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Enzyme Immunoassay in Which a Myeloma Protein Is Used for Detection of Salmonellae

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An enzyme immunoassay (EIA) in which an immunoglobulin A monoclonal antibody from a myeloma (MOPC 467) is used was developed to detect the presence of *Salmonella* organisms. This myeloma protein binds to a flagellar determinant of the organisms but is not directed toward the H antigens. Of 100 strains tested, 94% were detectable with this antibody. The EIA, used with MOPC 467, is quick, sensitive, and specific, showing virtually no cross-reactivity to other enteric organisms. Initial screening of antibody reactivity was performed by Ouchterlony gel diffusion with the supernatants of heat-treated *Salmonella* cultures. After this, an EIA was performed on the heat extracts with the myeloma protein, which had been directly coupled to alkaline phosphatase. A positive reaction was indicated by the production of a yellow color after the addition of a substrate (*p*-nitrophenylphosphate), and this was quantitated by determining the absorbance at 405 nm. The EIA proved to be slightly more sensitive than the Ouchterlony analysis. The sensitivity of the EIA is such that as few as 10^6 *Salmonella* organisms per ml were detected. This concentration was easily obtained after a 24-h preenrichment incubation of the sample. Mixtures of *Salmonella* strains with a 10× concentration of *Escherichia coli* did not prevent detection of the *Salmonella* strains. This EIA can be successfully used to detect contamination of foods, as it was used to detect the intentional contamination of infant formula in these studies. Indications are that the EIA is sensitive enough to detect *Salmonella* strains in M broth subcultures taken directly from a preenrichment culture. Testing of samples could thus be completed 36 h after culture initiation, rather than after 96 h, the time currently needed.

Salmonella organisms are gram-negative rods which cause food poisoning and which belong to the family *Enterobacteriaceae*. Their presence in raw ingredients and finished products in the United States is strictly prohibited by the U.S. Food and Drug Administration. The most common method to detect *Salmonella* strains in foods is a cultural procedure which depends on biochemical reactions and selective media to separate *Salmonella* strains from other enteric strains. The steps in the procedure include the following: (i) preenrichment in a suitable liquid medium, which allows the stressed organisms to recover and grow and which requires 24 h of incubation; (ii) selective enrichment in selenite-cystine (SC) and tetrathionate broths, which also requires a 24-h incubation period; and (iii) isolation of *Salmonella* strains by streaking the organisms in these selective broths on selective, differential plating media. The plates are checked after 24 and 48 h of incubation. Thus, negative results cannot be obtained until 96 h after test initiation.

In recent years, methods have been developed to shorten the time necessary for the assay. These include the fluorescent-antibody technique (13), enrichment serology (12), and, most recently, enzyme immunoassays (EIA) (6, 7). Various problems have been encountered in each of these methods, but the EIA appears to be the most promising because it is quick, sensitive, relatively inexpensive, and easy to perform.

The key to a successful *Salmonella* EIA is a specific antibody which will detect all common *Salmonella* strains but will not give false-positive reactions by reacting with other organisms. An EIA was developed in 1977 by Krysinski and Heimsch (6), but the antibodies which they used were not pure. Minnich (M.S. thesis, University of Idaho, Moscow, 1978) improved this procedure by purifying the immunoglobulin G portion of the antiserum and eliminating the immunoglobulin M fraction, thus reducing the amount of cross-reactivity of the antibody preparation. In 1982, Minnich et al. (7) developed an indirect

EIA procedure but stated that more potent antibody preparations would be preferable. Therefore, we studied a monoclonal antibody from a myeloma, MOPC 467. This myeloma was isolated in 1970 by Potter (8) and was later found to be specific for a flagellar determinant found on several *Salmonella* strains (9–11). This report describes a direct EIA with this antibody, which detected 94% of the *Salmonella* strains tested with virtually no cross-reactivity. The procedure may allow detection of *Salmonella* strains as early as 36 h after test initiation.

