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Detection and Serogroup Differentiation of *Salmonella* spp. in Food within 30 Hours by Enrichment-Immunoassay with a T6 Monoclonal Antibody Capture Enzyme-Linked Immunosorbent Assay

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We previously described an antigen capture enzyme-linked immunosorbent assay which makes use of monoclonal antibody T6, which recognizes an epitope on the outer core polysaccharide of *Salmonella* lipopolysaccharide molecules that is common to almost all *Salmonella* serovars. In this paper, we show that this assay can detect between 10^5 and 10^7 *Salmonella* cells per ml even in the presence of excess *Escherichia coli*. A total of 153 of 154 (99%) serogroup A to E strains and 51 of 78 (71%) serogroup F to 67 strains were reactive as determined by this assay. This corresponds to a detection rate of approximately 98% of all salmonellae known to affect humans. None of the 65 strains of non-*Salmonella* bacteria tested positive. Taking advantage of the O-factor polysaccharides also present on the antigen captured by the immobilized T6 antibody, we showed that 136 of 154 *Salmonella* serogroup A to E strains (88%) were correctly differentiated according to their serogroups by use of enzyme conjugates of a panel of O-factor-specific monoclonal antibodies. We evaluated this assay for the detection and serogroup differentiation of salmonellae directly from enrichment cultures of simulated food, eggs, pork, and infant formula milk. All 26 samples which had been contaminated with *Salmonella* spp. were detected by T6 (100% sensitivity), with only one false-positive result from 101 samples not contaminated by *Salmonella* spp. (99% specificity). The detection time was substantially reduced to between 17 and 29 h, depending on the enrichment methods used. Since there were no false-negative results, we concluded that this enrichment-immunoassay method can afford rapid screening for *Salmonella* spp. in food samples.

Salmonella is a diverse group comprising over 2,000 known serovars (10). These bacteria are widely distributed in nature, gaining entry to almost all aspects of the human food chain. As with other food-borne pathogens, control of human infection with *Salmonella* spp. depends primarily on maintenance of a high standard of food hygiene by both the producers and the consumers. The contribution of laboratories to this effort is to facilitate the prevention of infection through appropriate public health and management measures and timely intervention to contain the infection once it occurs. Timely intervention of the infection depends on rapid detection of these pathogens, while formulation of the appropriate preventive measures requires regular monitoring. In most countries, testing is done by different types of laboratories to achieve different objectives. Detection of these pathogens is done mainly by frontline diagnostic laboratories and routine food laboratories. Detailed identification and characterization of this large group of bacteria are difficult tasks requiring a combination of different approaches and are usually left to specialized public health and reference laboratories.

The conventional method for detection of salmonellae by isolation and identification of the organisms usually requires 3 to 5 days, and hence in most instances it cannot achieve the primary objective of timely intervention. On the other hand, enrichment-immunoassay allows a next-day result by eliminating the need for isolation. In this approach, the number of *Salmonella* organisms initially present is selectively expanded by enrichment culture and detected by immunoassay. This is not generally considered to be a definitive test for salmonellae because it detects antigens presented by these pathogens rather than isolating and identifying live bacteria. This can be overcome by treating enrichment-immunology as a screening process by which all samples are tested, after which the screened-positive samples are confirmed by conventional methods. This combination allows for rapid detection and thus timely intervention without the need to carry out laborious conventional methods for all samples.

An immunoassay suitable for reliable screening of samples must meet two criteria: the ability to detect most of the over 2,000 *Salmonella* serovars and sufficient sensitivity to detect these pathogens at a level that is usually present in enrichment cultures. Most assays developed to date can achieve a sensitivity on the order of 10^6 CFU/ml, but the specificity of some of these assays is restricted primarily to certain serovars (8, 9) and therefore they cannot be applied for the detection of the large diversity of salmonellae. Some assays make use of a combination of antibodies in order to achieve a broad spectrum of specificity for salmonellae (3), but this practice can potentially compound the cross-reactions or artifacts associated with each of the antibodies used in such assays. This difficulty was largely overcome by using a single monoclonal antibody (MAb) specific for the outer core oligosaccharide of *Salmonella* lipopolysaccharide (LPS) (16, 20). Previous studies showed that most, if not all, *Salmonella* serovars have a common outer core polysaccharide in their LPS molecules (11). Two types of immunoassays using such antibodies have been developed. One is a

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competitive enzyme immunoassay (15, 19), and the other is a capture enzyme-linked immunosorbent assay (ELISA) (4). Both types of assays are capable of specifically detecting a wide range of *Salmonella* spp. at levels of sensitivity comparable to those afforded by other immunoassays. Since the LPS molecules captured also bear somatic (O) factor-specific antigens of *Salmonella*, the application of the capture ELISA can potentially be extended to serogroup differentiation of *Salmonella* serovars by using a selected panel of *Salmonella* O-factor-specific MAbs.

