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Direct Immunoassay for Detection of Salmonellae in Foods and Feeds†

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A direct enzyme immunoassay (EIA) with polyclonal antibodies was developed for detecting salmonellae in foods and feeds. *Salmonella* cells were attached firmly to the wells of polystyrene microtitration plates with a capture-antibody technique. Spicer-Edwards anti-H immunoglobulin G was bound to protein A–β-D-galactosidase to serve as the signal; 4-methylumbelliferyl–β-D-galactoside was used as the substrate. The sensitivity threshold was 10^7 cells per ml. Direct EIA, indirect EIA, and pure-culture techniques were compared by using 48 samples of naturally contaminated foods and feeds. The direct EIA was more sensitive than the indirect EIA or pure-culture technique. Food samples were analyzed within 3 working days, and 32 samples were tested simultaneously in a single 96-well microtitration plate. False-positive or false-negative results did not pose a problem. This direct EIA is sensitive, rapid, and amenable to automation.

The food industry, regulatory agencies, and the public have a vital interest in decreasing the incidence of salmonellosis and other foodborne diseases. This is accomplished, in part, by quality control of raw materials and further processed foods.

Numerous methods exist for detecting the presence of salmonellae in foods. The pure-culture technique (PCT) (4), fluorescent antibody stain (14), and enrichment serology (12) are generally used for this purpose. The official method, recommended by the Food and Drug Administration (4), is the PCT. The PCT requires 4 days to obtain presumptive results and 5 to 7 days to obtain complete results. Fluorescent antibody (14) and enrichment serology (12) methods are rapid alternatives to the PCT. However, the fluorescent antibody method can yield high numbers of false-positive results, and the enrichment serology technique has not been widely accepted.

Krysinski and Heimsch (6) developed an enzyme immunoassay (EIA) for detecting salmonellae in foods. This method was improved (8) by using polystyrene microtitration plates as the solid phase and affinity-purified anti-immunoglobulin G antiserum. Although this improved EIA was sensitive and rapid, it was cumbersome for use in most quality control laboratories because of the need for centrifugation and inability to complete the assay in an 8-h workday.

In the present study, we developed a direct EIA procedure. The direct EIA was sensitive, rapid, and amenable to automation.

MATERIALS AND METHODS

Stock cultures, media, and samples. Bacterial cultures were obtained from the culture collection of the Department of Microbiology, Iowa State University and a co-worker. All media were commercial products of Difco Laboratories, Detroit, Mich., except M-broth, which was prepared as described by Sperber and Deibel (12). Samples of foods known to contain salmonellae were received from co-workers in the food-processing industry.

EIA procedure. Stocker and Heusser (13) and Voller et al. (15) described an indirect method for binding an antigen to a polystyrene microtitration plate by antibodies nonspecifically bound to the plate. The EIA developed in this study is a modification of these procedures.

To prepare capture antibody, a set of Spicer-Edwards anti-H antisera (Difco 2328-32-3) was combined and then diluted 1:100 in carbonate-bicarbonate buffer (1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$ per 1 liter of distilled water [pH 9.6]). Plates were washed with phosphate-buffered saline (PBS; 4.08 g of KH_2PO_4, 12.18 g of K_2HPO_4, 8.5 g of NaCl per liter of distilled water [pH 7.2]) containing 0.05% Tween 20 (PBS-Tween). Nonspecific binding to plates was blocked by using a 0.1% aqueous solution of bovine serum albumin (BSA; Sigma A-4503). An antibody–protein A–β-galactosidase conjugate was prepared by mixing 5 parts of pooled, undiluted Spicer-Edwards antiserum with 1 part of protein A–β-galactosidase (Sigma P-7650). This conjugate was stable for approximately 1 month. On each test day, antibody–protein A–β-galactosidase solution was made by diluting the antibody–protein A–β-galactosidase conjugate 1:100 in polyvinyl pyrrolidone-BSA-PBS (2.0 g of Sigma polyvinyl pyrrolidone-40, 1.0 g of BSA, 0.6 ml of formaldehyde in 100 ml of PBS [pH 7.2]). The substrate was prepared, just before it was used by warming 3.3 mg of β-MUGAL (4-methylumbelliferyl-β-D-galactoside; Sigma M-1633) in 0.2 ml of *N*,*N*-dimethyl formamide to about 80°C or until the β-MUGAL had dissolved; 9.8 ml of diluent (3.40 g of KH_2PO_4, 3.05 g of K_2HPO_4, 8.78 g of NaCl, and 0.95 g of $MgCl_2$ per liter of distilled water [pH 7.3]) was then added. This solution was diluted 1:10 to obtain a final concentration of 33 μg of β-MUGAL per ml.