MATERIALS AND METHODS

Stock cultures and media. Stock cultures of salmonellae and other enteric organisms were obtained from the Ohio Department of Health, Columbus. The *Salmonella* cultures selected (100 strains) represented all common antigenic types and included most strains commonly implicated in food poisoning outbreaks in the United States. All *Salmonella* cultures were streaked for purity on brilliant green (BG) agar and were tested biochemically on TSI and urea agars and in lysine decarboxylase and phenol red-sucrose broths.

Media were obtained from BBL Microbiology Systems, Cockeysville, Md., and Difco Laboratories, Detroit, Mich., with the exception of M broth, which was prepared as described by Sperber and Deibel (12). The organisms were maintained on Trypticase soy agar (TSA) slants at 4°C.

Flagellar antigen production. *Salmonella* strains and other enteric organisms were grown in 100-ml broth cultures at 37°C for 18 h before antigen extraction. M broth, reported by Sperber and Deibel (12) to enhance flagellar production by bacteria, was used in all preparations. The bacteria were harvested from the cultures by centrifugation at 1,000 × *g* for 25 min. The harvested cells were washed once in 60 ml of phosphate-buffered saline (PBS), pH 7.4, and again centrifuged at 1,000 × *g* for 25 min. The pellets were resuspended in PBS and heat treated for 1 h in a boiling water bath to remove the flagella from the organisms. After heating, the suspensions were centrifuged at 1,000 × *g* for 25 min to sediment whole cells and debris. The supernatants containing the flagellar antigens were stored at 4°C until needed. No further processing of the antigen preparations was found to be necessary.

Myeloma-produced monoclonal antibody (MOPC 467). The myeloma cells secreting monoclonal immunoglobulin A antibody were originally derived from a mineral oil-induced plasmacytoma in a BALB/c mouse (8). The cells were injected into BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) previously primed with 0.5 ml of Pristane (Sigma Chemical Co., St. Louis, Mo.). Each mouse received 10^6 cells intraperitoneally. Ascitic tumors were visually evident within 10 days after injection. Ascitic fluid containing MOPC 467 antibody was obtained by draining the peritoneal cavity of each tumor-bearing mouse every 3 days with a 20-gauge 1.5-in. (3.8-cm) hypodermic needle. Tumor passage among mice was accomplished by intraperitoneal injections of ascitic fluid containing 10^6 cells into new Pristane-primed mice.

When passage in mice was discontinued for any reason, the myeloma cells, washed free of ascitic fluid, were preserved in cryoprotectant (RPMI 1640 medium, 30% dimethyl sulfoxide, 20% fetal bovine serum) at −70°C. To reestablish a tumor line in mice, cells were thawed, and 10^6 viable cells (0.1% trypan blue excluded) were injected into the peritoneal cavity of each Pristane-primed mouse. Tumor cell passages in BALB/c mice were 99% successful.

Purification of the MOPC 467 antibody. Ascitic fluid harvested from tumor-bearing mice was pooled every 3 days, with each mouse yielding approximately 15 to 30 ml of fluid per week. The ascitic fluid was centrifuged at 1,000 × *g* for 20 min to pellet cells and debris. The clarified fluid was filtered through a 0.22-μm filter (Millipore Corp., Bedford Mass.). Antibody was precipitated from the filtered fluid by adding an equal volume of saturated ammonium sulfate dropwise and allowing the mixture to stand on ice for 1 h. The precipitate was pelleted by centrifugation at 5,000 × *g* for 15 min. The supernatant was discarded, and the precipitate was suspended in a minimal amount of distilled water. The solution was dialyzed against three changes of PBS, pH 7.4, over 48 h at 4°C. Calculations of the protein contents of the final preparations were based on readings of absorbance at 280 nm and the use of 1.34 as the extinction coefficient for mouse immunoglobulin A (14), as this immunoglobulin was by far the dominant protein in the precipitate. Each lot of purified protein was frozen until needed. For use in the EIA, alkaline phosphatase (type VII; Sigma) was coupled to the antibody by the method of Voller et al. (15).