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MATERIALS AND METHODS

Production and characterization of MAbs. MAbs were produced by the method of Kohler and Milstein (7). MAb T6 (16) was produced by immunizing BALB/c mice intraperitoneally with acetone-fixed *Salmonella typhi* 620Ty (5) and O-factor-specific MAbs. MO2 (14), MO4 (17), MO8 (13), MO9 (12), and MO10 (18) with acetone-fixed salmonellae belonging to serogroups A, B, C_2, D, and E_1, respectively. Two additional MAbs, MO7.1 (immunoglobulin G3) and MO15 (immunoglobulin G1), were produced by immunization with a phage 14-lysogenized *S. ohio* strain (6,7,14: b: 1,6) and a phage 15-lysogenized *S. london* strain (3,10,15; l,v; 1,6), respectively. Four doses of 0.5 ml of whole-cell suspension (incubated for 30 min at 100°C) containing 10^8 cells were injected intraperitoneally at weekly intervals, followed by a similar intravenous booster injection 1 week after the last intraperitoneal injection. Spleen cells from one of the mice that gave the strongest serum antibody response to the immunizing strain were fused with NSI myeloma cells by using polyethylene glycol 4000 (BDH, Poole, England) as the fusing agent. The resulting hybridomas produced were screened against the target *Salmonella* serovars in an indirect ELISA (14). Hybridomas producing specific antibodies were then cloned twice by limiting dilution, and the resulting hybridomas were expanded for antibody production by being injected intraperitoneally into mineral oil primed BALB/c mice to obtain ascitic fluid.

T6 capture ELISA. The T6 capture ELISA was performed with two test kits, Salmonella Test 1 and Salmonella Test 2, prepared and supplied by Sinoclone Ltd. (Hong Kong). The microtiter plates used in both of these test kits were coated with ascitic fluids of the T6 MAb (16), which had been diluted 1:750 in carbonate buffer (pH 9.6). In Salmonella Test 1, antigen captured on the immobilized T6 MAb was detected by using a horseradish peroxidase (HRP) (type VI; Sigma, St. Louis, Mo.) conjugate of the T6 MAb. In Salmonella Test 2, the captured antigens were differentiated according to their serogroups by using HRP conjugates of the panel of O-factor-specific MAbs: MO2 (14), MO4 (17), MO7.1, MO8 (13), MO9 (12), MO10 (18), and MO15. The MAbs were precipitated from ascitic fluids with 40% ammonium sulfate and conjugated to HRP by the method of Avrameas and Ternynck (2). The working dilution of each MAb conjugate was predetermined by a direct binding assay using microtiter plates coated with *Salmonella* serogroup A, B, C_1, C_2, D, and E_1 strains or the rough mutant *S. typhi* 620Ty (5) as described by Luk et al. (14).

The tests were performed according to the manufacturer's instructions. Briefly, the sample cultures were boiled with sample buffer (500 mM Tris-EDTA) for 10 min and incubated on the T6-coated microtiter plate at 37°C for 1 h. The plate was then washed four times with the wash buffer (0.05% Tween 20 in 10 mM phosphate-buffered saline), the enzyme-conjugated MAb was added, and the mixture was allowed to react for 1 h at 37°C. The plate was washed and then incubated with the substrate (tetramethyl benzidine) for 15 min at 37°C. The reaction was stopped by adding stop solution (2 M sulfuric acid), and the color developed was measured at an A_{450} with a 630-nm reference. A net optical density (OD) reading exceeding 0.3 was considered to be positive.

Bacterial strains and culture conditions. The bacterial strains used are listed in Tables 1 and 2. Six standard *Salmonella* strains were obtained from the American Type Culture Collection (Rockville, Md.), 1 *Salmonella paratyphi* A standard strain was obtained from National Collection of Type Cultures (Central Public Health Laboratory, London, England), and the remaining 225 *Salmonella* strains were either clinical isolates or kind gifts from S. Aleksic (Hygienisches Institut, Nationales Referenzzentrum für Enteritiserreger, Hamburg, Germany), A. Lindberg (Department of Clinical Bacteriology, Karolinska Institute, Stockholm, Sweden), and H. Lior (National Laboratory for Enteric Pathogens, Bureau of Microbiology, Laboratory Centre for Disease Control, Health Canada). Seventeen standard non-*Salmonella* strains were obtained from American Type Culture Collection, and the remaining 48 were clinical isolates. All clinical isolates were identified in the clinical microbiology laboratory of the Queen Mary Hospital, Hong Kong, by standard biochemical and serological methods (6).

