Enzyme Immunoassay - Membrane Filter Method for Detection of Salmonellae in Foods

JEFFREY M. FARBER*, PEARL I. PETERKIN, ANTHONY N. SHARPE and JEAN-YVES D’AOUST

Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, Health and Welfare Canada, Tunney’s Pasture, Ottawa, Ontario, Canada KIA 0L2

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) technique using a horseradish peroxidase-protein A-Spicer Edwards antiserum complex was developed for the detection of Salmonella colonies on membrane filters. In pure culture, 64 Salmonella species tested gave a positive reaction (purple stain). Of 22 naturally contaminated food samples, there was an exact correlation between the AOAC hydrophobic grid-membrane filter procedure and the ELISA technique (40.9% positives). This technique is simple, requires little equipment and can be completed in less than 2.5 h, thus allowing the detection of Salmonella spp. in foods within 48 h from initiation of sampling.

Conventional Salmonella spp. analysis from foods requires a minimum of 4 d after sample receipt to obtain presumptive results. To satisfy the growing needs of the food industry and government agencies for more rapid and automated Salmonella detection methods, researchers in the last decade have concentrated their efforts primarily on reducing the time required for this presumptive identification. Some of the novel techniques that have been used include fluorescent antibody (21), enrichment procedures (20), DNA-DNA hybridization (7) and enzyme-linked immunosorbant assay (ELISA; 9-11,13,14,16,19). Various problems have been encountered in each of these methods, although the last two techniques appear quite promising. The ELISA technique is simple to perform, and is relatively inexpensive, rapid, sensitive and amenable to automation.

Recently, a method based on ELISA was developed in which confirmed enterotoxin-producing Staphylococcus aureus strains could be enumerated within 27 h (12). In this method, cellular material was transferred from a hydrophobic grid-membrane filter (HGMF) to a nitrocellulose membrane (NCM), upon which the ELISA was performed. The potential of this method for the detection of Salmonella spp. in pure culture and in foods was investigated.

MATERIALS AND METHODS

Organisms

Bacterial cultures were obtained from collections maintained in research labs of the Health Protection Branch in Ottawa. A total of 64 strains of Salmonella representing an equal number of species was tested (Table 1). These isolates were obtained from foods or cases of human salmonellosis in Canada. Non-Salmonella test organisms included: Aeromonas hydrophila, Hafnia sp., Proteus morganii, Providencia sp., Shigella sonnel, Yersinia sp., Enterobacter agglomerans, Citrobacter freundii, Klebsiella pneumoniae, Serratia marcescens, Pseudomonas sp. and Escherichia coli.

Materials

Reagents and media were purchased from the following suppliers: protein A, horseradish peroxidase (HRP) type VI, sodium periodate and sodium borohydrdide from Sigma Chemical Co., St. Louis, MO; nitrocellulose membranes (NCM), 4-chloro-1-naphthol, Tris, gelatin and Tween 20 were contained in the Immuno-Blot assay kit from Bio-Rad Laboratories, Mississauga, Ontario, Canada; Salmonella H antisera Spicer-Edwards, nutrient broth (NB), tetrahydrate brilliant green (TBG) broth, triple sugar iron (TSI), lysine iron (LI), brain heart infusion (BHI), bismuth sulfite (BSA), brilliant green sulfa (BGS) and hektoen enteric (HE) agars from Difco Laboratories, Detroit, MI; selective lysine agar (SLA) and hydrophobic-grid membrane filters (ISO-GRID) from QA Laboratories, Toronto, Ontario, Canada. The monoclonal antibody MOPC-467 was kindly donated by Dr. J. A. Mattingly of Litton Bionetics, Inc. Foods were purchased locally or obtained from laboratory stocks of contaminated foods which had been stored at ambient temperature or at -20°C.

Conjugation of HRP to protein A or MOPC-467

The periodate method of Wilson and Nakane (22) was used to conjugate HRP to either MOPC-467 (7 mg) or protein A (5 mg) with Sephacryl S-200 chromatography being used to separate unreacted material. The pooled fractions of conjugate (HRP-protein A; HRP-MOPC-467) were stored in portions at -70°C and removed as required.

Preparation of HRP-protein A-Spicer-Edwards antiserum

A polyvalent H antiserum was prepared by mixing 0.1 ml of each of the seven sera comprising the Spicer-Edwards group.
TABLE 1. Salmonella serogroups tested by ELISA.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>No. of species tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>12</td>
</tr>
<tr>
<td>Other</td>
<td>12</td>
</tr>
</tbody>
</table>

This pooled antiserum (0.7 ml) was gently stirred with HRP-protein A (0.6 ml) for 1 to 1.5 h at 4°C and was then diluted 1:40 to 1:60 with 20 mM Tris-500 mM NaCl, pH 7.5 (TBS) containing 1% gelatin.

