Rapid Detection of Salmonellae by Immunoassays with Titanous Hydroxide as the Solid Phase

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Radioimmunometric and enzyme-immunometric assays were developed for the detection of salmonellae in pure and mixed cultures as well as in 59 food samples. The performances of titanous hydroxide suspension and microtiter plates as the solid phase for the immobilization of microorganisms were compared in these immunoassays. Detection of populations of salmonella cells in pure culture, diluted with saline, was 4- to 10-fold more sensitive with the microtiter plates. However, with mixed culture of salmonella and other enterobacterial species, the detection sensitivity with titanous hydroxide was 100- to 160-fold more sensitive than with microtiter plates. Good correlation existed between results of a standard cultural method for the detection of salmonellae in foods and those obtained from radioimmunometric and enzyme-immunometric assays utilizing titanous hydroxide. However, a high incidence of false-positive and false-negative results with food samples occurred with the enzyme-immunometric assay utilizing microtiter plates. The results provided strong evidence for the merits of substituting titanous hydroxide for microtiter plates as the solid phase for the immobilization of salmonellae for their detection by immunoassays. The immunoassays were rapid and enabled the analysis of a large number of selective enrichment cultures of food samples for salmonellae within 8 h.

An important factor necessary for the success of immunoassays which utilize radiolabeled or enzyme-labeled probes is the need for effective separation of bound and nonbound antibody moieties. In liquid-phase immunoassays, separation is facilitated by the utilization of immunoadsorbents, as is the case with macromolecular antigens (7, 13, 16), or by the use of charcoal, as is the case with micromolecules (5, 9).

The property of antibody adsorption to polymeric surfaces was used (3) as a basis to develop a solid-phase radioimmunoassay. This adsorptive characteristic allowed convenient and rapid removal of the free radioactive tracer antigen by washing of the solid phase with water, after completion of the immune reaction. Consequently, the use of solid-phase immunoassays has increased because of the simplicity of its separation technique. Also, other solid-phase substances for the immobilization of antibodies have been introduced such as commercial isothiocyanate-substituted plastic disks (17), polystyrene balls (22), nylon (8), activated thiol-Sepharose (21), and microparticulate cellulose (4).

Detection of salmonellae has been carried out by using solid-phase immunoassays, after coating plastic microtiter plates with suspensions or extracts of the organisms (1, 14, 19). This approach, however, has inherent deficiencies in that it is time-consuming as the coating requires from 1-h to overnight incubation; the coating is influenced by several factors (2, 6, 15) and only a proportion of cells may be adsorbed by this approach.

Kenny and Dunsmoor (12) investigated the adsorption characteristics of different microtiter plates for the detection of various antigens by enzyme-linked immunosorbent assay. They reported variation in the adsorptive capacities of the microtiter plates as well as variations in the background levels of nonspecific binding of the antibody-enzyme conjugate. They also observed adsorption competition between antigens and other substances as a function of the ratio of

competitor to antigen concentrations and not of the absolute amount. They concluded that the use of mixed or crude antigens in enzyme immunoassays presents significant problems concerning the sensitivity and specificity of tests.

Shekarchi et al. (18) also evaluated the binding properties of various plastic microtiter plates, using measles, toxoplasma, and human gamma globulin in enzyme-linked immunosorbent assays. They reported that most polystyrene or polyvinyl chloride plates gave acceptable binding. However, binding variations between lots, batches, and types of plastic were found in addition to well-to-well variations which were of greater statistical significance than the edge effect (wells located around the edge of microtiter plates). They concluded that batches of plates should be tested to determine the levels of plate-to-plate and well-to-well variations, using the particular antigen of interest.

The potential of titanous hydroxyoxide (hydroxide) for the immobilization of microorganisms for their diagnosis by immunoassays has been described before (11). The aim of this investigation was to develop radioimmunometric (RIMA) and enzyme-immunometric (EIMA) assays for the rapid detection of salmonellae, using titanous hydroxide as the solid phase. The immunoassays were evaluated with pure and mixed cultures of salmonella and food samples, naturally contaminated with salmonellae.