The bacterial strains tested were grown in brain heart infusion broth (CM225; Oxoid Ltd., London, England) at 37°C under static incubation conditions for 14 to 16 h before testing.

Evaluation of the T6 capture ELISA. The detection limit of each of the enzyme-conjugated MAbs was determined by titrating serially diluted cultures of 15 selected strains: *S. kiel*, *S. paratyphi* A, *S. sandiego*, *S. typhimurium*, *S. choleraesuis*, *S. infantis*, *S. livingstone* var. 14^+, *S. manhattan*, *S. newport*, *S. enteritidis*, *S. panama*, *S. typhi*, *S. anatum*, *S. orion*, and *S. london* var. 15^+. The assay was further evaluated against a large selection of test bacteria listed in Tables 1 and 2. The tests were done by using the T6 conjugate and the O-factor-specific MAb conjugates corresponding to the serogroups of the test strains. The number of viable organisms was determined by plate count.

Simulated-food samples. Eleven lyophilized simulated-food samples were kindly supplied by the Public Health Laboratory Service (Food Microbiology External Quality Assessment Scheme [FMEQAS] no. 0001, 0010, 0019, 0020, 0021, 0022, 0023, 0024, 0031, 0035, and 0037). These coded samples were reconstituted in 100 ml of prewarmed nutrient broth (CM1; Oxoid) and treated as equivalent to 100 g of food sample. For the T6 capture ELISA, a shortened enrichment method was employed. The reconstituted food samples were preenriched for 6 h at 37°C, and 100-μl portions were then transferred to 10 ml of Rappaport-Vassaliadis selective enrichment broth (RV; Oxoid) and further incubated for 14 to 16 h at 43°C. For postenrichment, 1-ml portions of selective enrichment broth culture were subsequently transferred to 10 ml of brain heart infusion broth (Oxoid) and incubated for 4 h before being tested by the T6 capture ELISA. For conventional bacteriological analysis, preenrichment and enrichment cultures were incubated for 24 h at 37 and 43°C, respectively (1). Isolation was done on MacConkey, xylose-lysine-deoxycholate, and deoxycholate-citrate agars, followed by biochemical and serological tests (6).

Ground pork. Twenty 100-g ground pork samples were purchased from 18 stores in nine wet markets and two supermarkets in Hong Kong. The samples were transported on ice and processed within 4 h of purchase. Three 25-g portions of each pork sample were mixed with 225 ml of prewarmed buffered peptone water (BPW) for preenrichment. For the T6 capture ELISA, a 6-h preenrichment was followed by 14 h of enrichment in 10 ml of RV (BPW/RV ratio, 1:100) and postenriched in 10 ml of BHI (RV/BHI ratio, 1:10) for 4 h. All incubations were carried out at 37°C except for RV, which was incubated at 43°C. For conventional bacteriological analysis, the enrichment, isolation, and identification procedures were as described for the simulated-food samples.

Fresh eggs. A total of 690 fresh hen eggs were obtained from different suppliers in Hong Kong. Those which were not visibly damaged were immersed for 15 min in 75% ethanol. The contents of 9 to 12 eggs were pooled, mixed with an equal volume of BPW, and incubated for 6 h at 37°C. Ten volumes of BPW were each inoculated with 1/10 of this preenrichment culture, further incubated for 14 to 16 h at 37°C, and then tested concurrently by the T6 capture ELISA and conventional bacteriology. To determine the detection limit of this two-step nonselective enrichment, eggs variously spiked with a rare serovar, *S. choleraesuis*, were similarly tested.

Infant formula milk. Forty-one infant formula milk samples (40 in powder form and 1 in liquid form) were tested. Twenty-five grams (or 25 ml) of the milk sample was added to 225 ml of BPW and the mixture was incubated for 14 to 16 h at 37°C before being tested by the T6 capture ELISA and the conventional bacteriological method.

RESULTS

Detection limit of the T6 capture ELISA. Of the 15 selected *Salmonella* strains tested, the detection limits of the T6 capture ELISA were on the order of 10^7 CFU/ml for 2 strains, 10^6 CFU/ml for 10 strains, and 10^5 CFU/ml for 3 strains, as determined by using the T6 antibody-enzyme conjugate. For the O-factor antibody-enzyme conjugates, the detection limits were on the order of 10^6 CFU/ml for nine strains and 10^5 CFU/ml for the other six. It may be expected that there is a greater abundance of the epitopes identified by the O-factor MAbs than that identified by the T6 MAb because O-factor antigens are present in repeating units. However, the detection limits with the two antibodies were found to be essentially similar. Presumably, the antigens captured by the T6 MAb are mainly cell associated, so that there is an abundance of targets to react with either the T6 MAb conjugate or the corresponding O-factor MAb conjugate.

