual Immunoassay. This method modification was adopted Final Action in 2002 as Method **2002.09**. This method was not intended to replace the original Method **995.22** but was offered as an alternative for the analysis of raw meats, processed meats, fresh produce/vegetables, fruit and fruit juices, seafood, and dairy products (cultured/noncultured). The Study Director reports that the field experience with this method has been favorable and recommends that the method be adopted Final Action. The co-General Referees concur. Continue study.

(32) **H66** Determination of *Escherichia coli* in Flesh Foods Using a Visual Immunoassay with a Modified Culture Procedure: Study Director, Ian Garthwaite. Continue study.

(33) **2000.07 MOD 2-15-01** TECRA Unique *Salmonella* Test: Study Director, Ian Garthwaite (see 30). A validation study was conducted to obtain approval of a specific method modification of the already approved UNIQUE *Salmonella* test, Official Method **2000.07**, to extend the applicability of

the method to fruit juices. In the modified method, the UNIQUE module is incubated at 42 C rather than at 37 C. In the validation study, 60 replicate test portions of orange juice (20 test portions each at high inoculum, low inoculum, and uninoculated levels) were analyzed with the modified alternative method and the reference culture method. No significant differences were observed in the numbers of positive test portions with the 2 methods. This modification of the alternative assay, Method **2000.07**, was approved Revised First Action in 2003. Continue study.

(34) **995.22 MOD 2/6/01 TECRA Listeria Visual Immunoassay (VIA) for Detecting Listeria spp. on Environmental Surfaces**: Study Director, Ian Garthwaite. The TECRA Listeria VIA was approved First Action in 1995 and Final Action in 1998. The Study Director plans to extend the applicability of the method to detect *Listeria* spp. on environmental surfaces. The precollaborative study has been completed, and a protocol for the collaborative study has been approved by the Committee. Continue study.

(35) **H17 Listeria in Selected Foods by TECRA UNIQUE 2000 Listeria Method**: Study Director, Ian Garthwaite. The precollaborative study is in progress, and a protocol for the collaborative study has been approved by the Committee. The manufacturer has removed "2000" from the name of the assay. Thus, the title of the topic should be changed to "*Listeria* in Selected Foods by TECRA UNIQUE Listeria Method." Continue study.

(36) **H71 Staphylococcus aureus in Foods, TECRA STAPH AUREUS Visual Immunoassay**: Study Director, Ian Garthwaite. The precollaborative study is nearing completion, and a protocol for the collaborative study has been approved by the Committee. Continue study.

(37) **2000.07 MOD 5/9/01 Salmonella in Foods, Rapid Colorimetric TECRA UNIQUE Test**: Study Director, Ian Garthwaite. The TECRA UNIQUE Salmonella Test was approved First Action in 2000 and Final Action in 2003. However, the test kit manufacturer has subsequently modified the module design so that the test may be read either visually or with the aid of a reader, the latter option allowing the assay to be performed either manually or automatically. A study was conducted to validate the modified UNIQUE assay for both manual and automated use. No significant differences were observed between the UNIQUE assays (either in the original format or with the minor modification in both manual and automated operations) and with the BAM reference method (3). A study report has been submitted for review. Continue study.

(38) **998.09 Salmonella in Foods, TECRA Salmonella Visual Immunoassay, Validation Study to Demonstrate Equivalence of a Minor Modification in 998.09 with Reference Culture Method**: Study Director, Ian Garthwaite. The TECRA Salmonella Visual Immunoassay (VIA), using Rappaport-Vassiliadis (RV) medium as the single selective enrichment broth, has Final Action approval. The test kit manufacturer has subsequently developed a more rapid protocol (TECRA ULTIMA) that includes a sample additive, thereby allowing direct analysis of the RV medium in the VIA

without subsequent post enrichment in M broth. An in-house validation study was conducted to compare modified Method **998.09** with the reference culture method (3). No significant differences were observed in the performance of the 2 methods. First Action approval of the minor modification of Method **998.09** has been granted so that the TECRA ULTIMA protocol is now an additional option within the method. The title of this topic should be changed to "**998.06 MOD3/13/03** Validation Study to Demonstrate Equivalence of a Minor Modification (TECRA ULTIMA Protocol) to AOAC Method **998.09** (TECRA Salmonella Visual Immunoassay) with the Reference Culture Method." Continue study.

(39) **H2000.07 MOD 2/15/00 Comparative Validation Study to Demonstrate Equivalence of a Modification of the TECRA UNIQUE Salmonella Test to the Reference Culture Method, 967.25-967.28, for the Detection of Salmonella in Foods**: Study Director, Ian Garthwaite. The TECRA UNIQUE Salmonella test was approved First Action in 2000 and Final Action in 2003 for the analysis of all foods except raw flesh foods. The test kit manufacturer plans to extend the applicability to all foods and to permit the module incubation temperature to be increased to 42 C for all foods. Protocols for precollaborative and collaborative studies have been approved by the Committee. Continue study.

(40) **Hg 130 VIDAS (LMO2) Immunoassay Method for Detection of Listeria Species in Foods**: Study Directors, Ronald L. Johnson and Deborah A. McIntyre. A collaborative study has been conducted, and the method has been approved First Action (see Collaborative Studies). Continue study.

(41) **2003.09 (Hg129) BAX System with Automated Detection of Salmonella in Foods**: Study Director, Deborah A. McIntyre. This system was approved First Action Method **2003.09** in 2003. Continue study.

(42) **2001.05 (H-77) Staphylococcus aureus in Foods, Dry Rehydratable Film Method**: Study Director, Deborah A. McIntyre. This method has been adopted Final Action; it is therefore recommended that this topic be discontinued.