MATERIALS AND METHODS

Organisms and growth conditions. The microorganisms used were *Salmonella waycross* 1312 (Salmonella Reference Laboratory, Adelaide, South Australia), *Citrobacter freundii* 748, *Edwardsiella tarda* 1136, *Enterobacter aerogenes* 1976, *Erwinia herbicola* 854, *Hafnia alvei* 1137, *Proteus rettgeri* 300, *Proteus vulgaris* 142, *Serratia marcescens* 169, *Shigella dysenteriae* 321 (Department of Microbiology, University of Queensland, Queensland, Australia), *Klebsiella pneumoniae* 248, *Yersinia pseudotuberculosis* 14 (Commonwealth Institute of Health, University of Sydney, N.S.W., Australia),

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Proteus mirabilis 12201, *Proteus morganii* 12301, *Shigella flexneri* 6201, *Shigella sonnei* 6401, *Yersinia enterocolitica* 16001 (Health Commission of New South Wales, Lidcombe, New South Wales, Australia), and *Escherichia coli* NTCC 8196.

All enterobacterial species other than salmonella were propagated overnight at optimum temperatures in brain heart infusion broth (BBL Microbiology Systems) and then mixed. The salmonella culture (in brain heart infusion broth) was diluted, using saline to produce appropriate populations which were determined by standard plate count. The same culture was also diluted by using the mixed enterobacterial cultures to produce the desired salmonella populations, in association with the other species. The pure and mixed cultures were assayed with solid-phase immunoassays.

Food samples. A total of 59 food samples were investigated for the presence of salmonellae. These included 11 samples of fresh soft cheeses (mozzarella, feta, ricotta, and cottage), 24 milk powders, 9 chicken livers, 10 chicken carcasses, and 5 egg products (egg powder and egg noodles).

Analysis of food samples with standard cultural method. Food samples were tested for the presence of salmonellae by a standard cultural method (20). Briefly, 25 g of the samples was homogenized in 225 ml of buffered peptone water and then preenriched by incubation for 16 h at 37°C. Chicken carcasses were placed individually in sterile polyethylene bags containing 200 ml of sterile buffered peptone water. After vigorous shaking for 2 min, the liquid rinse was poured into sterile bottles and preenriched by incubating for 20 h at 37°C. Preenrichment was followed by selective enrichment in both mannitol-selenite-cystine and tetrathionate broths (Oxoid) which were incubated for 18 to 24 h at 42 ± 1 and 37 ± 1°C, respectively. Streaking on selective agar media (Oxoid) was done on bismuth sulfite and xylose-lysine-desoxycholate agars. Suspect colonies were transferred to peptone water, which was incubated for checking the purity of isolates and for their confirmation. Biochemical confirmation was done by testing for lysine decarboxylase production and reactions in *o*-nitrophenyl-β-D-galacto-pyranoside broth. Slide agglutination tests were carried out for serological confirmation, using salmonella polyvalent O and H antisera (Wellcome Research Laboratories). Salmonella isolates were serotyped by the Microbiological Diagnostic Unit, Department of Microbiology, University of Melbourne, Melbourne, Australia.

Analysis of food samples with solid-phase immunoassays. After preenrichment and selective enrichment of food samples, salmonella detection was conducted in duplicate by immunoassays. Only mannitol-selenite-cystine broth was tested with the immunoassays. The antisera used for immunoassays was a mixture of the 10 individual antisera (PFA), produced previously (10) against purified salmonella flagellins. Each antiserum was present in the antiserum mixture at a final concentration of 1:4,000. In addition, pooled Spicer-Edwards salmonella H antisera (SEA), consisting of Spicer-Edwards sera 1, 2, 3, and 4 complexes EN, 1, and L (Difco Laboratories), were also used at a concentration of 1:200.