Detection in the presence of *E. coli*. The titration curves for *S. paratyphi* A (Fig. 1A) and *S. typhimurium* (Fig. 1B) obtained in the presence of 10^5 to 10^9 CFU of *E. coli* per ml were essentially the same as the control curves obtained in the absence of *E. coli*. The detection limits as determined from these curves were on the order of 10^5 and 10^6 CFU/ml. Compared with results obtained in the absence of *E. coli*, the detection limit was not affected (Fig. 1A) or only slightly affected (Fig. 1B) by the presence of excess *Escherichia coli*, indicating

TABLE 1. *Salmonella* strains tested[a]

Serogroup	Serovar	No. of strains[b]	Serogroup	Serovar	No. of strains
A	*S. kiel*	1		*S. newport*	2 (1)
	S. nitra	1		*S. tshiongwe*	2
	S. paratyphi A	8 (2)			
			D_1	*S. berta*	4
B	*S. agona*	3		*S. dublin*	3
	S. bredeney	1		*S. enteritidis*	6 (1)
	S. derby	2		*S. javiana*	1
	S. eko	1		*S. napoli*	2
	S. heidelberg	2		*S. panama*	7
	S. paratyphi B	3		*S. sendai*	1
	S. reading	1		*S. typhi*	5 (1)
	S. saintpaul	2			
	S. sandiego	2	D_2	*S. doba*	1
	S. stanley	1		*S. india*	1
	S. schwarzengrund	1		*S. salama*	5
	S. typhimurium	4 (1)		*S. zadar*	1
C_1	*S. braenderup*	1	$E_{1,4}$	*S. meleagridis*	1
	S. choleraesuis	(1)		*S. anatum*	4
	S. georgia	1		*S. anatum* var. 15^+	1
	S. infantis	5		*S. london*	2
	S. lille	1		*S. london* var. 15^+	2
	S. livingstone var. 14^+	2		*S. weltevreden*	2
	S. mbandaka	3		*S. sentfenberg*	4
	S. montevideo	1		*S. muenster*	2
	S. ohio	4		*S. vejle*	1
	S. ohio var. 14^+	3		*S. uganda*	1
	S. paratyphi C	1		*S. krefeld*	2
	S. potsdam	1		*S. orion* var. 15^+	1
	S. rissen	2			
	S. singapore	2			
	S. tennessee	1			
	S. thompson	1	F	*S. srinagar*	1
	S. virchow	2		*S. rubislaw*	1
C_2	*S. bardo*	1	G	*Unknown*	2
	S. blockley	2		*S. wichita*	1
	S. bovismorbificans	2		*S. arizona*	1
	S. chailey	3			
	S. glostrup	1	H	*S. ferlac*	1
	S. hadar	1		Unknown	1
	S. kottbus	1		*S. madelia*	1
	S. litchfield	3			
	S. manhattan	4	I	*S. hvittingfoss*	2
	S. muenchen	1		*S. gaminara*	1
				Unknown	1
J	*S. dahra*	1	W	*S. dugbe*	2
	S. berlin	1			
	S. bleadon	1	X	*S. luke*	1
				S. blitta	1
K	*S. cerro*	1		*S. bilthoven*	1
	S. sinthia	1			
	Unknown	1	Y	*S. toucra*	1
				S. fitzroy	1
L	*S. ruiru*	1			
	S. minnesota	2	Z	*S. bonaire*	1
				S. greenside	1
M	*S. babelsberg*	1		*S. arizona*	2
	S. nima	1			
	S. tel-aviv	1	51	*S. treforest*	1
				S. arizona	1
N	*S. godesberg*	1			
	S. matopeni	1	52	*S. utrecht*	1
				S. arizona	1