(43) **2003.07 (Hg128) 3M Petrifilm Staph Express Count Plate Method for the Enumeration of Staphylococcus aureus in Selected Processed and Prepared Foods**: Study Director, Deborah A. McIntyre. This system was approved First Action Method **2003.07** in 2003. Continue study.

(44) **Hg 125 BAX System for Detection of Listeria monocytogenes in Foods**: Study Director, Deborah A. McIntyre. Continue study.

(45) **2000.15 (H48) Coliform Counts in Foods, Dry Rehydratable Film Method**: Study Director, Deborah A. McIntyre. This method was adopted First Action in 2000. In the absence of any adverse reports concerning this method, the General Referee recommends that this method be adopted Final Action. Continue study.

AOAC Research Institute Studies

The following studies have recently been approved by the AOAC Research Institute (RI):

(1) *Microwell DNA Probe Assay for Detection of Listeria spp. in Foods*: The GENE-TRAK *Listeria* Microwell Test (Neogen Corp., Lansing, MI) is a DNA probe-based diagnostic in kit format, which permits rapid detection of *Listeria* spp. in foods. Following test portion preenrichment and selective enrichment, target bacteria are lysed enzymatically at 37 C, and *Listeria*-specific oligonucleotide probes are added for a 60 min hybridization incubation at 45 C. If *Listeria* ribosomal RNA (rRNA) is present in the test portion, the detector probe, labeled with horseradish peroxidase (HRP), and a polydeoxyadenylic acid (poly dA)-tailed capture probe will hybridize to the target organism rRNA sequences. Concurrently, base pairing between the poly dA-tailed capture probe and the polydeoxythymidylic (poly dT) coated polystyrene microwells facilitates solid phase capture of probe-target molecules. Unbound probe is removed by washing, and then substrate chromogen is added to react with HRP to yield a blue color. The reaction is stopped by the addition of sulfuric acid, which changes the color of the substrate from blue to yellow. A microwell plate or microwell strip reader (A₄₅₀) measures absorbance. An absorbance in excess of the threshold value indicates the presence of *Listeria* in the test portion. Positive assay results must be confirmed by standard culture methods.

Inclusivity testing was conducted with 52 strains representing all of the *Listeria* species, and all of the strains were reactive with the assay. Exclusivity testing was conducted with 33 strains other than *Listeria* spp., and all of these strains were nonreactive.

An in-house comparative study was conducted comprising 15 food types and a total of 920 test portions. Performance of the DNA hybridization method was compared with that of the USDA reference culture method (2) for raw and cooked meat and poultry products. The performance of the alternative method was compared with that of the BAM method (3) for the analysis of dairy products, seafoods, and vegetables. In comparison to the USDA method, sensitivity of the DNA method was 96.0 versus 98.7% for the culture method. Agreement between the 2 methods was 97.1%. In comparison to the BAM method, sensitivity of the DNA method was 98.1 versus 92.3% for the culture method. Agreement was 94.0%. The overall specificity of the DNA assay was 98.7%. Chi square analysis indicated no statistically significant differences in performance between the DNA hybridization method and the appropriate reference culture method for any of the 15 foods tested, with the single exception of the Brie test portions inoculated with the target analyte at the high level. In this instance, the DNA hybridization method showed a higher detection rate than did the reference culture method. Results of an independent laboratory study with 2 food types provided additional data supporting product claims.

With respect to ruggedness testing, the following parameters were investigated: (1) number of mixing steps of the lysed test portion and the probe/hybridization solution; (2) volume of the premixed probe/hybridization solution; (3) number of wash steps; (4) hybridization incubation temperature; and (5) hybridization incubation time. Even

though mixing did not seem to be an important contributor to the appearance of background, the manufacturer recommends mixing the lysed test portions with the probe/hybridization solution 5 times to ensure complete distribution of the formamide in the solution. Ruggedness testing of a variation in the volume of the premixed probe/hybridization solution demonstrated that a volume of 0.125 mL probe/hybridization solution is needed. Without the proper concentration of formamide (hybridization solution) in the hybridization reaction, the assay conditions do not have the proper stringency, and false-positive reactions may occur due to mismatching of the probe sequences with non-target rRNA sequences. With respect to variation in the number of wash steps, the manufacturer recommends that the wells be washed 5 times before addition of the substrate chromogen solution. With respect to ruggedness testing of a variation in hybridization incubation temperature, the manufacturer recommends a temperature of 45 C. Ruggedness testing of a variation in hybridization incubation time indicated that a variation of up to 15 min above or below the recommended time of 60 min does not affect the performance of the test.

(2) *RapidChek Methods for the Detection of Listeria species in a Variety of Foods and on Selected Environmental Surfaces*: Two RapidChek methods have been validated. The RapidChek *Listeria* lateral flow device (LFD) method uses proprietary enrichment media for a 1-step 40 h enrichment, and detects *Listeria* spp. organisms on the LFD in 10 min. The RapidChek *Listeria* culture method also uses the same proprietary media as a 1-step enrichment, and detects *Listeria* spp. by streaking on selective agar plates as recommended by standard reference culture methods (2, 3). With the LFD, the test portion is enriched, boiled, and cooled. The test strip is added directly to the tube containing the cooled, boiled enrichment. The test portion flows up the strip through a zone containing antibody coated with colloidal gold reagents specific to *Listeria* species. If antigens are present in the test portion, they will bind with the antibody conjugates to form an antigen-antibody complex. As this complex migrates through the nitrocellulose matrix, it passes a zone of anti-*Listeria* antibody. If *Listeria* antigen is present, the complex is captured in this zone and is visualized by the formation of a red line. A second zone on the membrane is designed as a control to capture any antibody-gold complex not bound in the first zone. As a result, when *Listeria* antigen is present, the formation of 2 red lines is observed. When no *Listeria* antigen is present, only one line forms.