RIMA. Portions of 50 μl of titanous hydroxide suspension were added to polystyrene tubes (77 by 8 mm; Kayline Plastics, Australia). Samples of salmonella or enrichment cultures (100-μl quantities) were then added. After immobilization of microorganisms by agitation for 10 min (11), 100-μl quantities of 0.05 M phosphate buffer containing 0.85% NaCl, 0.1% NaN$_3$, and 2% gelatin (PSAG) were added. Subsequently, 100-μl quantities of the antiserum pool

PFA or SEA were added after dilution in PSAG. The mixtures were incubated for 2 h at 37°C in an orbital shaker incubator (Gallenkamp) at 180 rpm. The unbound antibodies were eliminated from the tubes by dilution with 3.0 ml of saline, centrifugation at 1,700 × *g* for 5 min, and aspiration. Next, 100 μl of ^{125}I-labeled protein A (Radiochemical Centre), diluted in PSAG to give approximately 25,000 cpm/100 μl, was added. The tubes were placed in the shaker incubator for 1 h at 30°C. The unbound radioactive reagent was removed by dilution, centrifugation, and aspiration, and the residual radioactivities were counted with a Packard Selectronic Autogamma Spectrometer. Control tests were conducted as above for each sample, whereby quantities of 100 μl of PSAG were added in place of salmonella H antisera. The net radioactivities bound were determined by deducting the residual radioactivities of the tubes containing no H antisera from those containing the antisera.

RIMA was also conducted with round-bottom microtiter plates (polyvinyl chloride; Dynatech Laboratories) as follows. Quantities of 100 μl of 0.1 M carbonate buffer, pH 9.6, and 100 μl of test samples were added to the wells. The mixture was incubated at room temperature for 1 h to immobilize the bacterial cells onto the wells' interior. The contents of the wells were discarded, and after three washes with saline, 100-μl quantities of buffer (PSAG) were added. Subsequently, 100 μl of the antiserum pool PFA or SEA was added to each well. After incubation for 2 h at 37°C, the contents of the wells were discarded and the wells were washed three times with saline. To each well, 200 μl of ^{125}I-labeled protein A, diluted in PSAG to give approximately 25,000 cpm/200 μl, was added and incubation was for 1 h at 30°C. After the unbound radioactive reagent was discarded, the wells were washed three times with water, cut, and inserted into polystyrene tubes, and the residual radioactivities were counted.

EIMA. EIMA were performed with titanous hydroxide suspension in polystyrene tubes and in microtiter plates as the solid phases, as described above except that the use of the antibacterial agent NaN$_3$ was omitted and, in place of ^{125}I-labeled protein A, 200 μl of anti-rabbit immunoglobulin G (whole molecule)-alkaline phosphatase conjugate antibody raised in goats (Sigma Chemical Co.) was used. The optimum concentration of the enzyme conjugate was found to be 1:250 as determined by checkerboard titration. After incubation for 1 h at 37°C and the elimination of the unbound enzyme conjugate fraction, quantities of 200 μl of alkaline phosphatase substrate were added. The enzyme substrate was prepared on the same day of use by adding one tablet (5 mg) of Sigma phosphatase substrate 104 to 5 ml of 0.1 M glycine buffer containing 1.0 mM zinc and 1.0 mM magnesium chloride (pH 10.4). After incubation for 1 h at 37°C, 200-μl quantities were removed after the centrifugation of titanous hydroxide, or directly from microtiter plates, and transferred to a polystyrene microtiter plate with a flat bottom (Linbro; Flow Laboratories, Inc.). The absorbance was then measured with a Titertek Uniskan spectrophotometer (Flow Laboratories) at 405 nm. The net absorbance was then calculated for each sample by deducting the control absorbance value (where 100 μl of PSAG was added in place of salmonella H antisera).

RESULTS

Detection of salmonella in pure and mixed cultures by immunoassays. With RIMA (utilizing PFA antisera), the pure culture of *S. waycross* diluted in saline resulted in dose-

response curves with similar shapes when titanous hydroxide and the microtiter plates were used for immobilization (Fig. 1). The generally higher radioactivity-bound values of the titanous hydroxide dose-response curve only reflect a higher incidence of nonspecific binding, which at that level did not appreciably affect the assay's sensitivity. However, with EIMA, the dose-response curve of salmonella cells in saline immobilized on the microtiter plates was steeper than that obtained when titanous hydroxide was used (Fig. 2).