Continued on following page

TABLE 1—*Continued*

Serogroup	Serovar	No. of strains[b]	Serogroup	Serovar	No. of strains
O	*S. widemarsh*	1			
	S. ealing	1	53	*S. humber*	1
	S. adelaide	1			
			54	*S. uccle*	1
P	*S. inverness*	2			
			55	*S. tranoroa*	1
Q	*S. mara*	1			
	S. wandsworth	1	56	*S. arizona*	1
	S. abidijan	1			
			57	*S. locarno*	1
R	*S. duval*	1			
	S. johannesberg	1	58	*S. basel*	1
	S. bern	1		Unknown	1
S	*S. waycross*	2	60	*S. luton*	1
T	*S. weslaco*	1	61	*S. arizona*	1
	S. arizona (IIIb)	1		*S. arizona*	3
	S. ursenback	1	65	*S. arizona*	3
U	*S. mbao*	1	66	*S. malawi*	1
	S. bunnik	1			
	S. milwaukee	1	67	*S. crossness*	1
V	*S. madigan*	1			
	S. niarembe	1			
	S. christiansborg	1			

[a] For serogroups A to E, a total of 67 serovars and 154 strains were tested; overall, 136 serovars and 232 strains were tested.
[b] Numbers of standard strains are shown in parentheses.

that this assay is capable of detecting *Salmonella* spp. in the presence of excess *E. coli.*

***Salmonella* detection (Salmonella Test 1).** We tested overnight broth cultures of 232 *Salmonella* (Table 1) and 65 non-*Salmonella* (Table 2) strains by the T6 capture ELISA. The results obtained for *Salmonella* serovars belonging to the different serogroups are shown in Table 3. The majority of the serogroup A to E strains were strongly reactive with the T6 conjugate. The mean ELISA OD obtained for each of these serogroups exceeded 2.0. The greater variance of ODs in serogroups C_1, C_2, and D was due to the fact that a few strains in these serogroups showed only moderate reactivities, with ODs between 0.3 and 2.0. One strain from serogroup D_1 (*S. napoli*) was not detected by the T6 conjugate, with an OD of 0.20. The strong reactivity of the majority of the serogroup A to E strains was in contrast to that of *Salmonella* strains belonging to the higher O serogroups, of which only 55 of the 78 strains tested (71%) were reactive, and most of the reactive strains were only moderately reactive against the T6 conjugate (OD = 0.3 to 2.0). The mean OD (± standard deviation) obtained for the 55 reactive strains belonging to serogroups F to 67 was 1.31 ± 0.74. As the assay was carried out under the condition of antigen excess, the difference in the reactivity with T6 antibody conjugate suggests that the epitopes presented by the strongly reactive serovars might be related, but not identical, to those presented by the moderately reactive serovars. The results obtained with 65 strains of the non-*Salmonella* bacteria are also shown in Table 3. All 65 non-*Salmonella* strains gave a negative result in this assay (mean OD ± standard deviation = 0.05 ± 0.07).

Since over 95% of natural isolates of *Salmonella* known to

TABLE 2. Non-*Salmonella* strains tested[a]

Bacterium	No. of strains tested[b]
Acinetobacter anitratus	(1)
Acinetobacter lwoffii	(1)
Aeromonas hydrophila	2
Aeromonas caviae	2
Alcaligenes faecalis	(1)
Campylobacter jejuni	1
Citrobacter diversus	7
Citrobacter freundii	5 (1)
Citrobacter koseri	1
Citrobacter sp.	3
Citrobacter amalonaticus	1
Enterobacter agglomerans	(1)
Enterobacter cloacae	(1)
Escherichia coli	3 (2)
Klebsiella pneumoniae	4 (1)
Klebsiella ozaenae	1
Morganella morganii	1 (1)
Plesiomonas shigelloides	1 (1)
Proteus mirabilis	(1)
Proteus vulgaris	3
Providencia rettgeri	(1)
Pseudomonas aeruginosa	(1)
Shigella boydii	2
Shigella flexneri	2 (1)
Shigella sonnei	6 (1)
Vibrio parahaemolyticus	2
Yersinia enterocolitica	1 (1)

[a] A total of 27 species (65 strains) were tested.
[b] Numbers of standard strains are shown in parentheses.

