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## FOOD BIOLOGICAL CONTAMINANTS

# ***Salmonella* in Foods—A New Enrichment Procedure for Use with the TECRA *Salmonella* Visual Immunoassay: Collaborative Study**

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**A collaborative study was conducted to compare a new enrichment procedure for the TECRA *Salmonella* Visual Immunoassay with the reference method given in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM 7th Ed.). Three food types (milk powder, black pepper, and soy flour) were analyzed in Australia, and 3 food types (milk chocolate, dried egg, and raw turkey) were analyzed in the United States. Thirty-eight collaborators participated in the study. No significant differences ( $p > 0.05$ ) were observed for the pairwise comparison of the proportion of positive samples for the TECRA method with that for the reference method. The new enrichment procedure for the TECRA method has been adopted First Action by AOAC INTERNATIONAL.**

The TECRA *Salmonella* Visual Immunoassay (TSVIA) is an enzyme-linked immunosorbent assay performed in the sandwich configuration. High-affinity "capture" antibodies specific to *Salmonella* spp. are adsorbed onto the surface of Removawell strips. If *Salmonella* antigens are present in the sample, they are captured by the antibodies. All other material in the sample is washed away. The sandwich is completed by the addition of enzyme-labeled antibodies (conjugate) specific for *Salmonella* spp. The presence of salmonellae is indicated when the bound conjugate converts the substrate to a green color.

Method 989.14, *Salmonella* in Foods (Using the TSVIA), was granted Association of Official Analytical Chemists (AOAC) First Action approval in 1989 and Final Action approval in 1990. In 1996, a modified version of the TSVIA, which had been supplemented with antibodies to *S. pullorum* and *S. gallinarum*, was adopted First Action (1).

For the previous AOAC studies on TSVIA, a cultural protocol using selective enrichment in selenite cystine (SC) and tetrathionate (TT) broths at 35°C was used. However, there has been a recent trend to use elevated incubation temperatures and to replace SC broth with the Rappaport-Vassiliadis (RV) medium (2).

This study was undertaken to validate a new enrichment procedure for use with the latest version of the TSVIA (approved in 1996). The new enrichment procedure for the assay incorporates Rappaport-Vassiliadis broth (R10) and TT broth at 42°C for selective enrichment. The reference method for the study was the 7th edition of the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM; 3), because this method was current at commencement of the precollaborative study. Since then, an 8th edition of BAM has been published, in which the method for raw flesh foods has been changed.

## **Collaborative Study**

The collaborative study was conducted in 2 parts: with milk powder, ground black pepper, and soy flour analyzed in Australia and New Zealand, and with milk chocolate, dried egg, and raw turkey analyzed in the United States. The 6 food types chosen were the same as those in the original collaborative study of the TSVIA (4). All foods were artificially contaminated with the exception of raw turkey, which was naturally contaminated.

To obtain the required number of collaborators in a small country such as Australia, some modifications were included in

Submitted for publication May 6, 1997.

The recommendation was approved by the Methods Committee on Microbiology and Extraneous Materials and was adopted by the Official Methods Board of AOAC INTERNATIONAL. See "Official Methods Board Actions" (1998) *Inside Laboratory Management*, July issue.

the collaborative study protocol. Two or more analysts working at the same institution were regarded as separate collaborators, provided that they worked independently and used separate media and reagents. In addition, a small number of collaborators sent their isolates to the organizing laboratory for confirmation.

#### *Preparation of Inoculum*

The cultures to be used as inocula were grown for 24 h at 35°C in Brain Heart Infusion (BHI) broth. Cultures were centrifuged to cell pellets and washed twice with 0.1M phosphate buffer (pH 7). The cell pellets were then resuspended in sterile nonfat milk and lyophilized at room temperature for 24 h. Freeze-dried inocula were ground to a fine powder prior to use.

#### *Inoculation of Samples*

For dried products (milk powder, ground pepper, soy flour, and dried egg), a concentrated "seed" was made by addition of freeze-dried inoculum to ca 500 g of the test product. This seeded product was mixed well and stored at room temperature for at least 2 weeks to allow cell levels to stabilize, prior to estimation of cell count by serial dilution and plating on Xylose Lysine Desoxycholate Agar. According to the estimated *Salmonella* count, an appropriate amount of the concentrated seed was added to the test product with the aim of achieving a high level (10–50 cells/25 g) or a low level (1–5 cells/25 g) of inoculation. A most probable number (MPN) determination was performed prior to sample dispatch, and levels of salmonellae were adjusted by addition of more seed or more product if necessary.