ELISA detection of Salmonella

HGMF inoculated with single lines of pure cultures of Salmonella or other Enterobacteriaceae were placed onto the surface of BHI agar and incubated for 18 to 24 h at 35°C, whereas filters with presumptive Salmonella isolates from artificially-contaminated foods were incubated on BHI, BSA and BGS agar media. For naturally-contaminated food, the sample (100 g) was dispersed in sterile NB by blending in 900 ml for 1 min at high speed in a Waring blender or, for raw poultry, bird and giblets were rinsed in a heavy sterile plastic bag containing 1 l of NB. The inoculated broth was incubated overnight at 35°C. The AOAC procedure for the HGMF method for Salmonella detection (5) was then followed. A portion (0.1 ml) of the preenrichment culture was transferred to 10 ml of tempered NB. The inoculated broth was incubated overnight at 35°C. The AOAC procedure for the HGMF method for Salmonella detection (5) was then followed. A portion (0.1 ml) of the preenrichment culture was transferred to 10 ml of tempered (35°C) TBG broth that was mixed and incubated 6 to 8 h in a 35°C waterbath. Portions (1 ml), or for raw poultry 1 ml of a 10^-2 dilution of TBG culture, were then filtered through sterile HGMF that were incubated on HE agar and SLA at 35 and 43°C, respectively, for 24 h. Growth on HGMF was screened for Salmonella by using the following ELISA or colony-lifting enzyme-linked antibody reaction (CLEAR) technique.

Dry NCM were either laid onto or rolled over colonies lying on the surface of the HGMF, thus transferring colony material from the HGMF to the NCM ("colony lifting" or blotting). The blots obtained from colonies grown on either BGS or BS agar were bleached by placing the NCM (blot up) on a filter development solution consisting of 0.05% 4-chloro-1-naphthol, 0.015% H_2O_2 and 16% methanol in TBS (2). To terminate the reaction, membranes were rinsed in distilled water and air dried. Isolates from three grid cells on HE and SLA agars giving positive reactions by the ELISA technique were then inoculated onto TSI and LIA; Salmonella-presumptive cultures were confirmed serologically by somatic and flagellar agglutination reactions (8).

RESULTS

ELISA method with pure cultures

Initial experiments performed with Salmonella Spicer-Edwards poly H antisera demonstrated cross-reactivity with other Enterobacteriaceae (results not shown). After addition of protein A to the antiserum (3,11), little or no cross-reactivity was observed. All 64 species of Salmonella tested gave positive purple reactions on the NCM (Fig. 1) and of the 13 non-salmonellae organisms tested, A. hydrophila and Providencia sp. gave a weak reaction. No endogenous peroxidase or catalase activity was noted as demonstrated by the lack of purple color development upon placing blotted NCM in the substrate solution. Additional ELISA experiments were performed on pure cultures by using MOPC-467 labelled with HRP-protein A as described previously. Untreated NCM failed to stain, whereas those dipped in 25% (vol/vol) sulfuric acid gave a weak, diffuse purple stain (see Discussion).

ELISA method with artificially-contaminated foods

In initial trials, HGMF were inoculated directly with 0.5 and 1.0 ml of a stomached decimal dilution of food (chicken, turkey and tuna flakes, raw egg, chocolate and chili containing 10^7 Salmonella infantis/ml) and then incubated on BGS, BS and BHI agars for 24 h at 35°C. The presence of food did not interfere with the ELISA reaction on the membrane filter (Fig. 2). Subsequent experiments using a low level inoculum (1 to 3 Salmonella/ml of diluted food) were equally encouraging.

ELISA method with naturally-contaminated foods

Twenty-two food samples were analyzed for the presence of Salmonella. In all cases, there was an exact correlation between the net result of the CLEAR technique and the AOAC/HGMF procedure (Table 2). In some instances, however, not all six grid cells (three grids each picked off HE agar and SLA) agreed with the results of the ELISA technique. For example, with Cheddar cheese samples, some grid cells that were positive by the CLEAR technique were negative by the conventional method. However, for all foods, a minimum of two and a mean of four grid cells were Salmonella-positive by both methods.