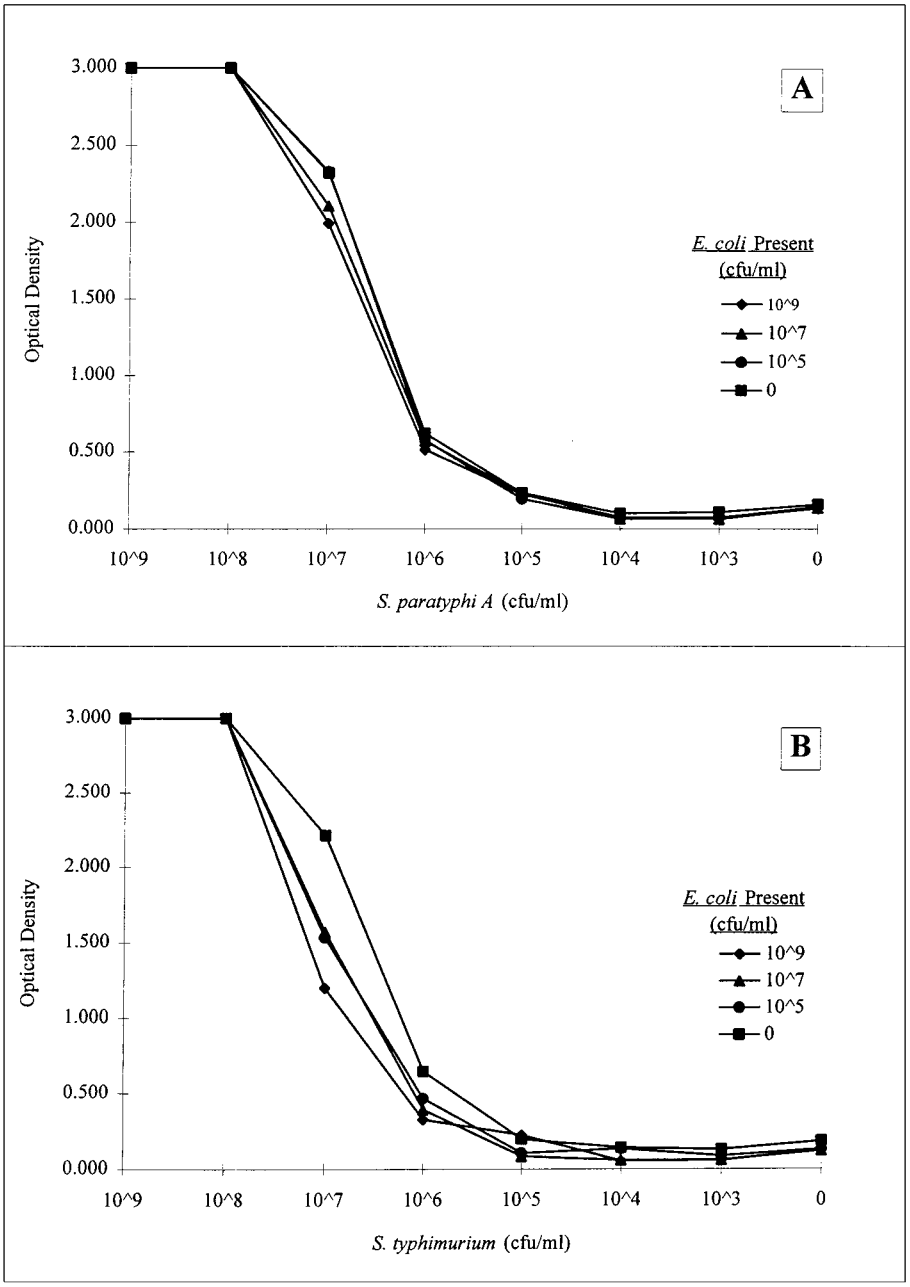

FIG. 1. Titration of *Salmonella* organisms in the presence of *E. coli* by the T6 capture ELISA. (A) *S. paratyphi* A; (B) *S. typhimurium.*

affect humans belong to serogroups A to E (19, 21), it may be expected from the above results that 98% of these pathogens naturally affecting humans may be detected by the T6 capture ELISA, i.e., 99% × 95% of serogroup A to E strains plus 71% × 5% of the higher-serogroup strains.

Serogroup differentiation (Salmonella Test 2). The majority of *Salmonella* serovars exhibit characteristic patterns of reactions corresponding to their respective serogroup specificity (Table 4). Serogroup A strains can be differentiated from other *Salmonella* serogroups by their reaction with the O-factor 2 MAb (MO2), serogroup B strains can be differentiated by their reaction with the O-factor 4 MAb (MO4), serogroup C_1 strains can be differentiated by their reaction with the O-factor 7 MAb (MO7.1), serogroup C_2 strains can be differentiated by their reaction with the O-factor 8 MAb (MO8), serogroup D_1 and some strains of serogroup D_2 can be differentiated by their reaction with the O-factor 9 MAb (MO9), serogroup E_1 and some strains of serogroup E_4 can be differentiated by their reaction with the O-factor 10 MAb (MO10), and the phage 15-lysogenized serogroup E_1 (previously E_2) strains can be differentiated by their reaction with the O-factor 15 MAb (MO15).