For milk chocolate, the seed was prepared by inoculating freeze-dried *Salmonella* culture into milk chocolate tempered at 55°C. It was then mixed with an electric mixer and held at room temperature for 2–3 weeks to stabilize. The stabilized seed was mixed with tempered chocolate to obtain target levels.

Naturally contaminated raw ground turkey was used for inoculation at high or low levels. Different batches of turkey were tested and, on the basis of contamination levels, used as uninoculated control or inoculated sample at high or low level. Samples were frozen prior to shipment.

#### *Sample Dispatch*

Dried foods and chocolate were shipped at ambient temperatures. Raw turkey was shipped frozen on dry ice.

Each collaborator received 15 samples of the food to be analyzed: 5 inoculated at high level, 5 inoculated at low level, and 5 uninoculated.

The appropriate samples were dispatched to participating laboratories the week prior to analysis. On arrival at the laboratory, samples were stored at room temperature with the exception of raw turkey, which was stored frozen.

#### *Sample Analysis*

A different food product was scheduled for testing each week. On the day when sample analysis was initiated by collaborators, an MPN determination also was performed by the organizing laboratory.

Each sample was analyzed by the BAM method (7th Ed.) and the TSVIA method using the enrichment protocol described in section F of the method, *Preparation of Sample*. Equal portions (0.5–1 mL) from each of the 2M broths arising from the 2 selective-enrichment broths were combined in a small screw-capped tube and heat treated in boiling water for 15 min prior to performing the TSVIA.

Confirmation for the immunoassay was by streaking from the M broth culture onto selective agar, as specified in the BAM reference culture method.

#### *Analysis of Data*

Data from each food type were collated, and numbers of false-negative results were determined for the TECRA method and for the reference culture method. The false-negative rate (the number of false-negative results divided by the total number of confirmed positive samples) and the false-positive rate (the number of false-positive results divided by the number of method negative results) were determined. A false-positive result was determined at the first assay or decision point for the procedure, that is, at the immunoassay result for the TECRA method and at the plate reading stage for the cultural method. The percentage agreement was determined as 100 times the number of samples giving identical results for both methods divided by the total number of samples. A pairwise comparison of the proportion of positive samples for the TECRA method with the reference method was made. McNemar's test ( $p > 0.05$ ) was used to determine whether the 2 methods were significantly different.

### **998.09 *Salmonella* in Foods, Colorimetric Polyclonal Enzyme Immunoassay Screening Method with Rappaport-Vassiliadis (R10) Broth and Tetrathionate Broth (TECRA *Salmonella* Visual Immunoassay)**

#### **First Action 1998**

The method is a screening procedure for the presence of *Salmonella* in all foods; it is not a confirmatory test because polyclonal antibodies used in the test may cross-react with a small percentage of non-*Salmonella* organisms. Enrichment broths and M broths from samples positive by an enzyme immunoassay (EIA) method must be streaked on selective media as in 967.26B, and typical or suspicious colonies must be identified as in 967.26C, 967.27, and 967.28.

Determination of positive results may be performed (1) visually by aid of a color comparator card, where a result is valid when negative and positive controls match those described on card, or (2) instrumentally, with a filter photometer, where a result is valid only when negative and positive controls possess acceptable optical density readings.

#### *A. Principle*

Detection of *Salmonella* antigens is based on an EIA using highly purified antibodies prepared from antigens unique to *Salmonella* spp. Polyclonal antibodies to *Salmonella* antigen are adsorbed onto the internal surface of a 96-well microtiter

tray. A sample to be assayed is placed into a well of the tray. If *Salmonella* antigens are present, they will attach to the antibody adsorbed on the well. All other material in sample is washed away.

A conjugate is added that will bind to *Salmonella* antigens if they are attached to the antibody adsorbed on the surface of the well. Wells are washed to remove unbound conjugate and enzyme substrate is added. A dark blue-green color indicates the presence of *Salmonella* antigen in sample.

## B. Method Performance

See Table 998.09 for method performance data.

## C. Reagents

Items (a)–(m) are available as TECRA *Salmonella* Visual Immunoassay from BioEnterprises Pty. Ltd. (28 Barcoo St, Roseville, NSW 2069, Australia) and International BioProducts, Inc. (Redmond, WA). Substitutions must be pretested for equivalency.

