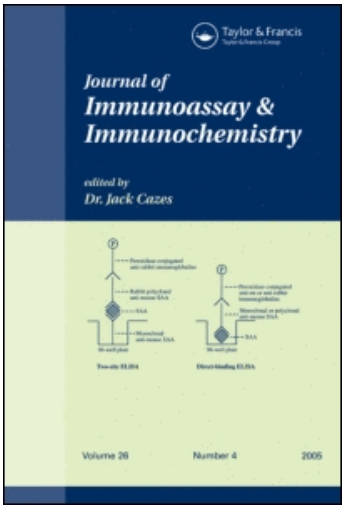

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A Fluorescent Enzyme Immunoassay for *Salmonella* Detection

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A FLUORESCENT ENZYME IMMUNOASSAY FOR SALMONELLA DETECTION

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ABSTRACT

A double antibody sandwich immunoassay (EIA) was developed for the detection of Salmonella. The assay utilizes a β-galactosidase-murine myeloma monoclonal antibody (M467) conjugate prepared with the heterobifunctional coupling reagent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and uses 4-methyl umbelliferyl β-D-galactoside as a fluorogenic substrate for the enzyme. The EIA is sensitive and rapid, and compared favorably with the conventional cultural technique in the analysis of 60 feed samples naturally contaminated with Salmonella. Proteins or natural contaminants from the sample did not interfere in the assay.

(KEY WORDS: Fluorescence, enzyme immunoassay, Salmonella, M467, feeds, monoclonal antibody, heterobifunctional, SPDP)

INTRODUCTION

The use of an enzyme immunoassay (EIA) for the detection of salmonellae in foods was first advocated by Krysinsky and Heimsch (1). Minnich et al (2) developed an EIA procedure which employed a commercial polyclonal polyvalent flagellar antiserum purified by protein A affinity chromatography and microtitration plates. A polyclonal polyvalent Salmonella flagellar antiserum and a

protein A-Alkaline phosphatase conjugate were used by Aleixo et al (3) for the detection of salmonellae in foods and feeds. Although it was possible to detect salmonellae after non-selective enrichment of the samples, cross-reactions with other bacteria and with proteins from some specimens were two of the major drawbacks of the assay. Robison et al (4) reported on the use of a mouse myeloma immunoglobulin a (M467 antibody) in an EIA for Salmonella. This monoclonal antibody, developed by Potter (5), recognizes a flagellar antigenic determinant common to many salmonellae (6,7).

This report describes the use of a double-antibody sandwich EIA for detection of salmonellae which takes advantage of the reactive properties of M467 antibody towards the flagellar protein of Salmonella serotypes, and of the increase which can be obtained in the sensitivity of an EIA by using a fluorogenic substrate for the enzyme.

MATERIALS AND METHODS

Bacterial Strains and Media

All strains of bacteria utilized in pure culture studies were obtained from the culture collection maintained at the Department of Foods and Nutrition, Purdue University. The media used for cultivation of bacteria were from Difco Laboratories, Detroit, Michigan.

Preparation of M467 Antibody

The mineral oil-induced mouse plasmacytoma (MOPC 467), which produces the M467 antibody, was kindly provided by Dr. M. Potter,

Laboratory of Cell Biology, National Cancer Institute, Bethesda, Maryland. The M467 antibody (Mol. wt: 380 kd) was prepared according to the procedure of Robison et al (4).

Reactivity of M467 Antibody With Enterobacteriaceae

A colorimetric direct EIA was employed to test the reactivity of M467 antibody against several serotypes of Salmonella and other enteric bacteria. Overnight cultures of the bacteria in brain heart infusion (BHI) were steamed for 45 minutes and passively adsorbed (0.1 ml) to the wells of Immulon 2 microtitration plates (Dynatech Laboratories, Alexandria, Virginia) for 1 h at 37°C. Unadsorbed material was removed by washing twice with phosphate buffered saline (PBS, 0.02M, pH 7.4) containing 0.05% Tween 20 (PBST). One hundred microliters of a M467 antibody-alkaline phosphatase conjugate, prepared by one-step glutaraldehyde procedure (8), was added to each well and the plate incubated at 37°C for 1 h. After five washes with PBST, 0.2 ml of p-nitrophenyl phosphate solution (SIGMA 104) in diethanolamine buffer (9) was added to each well and the plate was incubated at 37°C for 30 minutes. The reaction was stopped by adding 0.05 ml of 5 M NaOH solution, and the absorbance was read at 410 nm in a MicroElisa Minireader (Dynatech Laboratories) after zeroing the instrument against the substrate solution in an untreated well of the microtitration plate.