For the inclusivity study, all of 50 *Listeria* spp. strains were reactive with the LFD. With respect to the exclusivity study, only one (*Staphylococcus aureus*) of 30 non-*Listeria* strains was reactive with the LFD when grown in brain heart infusion broth. However, this same culture was nonreactive with the LFD when grown in the proprietary media.

The 2 alternative methods were compared to the USDA reference method (2) for the analysis of deli turkey, pepperoni, roast beef, and frankfurters and to the FDA reference method (3) for the analysis of milk, soft cheese, ice cream, potato salad, cooked shrimp, and smoked fish. Moreover, the

2 alternative methods were also compared to the USDA reference method for the analysis of 3 types of environmental surfaces (stainless steel, painted concrete, and rubber). A total of 260 spiked test portions were tested, with 189, 191, and 169 test portions positive with the RapidChek LFD method, RapidChek culture method, and the reference culture methods, respectively. The overall agreement of the RapidChek LFD method and the reference culture methods was 90%, whereas the agreement between the RapidChek culture method and the reference methods was 89%.

Ruggedness testing involved determination of variation of the following factors on the performance of the alternative method: (1) temperature of incubation of the LFD (4, 25, and 45 °C); (2) period of incubation (5, 8, 10, 15, and 20 min); (3) volume of test portion (300, 400, and 500 µL); (4) autoclaving versus nonautoclaving of proprietary media; (5) boiling time of incubated enrichment media (5, 10, 15, and 30 min); and (6) enrichment volume for sponges (60 and 100 mL). The specified variations in these parameters had no significant effect on the performance of the LFD.

(3) *RAPID L. Mono Agar for Identification of Listeria monocytogenes in Selected Foods*: This agar (Bio-Rad Laboratories, Hercules, CA) relies on the specific detection of phosphatidylinositol phospholipase C (PIPLC) activity of *L. monocytogenes*, resulting in a blue colony, and the inability of this species to metabolize xylose. The only species of *Listeria* to demonstrate PIPLC activity are *L. monocytogenes* and *L. ivanovii*. The addition of xylose to this medium allows for differentiation of these 2 species, because *L. monocytogenes* does not metabolize xylose. *L. ivanovii* produces colonies with a distinct yellow halo based on its ability to metabolize xylose, whereas *L. monocytogenes* will lack this halo. The other nonpathogenic species of *Listeria*, namely *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*, do not exhibit PIPLC activity, and produce white colonies on this agar. *L. welshimeri* will metabolize xylose and will therefore produce a white colony with a yellow halo. The selective solution contained in this medium inhibits the majority of interfering bacterial microflora, yeasts, and molds.

For the inclusivity study, 171 of 172 *L. monocytogenes* strains produced typical blue colonies on this agar, for a sensitivity of 99.4%. For the exclusivity study, all of 74 non *L. monocytogenes* cultures produced no typical colonies on this agar, for a specificity of 100%.

For the repeatability studies, 3 food types (Brie, surimi, and mixed salad) were examined by the internal laboratory. The foods were analyzed by the alternative method and either of 2 reference methods: AOAC (1) or FDA (3). The independent laboratory analyzed deli turkey. Method agreement for the alternative and reference culture methods ranged from 80 to 100%. There were no statistically significant differences between the alternative and reference methods for all of the 4 food types tested.

Ruggedness testing involved 3 variations: (1) type of enrichment broth (Demi-Fraser broth versus *Listeria* enrichment broth); (2) incubation temperature of the alternative agar; and (3) incubation time for the alternative

agar. None of these variations affected the performance of this agar.

(4) *Roche/BIOTECON Diagnostics LightCycler-Salmonella Detection Kit for Salmonella spp. in Combination with Salmonella ShortPrep Kit*: This method is based on a real-time polymerase chain reaction (PCR). This method has been designed to reduce the time necessary to achieve results from PCR reactions and to enable the user to monitor the amplification of the PCR product simultaneously, in real time. After DNA isolation using the Roche/BIOTECON *Salmonella* ShortPrep kit (BIOTECON, Potsdam, Germany) designed for rapid preparation of *Salmonella* DNA for direct use in PCR, the real-time detection of *Salmonella* DNA is performed with the Roche/BIOTECON LightCycler-*Salmonella* Detection kit.

For the inclusivity study, 707 *Salmonella* strains were tested, and all reacted positively with the assay. For the exclusivity study, 51 *Enterobacteriaceae* and other bacterial strains were tested, and none of these isolates reacted with the assay.

For the repeatability studies, 20 different food types, representing 15 food categories recommended by the RI, were analyzed. The foods were inoculated to achieve low (1–10 CFU/25 g) and high (10–50 CFU/25 g) levels of *Salmonella* organisms on the day of initiation of analyses. The foods were analyzed by the alternative method and either of 2 reference methods, USDA (2) or FDA (3), depending on the matrix. The sensitivity of the PCR method compared favorably with those of the reference culture methods at the low inoculation level (100 versus 97.0%, respectively) and at the high inoculation level (100 versus 99.7%, respectively). Results from an independent laboratory analyzing a single food type provided additional data to support product claims.

The ruggedness tests showed no influences to any variation of the following parameters: time for settlement of enriched food test portion, volumes for test portion, and (3) incubation temperatures for the DNA preparation procedure and for the PCR set up.