The minimum detectable population of salmonella cells was considered in this investigation as the corresponding populations of $A_0 + 2 \times$ the standard deviation of A_0, where A_0 is the mean of the response (percent bound radioactivity or absorbance) of 10 control tests conducted without the addition of salmonella H antisera. The minimum detectable population of salmonella cells in pure culture immobilized on microtiter plates was lower than that obtained by the immobilization with titanous hydroxide. Such difference amounted to 4- to 10-fold with RIMA and EIMA (Table 1).

Different results were obtained when the salmonella culture was diluted with the mixed culture of enterobacterial species. The dose-response curves obtained by immobilization with titanous hydroxide were much steeper than those obtained with the microtiter plates in both RIMA and EIMA (Fig. 1 and 2). Consequently, the minimum detectable population obtained with titanous hydroxide was 100- to 160-fold lower than that obtained with microtiter plates (Table 1). Moreover, the nonspecific absorbance of the phosphatase-antibody conjugate in EIMA increased dramatically with the microtiter plates, reaching 4.1 (Fig. 2).
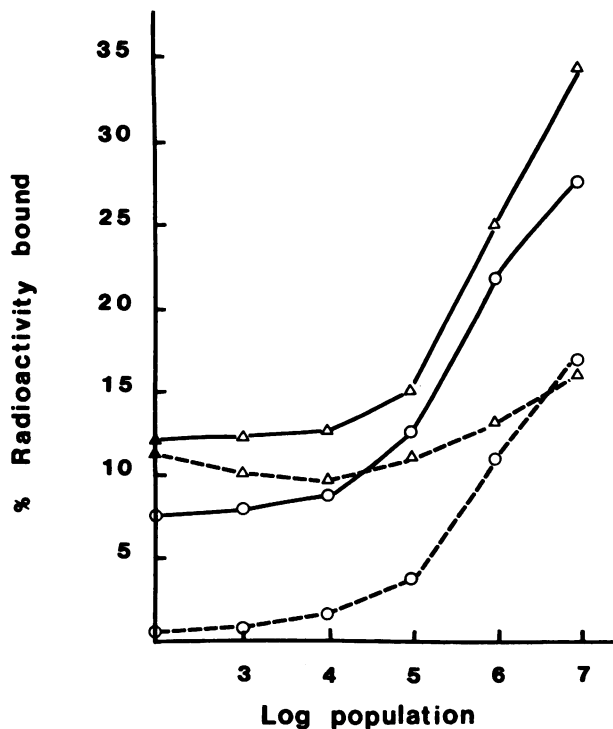

FIG. 2. Dose responses of EIMA for *S. waycross* in pure culture, immobilized on titanous hydroxide (O——O) and on microtiter plates (O——O), and in mixed culture, immobilized on titanous hydroxide (△——△) and on microtiter plates (△---△).

FIG. 1. Dose responses of RIMA for *S. waycross* in pure culture, immobilized on titanous hydroxide (O——O) and on microtiter plates (O---O), and in mixed culture, immobilized on titanous hydroxide (△——△) and on microtiter plates (△---△).
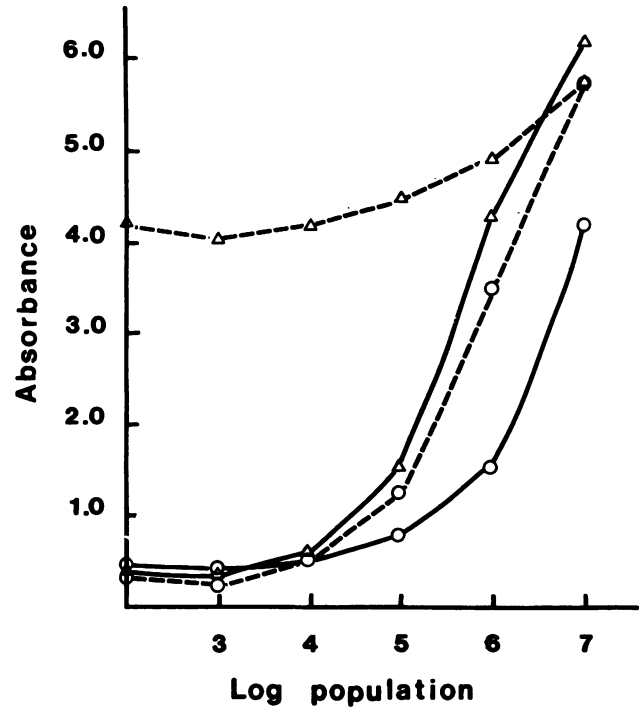
Detection of salmonellae in food samples. With the standard cultural method, the presence of salmonella was detected in a total of 12 food samples (Table 2). The same samples were also positive for salmonella when tested by RIMA (titanous hydroxide immobilization), using the flagellin antiserum mixture PFA. With the Spicer-Edwards antiserum mixture (SEA), only 11 of these food samples were positive for salmonella by RIMA as one chicken carcass gave a false-negative result (Table 2). Further, no false-positive results were obtained with RIMA when titanous hydroxide immobilization and either PFA or SEA antisera were used.