Detection of salmonellae with MOPC 467. (i) Ouchterlony gel diffusion. Initial screening of *Salmonella* strains with MOPC 467 antibody was performed by gel diffusion as reported by Potter (8). Microscope slides for agglutination testing (3 by 2 in. [7.6 by 5 cm]) were cleaned, and 11 ml of molten agarose (Baker Chemical Co., Phillipsburg, N.J.) was placed on each slide and allowed to solidify. Slides were kept refrigerated in a humidity chamber until use. The MOPC 467 antibody preparation, which had a protein concentration of 10 mg/ml, was placed in the center well, and the undiluted flagellar extracts were placed in the three outer wells. After inoculation, the gels were incubated in a humidified petri dish at room temperature overnight.

(ii) EIA. All EIA were performed in MICROELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.), which are 96-well flat-bottom microtiter plates. We added 100 μl of flagellar extract to 100 μl of coating buffer (1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 per liter of water, pH 9.6) in each well. In most cases, samples were tested in triplicate. Negative control wells, which contained no antigen or antibody but were otherwise treated the same as test wells, were included on each plate. A similarly treated *Escherichia coli* control was also included on each plate, and the plate was incubated at room temperature for 1 h. Washing was performed on a MINIWASH plate washer (Dynatech) with a solution of PBS containing 0.05% Tween 20 and 1% fetal bovine serum. The plates were washed three times and then incubated on a MICROSHAKER (Dynatech) for 1 h, which allowed any exposed plastic surfaces to be sealed by the fetal bovine serum, eliminating nonspecific binding sites. The plates were then washed twice more and aspirated dry. We added 200 μl of alkaline phosphatase-labeled

MOPC 467 antibody at a concentration of 3 μg/ml to each well and incubated the plates again at room temperature for 1 h. Unbound conjugate was removed by washing as described above, except that the plates remained on the shaker for only 15 min. Finally, 200 μl of alkaline phosphatase substrate (*p*-nitrophenylphosphate; Sigma) was added to each well. The substrate was prepared on the day of use by adding 5 mg to 5 ml of diethanolamine buffer (97 ml of diethanolamine, 0.2 g of NaN_3, and 100 mg of $MgCl_2 \cdot 6H_2O$ in 800 ml of distilled water; pH adjusted to 9.6 with 1 M HCl; volume brought to 1 liter). After incubation at room temperature for 0.5 h, the reaction was stopped by adding 100 μl of 3 M NaOH to each well. Positive wells were yellow and were quantitated by measuring absorbance at 405 nm on a MICROELISA reader (Dynatech). Absorbance levels for *E. coli*, which did not react with MOPC 467 antibody, were used as a baseline for comparison with absorbance levels of all test samples.

Cross-reactivity of MOPC 467. Twenty-eight strains of motile enteric organisms representing nine genera were tested against MOPC 467 antibody by Ouchterlony gel diffusion and EIA in the same manner used to test the *Salmonella* strains.

Sensitivity of EIA. Studies with five *Salmonella* strains were conducted to determine the level of sensitivity of the EIA. Serial decimal dilutions of overnight M broth cultures were made in phosphate-buffered water. A viable cell count was made by plating appropriate dilutions in tempered TSA. The undiluted cultures were centrifuged at 1,000 × *g* for 25 min, and the pellet was suspended in 9 ml of PBS. The washed culture and dilutions of 10^{-1} through 10^{-4} were then heated as described above and subjected to EIA.

Ability of EIA to detect *Salmonella* strains in mixed culture. Two *Salmonella* strains, *S. korovi* and *S. kingston*, were mixed with *E. coli* in M broth to obtain initial ratios of 1:10, 1:100, and 1:1,000. We also transferred 0.1 ml of each pure culture into M broth. After incubation at 37°C for 5.5 h, all tubes were streaked on BG agar to determine if the salmonellae were detectable by cultural procedures. All pure cultures and mixtures were centrifuged at 1,000 × *g* for 25 min, suspended in 1 ml of PBS, heated as described above, and then analyzed by EIA.