(5) *BAX System for the Detection of Escherichia coli O157:H7 in Apple Cider, Orange Juice, and Raw, Ground Beef*: The BAX system (DuPont Qualicon, Wilmington, DE) uses the PCR to amplify a specific fragment of bacterial DNA, which is stable and unaffected by the growth environment. The fragment is a genetic sequence that is unique to the *E. coli* O157:H7 serovar, thus providing a reliable indicator that the organism is present. This alternative method simplifies the PCR process by combining the requisite primers, polymerase, and nucleotides into a stable tablet already packaged inside the PCR tubes. After amplification, these tubes remain sealed for the detection phase, thereby significantly reducing the potential for contamination with one or more molecules of amplified PCR product.

This system uses fluorescent detection to analyze the amplified PCR product. Each PCR tablet contains a fluorescent dye which binds with double-stranded DNA and emits a signal in response to excitation light. During the detection phase, the temperature of the test portions is slowly

increased to denature the DNA. This action releases the dye and causes a drop in the emission signal. This system measures the denaturation temperature and the magnitude of the fluorescent signal change, and then analyzes those data to determine a positive or negative result.

For the inclusivity testing, the alternative method was reactive with all of 50 *E. coli* O157:H7 strains tested. Exclusivity results were 100% accurate for 10 non-*E. coli* strains, 10 *E. coli* non-O157 strains, and 11 *E. coli* O157:non-H7 strains.

The internal repeatability study for the analysis of apple cider and orange juice showed no significant difference between the alternative method and the BAM method (3). The external study for the analysis of ground beef included variations in enrichment media, as well as for time and temperature of incubation for the alternative method. All of the alternative method variation treatments demonstrated greater or comparable sensitivity and specificity than did the USDA reference culture method (2).

(6) *Genevision Method for Detection of Escherichia coli O157:H7 in Ground Beef*: The Genevision system (Warnex Diagnostics, Inc., Laval, Quebec, Canada) uses customized microplates and real-time PCR technology for detection of the target analyte. Each microplate contains all the ingredients needed to detect the target analyte. An assessment of target analyte contamination is made by specifically identifying the DNA signature of the target analyte organism in a series of sequential steps that include test portion collection, enrichment, DNA extraction, and DNA amplification by real-time PCR.

The fluorescence of the molecular beacons occurs once they recognize the target bacterial DNA sequence. The amount of fluorescence produced is proportional to the amount of DNA amplified and recognized by the beacon. In the absence of the target bacteria in food test portions, no fluorescence signal is detected.

For the inclusivity study, all of 62 *E. coli* O157:H7 strains were reactive with the assay. For the exclusivity study, 447 of 450 (99.3%) non-target analyte strains were nonreactive with the assay.

An independent laboratory study of this method was conducted in which the performance of the alternative method was compared with that of the reference culture method (2). Each test portion set consisted of forty-five 25 g ground beef test portions inoculated as follows: 20 replicates at the high level of inoculation with the target analyte (37.5 CFU/25 g), 20 replicates at the low level of inoculation (2.3 CFU/25 g), and 5 uninoculated control replicates. In the independent study, the sensitivity of the alternative method was equal to, or exceeded, that of the reference culture method at both levels of inoculation.

Ruggedness testing involved the determination of variation of the following 3 factors on the performance of the alternative method: (1) rehydrating the lyophilized component of extraction buffer with half the required amount of rehydrating fluid; (2) transferring in the detection plate 20% of the recommended amount of extracted DNA; and

(3) inverting the volumes of component of PCR master mix and DNA (10 L component of PCR master mix and 15 L DNA test portion instead of 15 L component of PCR master mix and 10 L DNA test portion). All deviations had no significant effect on the performance of the alternative method.

(7) *Genevision Method for Detection of Escherichia coli O157 in Ground Beef*: The mode of action for the Genevision system (Warnex Diagnostics, Inc.) for the detection of *E. coli* O157 in ground beef is the same as the Genevision system for the detection of *E. coli* O157:H7 in ground beef (see 6).

For the inclusivity study, all of 70 *E. coli* O157 strains were reactive with the assay. For the exclusivity study, all of 456 non-target analyte organisms were nonreactive with the assay.

An independent laboratory of this method was conducted as described previously (see 6). In this study, the sensitivity of the alternative method was equal to, or exceeded, that of the reference culture method at both levels of inoculation.

Ruggedness testing was performed as described previously and with similar results (see 6).

(8) *SINGLEPATH E. coli O157 for the Detection of Escherichia coli O157 in Pasteurized Whole Milk and Raw, Ground Beef*: The Singlepath *E. coli* O157 system (Merck KgaA, Darmstadt, Germany) is an immunochromatographic test which uses gold-labeled antibodies. The test device has a circular sample port and an oval-shaped test and control window. After appropriate enrichment, the test portion broth is applied to the nitrocellulose membrane by way of the sample port. The test portion is absorbed through the pad to the reaction zone containing colloidal, gold-labeled antibodies specific to *E. coli* O157 (including *E. coli* O157:H7). Any *E. coli* O157 antigen present complexes with the gold-labeled antibody and migrates over the membrane until it encounters the binding zone in the test area. The binding zone contains another anti-*E. coli* O157 antibody, which immobilizes any *E. coli* O157 antibody complex present. Because of the gold labeling, a distinct red line is formed. The remainder of the test portion continues to migrate to a second binding reagent zone within the control zone, where it forms a second distinct red line which serves as the positive control. In the control zone, an antibody, directed against the gold-labeled antibody, is immobilized onto the membrane. Irrespective of whether any *E. coli* O157 organisms are present, this distinct red line is always formed in the control zone, thereby ensuring that the test has been performed properly. When *E. coli* O157 organisms are present in the test portion, the formation of 2 distinct red lines is observed. When only one red line (control) is observed, there are no *E. coli* O157 organisms in the test portion.