The same 12 salmonella-positive samples were also salmonella positive when tested by EIMA, using titanous hydroxide immobilization and either PFA or SEA. However, under the same conditions and with EIMA with microtiter plate immobilization, only 4 of the 12 samples were salmonella

TABLE 1. Log minimum detectable populations[a] of *S. waycross* culture diluted with saline (alone) and with a mixed culture of enterobacterial species (in association), as determined by RIMA and EIMA

S. waycross	RIMA with given immobilization		EIMA with given immobilization	
	Titanous hydroxide	Microtiter plates	Titanous hydroxide	Microtiter plates
Alone	3.60	3.00	4.75	4.10
In association	4.70	6.70	3.75	6.30

[a] Determined by using pooled antisera produced against 10 purified salmonella flagellins. The data were calculated by using the formula, $A_0 + 2 \times$ the standard deviation of A_0 (see text).

TABLE 2. Salmonella detection in foods with a standard cultural method, RIMA, and EIMA, utilizing titanous hydroxide, microtiter plates, SEA, and PFA

Food sample	No. tested	No. positive by SCM[a]	RIMA (titanous hydroxide)				EIMA							
			Positive		False-positive		Titanous hydroxide				Microtiter plates			
							Positive		False-positive		Positive		False-positive	
			SEA	PFA	SEA	PFA	SEA	PFA	SEA	PFA	SEA	PFA	SEA	PFA
Soft cheese	11	1	1	1	—[b]	—	1	1	—	—	—	1	1	—
Milk powder	24	3	3	3	—	—	3	3	—	—	1	2	2	—
Chicken carcass	10	3	2	3	—	—	3	3	—	—	1	2	—	—
Chicken liver	9	4	4	4	—	—	4	4	—	—	1	3	1	—
Egg products	5	1	1	1	—	—	1	1	—	—	1	1	—	—

[a] SCM, Standard cultural method. Salmonella isolates were serotyped as *S. havana*, *S. nienstedten*, *S. ohio*, *S. sofia*, and *S. typhimurium*.
[b]—, Nil.

positive when SEA was used and only 9 were positive when PFA was used. In addition, four false-positive results were obtained with EIMA, using microtiter plates and SEA, although no false-positive results were obtained with PFA (Table 2).

The data obtained from the immunoassays of food samples are given in Table 3, which shows the net bound radioactivity or absorbance (i.e., $A - A_0$, where $A =$ the values obtained when salmonella H antisera were added and $A_0 =$ the values obtained when the H antisera were not added to the system). As can be seen, these values varied considerably depending upon the food type and the immunoassay system used. Consequently, a salmonella-positive result can be considered as any net value above a certain limit (the cutoff value) which can be defined as the mean of $(\bar{A} - \bar{A}_0)$ + 2 × the standard deviation of $(\bar{A} - \bar{A}_0)$, where $(\bar{A} - \bar{A}_0) =$ the net response obtained from a number of known salmonella-negative food samples (by cultural procedure) of the same food type under investigation.

Table 3 shows the possibility of using such cutoff values as a criterion for deciding the presence or absence of salmonellae in foods, with the immunoassays utilizing titanous hydroxide. For example, the cutoff value for soft cheese obtained from testing with RIMA, using titanous hydroxide and SEA, was 4.7%. Under the same conditions the salmonella-positive cheese sample gave 11.5%.