For each sample tested, 0.2 ml of the capture antibody preparation was added to two test wells in a 96-well microtitration plate (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) One control well was also prepared for each sample by adding 0.2 ml of carbonate buffer without antiserum. The plate was incubated overnight at 4°C or for 4 h at room temperature. Excess antiserum was removed by immersing the plate in a beaker of PBS-Tween. The plate

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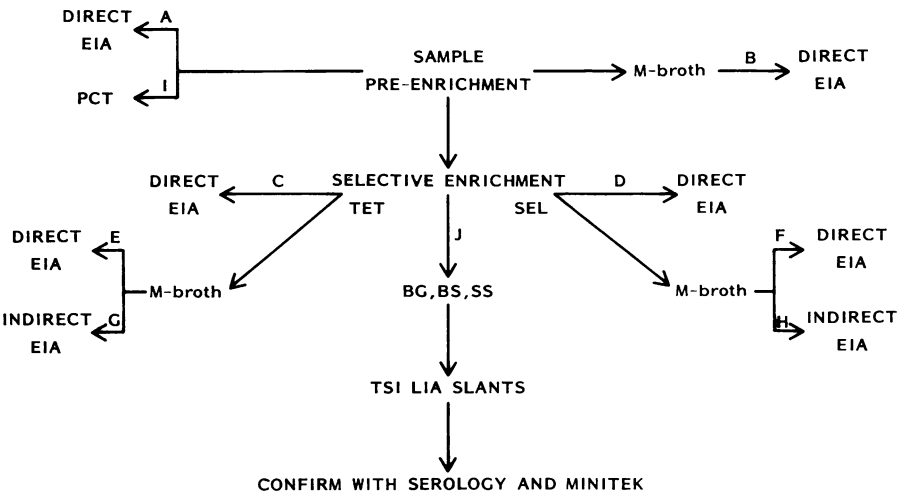

FIG. 1. Protocol followed during the examination of the food and feed samples. Method: A, direct EIA conducted on preenrichment; B, direct EIA conducted on secondary enrichment broth of preenrichment; C, direct EIA conducted on tetrathionate broth; D, direct EIA conducted on selenite cysteine broth; E, direct EIA conducted on the secondary enrichment broth of tetrathionate broth; F, direct EIA conducted on the secondary enrichment broth of selenite cysteine broth; G, indirect EIA conducted on the secondary enrichment broth of tetrathionate broth; H, indirect EIA conducted on the secondary enrichment broth of selenite cysteine broth; I, PCT conducted on the preenrichment broth; J, PCT conducted on the selective enrichment broths.

was removed and flicked gently to remove the wash solution. This procedure was repeated for a total of five times. Nonspecific binding was eliminated by filling the wells with BSA blocking solution and incubating for 15 min at room temperature. Excess BSA was removed by washing the plate five times in PBS-Tween. The test samples were then added as 0.1 ml of each to two test wells and 0.1 ml to a control well. The plate was incubated for 1 h at room temperature and then washed five times in PBS-Tween. The test and control wells received 0.1 ml of antibody–protein A–β-galactosidase and the plate was incubated for 1 h at room temperature. Unbound conjugate was removed by washing five times with PBS-Tween, and then 0.2 ml of β-MUGAL was added to each test and control well. The microtitration plate was incubated at 45°C for 1 h and examined visually under long wave length UV light for the presence of *Salmonella* spp., as indicated by fluorescence.

Preliminary studies. Twelve cultures of *Salmonella* spp. (*S. anatum*, *S. blockney*, *S. bredeney*, *S. heidelberg*, *S. illinois*, *S. miami*, *S. paratyphi* B, *S. rubislaw*, *S. salinatus*, *S. sandiego*, *S. schottmuelleri*, and *S. zuerich*) were grown in M-broth at 37°C overnight and analyzed by using the direct EIA procedure. Because all tests were positive (data not shown), two batches of hamburger were tested by using the direct EIA, indirect EIA (8), and a modified PCT (4) procedure to determine whether false-positives might be a problem. Hamburger was used because it contains a diverse population of members of the family *Enterobacteriaceae* and other gram-negative organisms (9). Hamburger samples (25 g) were homogenized with 225 ml of lactose broth. The homogenates were incubated for 18 h at 37°C, and then 1 ml of each homogenate was transferred to 10 ml of selenite cysteine broth. An additional 1 ml of each homogenate was transferred to 10 ml of tetrathionate broth. After incubation for approximately 24 h at 37°C, separate tubes containing 9 ml of M-broth (12) were inoculated with 1 ml from the selective enrichment broths; these were analyzed, after incubation for 6 h at 37°C, by using the direct EIA and indirect EIA (8) procedures. Streak plates of brilliant green, bismuth sulfite, and salmonella-shigella agars were also made from each selective enrichment broth. Hektoen enteric and xylose lysine agars (4) were not used because an abundant supply of the other media was available. Subsequent tests for the PCT were followed as outlined by the Food and Drug Administration (4). Separate tubes containing 9 ml of M-broth (12) were also inoculated with 1 ml from the selective enrichment broths; these were analyzed, after incubation for 6 h at 37°C, by using the direct and indirect EIA procedures.