Inclusivity testing was conducted with 60 *E. coli* O157 isolates, and all of these isolates were positive with this assay. Exclusivity testing was conducted with 36 bacterial isolates that included members of the *Enterobacteriaceae* (other than *E. coli* O157), other gram-negative organisms, and gram-positive organisms. All of these 36 cultures were negative with the assay.

In the internal laboratory study of raw, ground beef inoculated at a level of 0.23 *E. coli* O157 CFU/25 g, there was 100% agreement between the alternative and reference culture methods (2). In the independent laboratory study of pasteurized whole milk inoculated at a level of 1 *E. coli* O157 CFU/25 g, the alternative method detected 15% more positive test portions than did the reference culture method (3).

With respect to ruggedness testing, variation of the following parameters on the performance of the alternative method was investigated: lapse of time between the opening of the foil pouch containing test devices and the initiation of test (2 and 2.5 h); incubation of test device (18, 20, and 25 °C); and period of incubation (15, 20, and 25 min). The performance of the alternative method was not significantly affected by variation in any of these parameters.

(9) *Transia Card E. coli* O157 for Detection of *Escherichia coli* O157 in Raw, Ground Beef: This system (Diffchamb AB, Vasta Frolunda, Sweden) is based on an immunochromatographic test using colloidal gold-labeled antibodies as tracer and another anti-*E. coli* O157 antibody coated on a nitrocellulose membrane as the solid phase. The test device has a sample port and a result window containing test and control zones. The test portion is applied to the device by way of the sample well and is absorbed in the reaction pad containing colloidal gold-labeled antibodies specific to *E. coli* O157. Any *E. coli* O157 antigens present will complex with the gold-labeled antibodies and migrate to the test zone. The test zone contains another anti-*E. coli* O157 antibody which immobilizes any *E. coli* O157-colloidal gold antibody complex present. Because of the gold-labeling, a distinct reddish-purple line is then formed. The test portion continues to migrate to the control zone which contains another binding reagent. A distinct reddish-purple line (positive control) is formed in the control zone regardless of the presence or absence of *E. coli* O157 antigen. This control assures the analyst that the test is working properly.

For the inclusivity study, all of 50 *E. coli* O157 cultures were reactive with the assay. For the exclusivity study, 57 of 58 non-*E. coli* O157 cultures were nonreactive with the assay.

An independent laboratory study of this method was conducted in which the performance of the alternative method was compared with that of the reference culture method (2). Each test portion set consisted of forty-five 25 g raw, ground beef test portions inoculated as follows: 20 replicates at the low level of inoculation (6 CFU *E. coli* O157/25 g) examined by the alternative method; 20 replicates at the low level of inoculation (6 CFU *E. coli* O157/25 g) examined by the reference culture method (2); and 5 uninoculated control replicates. In this study, the performance of the 2 methods was equivalent.

Ruggedness testing involved determination of variation of the following 2 factors on the performance of the alternative method: volume of test portion added to the sample well (80, 120, and 160 µL), and incubation time (3, 5, 10, 15, and 20 min). The results demonstrated that all 3 test portion volumes resulted in a positive assay result, but that a minimum

incubation time of 5 min is needed to obtain a clearly positive signal in the alternative method assay.

(10) *BioSys/MicroFoss COLIFORM Test for Enumeration of Coliforms in Chocolate, Raw Eggs, Whole Chicken, Ground Beef, and Pork Sausage*: The BioSys/MicroFoss system (BioSys, Inc., Ann Arbor, MI) measures microbial growth by monitoring changes in pH or other biochemical reactions that result in a color change as the microorganisms in the broth grow and metabolize. Test portions are inoculated in ready-to-use vials that contain broth specific to the assay. This broth contains a peptone-yeast extract broth base with lactose as the carbon source. The selective agents include bile salts, sodium lauryl sulfate, and other gram-positive inhibitors. Acidification of the medium caused by lactose utilization by coliforms changes the pH. Bromocresol purple, the pH indicator, changes from purple to yellow. The vials contain, at the bottom, an agar barrier layer that separates the test portion containing liquid broth from the optical measuring area. The color changes in the agar mirror the color change in the broth, without letting the test portion particles or turbidity influence the measurements.

Light from light-emitting diodes passes through the agar, and a photo diode on the other side of the vial reads the color change as microbial growth occurs. A measurement is taken every 6 min. As soon as a color change is detected, the time of such detection is recorded. Detection times are inversely related to the number of organisms in the test portion.

With respect to the inclusivity study, all of 55 coliform cultures were reactive with the assay. For the exclusivity study, 51 of 53 noncoliform cultures were nonreactive with the assay. The 2 noncoliform cultures that were reactive with the assay were *S. Typhimurium* and *S. Senftenberg*.

For the repeatability study, chocolate and liquid eggs were artificially contaminated, whereas whole chicken, ground beef, and pork sausage were naturally contaminated. Four levels of paired samples were prepared so that the following levels of coliforms were used in the artificially contaminated test portions: 0, 10–100, 100–1000, and 1000–10 000 CFU/g. The naturally contaminated foods contained naturally occurring coliforms at each of 3 levels. Test portions were analyzed for coliforms by the alternative method and the solid medium procedure (violet red bile agar) of the reference culture method (3). There was close agreement between the 2 methods with correlation coefficients ranging from 0.92 to 0.99.