Detection of salmonellae inoculated into reconstituted infant formula. To determine if *Salmonella* strains could be detected directly in samples from the preenrichment mixtures as suggested by Minnich et al. (7), five *Salmonella* strains and 1:1 mixtures of the five strains and *E. coli* were inoculated at low levels (<50 salmonellae per liter) into 10% solutions of reconstituted infant formula. Plate counts were made of the initial inocula with TSA. After incubation at 37°C for 24 h, 0.1 ml of preenrichment solution was inoculated into 10 ml of M broth. The M broth was incubated at 37°C for 6 h, and then the preparation steps necessary for EIA were peformed. We also transferred 1-ml quantities of the preenrichment solutions to 10-ml portions of tetrathionate and selenite-cystine broths. These broths were incubated at 37°C for 24 h. After incubation, BG agar plates were streaked with the selective enrichments, and 0.1-ml quantities were also transferred into M broth. After incubation at 37°C for 6 h, all M broth cultures were subjected to EIA. Plate counts with TSA (for samples inoculated with *Salmonella* strains only) and violet-red bile agar (for samples inoculated with mixed cultures) were done on all M broth subcultures after incubation to determine the numbers of *Salmonella* and *E. coli* organisms present.

RESULTS

In the initial Ouchterlony gel diffusion assays with MOPC 467 antibody in the center well, 92 of the 100 *Salmonella* strains tested were positive, forming distinct lines of precipitation in the gel. There was no relationship between O or H antigens and positive reactions to the antibody. The eight negative strains were *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *S. kirkee*, *S. javiana*, *S. tennessee*, *S. reading*, and *S. newington*.

To make the test rapid and quantitative, an EIA was performed with alkaline phosphatase-coupled MOPC 467 antibody. In the EIA, absorbance values at 405 nm were measured, and a positive sample was determined to be one which had an optical density at least 0.10 above that of a similarly treated extract of motile *E. coli*. All organisms positive by gel diffusion were also positive by EIA. In addition, two of the eight Ouchterlony-negative strains (*S. javiana* and *S. reading*) were positive by the EIA. Typical absorbance values of selected *Salmonella* and *E. coli* strains are shown in Table 1.

The cross-reactivity of MOPC 467 antibody with determinants of other organisms was tested. Heat extracts were made from 28 flagellated enteric organisms, including three *Escherichia* strains, four *Citrobacter* strains, one *Edwardsiella* strain, five *Klebsiella* strains, five *Enterobacter* strains, five *Serratia* strains, three *Providencia* strains, one *Arizona* strain, and one *Proteus* strain. Only one organism, *Arizona*

TABLE 1. EIA absorbance readings of pure cultures

Organism	Absorbance (405 nm)
S. enteritidis	2.165
S. heidelberg	0.612
S. newport	2.010
S. saint paul	1.864
S. infantis	0.598
S. agona	2.311
S. derby	1.688
S. montevideo	2.311
S. typhimurium	0.533
S. typhi	0.029[a]
S. tennessee	0.029[a]
E. coli	0.021
Negative control	0.023

[a] Negative by EIA. Positive wells were considered to be those which gave readings 0.10 absorbance units higher than *E. coli* readings.

TABLE 2. Sensitivity of the EIA

Organism	Absorbance (405 nm) at indicated cell concn (per ml)			
	10^8	10^7	10^6	10^5
E. coli	0.228	0.220	0.144	0.142
S. korovi	2.232	2.232	1.695	0.262
S. infantis	2.068	1.872	0.336	0.196
S. minnesota	1.475	1.448	0.273	0.079
S. anatum	2.210	2.232	0.771	0.245
S. tennessee	0.045	0.100	0.195	0.116

hinshawii, was reactive to the antibody in the EIA; this organism was formerly included in the genus *Salmonella* as *S. arizonae* (1). All other common flagellated enteric organisms showed a complete absence of the determinant recognized by MOPC 467 antibody.

Selected *Salmonella* strains, including a strain negative by EIA, *S. tennessee*, were used for determining the sensitivity of the EIA. Results of absorbance readings indicate that all four positive strains tested were positive by EIA when at least 10^6 organisms per ml were present in the growth medium (Table 2); two of the four strains, *S. korovi* and *S. anatum*, could be identified as positive at a concentration of only 10^5/ml. It was also readily apparent that an organism which was negative (*E. coli* and *S. tennessee*) was not identified as positive even at 10^8/ml. These organisms were tested at concentrations as high as 10^{10}/ml, and similar results were obtained (data not shown). The concentration of 10^6 organisms per ml should easily be reached in preenrichment broth after 24 h of incubation.