M467 Antibody-β-Galactosidase Conjugate Preparation

The procedure described by Carlsson et al (10) for protein-protein conjugation was followed with modifications.

Briefly, purified M467 antibody (2.55 mg, 6.7×10^{-6} millimoles in PBS) was treated with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Sigma Cehmical Co., St. Louis, MO, 20 mM in absolute ethanol) in a ratio of 60 moles of SPDP per mole of M467 antibody to introduce 2-pyridyl disulfide groups in the antibody molecule. The reaction mixture (2 ml) was left for 30 minutes at room temperature with occasional shaking. The resulting degree of substitution (moles 2-pyridyl disulfide/moles M467 antibody) was 8.8, as estimated by determining the increase in absorbance at 343 nm after reduction of the 2-pyridyl disulfide structures with a 50 mM dithiothreitol solution (10).

Modified M467 antibody and β-Galactosidase (Mol. wt: 135,000, Boehringer Mannheim, Indianapolis, IN) containing free sulfhydryl groups were mixed in a molar ratio of 1:2. The reaction volume was made up to 1.9 ml with PBS and left 20 h at room temperature. Unreacted sulfhydryl groups were blocked by adding 0.1 ml of a 20 mM L-cysteine solution and left to react 1 h at room temperature. The conjugate thus obtained was then aliquoted and stored at $-20^{\circ}C$. For use, the optimal dilution of the conjugate was determined by checkerboard titration (9).

The Fluorescent EIA Procedure

The fluorescent double-antibody sandwich EIA was performed as follows. M467 antibody diluted to 2 μg/ml in carbonate buffer, pH 9.6 was added (0.2 ml) to the wells of an Immulon II microtitration plate (Dynatech). After incubation overnight at $4^{\circ}C$, the plate was washed twice with PBST and 0.1 ml of a steamed

antigen preparation, obtained either from a post-enrichment culture of a test sample or from cultures of reference strains of Salmonella, was added to each well and the plate was incubated for 30 min at 37°C with gentle shaking. The washing procedure was repeated to remove unreacted material and 0.1 ml of the M467 antibody-β-galactosidase conjugate was added and the plate was incubated for 30 min at 37°C. After five washes with PBST, 0.2 ml of the fluorogenic substrate for β-galactosidase (0.1 mM 4-methylumbelliferyl-β-D-galactoside dissolved in 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1%NaN_3, and 0.1% bovine serum albumin) was added to each well. After incubating for 15 min at 37°C, fluorescent intensity was measured in a MicroFluor reader (Dynatech). Test samples were scored as positive for Salmonella when the fluorescent intensity was greater than 3.5 standard deviations of the mean of negative controls prepared from a culture of Escherichia coli.

EIA Sensitivity

To determine the sensitivity of the fluorescent assay, overnight cultures of five Salmonella serotypes in BHI were harvested by centrifugation at 13,000 RPM for 1 min (Fisher microcentrifuge, Model 235A). The supernatant was discarded and the cells were resuspended in PBS. An aliquot was removed for plate count and the remainder of the cell suspension was steamed for 45 min. Decimal dilutions of the steamed antigen preparation were made in PBS and 0.1 ml of the 10^{-1} through 10^{-7} dilutions was added to the wells of a microtitration plate previously sensitized with M467 antibody. EIA was then performed as described above.

Analysis of Feed Samples

Sixty feed samples naturally contaminated with salmonellae were obtained from various sources and were examined by the fluorescent EIA procedure and by the conventional culture technique (CCT). The CCT was carried out according to standard procedures described in the Bacteriological Analytical Manual (11). The EIA was performed at two different stages of the CCT: (i) at the nonselective enrichment step, after a 6 h post-enrichment period and (ii) at the selective enrichment step, after a 6 h post-enrichment period. For the post-enrichment, a tube containing 5 ml of M broth was inoculated with 0.5 ml of the nonselective or selective enrichment culture, and incubated at 37°C with vigorous agitation. The bacterial cells were havested by centrifugation for 1 min at 13000 rpm (Fisher microcentrifuge, Model 235A), resuspended in 1 ml of PBS and steamed for 45 min. The steamed antigen preparation was used in the EIA as described above.

RESULTS

Table 1 shows representative results of the study on the reactivity of M467 antibody with members of the family Enterobacteriaceae. Of approximately 800 serotypes of Salmonella tested by the colorimetric EIA, 93.2% yielded positive reactions. A few serotypes of Yersinia enterocolitica and one strain of Citrobacter freundii showed positive reactions in the assay.