EIA sensitivity threshold. *S. anatum* and *S. schottmuelleri* were used. Decimal dilutions of 10-h cultures in M-broth were made in PBS. Viable cell numbers were determined by using Trypticase soy agar pour plates, and appropriate dilutions of the cultures in PBS were analyzed by using the direct EIA.

Analysis of food samples. Foods and feeds known to harbor low numbers of salmonellae were analyzed with the direct EIA, indirect EIA (8), and a PCT method (4) modified as described previously. Each sample was subjected to 10 tests. An 18-h preenrichment (50 g in 250 ml of lactose broth) was examined by direct EIA and PCT (Fig. 1, arrows A and I). The preenrichment was further enriched for 4 to 6 h in M-broth and examined by using the direct EIA (Fig. 1, arrow B). Selective 24-h enrichments were examined by both direct EIA and PCT (Fig. 1, arrows C and D). The selective enrichments were further enriched for 4 to 6 h in M-broth and examined by using both the direct and indirect EIA procedures (Fig. 1, arrows E to H). *Salmonella*-like isolates obtained from the PCT were identified serologically by tube agglutination (12) and biochemically with the Minitek system (BBL Microbiology Systems, Cockeysville, Md.).

RESULTS

Preliminary studies. Hamburger samples were tested by direct EIA, indirect EIA, and PCT to determine the effects of non-salmonellae organisms and food proteins. All tests for salmonellae were negative, indicating that food microflora, at least that of hamburger, will not give false-positive results.

EIA sensitivity threshold. Two *Salmonella* serotypes, *S. anatum* and *S. schottmuelleri*, were used to determine the

TABLE 1. Comparative analysis of naturally contaminated foods and feeds containing low numbers of salmonellae[a]

Assay method[b]	Positive samples (group)		Total positive	% Positive
	I	II		
Direct EIA on preenrichment (A)	3	0	3	9
Direct EIA on M-broth from preenrichment (B)	8	4	12	34
Direct EIA on tetrathionate broth (C)	13	4	17	49
Direct EIA on selenite-cysteine broth (D)	4	7	11	31
Direct EIA on M-broth from tetrathionate (E)	14	2	16	46
Direct EIA on M-broth from selenite-cysteine (F)	14	8	22	63
Indirect EIA on M-broth from tetrathionate (G)	4	1	5	18[c]
Indirect EIA on M-broth from selenite-cysteine (H)	6	0	6	22[c]
PCT on preenrichment (I)	3	0	3	11[d]
PCT on selective enrichments (J)	8	1	9	32[d]

[a] Group I (20 samples): 1 fish meal, 2 meat meal, 17 mixed fish and meat meal. Group II (15 samples): 2 cocoa powder, 6 chocolate chip, 4 candy, 3 vegetable protein.

[b] The letters in parentheses refer to the steps designated in Fig. 1.

[c] This percentage is based on 27 samples; 8 samples were not tested by this method.

[d] This percentage is based on 28 samples; 7 samples were not tested by this method.

sensitivity threshold. A concentration of 10^7 cells per ml (10^6 cells per well) always gave a positive result. Variable results were obtained with 10^6 cells per ml (10^5 cells per well). A negative result was always obtained with 10^5 cells per ml (10^4 cells per well).

Examination of food and feed samples. Of 48 food and feed samples analyzed, 35 yielded positive results by one or more of the assays. Of these 35 positive samples, only 22 were detected by a single method (Table 1, method F). Salmonellae were detected from the preenrichment broth of only 9% of the samples and from the secondary enrichment M-broth of the preenrichment broth in only 34% of the samples by the direct EIA (Table 1, methods A and B). However, 31 to 63% of the samples were positive when the direct EIA was used to analyze selective enrichment broths or secondary enrichments in M-broth of the selective enrichment broths (Table 1, methods C through F). The indirect EIA detected salmonellae in only 18 to 22% of the samples (Table 1, methods G and H). The PCT detected salmonellae in 32% of the samples after selective enrichment; when preenrichments were examined by PCT, only 11% of the samples were positive.