With respect to ruggedness testing, variation in test portion volume, broth volume, and incubation temperature demonstrated insignificant changes in detection times.

(11) *Compact Dry TC for Total Microbial Counts in Raw Meats*: This system (Nissui Pharmaceutical Co., Tokyo, Japan) consists of a plastic device containing a sheet of dehydrated non-selective medium containing peptone, yeast extract, glucose, phosphate buffer, magnesium chloride, algae polysaccharides (solidifying agent), and 2, 3, 5 triphenyl tetrazolium chloride as a redox indicator. A buffering solution is added to the test portion and homogenized. Serial 10-fold dilutions are made, and 1 mL inoculum is pipetted into the

middle of the dry sheet. The inoculum diffuses evenly throughout the sheet, transforming the sheet to a gel within seconds. During incubation, viable organisms react with the indicator dye to produce red colonies. These colonies are counted as in the conventional pour plating procedure in Official Method **966.23** (1).

This method was validated in the internal laboratory with 4 different types of raw meat (raw pork; raw, ground pork; raw lamb; and raw veal). This method was validated at both 35 C (temperature primarily used in North American laboratories) and at 30 C (temperature primarily used in European laboratories). No significant differences were observed in the performance of the 2 methods. With respect to accuracy for all pooled test portion data, correlation factors of $R^2_{35} = 0.9977$ (35 C incubation temperature) and of $R^2_{30} = 0.9932$ (30 C incubation temperature) were observed. In the independent study using raw, ground beef, no significant differences were observed between the alternative and reference methods.

For ruggedness testing, the effect of variations in inocula volumes (0.8, 1.0, and 1.2 mL); incubation temperature (30, 35, and 37 C), and incubation periods (18, 24, 27, 42, 48, 51, 66, and 72 h) on the performance of the alternative method was determined. Variations in inocula volume had no effect on method performance. Variations in incubation temperatures and incubation periods, however, resulted in different levels of aerobic plate counts, thus verifying the importance of using the alternative method plates at a defined incubation temperature and period.

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Efficacy Testing of Disinfectants

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Summary

The U.S. Environmental Protection Agency's (EPA) Office of Pesticide Programs (OPP) has the responsibility for

regulating antimicrobial products, including sporicides, used to treat and decontaminate inanimate surfaces. In response to the anthrax (*Bacillus anthracis*) attacks of 2001 and the associated need for verifying the performance of chemicals for building decontamination, the EPA initiated research in late 2003 to evaluate and improve efficacy test methods for sporicides. The OPP Microbiology Laboratory located at the Environmental Science Center, Ft. Meade, MD is the lead laboratory. Through funding provided by EPA's Office of Research and Development (Safe Buildings Program), a collaborative research plan has been established to address several key issues. Research is currently being conducted on 2 fronts: (1) the evaluation of quantitative methodology for assessing the efficacy of sporicides, and (2) the development and comparative testing of selected modifications to improve the AOAC Sporicidal Activity Test (AOAC Method **966.04**). Future studies will include the evaluation of candidate surrogates of *B. anthracis* using a quantitative method, and a multilaboratory validation study of a quantitative method—surrogate combination.

The General Referee is serving as the Principal Investigator for all research described in this report, and has the overall responsibility for the technical conduct of the projects. In cases where the General Referee has oversight of projects that involve official collaborative studies and validation support from AOAC INTERNATIONAL, AOAC officials and the Committee Chair will determine the appropriate mechanism for formal study review. The 2003 General Referee report provides the background on the development and direction of the research projects. The preliminary data, general conclusions, next steps, and recommendations are provided in this report.

Selected Study Director Topics

Currently, the EPA accepts the AOAC Sporicidal Activity Test, AOAC Method **966.04**, for generating efficacy data for sporicides. AOAC Method **966.04** provides a qualitative measure of product efficacy against spores of *Bacillus subtilis* and *Clostridium sporogenes*. The suitability of AOAC Method **966.04** for evaluating sporicidal chemicals has been challenged because of its limitations (e.g., qualitative nature, applicable for liquid and gaseous chemicals only), and lack of standardization in several critical steps. The purpose of the initial research is to evaluate quantitative efficacy test methods for liquid sporicidal chemicals on hard surfaces. In addition, modifications to the AOAC method are being investigated as a shorter-term/interim approach to improve the efficacy assessment of liquid sporicides. The data generated from these studies will be used to develop additional research projects and aid in the validation of a selected test method and surrogate for *B. anthracis*. Ultimately, the data, analyses, and study conclusions will be used to develop regulatory guidelines (i.e., new performance standards) for sporicidal products to be used in the treatment of buildings and contents contaminated with spores of *B. anthracis*.

Test Method Comparison Study

Three methods were selected for comparative investigation: AOAC Method **966.04** (1); Standard Quantitative Carrier Test Method-ASTM E 2111-00 (2); and Three Step Method (TSM), an adaptation of a quantitative micro-method as reported by Sagripanti and Bonifacino (3). AOAC Method **966.04** was included in the study as the gold standard due to its acceptance by EPA and the U.S. Food and Drug Administration (FDA) for regulatory performance standards for sporicides/sterilants. In early 2004, 3 federal laboratories conducted the studies in a standardized fashion: OPP Microbiology Laboratory, Ft. Meade, MD; FDA Denver District Laboratory, Denver, CO; and the U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. Prior to initiating the research, an on-site readiness review was conducted at each participating laboratory by the OPP Microbiology Laboratory Quality Assurance Unit to ensure compliance with a Quality Assurance Project Plan (QAPP), i.e., each laboratory was required to perform the necessary quality control activities consistent with EPA Good Laboratory Practices. Hands-on training was conducted for each test method to provide greater consistency of expertise from laboratory to laboratory, and precollaborative studies were conducted to establish test variables. A randomized test matrix, forms, data sheets, test chemicals, and selected reagents were provided by the lead laboratory (OPP Microbiology Laboratory).