The sensitivity of the fluorescent EIA was tested using five serotypes of Salmonella to which M467 antibody has different binding avidities (Table 2). For S. milwaukee, S. montevideo, and

TABLE 1

Reactivity of M467 Antibody with Enterobacteriaceae in a Direct Enzyme Immunoassay.

Organism[a]	Absorbance (410 nM)
Salmonella senftenberg	1.43
S. berkeley	1.38
S. havana	1.44
S. heidelberg	0.58
S. typhimurium	1.39
S. milwaukee	1.43
S. montevideo	1.42
S. minnesota	0.71
S. butantan	1.03
S. typhi	0.06
S. paratyphi A	0.08
S. paratyphi B	0.09
S. paratyphi C	0.06
S. potsdam	0.04
S. tennessee	0.07
S. gallinarum	0.12
S. remo	0.02
S. tucson	0.03
S. willemstad	0.02
S. ballerup	0.03
S. del plata	0.02
S. sendai	0.01
Yersinia enterocolitica (ser 0:8)	1.43
Y. enterocolitica (ser 0:9)	1.56
Y. enterocolitica (ser 0:3)	0.45
Y. enterocolitica (ser 0:6,31)	1.55
Y. enterocolitica (ser 0:6,30)	1.55
Y. enterocolitica (ser 0:17)	0.01
Y. enterocolitica (ser 0:28)	0.02
Escherichia coli ≠ 104	0.29
E. coli ≠	0.09
E. coli ≠ 119	0.08
Citrobacter freundii ≠ 3	0.06
C. freundii	1.09
Proteus vulgaris	0.11
Enterobacter aerogenes	0.09

[a]number of organisms/ml, approx. 10^8

TABLE 2

Sensitivity of the Fluorescent Enzyme Immunoassay for Five Serotypes of Salmonella

Organism	Intensity of fluorescence at indicated dilutions[a]				
	10^7/ml	10^6/ml	10^5/ml	10^4/ml	10^3/ml
S. milwaukee	1629±93	1041±106	205±7	158±4	120±4[b]
S. montevideo	1735±166	1183±169	209±17	153±8	122±3[b]
S. typhimurium	1299±70	833±65	247±5	149±11	119±4[b]
S. heidelberg	573±26	332±13	133±3	92±7[b]	89±6[b]
S. infantis	539±23	320±15	131±3	88±2[b]	85±4[b]

[a]Values are mean ± STD DEV of wells. Background fluorescence of negative controls made from a culture of Escherichia coli (ca. 10^9 cells/ml) was 109±5.
[b]Negative values.

S. typhimurium, a concentration of about 10^4 cells/ml was adequate to yield a positive result. However, at least 10^5 cells/ml were requried for positive reactions with S. heidelberg and S. infantis, and the fluorescence of the wells containing a higher concentration of cells was much lower than that observed with equivalent number of cells of the other three serotypes tested.

Results of the analysis of 60 feed samples for Salmonella by the EIA procedure and by the CCT are shown in Table 3. Thirty-nine samples were positive by the CCT and 40 were positive by the EIA. No sample was positive only by the CCT. Samples positive by the EIA performed at the nonselective enrichment step were 77% (30/39) of the samples positive by CCT. The nine remaining positive samples were detected in the EIA performed after the selective enrichment step.

DISCUSSION

The application of the monoclonal antibody M467 to the detection of salmonellae in foods was first reported in 1983 (4). Of 100 strains of salmonellae tested, 94% were found to react with M467 antibody. This number is close to what we have found after testing about 800 different serotypes of Salmonella. Our studies have shown, that the M467 antibody reacts with certain serotypes of Y. enterocolitica and, on rare occasions, with other enterics such as C. freundii.