It was shown that 136 (88%) of the 154 test strains gave a typical reaction pattern and could thus be correctly differentiated into their respective serogroups (Table 4). The other 18 strains showed either a negative reaction with their corresponding MAbs or atypical reaction patterns. We similarly tested 65 strains of non-*Salmonella* bacteria. One strain of

TABLE 3. Detection of *Salmonella* spp. by the T6 capture ELISA

Test bacteria	No. tested	% Positive	Mean OD[a]
Salmonella serogroups			
A	12	100	2.73
B	24	100	2.79 ± 0.10
C_1	32	100	2.27 ± 0.83
C_2	24	100	2.10 ± 0.87
D_1	31	97	2.31 ± 0.72 (0.20)
D_2	8	100	2.52 ± 0.67
$E_{1,4}$	23	100	2.76 ± 0.06
Total, A–E	154	99	2.46 ± 0.66 (0.20)
F–67 (higher serogroups)	78	71	1.31 ± 0.74 (0.07 ± 0.10)
Non-*Salmonella* spp.	65	0	(−0.05 ± 0.07)

[a] Standard deviations provided where calculation is possible. Values in parentheses are ODs of negative strains.

Citrobacter species was reactive with MO2 and one strain of *Shigella sonnei* was reactive with MO7.1, but neither of these test strains was reactive with the T6 antibody. The other 63 strains (97%) of non-*Salmonella* bacteria were not reactive with any of the O-factor antibodies.

Simulated-food samples. Eleven coded and spiked samples were tested blindly by the T6 capture ELISA. The bacterial contents of these samples were revealed only after the test had been completed. Table 5 shows that six of these samples were spiked with between 50 and 4,000 *S. typhimurium*, *S. enteritidis*, or *S. virchow* organisms and different mixtures of between 10^5 and 10^7 *Campylobacter*, *Citrobacter*, *Enterobacter*, *Klebsiella*, or *Pseudomonas* organisms. The other five samples did not contain salmonellae but were similarly spiked with the non-*Salmonella* bacteria. All the tests of samples by enrichment-immunoassay were completed in 29 h. All six samples previously spiked with salmonellae gave a positive reaction by the enrichment-immunoassay method using the enzyme conjugate of T6 MAb. This was confirmed by performing the same assay with enzyme conjugates of a panel of O-factor-specific MAbs as the detecting antibodies. *S. typhimurium* present in three samples reacted with MO4 but not the other O-factor MAbs. Similarly, *S. enteritidis* present in two samples was selectively reactive with MO9, and the *S. virchow* in one sample was selectively reactive with MO7.1. The other five samples were not reactive with the T6 conjugate, with ODs below the cutoff value of 0.3 (0.05 to 0.09).

Pork samples. Twenty ground pork samples were tested by the same enrichment-immunoassay method. Conventional bacteriology was performed concurrently on the standard enrichment cultures (Table 6). As with the simulated-food samples, the pork samples contained a highly mixed growth of bacteria (results not shown). Conventional bacteriology showed that 1 to 3 serovars of *Salmonella* were present in each of the 19 samples, and a total of 31 serovars were identified. Only one sample did not contain *Salmonella* organisms. The results obtained by the enrichment-immunoassay using the T6 conjugate completely agreed with the results of conventional bacteriology. The O-factor-specific MAb conjugates detected and identified 12 of these serovars. Results obtained with the O-factor-specific MAb conjugates detected additional serovars in seven samples which were not originally identified by conventional bacteriology, but all of these serovars were subsequently isolated from the corresponding enrichment cultures when they were retested.

Egg samples. For the first 10 test batches of eggs, both the shortened two-step and the standard enrichment cultures were performed before testing by the T6 capture ELISA and conventional bacteriology (see Materials and Methods). There was no discrepancy between the two enrichment methods, and the T6 capture ELISA correctly detected the presence of *Salmonella* spp. from cultures of both enrichment methods in 1 of the 10 test batches. Detection of *Salmonella* spp. by the T6 MAb conjugate was confirmed by using the panel of O-factor-specific MAb conjugates, showing that the *Salmonella* strain present in this sample reacted exclusively with MO9, indicating serogroup D. The T6 MAb conjugate gave an average OD of 1.80 from duplicate determinations, and the MO9 conjugate yielded an average OD of 1.04. The *Salmonella* strain was later isolated from the enrichment medium by conventional bacteriological procedures and identified as *S. enteritidis* (serogroup D_1). None of the other nine test batches gave a positive result by culture or the T6 capture ELISA, although these samples were contaminated with scanty growth of other bacteria, such as *Citrobacter*, *Edwardsiella*, and *Bacillus* spp. The detection limit of the two-step enrichment procedure determined by using egg samples variously spiked with *S. choleraesuis* was <1 CFU/100 g.