Selective enrichment broths and their respective M-broths were positive for salmonellae at a frequency higher than were the preenrichment broths when the direct EIA (Table 1) was used. Emswiler-Rose et al. (3) also found that ELISA tests on preenrichment broths resulted in substantial numbers of false-negative results. The two selective enrichment broths, when tested by using the direct EIA (Table 1, methods C and D, combined), yielded 63% total positive results (data not shown). The direct EIA, when performed on M-broths prepared from both selective enrichment broths (Table 1, methods E and F, combined), yielded 69% total positive results (data not shown). When both the selective enrichment broths and their respective M-broths were examined (Table 1, methods C through F, combined), however, 97% of the subsamples that were positive by one or more tests were positive with the direct EIA (data not shown). Thus, optimum detection of salmonellae was obtained when both the selective enrichment broths and M-broths prepared from them were examined by using the direct EIA. Only one sample was negative by the direct EIA when the PCT result was positive.

DISCUSSION

D'Aoust (2) suggested that immunological methods, especially EIA and radioimmunoassay techniques, hold promise for the rapid detection of salmonellae in foods and feeds. Minnich et al. (8) developed a sensitive EIA, but this assay was not suitable for use in quality control laboratories because the test required a 9-h period from start to finish, and centrifugation was necessary. The EIA reported here is a direct EIA that can be completed within an 8-h work day and does not require centrifugation. Furthermore, all reagents are commercially available.

Staphyloccal protein A acted as the cross-linking reagent in the antibody-enzyme conjugate used in the present method. Because protein A binds primarily to the Fc region of immunoglobulin G (5), the possibility of cross-reactions and false-positive results caused by immunoglobulin M antibodies in the antiserum was reduced (8). The conjugate was easy to prepare, but precautions were taken to ensure the proper ratio of antibody to protein A–β-galactosidase. Excess protein A–β-galactosidase could attach to the bound capture antibody and cause false-positive results. Free antibody would decrease the sensitivity of the assay by competing with conjugated signal antibody for binding sites on the flagella.

The sensitivity threshold of the direct EIA was 10^7 salmonellae cells per ml or 10^6 cells per microtitration well. This level of sensitivity is one order of magnitude higher than that reported by other workers (8, 11). Our reactions were determined visually, however, and use of a microtitration plate reader, which detects fluorescent reaction products, would lower the sensitivity threshold of the direct EIA by one order of magnitude or more.

Mattingly and Gehle (7) stated that the antibodies used by Minnich et al. (8) showed "cross-reactivity to miscellaneous antigens." Cross-reactivity to miscellaneous antigens was not detected, however, either in the present study or in that of Minnich et al. (8). Positive direct EIA results were never obtained on numerous subsamples of foods in which other subsamples gave uniformly negative results by using other *Salmonella* detection methods. Rather, the increased sensitivity of the direct EIA may be a result of the selective binding of *Salmonella* cells by the capture antibody, which could increase the selectivity and sensitivity of subsequent steps of the assay. Alexio et al. (1) developed a method similar to the direct EIA proposed in this study. A major difference between their method and ours is that they adsorbed cells directly to polystyrene microtitration plates, whereas we used a capture-antibody technique. They suggested to us the potential use of capture antibody but abandoned its use; we continued using capture antibody because preliminary tests, not reported here, indicated that it was useful. If the majority of the cells attached to the microtitration plate by the capture antibody are *Salmonella*, it would decrease the possibility of false-positive results, compared with nonspecific adsorption methods. The capture antibody procedure also eliminates interference by food or

feed components, which could be a problem when nonspecific adsorption is used.

Robison et al. (11) developed an EIA for detecting *Salmonella* species in foods by using a monoclonal antibody (M. Potter, Fed. Proc. **29**:85–91, 1970). This EIA was subsequently improved (3, 7) and is now rapid, sensitive, and specific. An inhibition enzyme-linked immunosorbent assay method also has been reported (10). In this method, salmonellae in specimens treated with protease inhibit binding of polyvalent anti-O antiserum to wells of microtitration plates coated with *Salmonella* lipopolysaccharide. This method shows promise, but in its present form (10), some salmonellae present in foods might not be detected.

The direct EIA is sensitive, specific for *Salmonella* species, inexpensive, easy to perform, and amenable to automation. Commercially available reagents can be used without purification. Although monoclonal antibodies are available for *Salmonella* detection by EIA (3, 7, 11), our work and that of Alexio et al. (1) demonstrated that polyclonal antibodies also are satisfactory for *Salmonella* EIA procedures. The examination of food and feed samples with our direct EIA could be completed in 3 work days, and 32 samples could be tested simultaneously in a single 96-well microtitration plate. The results from the comparative study showed that this direct EIA procedure is a better alternative than the PCT.

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