However, the use of the cutoff value concept was not always possible with EIMA utilizing microtiter plates due to the incidence of high nonspecific absorbance (Table 3). This resulted in a lack of precision that produced high incidences of false-negative and false-positive results.

DISCUSSION

This investigation reports a novel, rapid, and reliable procedure for the detection of salmonellae by immunoassay. The procedure utilizes a suspension of titanous hydroxide as the solid support for immobilizing cells of salmonellae. After immobilization, the cells are allowed to react with flagellar antisera, the binding of which is detected radiometrically or enzymetrically. Radioimmunometric detection (RIMA) is based upon the reaction of bound flagellar antibodies with ^{125}I-labeled protein A. Enzyme-immunometric detection (EIMA) is based upon the reaction of bound flagellar antibodies with anti-rabbit-alkaline phosphatase conjugate, followed by the addition of enzyme substrate to determine the bound enzyme activity.

Pilot studies with pure cultures of *S. waycross* diluted with saline showed that the sensitivity of the immunoassay that uses titanous hydroxide was less than that obtained by the immunoassay that uses the more conventional microtiter plate method for immobilization. In this case, the dilution of the culture with saline resulted in reducing the concentration of proteinaceous compounds and other macromolecules, resulting in a decreased competition between such compounds and salmonella cells for adsorption sites on the

TABLE 3. RIMA and EIMA results for salmonella detection in food samples, using titanous hydroxide and microtiter plates as immobilization supports

Food sample	Antisera	RIMA [mean (SD) % radioactivity] with titanous hydroxide		EIMA [mean (SD) absorbance]			
				Titanous hydroxide		Microtiter plates	
		Positive	Negative	Positive	Negative	Positive	Negative
Soft cheese[a]	SEA	11.50	0.70 (2.00)	0.98	0.18 (0.14)	0.54	0.49 (0.32)
	PFA	15.50	0.50 (2.20)	1.93	0.10 (0.26)	2.25	0.83 (0.26)
Milk powder	SEA	11.90 (0.90)	0.46 (1.90)	1.25 (0.76)	−0.05 (0.14)	1.72 (0.85)	0.63 (0.56)
	PFA	13.70 (7.46)	−0.37 (2.20)	1.12 (0.28)	0.06 (0.10)	2.21 (1.46)	0.73 (0.31)
Chicken carcass	SEA	6.20 (4.79)	1.30 (0.59)	0.55 (0.03)	0.06 (0.06)	2.85 (0.40)	2.33 (0.27)
	PFA	22.37 (3.97)	1.29 (0.79)	0.58 (0.06)	0.01 (0.05)	3.55 (1.10)	1.85 (0.30)
Chicken liver	SEA	10.25 (1.26)	1.16 (2.05)	0.66 (0.16)	0.06 (0.14)	4.39 (1.43)	2.13 (1.84)
	PFA	12.75 (2.64)	2.34 (3.56)	0.65 (0.16)	0.08 (0.15)	5.34 (1.37)	1.60 (0.89)
Egg products[a]	SEA	8.50	−1.05 (1.04)	1.11	−0.15 (0.21)	1.47	0.70 (0.28)
	PFA	30.60	0.28 (1.60)	1.86	−0.05 (0.15)	4.59	1.07 (0.69)

[a] Only one salmonella-positive sample was detected by the standard cultural procedure.

microtiter plates. It is also conceivable that the low density of salmonella cells in the diluted culture contributed to more efficient adsorption and, thus, better detection. However, with mixed cultures of salmonella and other species of *Enterobacteriaceae*, the immunoassay with titanous hydroxide proved to be more sensitive, and as few as 4,000 to 5,000 salmonella cells could be detected. The mixed culture was used to simulate conditions likely to occur in selective enrichment cultures of food samples. Moreover, unacceptable high levels of nonspecific binding of the enzyme conjugate were obtained when EIMA was performed with microtiter plates, resulting in reduction in sensitivity. These results are consistent with the findings of a previous investigation (12) which showed that the sensitivity and specificity of antigen detection by enzyme immunoassay in microtiter plates were reduced when mixed or crude antigens were present.