AOAC Method **966.04** calls for treating 60 inoculated porcelain penicylinders (carriers) with the sporicidal agent, neutralizing the agent, and recovering viable spores in a liquid growth medium. Following treatment, each of the 60 carriers (EPA requirement) is evaluated for growth following incubation (21 days). Efficacy results from the AOAC Method **966.04** are scored as positive (tubes with growth) or negative (tubes without growth). Although currently not part of AOAC Method **966.04**, control carrier spore titer was evaluated and used to estimate log reduction (LR) of spores to allow for comparison between the AOAC Method **966.04** and the quantitative methods. The AOAC data were subjected to calculations per Hamilton et al. (4) to estimate the LR.

ASTM Method E 2111-00 uses a glass vial as the carrier, with 10 carriers per test. Following exposure, the sporicidal agent (1 mL) is neutralized, the spores are recovered using stirring and vortexing, and viable spores are recovered on filters.

TSM uses 5 × 5 mm glass coupons to deliver spores into the sporicidal agent (400 L) contained in 1.5 mL microcentrifuge tubes, 3 coupons per test. Following exposure, spores are removed from the carriers in 3 fractions by sonication and vortexing. Liquid from each fraction is plated on recovery medium for enumeration. For both quantitative tests, control counts were compared to the treated counts, and the degree of efficacy was determined by calculating the LR; $LR = \log_{10}(\text{CFU}/\text{control carrier}) - \log_{10}(\text{CFU}/\text{treated carrier})$.

Spores of *Bacillus subtilis* (ATCC 19659) were used. Inoculated porcelain penicylinders and spore suspensions were purchased from Presque Isle Cultures, 3804 West Lake Rd, PO Box 8191, Erie, PA 16505. In this study, the target spore load for the AOAC Method **966.04** was 1.0×10^6 spores/carrier with a minimum of 2.0×10^5 spores/carrier. The spore suspension used to inoculate carriers for the quantitative tests was generated by concentrating spores from the soil extract nutrient broth (per the AOAC Method **966.04**). The target carrier load for both quantitative tests was 1.0×10^7 spores/carrier. For each test method, horse serum (5%, v/v) was added to spores prior to inoculation to provide organic burden. Three test chemicals representing strong and weak sporicides were evaluated. The strong sporicidal treatments were sodium hypochlorite (3000 ppm, adjusted to pH 7.0) and a commercial sporicide containing 0.8% hydrogen peroxide and 0.06% peracetic acid. The weak sporicide treatment was sodium hypochlorite at 3000 ppm with an unadjusted pH. In addition, sodium hypochlorite was tested at 6000 ppm, adjusted to pH 7.0 in the AOAC Method. The contact time was 10 min for all treatments except the 6000 ppm sodium hypochlorite (30 min). Each laboratory tested each chemical 3 times (1 replication per day) with each test method. Analysis of variance for each combination of test method and test chemical was conducted to estimate the repeatability (within laboratory) standard deviation of Log_{10} reduction (LR) values, the reproducibility (between laboratory) standard deviation (SD) of LR values, and the within-test, intralaboratory, and interlaboratory components of variance.

The preliminary data analysis indicates that mean control counts (spores/carrier) for each test method fell within the expected target level; 1.5×10^6 for AOAC Method **966.04**, 1.2×10^7 for the ASTM method, and 3.1×10^7 for the TSM. Control carrier counts for the quantitative methods exhibited low SD within and between laboratories, thus indicating a high degree of reproducibility. The AOAC Method **966.04** exhibited consistent counts as well; however, only one set of carrier counts was required for each laboratory, and the repeatability SD was not calculated. Compared to the SD associated with other antimicrobial test methods (5), the ASTM and the TSM exhibited small and acceptable repeatability SD and reproducibility SD when challenged against the strong and weak sporicides. For the combined LR data for effective treatments, the reproducibility SD values associated with the ASTM and TSM were 0.46 and 0.49, respectively. The LR values for each test method treatment combination are presented in Table 1. For the AOAC Method, 60 out of 60 carriers were positive in 4 of 6 tests for the sodium hypochlorite (3000 ppm with unadjusted pH); the number of positive carriers ranged from 16 to 56 across 6 tests for the sodium hypochlorite (3000 ppm, adjusted to pH 7.0). For the commercial sporicide containing 0.8% hydrogen peroxide and 0.06% peracetic acid, the number of positive culture tubes ranged from 5 to 60 across 6 tests. In 4 of 6 AOAC tests, the 6000 ppm, adjusted pH treatment exhibited 0 positives.

Because the statistical similarities between the quantitative methods, other attributes such as ease of using the protocols,

amount of training necessary, logistics for setting up the test, number of technique-sensitive steps, and the human resources needed to conduct the test will be considered. A questionnaire was submitted to the analysts who conducted testing, and their responses were considered prior to selecting a method. Overall, TSM was determined to be the most suitable method, and was selected for further research.

The General Referee recommends continued research in method development for disinfectant testing. This initiative represents the first in a series of projects designed to advance our knowledge of quantitative sporicidal efficacy testing and surrogates for *B. anthracis*. The TSM will be advanced for future study (e.g., testing of surrogates of *B. anthracis*) and will be subjected to validation testing across multiple independent laboratories. Conducting the tests side-by-side in a comparative, standardized fashion has provided information on various method attributes such as throughput, technical expertise, within- and between-laboratory variability, and the use of LR as a measure of sporicidal efficacy. The EPA has entered into a contractual relationship with AOAC INTERNATIONAL to support this effort.