Caution: Never pipette by mouth; reagents may contain low levels of toxic substances. Use appropriate chemical and microbiological safety procedures.

(a) *Antibody adsorbed strips*.—Removawell (Dynatech Laboratories, Inc.) strips with polyclonal antibodies to *Salmonella*. Store at 2°–8°C when not in use.

(b) *Tray*.—Sufficient to secure individual wells or strips.

(c) *Control antigen*.—Positive control (lyophilized). Purified *Salmonella* antigen, which reacts with antibodies to *Salmonella*, one vial. Reconstituted control antigens are stable 2 months when stored at 2°–8°C.

(d) *Control diluent*.—One vial (6 mL/vial). Contains Tris–saline–Tween with 0.01% thimerosal. Also used as negative control.

(e) *Conjugate*.—Two vials (lyophilized). Contains anti-*Salmonella* antibodies (from sheep) conjugated to horseradish peroxidase in borate buffer (0.001M) with 0.01% thimerosal. Reconstituted conjugate is stable 28 days when stored at 2°–8°C.

(f) *Conjugate diluent*.—Two vials (13.5 mL/vial). Contains borate buffer (0.005M) with 0.01% thimerosal.

(g) *Substrate*.—One vial (lyophilized). Contains 0.011 g 2,2'-azinodi(3-ethylbenzthiazoline sulfonate) and 0.123 g NaH<sub>2</sub>PO<sub>4</sub>. Reconstituted substrate is stable 2 months when stored at 2°–8°C.

(h) *Substrate diluent*.—One vial (22 mL/vial). Contains 0.116 g citric acid, 0.0011 g H<sub>2</sub>O<sub>2</sub>, and 0.0185 g NaOH in H<sub>2</sub>O.

(i) *Stop solution*.—One vial (6 mL/vial). Contains 0.15 g NaF in H<sub>2</sub>O. Caution: Avoid contact with skin. If contact occurs, wash area with H<sub>2</sub>O. If spill occurs, sweep or pipette contents into beaker and dilute with water. Wash area with copious amounts of water.

(j) *Wash solution concentrate*.—One vial (25 mL/vial). Contains Tris–saline–Tween (58 g Tris/L, 234 g NaCl/L, 40 mL Tween 20/L) in 0.01% thimerosal. Reconstituted wash solution is stable 2 months when stored at 2°–8°C.

(k) *Color comparator card*.—For visual interpretation of positive and negative tests.

(l) *M broth*.—5.0 g yeast extract, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g sodium citrate, 5.0 g NaCl, 5.0 g K<sub>2</sub>HPO<sub>4</sub>,

0.14 g MnCl<sub>2</sub>, 0.8 g MgSO<sub>4</sub>, 0.04 g FeSO<sub>4</sub>, and 0.75 g Tween 80. Suspend ingredients in 1 L H<sub>2</sub>O and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°C. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(m) *Diagnostic reagents*.—Necessary for culture confirmation of presumptive EIA tests. Enrichment broths and M broths from samples positive by EIA method must be streaked on selective media as in 967.26B, and typical or suspicious colonies must be identified as in 967.26C, 967.27, and 967.28.

## D. Apparatus

(a) *Incubators*.—35°–37°C, 41°–43°C.

(b) *Pipets*.—Capable of delivering 2 and 200 µL volumes for immunoassay, and 1 and 0.1 mL volumes for sample enrichment.

(c) *Water bath*.—Capable of maintaining 100°C. Autoclave set at 100°C is an acceptable alternative, as are generators of flowing steam.

(d) *Plastic squeeze bottle*.—500 mL, for dispensing wash solution. Automatic washer may be used.

(e) *Plastic film wrap*.—To cover wells during incubation.

(f) *EIA reader*.—Optional. Photometer with 414 ± 10 nm screening filter that will read through microtiter plates, or dual-wavelength reader with filters at 414 ± 10 nm and 490 ± 10 nm.

## E. General Instructions

(1) Components of kit must be refrigerated when not in use. Kit is intended for one-time use only; do not reuse wells containing sample, reagents, or wash solution.

(2) Include single positive and negative control antigens with each group of test samples. All controls must function properly for test to be valid.

(3) Use data record sheet to identify location of each test sample.

(4) Use separate pipets for each sample and kit reagent to avoid cross-contamination. If plastic troughs are used to dispense conjugate and substrate, ensure that they are always kept separate.