As it would be considered rare to obtain a pure *Salmonella* culture from a food sample or any other source to be analyzed, we tested the ability of MOPC 467 to detect these organisms in a mixed culture. *S. korovi* and *S. kingston* were detectable by EIA when outnumbered 10:1 by *E. coli* in the initial seed culture (Table 3). No detection of *Salmonella* strains was possible when they were initially outnumbered by 100:1 or more. When the same cultures were transferred to BG agar plates, *Salmonella* strains grew from the culture initially seeded at an *E. coli*/*Salmonella* ratio of 10:1 but not from cultures seeded at 100:1 or 1,000:1. It appears, therefore, that the EIA had a *Salmonella* sensitivity identical to that of the conventional cultural procedure.

TABLE 3. *Salmonella* detection in mixed cultures

Organism	Absorbance (405 nm) at indicated *Salmonella*/*E. coli* ratio[a]			
	1:0	1:10	1:100	1:1,000
S. korovi	2.232	0.321	0.097	0.043
S. kingston	1.262	0.238	0.099	0.070

[a] *E. coli* pure culture absorbance reading, 0.095.

TABLE 4. *Salmonella* detection in infant formula after direct transfer from preenrichment broth to M broth

Sample inoculum	Count (per ml)		Absorbance (405 nm)
	Salmonella strains	*E. coli*	
1. *S. korovi*	1×10^8		2.165
2. *S. korovi* + *E. coli*	1×10^9	3×10^8	1.536
3. *S. typhimurium*	7×10^8		1.613
4. *S. typhimurium* + *E. coli*	4×10^8	$<10^6$	1.048
5. *S. heidelberg*	1×10^9		0.721
6. *S. heidelberg* + *E. coli*	1×10^9	$<10^6$	1.053
7. *S. infantis*	1×10^9		0.272
8. *S. infantis* + *E. coli*	1×10^8	7×10^8	0.130
9. *S. javiana*	1×10^9		0.181
10. *S. javiana* + *E. coli*	$<10^7$	1×10^9	0.037[a]
E. coli		2×10^9	0.007

[a] Negative by EIA.

In a series of experiments designed to determine if the EIA could be used to detect *Salmonella* strains in food products directly in samples from preenrichment broths, reconstituted powdered infant formula was intentionally contaminated with *Salmonella* strains, *E. coli*, or both at <100 organisms per liter. After a 24-h incubation in preenrichment broth, 0.1 ml was transferred to M broth; 6 h later, these cultures were plated to obtain *Salmonella* and *E. coli* counts and also screened by EIA (Table 4). All samples were positive by EIA except one (a 1:1 mixture of *E. coli* and *S. javiana* [sample 10]), and the *Salmonella* strains in this one were not detectable by plate count ($<10^7$/ml on violet-red bile agar). All EIA were completed within 36 h of culture initiation.

When these cultures were given an additional 24 h of growth in tetrathionate broth and then inoculated into M broth, all samples originally inoculated with *Salmonella* strains were positive by EIA (Table 5), including sample 10. When selenite-cystine broth was used for 24-h selective enrichment, 3 of the 10 samples were negative by EIA; all samples from both preenrichment broths were positive on BG agar plates. In these experiments, all EIA were completed within 60 h of culture initiation.

DISCUSSION

A quick, simple, sensitive, and inexpensive method to detect *Salmonella* strains is needed by many health-related industries, including the food industry. The EIA described above fits

TABLE 5. *Salmonella* detection in infant formula after transfer from selective selenite-cystine (SC) and tetrathionate (TET) broths to M broth