Modifications to AOAC Method 966.04: Collaborative studies will be conducted to officially modify and improve AOAC Method 966.04 to eliminate and/or reduce variability associated with important steps. Data comparing the current method and the modified version will be required to support the modifications. Three federal laboratories are participating in this effort: OPP Microbiology Laboratory, Ft. Meade, MD; FDA Winchester Engineering and Analytical Center, Winchester (WEAC), MA; and the U.S. Air Force Research Laboratory, Aberdeen Proving Ground, MD. Anticipated modifications are listed below. Several of the modifications listed (Nos. 1, 2, and 4) were previously proposed by scientists at FDA-WEAC. Modifications 1–7 are associated with *B. subtilis* only and will be limited to liquid sporicides and to hard surfaces (porcelain carriers). For *C. sporogenes*, only modification 8 applies. (1) *Sporulation medium*.—The current sporulation medium, the AOAC soil extract nutrient broth, is highly variable and uses raw garden soil as the source

of minerals. The use of a synthetic, standardized sporulation medium (e.g., nutrient agar amended 5 g/L manganese sulfate) is being evaluated. (2) *Carriers*.—The porcelain carriers are somewhat porous and the surface can become variable upon reuse. Stainless steel carriers will be used as an alternative hard surface material. (3) *Spore enumeration*.—The method does not provide instructions for performing carrier counts. Thus, the addition of a carrier count procedure for enumeration of spore inoculum is an essential modification and will be necessary to achieve items 4 and 6. (4) *Spore titer*.—The establishment of a minimum spore titer per carrier is necessary. The method does not indicate a lower limit; 2.0×10^5 spores/carrier will be proposed. (5) *Neutralization*.—The method does not include a procedure to confirm neutralizer effectiveness. A neutralization confirmation procedure is being evaluated. (6) *Data transformation*.—The application of a data transformation procedure (control counts and qualitative data used to estimate Log_{10} reduction) will be proposed. (7) *Wash-off*.—Wash-off of inoculum from carriers into the sporicide during exposure may lead to the loss of viable, recoverable spores. The method will be modified to include a step to assess the sporicide for viable spores following carrier exposure. (8) *Egg Meat Medium*.—A replacement for the Egg Meat Medium used for spore production of *C. sporogenes* will be pursued (e.g., Cooked Meat Medium). The commercial source, Becton Dickenson, has discontinued production of Difco Egg Meat Medium because of lack of sales.

For this study, the General Referee will serve as the Study Director. To avoid even the appearance of any bias, an Acting General Referee will be appointed. A Topic Advisor appointed from the membership of the AOAC Presidential Task Group on Disinfectants will provide additional technical input. The EPA recommended that the *Peer-Verified Methods*SM program approach be used for the modification of AOAC Method 966.04. However, because AOAC Method 966.04 is an *Official Method*SM, a modification through the *Official Methods*SM program will provide a higher level of confidence. A multilaboratory validation may be sufficient to make the modifications to an AOAC *Official Method*SM within the *Official Methods*SM program. This approach has been confirmed to be acceptable by Darryl Sullivan, Chairman, Official Methods Board.

In advance of submitting an official study to AOAC, studies are being conducted to establish test parameters and procedures for each modification. Several comparative studies have generated encouraging results to support modifications 1–4 listed above. The use of amended nutrient agar to generate spores for inoculation of test carriers (porcelain and stainless steel) has been very successful; carrier counts 2.0×10^5 spores/carrier have been achieved for both type of carriers. In addition, HCl resistance results from carriers inoculated with spores generated from soil extract nutrient broth and amended nutrient agar have been very similar (i.e., carriers must pass the HCl resistance test prior to use in efficacy testing; resist 2.5M HCl 2 min). Preliminary efficacy results for peracetic acid/hydrogen peroxide,

Table 1. Mean log reduction of *Bacillus subtilis* spores in test method comparison study

Test method ^a	Sodium hypochlorite, 3000 ppm, unadjusted pH	Sodium hypochlorite, 3000 ppm, adjusted pH	Peracetic acid/hydrogen peroxide product
ASTM	3.6	7.1	6.7
TSM	1.2	7.5	7.3
AOAC ^{b,c}	5.5	6.5	6.8

^a For ASTM and TSM, 200 was substituted for plates or filters with colonies too numerous to count and 0.5 for plates or filters with 0 colonies.

^b For estimate of LR calculation, see Hamilton et al. (4).

^c The 6000 ppm sodium hypochlorite adjusted pH treatment comprised 20 carrier screens; mean LR was estimated at 7.5.

gluteraldehyde, and sodium hypochlorite-based products have shown consistent performance results across each carrier type sporulation medium combination.

Once the precollaborative data have been generated, compiled, and analyzed, an official study will be submitted to AOAC by the Study Director for review. Upon approval, an official validation study will be conducted. The EPA has entered into a contractual relationship with AOAC INTERNATIONAL to support this effort. The General Referee recommends continued research and effort to officially modify AOAC Method **966.04**.

References

- (1) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD
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- (3) Sagripanti, J.L., & Bonifacino, A. (1996) *Am. J. Infect. Control* **24**, 364–371
- (4) Hamilton, M.A., Devries, T.A., & Rubino, J.R. (1996) *J. AOAC Int.* **78**, 1102–1109
- (5) Tilt, N., & Hamilton, M.A. (1999) *J. AOAC Int.* **82**, 384–389