(5) Components in kit are intended for use as integral unit.

## F. Preparation of Sample

(a) *Pre-enrichment*.—Pre-enrich product in noninhibitory broth to initiate growth of *Salmonella* spp. Methods used may vary with product and should be performed as indicated in 967.26A or BAM, 7th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, chapter 5, section C. Pre-enrichment broths should be incubated at 35°–37°C.

(b) *Selective enrichment*.—Transfer 0.1 mL incubated pre-enrichment mixtures to Rappaport-Vassiliadis R10 broth (9.9 mL) and 1 mL to TT broth (9 mL). For all foods other than raw foods or foods having a high microbial load, incubate 6–8 h at 41°–43°C. Selective enrichments of raw foods or foods having a high microbial load must be incubated 16–20 h at 41°–43°C. An air incubator is suitable for this purpose.

(c) *Post-enrichment*.—Remove selective broths from incubation and mix by hand or by Vortex mixer. Remove 1 mL from TT tube and transfer to separate tube of sterile M broth (10 mL)

Table 998.09. Statistical analysis of collaborative study results for comparison of TECRA assay with method in BAM, 7th Ed.

| Food<br>(No. labs)         | MPN <sup>a</sup> ,<br>cfu/g | Method<br>agreement, % <sup>b</sup> | Total<br>samples | Samples positive |                                  |               |               | $\chi^2$ <sup>c</sup> | Incidence of false<br>negatives among<br>total positive<br>samples, % <sup>d</sup> |                        |                  |                 | Sensitivity rate, % <sup>e</sup> |                       |                        |                          | Incidence of false<br>positives among<br>total negative<br>samples, % <sup>f</sup> |                         |                   |                | Specificity rate, % <sup>g</sup> |  |
|----------------------------|-----------------------------|-------------------------------------|------------------|------------------|----------------------------------|---------------|---------------|-----------------------|------------------------------------------------------------------------------------|------------------------|------------------|-----------------|----------------------------------|-----------------------|------------------------|--------------------------|------------------------------------------------------------------------------------|-------------------------|-------------------|----------------|----------------------------------|--|
|                            |                             |                                     |                  | ELISA            |                                  | Culture       |               |                       | ELISA                                                                              |                        | Culture          |                 | ELISA                            |                       | Culture                |                          | ELISA                                                                              |                         | Culture           |                |                                  |  |
|                            |                             |                                     |                  | Pres.            | Conf.                            | Pres.         | Conf.         |                       | Pres.                                                                              | Conf.                  | Pres.            | Conf.           | Pres.                            | Conf.                 | Pres.                  | Conf.                    | Pres.                                                                              | Conf.                   | Pres.             | Conf.          |                                  |  |
|                            |                             |                                     |                  |                  |                                  |               |               |                       |                                                                                    |                        |                  |                 |                                  |                       |                        |                          |                                                                                    |                         |                   |                |                                  |  |
| Milk<br>chocolate<br>(14)  | <0.003<br>0.009<br>0.093    | 100<br>100<br>100                   | 70<br>70<br>70   | 0<br>12<br>45    | 0<br>12<br>44                    | 0<br>12<br>45 | 0<br>12<br>45 | 6<br>15<br>47         | 0<br>12<br>45                                                                      | — <sup>h</sup>         | —<br>0.0<br>0.0  | —<br>0.0<br>0.0 | —<br>0.0<br>0.0                  | —<br>100<br>100       | —<br>100<br>100        | —<br>100<br>100          | 0.0<br>1.72<br>0.0                                                                 | 8.6<br>2.3<br>2.1       | 100<br>100<br>100 | 91<br>98<br>98 |                                  |  |