Figure 1. ELISA reaction on NCM. Organisms were grown up on an HGMF overlying BHI agar. An imprint of the colonies was taken with NCM, upon which the ELISA was performed.
On HE agar, *Salmonella* colonies appear black due to the production of H₂S. In Figure 3, it is seen that wherever black colonies appeared on HE agar, a perfect mirror image of purple dots (signifying positive *Salmonella* colonies) became visible on the NCM after the ELISA reaction was performed.

**TABLE 2.** Detection of *Salmonella* in foods by cultural and ELISA techniques.

<table>
<thead>
<tr>
<th>Food type</th>
<th>No. of samples tested</th>
<th>Culture&lt;sup&gt;a&lt;/sup&gt; Positive</th>
<th>Culture&lt;sup&gt;a&lt;/sup&gt; Negative</th>
<th>ELISA Positive</th>
<th>ELISA Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumen</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Egg</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pork liver</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pork sausage</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Poultry</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>AOAC procedure for a HGMF method for *Salmonella* detection (5).

**DISCUSSION**

As part of a development program for hydrophobic-grid membrane filter techniques in food microbiology (4,6,12,15), methods are needed which allow optical discrimination of colonies for manual or automated recording. The ELISA technique for *Salmonella* detection described here satisfies this goal and is simple, relatively rapid and inexpensive.

Development of the ELISA reaction directly on colonies growing on different membrane filter brands leads to diffuse and unsatisfactory stained areas. The reaction site is much more sharply defined when carried out on nitrocellulose membrane “blots” from the surface of the HGMF. The effect was also noted by Peterkin and Sharpe (12) working with enterotoxigenic *Staphylococcus aureus*. They attributed the effect to an accumulation of bacterial metabolites in the membrane filter at the site of the colony. Bio-Rad NCM yielded the best blots and Millipore HAWP (mixed cellulose esters) also performed satisfactorily; however, no reactions were obtained on Tuffryn HT-450 MF (polysulfone). This result is also consistent with that of Peterkin and Sharpe (12) who demonstrated minimal binding of protein to the surface of a polysulfone MF.

The IgA isotype monoclonal antibody derived from MOPC-467 mouse plasma-cytoma presumably recognizes a sequence of *Salmonella* flagellin molecule. Although early work performed by Robison et al. (14) indicated that MOPC-467 only bound to heated *Salmonella* cells, it was later demonstrated that heating was unnecessary (16). Our own attempts to use this monoclonal antibody on unheated *Salmonella* cells were unsuccessful. Because NCM does not withstand boiling, alternative approaches, such as dipping NCM into solutions of ethanol, formaldehyde, hydrochloric acid, sodium dodecyl sulfate, sodium hypochlorite, sulfuric acid and urea were tested. Dipping the MF into 25% sulfuric acid yielded the most satisfactory result; however, the reaction was still weak and diffuse compared with that obtained with Spicer-Edwards antisera, possibly due to failure to fully expose the antigen. All further work was performed using the Spicer-Edwards antisera.

On occasion, colony material from HGMF grid cells which had given a positive ELISA reaction on the NCM, could not be confirmed as *Salmonella* using conventional biochemical tests. The problem may be due to the presence of mixed growth in HGMF grid cells and masking.
of the Salmonella cells present. Occurrence of false-positive ELISA reactions or false-negative results by the conventional method cannot be ruled out. Cross-reactions with the HRP-protein A-Spicer-Edwards complex could potentially pose a problem upon examining a larger cross-section of samples, although in our hands it performed very well.

Three attempts were made to detect Salmonella in chicken without cultural enrichment. Whole chickens were shaken in 1000 ml of peptone water and 100 ml of the rinse was filtered. The ELISA reaction was developed directly from the incubated HGMF. In two experiments, grid cells were heavily overloaded by the flora which prevented confirmation of Salmonella. In the third, a presumptive positive by ELISA was later confirmed biochemically and serologically as Salmonella. Similarly, an unenriched sample of naturally-contaminated Cheddar cheese inoculated directly onto the HGMF was found to give a presumptive positive by ELISA was later confirmed biochemically and serologically. Further work to detect Salmonella in foods without cultural enrichment is in progress.

The ELISA membrane filter technique has several attractions: (a) filtration of a food suspension reduces or eliminates possible interference by inhibitors, (b) no centrifugation is required, (c) the distinctive colony-sized colored deposit minimizes ambiguity in recording results and (d) no minireader or microtitration plates are needed, and potential variations due to this (11) are eliminated. Additionally, the technique should be amenable to automation and lends itself readily to adaptation to more specific and sensitive antibodies.

REFERENCES