The success of immunoassays that use titanous hydroxide was extended to the detection of salmonellae naturally present in food samples. In this case, food samples were tested for the presence of salmonellae at the conclusion of selective enrichment. Both RIMA and EIMA gave results comparable to those of a standard cultural method, the only difference being that immunoassay data were obtained within 8 h with selective enrichment cultures, whereas 3 to 4 days were required to obtain the same data by the cultural method. Furthermore, RIMA and EIMA utilizing titanous hydroxide lend themselves to the routine examination of a large number of samples for salmonellae.

Our data showed that the use of 2 standard deviations to determine the cutoff values resulted in diminishing the incidence of false-positive results without producing false-negatives. The use of 2 standard deviations of the zero-dose binding (salmonella-negative sample) represents 95% of the variation around the mean, and therefore any variation greater than such a value could not be considered a normal variation. The use of salmonella-negative samples (controls) of the same food sample under investigation by immunoassays is indispensable to establish the cutoff value for such food. This is due to variation in the nonspecific binding of the reagents used in immunoassays with different foods. In addition, a cutoff value may vary between assays conducted on different occasions.

This investigation has also compared the use of two preparations of salmonella polyvalent H antisera in immunoassays for the detection of salmonellae in food samples. One of these preparations (PFA) was a mixture of 10 antisera raised in rabbits against polymeric flagellins from 10 *Salmonella* serotypes. The other preparation (SEA) was a commercial product. The data (Tables 2 and 3) showed that the performance of PFA was superior to that of SEA. The reason for this may be attributed to compositional differences and characteristics of each preparation as a result of the different methods used for the production of each polyvalent antisera (10). Although PFA consisted of a mixture of only 10 antisera, it was capable of detecting salmonellae on the basis of the presence of common antigenic determinants on flagella from different *Salmonella* serotypes (G. F. Ibrahim, G. H. Fleet, M. J. Lyons, and R. A. Walker, Med. Microbiol. Immunol., in press).

For comparison, both RIMA and EIMA were conducted on the food samples, using microtiter plates. With the RIMA-microtiter plate procedure, the response (percent specific binding due to salmonella presence) was, for unknown reasons, very low, thus producing unreliable results. The same phenomenon occurred, although to a lesser extent,

with the EIMA-microtiter plate procedure (Table 3). The response difference between salmonella-positive and salmonella-negative samples was not sufficiently large to make unequivocal decisions in relation to the presence or absence of the pathogen when the cutoff value concept was applied, due to high nonspecific binding and high standard deviation values. This accounted for the poor correlation between the results of the EIMA-microtiter plate procedure and the cultural method (Table 2). The deficiencies of the use of microtiter plates as a solid phase for enzyme immunoassays have been reported by other researchers and relate to two basic problems. These are variation in adsorptive capacity for antigens and the high levels of nonspecific binding tracer reagents (i.e., antibody-enzyme conjugates). These problems can vary with the plates in the same batch and even with the wells in one plate (12, 18).

Immunoassays utilizing microtiter plates for immobilization have been proposed for the detection of salmonellae in foods (1, 14, 19). These studies did not draw attention to the deficiencies encountered in the present investigation, in relation to the use of microtiter plates. Also, the use of microtiter plates for immobilization was not compared with other immobilization supports in these studies. However, the results of the present investigation provide strong evidence for the merits of substituting titanous hydroxide for the microtiter plates as a solid phase for the immobilization of salmonellae for their detection by immunoassay. Apart from producing more reliable results, other advantages of titanous hydroxide include simplicity, rapidity, and efficiency of immobilization in the presence of high levels of proteinaceous compounds and other macromolecules, as well as low levels of nonspecific binding of antibodies and tracer reagents used in immunoassays (11). Moreover, as shown previously (11), titanous hydroxide effectively immobilizes other gram-negative and gram-positive bacteria. Consequently, the use of titanous hydroxide as a solid phase may be extended for developing immunoassays for the specific detection of other bacteria.

ACKNOWLEDGMENT

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