| Dried<br>whole egg<br>(13) | <0.003<br>0.009<br>0.430    | 98.5<br>98.5<br>100                 | 65<br>65<br>65   | 0<br>36<br>60    | 2<br>37 (35r) <sup>i</sup><br>56 | 1<br>36<br>60 | 0<br>35<br>60 | 0<br>35<br>60         | 0<br>35<br>60                                                                      | 0.0<br>0.0 (2.8r)<br>— | —<br>2.8<br>0.0  | —<br>2.8<br>0.0 | —<br>97<br>100                   | —<br>100 (97r)<br>100 | —<br>97<br>100         | 1.5<br>1.3 (0.0r)<br>0.0 | 0.0<br>0.0<br>0.0                                                                  | 99<br>99 (100r)<br>100  | 100<br>100<br>100 |                |                                  |  |
| Nonfat dry<br>milk (18)    | <0.003<br>0.24<br>2.4       | 100<br>98.8<br>100                  | 80<br>80<br>80   | 0<br>75<br>75    | 0<br>74<br>75                    | 0<br>74<br>75 | 0<br>75<br>75 | 0<br>75<br>75         | 0<br>75<br>75                                                                      | —<br>0.0<br>0.0        | —<br>1.3<br>0.0  | —<br>0.0<br>0.0 | —<br>100<br>100                  | —<br>99<br>100        | —<br>100<br>100        | 0.0<br>0.0<br>0.0        | 0.0<br>0.0<br>0.0                                                                  | 100<br>100<br>100       | 100<br>100<br>100 |                |                                  |  |
| Black<br>pepper<br>(15)    | <0.003<br>0.014<br>0.088    | 100<br>97.3<br>100                  | 75<br>75<br>75   | 0<br>37<br>66    | 0<br>35<br>66 (64r)              | 0<br>35<br>66 | 0<br>37<br>66 | 3<br>38<br>67         | 0<br>37<br>66                                                                      | —<br>0.5<br>—          | —<br>5.4<br>0.0  | —<br>0.0<br>0.0 | —<br>100<br>100                  | —<br>95<br>100 (95r)  | —<br>100<br>100        | 0.0<br>0.0 (1.2r)<br>1.2 | 4.0<br>0.9<br>1.2                                                                  | 100<br>100<br>100 (99r) | 96<br>99<br>99    |                |                                  |  |
| Soy flour<br>(15)          | <0.003<br>0.24<br>2.4       | 100<br>100<br>100                   | 75<br>75<br>75   | 0<br>69<br>68    | 0<br>69<br>68                    | 0<br>69<br>68 | 0<br>69<br>68 | 1<br>69<br>68         | 0<br>69<br>68                                                                      | —<br>—<br>—            | —<br>0.0<br>0.0  | —<br>0.0<br>0.0 | —<br>100<br>100                  | —<br>100<br>100       | —<br>100<br>100        | 0.0<br>0.0<br>0.0        | 1.3<br>0.0<br>0.0                                                                  | 100<br>100<br>100       | 99<br>100<br>100  |                |                                  |  |
| Raw turkey<br>(12)         | <0.003<br>0.043<br>0.46     | 100<br>85<br>90                     | 60<br>60<br>60   | 0<br>57<br>59    | 3 (4v) <sup>j</sup><br>52<br>53  | 0<br>51<br>54 | 0<br>54<br>56 | 33<br>59<br>59        | 0<br>54<br>56                                                                      | —<br>0.4<br>0.16       | —<br>10.5<br>6.9 | —<br>5.3<br>3.4 | —<br>89.4<br>93.1                | —<br>94.7<br>96.5     | —<br>95 (93.3v)<br>100 | 5.0 (6.6v)<br>1.6<br>0.0 | 55<br>7.9<br>4.8                                                                   | 45<br>98.4<br>95.2      |                   |                |                                  |  |

<sup>a</sup> Most probable number of colony-forming units per gram of food.<sup>b</sup> Rate reflects the number of confirmed determinations that were equivalent between the TECRA and the culture methods.<sup>c</sup>  $\chi^2$  is defined by McNemar as  $(a - b)^2 / (a + b)$  where  $a$  = samples positive by TECRA and negative by culture method, and  $b$  = samples negative by TECRA and positive by culture method. An  $\chi^2$  value greater than 3.84 indicates significance at  $p < 0.05$ .<sup>d</sup> Incidence of false negatives is  $100 - \text{sensitivity rate}$ .<sup>e</sup> Sensitivity rate is defined as 100 times the total number of analyzed positive test portions among "known" positive test portions divided by total number of "known" test portions, where "known" positive is defined as samples confirmed positive by the reference method.<sup>f</sup> Incidence of false positives is  $100 - \text{specificity rate}$ .<sup>g</sup> Specificity rate is defined as 100 times the total number of analyzed negative test portions among "known" negative test portions divided by the total number of "known" negative test portions, where "known" negative is defined as samples confirmed negative by the reference method and negative controls.<sup>h</sup> —, statistical analysis not applicable.<sup>i</sup> r, reader only.<sup>j</sup> v, visual only.