Sample[a]	Count (per ml)				Absorbance (405 nm)	
	Salmonella strains		*E. coli*			
	TET	SC	TET	SC	TET	SC
1	1×10^9	7×10^8			2.232	1.682
2	6×10^8	3×10^8	2×10^8	$<10^6$	2.210	2.010
3	2×10^9	7×10^8			0.612	0.702
4	2×10^9	5×10^8	$<10^6$	$<10^6$	1.436	0.760
5	2×10^9	1×10^9			0.646	0.695
6	1×10^9	9×10^8	$<10^6$	$<10^6$	0.864	0.534
7	2×10^9	5×10^8			0.266	0.161[b]
8	1×10^9	6×10^8	3×10^8	$<10^6$	0.256	0.145[b]
9	2×10^9	6×10^8			0.487	0.069[b]
10	2×10^9	2×10^8	4×10^8	$<10^6$	0.457	1.033
E. coli			$<10^6$	2×10^7	0.103	0.150

[a] See Table 4.
[b] Negative by EIA.

these criteria and, in addition, could be automated for more speed and convenience. Minnich et al. (7) have recently developed an indirect EIA, but indirect methods require more time, expense, and equipment. Also, the commercial antibody which they have used has to be purified over a staphylococcal protein A column and is of relatively low affinity. We improved their method by directly coupling the antigen to the plate and using a high-affinity monoclonal antibody which was directly coupled to the enzyme. No centrifugation of microtiter plates or affinity column chromatography is necessary in our EIA.

We initially attempted to bind whole bacterial cells to the walls of the microtiter plate, but this technique was unsuccessful. We then tested the heated flagellar extracts that have been shown (9–11; this study) to be recognized by MOPC 467 antibody in Ouchterlony analysis. Excellent results were obtained by EIA with these extracts. Most strains gave color reactions which were easily discernible without equipment; however, we elected to use the MICROELISA reader to quantitate the results, thus obtaining objective numerical data.

In developing the assay, we found that Ouchterlony-positive *Salmonella* cultures consistently gave readings which were at least 0.10 absorbance units above those of *E. coli* and other negative enteric organisms. We therefore chose to use *E. coli* absorbance values as our baseline for comparison with the values for all test samples, and an *E. coli* culture was included on each microtiter plate. Typically, the reader was set so that negative control wells (no antigen or antibody) gave values of between 0.015 and 0.030 absorbance units at 405 nm.

The EIA procedure with MOPC 467 antibody is sensitive and highly specific. The lack of cross-reactivity of the antibody is a definite advantage, since false-positive reactions are virtually eliminated. In addition to *A. hinshawii*, which we found to react with the antibody, the only other organisms which have been reported to cross-react with MOPC 467 antibody are *Herellea vaginicola* and *Pasteurella pneumotropica* (9), which are not commonly encountered in most laboratory analyses.

Other attractive aspects of the EIA are the savings in cost and time. The cost of the EIA with a monoclonal antibody (less than 25¢ per assay) is less than the cost of the existing cultural assays, and even the maximum time of the assay (60 h) is 36 h less than the time required for the conventional procedure. Approximately 30 triplicate samples can be put on one microtiter plate.

Only limited studies of food samples were performed and no naturally contaminated samples were studied. These studies indicate, however, that *Salmonella* detection is feasible when samples are taken directly from a 24-h preenrichment broth. Interference from other organisms did not affect the ability of the assay to detect *Salmonella* strains.

In 1979, the Center for Disease Control reported that 31,123 cases of human salmonellosis occurred in the United States (2). A total of 207 strains were implicated, and our study used 94 of them, 5 of which were negative by EIA with MOPC 467 antibody. The 89 positive strains accounted for 27,745 salmonellosis cases (89% of the total), and the 5 negative strains accounted for only 1,076 cases (3.5% of the total). The strains responsible for the remaining 7.5% of

reported cases were not tested in our study. The strains which were not identified with MOPC 467 antibody and which have been involved in food outbreaks in the United States recently are *S. typhi* (3), *S. tennessee* (4), and *S. newington* (5).

The only problem which remains in the complete and successful use of this EIA is the presence of these few undetectable strains. Additional monoclonal antibodies which will detect these negative strains are highly desirable. Our future efforts will concentrate on developing the necessary antibodies by immunization of rabbits with the flagella of *Salmonella* strains which are not detected with MOPC 467 and developing the necessary hybridoma antibodies from immunized mice.

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