Two primary requirements of any EIA are specificity and sensitivity. The recent introduction of the monoclonal antibody technology provide a means to fulfill the specificity requirement. Use of fluorogenic substrates for the enzyme can provide for the development of highly sensitive assays. For example, it was possible to detect as low as 3-10 attograms (10^{-18} grams) of antigen per ml by optimizing an EIA system and using a fluorogenic substrate (12). Yolken and Stopa (13) and Yolken and Leister (14) obtained 60-100 fold increases in sensitivity in EIA by using a fluorogenic substrate in place of a colorigenic substrate. Using the present fluorogenic EIA it is possible to detect about 10^4 cells per ml of different serotypes of Salmonella in pure culture (Table 2). This represents a 100-fold increase in sensitivity over a previous protocol for detection of Salmonella by an EIA (3). However, this level of sensitivity was not achieved for S. heidelberg and S. infantis, and will probably not be attained with certain other salmonellae, because of the differences in the

TABLE 3

Comparison of Fluorescent EIA and Conventional Cultural Technique (CCT) for Detection of Salmonella in Naturally Contaminated Feed Samples[a]

Feed Sample	EIA Performed at Nonselective Broth	EIA Performed at Selective Broth	Cultural Technique	Salmonella "O" Group
1	765(+)[b]	598(+)	+	B
2	603(+)	1251(+)	+	E_2
3	712(+)	1202(+)	+	B
4	744(+)	404(+)	+	E_2
5	629(+)	1019(+)	+	E_2
6	1230(+)	1329(+)	+	B
7	85	95	-	-
8	85	61	-	-
9	84	62	-	-
10	80	304(+)	+	C_1
11	87	85	-	-
12	325(+)	545(+)	+	C_1
13	78	222(+)	+	C_1
14	168(+)	185(+)	+	E_2
15	127(+)	281(+)	+	E_2
16	195(+)	259(+)	+	E_2
17	159(+)	825(+)	+	G_1
18	290(+)	123(+)	-	-
19	248(+)	519(+)	+	E_2
20	63	56	-	-
21	56	55	-	-
22	64	59	-	-
23	853(+)	135(+)	+	C_1
24	56	58	-	-
25	180(+)	574(+)	+	G_1
26	51	62	-	-
27	54	59	-	-
28	140(+)	941(+)	+	C_1
29	103	176(+)	+	E_2
30	84	58	-	-
31	79	91	-	-
32	75	62	-	-
33	81	202(+)	+	E_2
34	85	130(+)	+	E_2
35	81	200(+)	+	E_2
36	86	62	-	-
37	146(+)	891(+)	+	E_4
38	95	85	-	-
39	165(+)	525(+)	+	C_1

TABLE 3 (continued)

Feed Sample	EIA Performed at Nonselective Broth	EIA Performed at Selective Broth	Cultural Technique	Salmonella "O" Group
40	131(+)	502(+)	+	C_1
41	130(+)	422(+)	+	C_1
42	98	73	-	-
43	280(+)	442(+)	+	C_1
44	98	387(+)	+	C_1
45	87	471(+)	+	C_1
46	581(+)	170(+)	+	E_2
47	86	80	-	-
48	242(+)	264(+)	+	C_1
49	132(+)	224(+)	+	C_1
50	249(+)	221(+)	+	C_1
51	164(+)	351(+)	+	C_1
52	133(+)	262(+)	+	C_1
53	89	78	-	-
54	85	72	-	-
55	87	200(+)	+	C_1
56	117	119	-	-
57	170(+)	288(+)	+	C_1
58	132(+)	295(+)	+	C_1
59	159(+)	239(+)	+	C_1
60	166(+)	238(+)	+	C_1

[a]EIA results are fluorescent intensities. Control for background fluorescence, made from a culture of *Escherichia coli* (ca. 10^9 cells/ml), was 109±5 (X ± STD DEV, N=3).
[b]Positive by EIA. Sample 18 was positive by EIA only.

avidity with which the M467 antibody binds to the various *Salmonella* flagellins (7).

Passive adsorption of *Salmonella* antigens to polystyrene plates was as effective as capture antibody mediated immobilization in polyclonal EIA for salmonellae (3). However, proteins from the test sample and from the other organisms growing in the enrichment culture compete with *Salmonella* antigens for binding sites in the solid phase and can interfere in the results

of the EIA. Therefore, we decided on a sandwich assay where M467 antibody is utilized both for capture and for detection of the Salmonella antigens. The evaluation of this procedure against the conventional cultural method for detection of salmonellae in naturally contamined feed samples indicated that the test is sensitive and specific. Of sixty samples tested, 59 yielded the same results by both methods. One sample was positive only by EIA. Although most samples were positive by the EIA after the nonselective enrichment step (77% of positives by CCT), thus being detectable one day after the initiation of analysis, nine samples were positive by EIA only after the selective enrichment. It is likely that the number of salmonellae in the nonselective enrichment broth of these samples was not within the range of sensitivity of the assay. Enrichment protocols that selectively enhance the growth of Salmonella need to be devised to further reduce the time required for enrichment of a sample before the application of the EIA.

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