The above results showed that the level of contamination of eggs with non-*Salmonella* bacteria is substantially lower than that of the pork samples. This suggested that selective enrichment may not be necessary for the detection of *Salmonella* spp. in eggs, and it was therefore omitted from the testing of the remaining 45 batches. Some of these samples gave scanty to

TABLE 4. *Salmonella* serogroup differentiation by the T6 capture ELISA

Serogroup or species	No. of strains tested	No. detected by the indicated MAb conjugate							No. identified
		MO2	MO4	MO7.1	MO8	MO9	MO10	MO15	
A	12	12	2	0	0	0	0	0	10
B	24	1	24	0	0	0	0	0	23
C_1	32	1	0	32	0	0	0	0	31
C_2	24	0	0	0	23	0	0	0	23
D_1	31	1	0	0	0	30	0	0	29
D_2	8	0	0	0	0	3	0	0	3
$E_{1,4}$	19	1	0	0	0	0	15	0	14
E_2	4	1	0	0	0	0	0	4	3
Total	154								136
Non-*Salmonella* spp.	65	1	0	1	0	0	0	0	

TABLE 5. Detection and serogroup differentiation of *Salmonella* spp. in simulated-food samples by the T6 capture ELISA

FMEQAS no.[a]	Bacterium in sample[b]		Mean OD[c] with detecting enzyme conjugate of:			
			T6 MAb		O-factor-specific MAb	
	Species	Concn (CFU/100 g)	Postenriched	Not postenriched	Postenriched	Not postenriched
0001	*Salmonella typhimurium*	3.7×10^3	1.825	0.004	0.575 (MO4)	−0.024 (MO4)
	Citrobacter freundii	10^5–10^6				
	Enterobacter cloacae	10^6–10^7				
	Pseudomonas fluorescens	10^5–10^6				
0020	*Salmonella typhimurium*	50	0.910	−0.005	0.955 (MO4)	−0.046 (MO4)
0022	*Salmonella typhimurium*	50	0.459	0.005	0.389 (MO4)	−0.066 (MO4)
	Citrobacter freundii	10^5				
	Enterobacter cloacae	10^5				
	Pseudomonas aeruginosa	10^5				
0037	*Salmonella virchow*	100	0.980	0.092	0.818 (MO7)	0.027 (MO7)
	Citrobacter jejuni	10^5				
	Klebsiella oxytoca	10^4–10^5				
	Enterococcus faecalis	10^5–10^6				
0019	*Salmonella enteritidis*	50	2.902	0.267	0.772 (MO9)	−0.109 (MO9)
	Citrobacter freundii	10^5				
	Enterobacter cloacae	10^5				
	Pseudomonas aeruginosa	10^5				
0023	*Salmonella enteritidis*	400	2.902	0.102	0.534 (MO9)	−0.120 (MO9)
	Citrobacter freundii	10^5				
	Enterobacter cloacae	10^5				
	Pseudomonas aeruginosa	10^5				
0010	*Citrobacter freundii*	10^5	0.051	−0.009	ND[d]	ND
	Enterococcus faecalis	10^5				
	Klebsiella aerogenes	10^4				
	Pseudomonas aeruginosa	10^5				
0021	*Citrobacter freundii*	10^5	0.088	−0.013	ND	ND
	Enterobacter cloacae	10^5				
	Pseudomonas aeruginosa	10^5				
0024	*Enterococcus faecalis*	10^5	0.052	−0.019	ND	ND
	Escherichia coli	10^4				
	Hafnia alvei	10^4				
	Pseudomonas putida	10^4				
0031	*Citrobacter freundii*	10^5	0.059	−0.001	ND	ND
	Citrobacter jejuni	10^4–10^5				
	Escherichia coli	10^5				
	Pseudomonas aeruginosa	10^4				
0035	*Citrobacter freundii*	10^5	0.065	−0.014	ND	ND
	Klebsiella aerogenes	10^5				
	Escherichia coli	50–100				

[a] Supplied by the Public Health Laboratory Service.
[b] These data were supplied by the Public Health Laboratory Service after completion of the trials.
[c] Mean OD of duplicate determinations.
[d] ND, not done.

moderate growth of other bacteria, but *Salmonella* strains were not isolated from any of them. These results agree with those obtained by the T6 capture ELISA. The mean OD (± standard deviation) for the 54 batches of *Salmonella*-negative eggs was 0.08 ± 0.06.

Infant formula milk samples. Similar to the egg samples, the infant formula milk samples were not expected to contain a high bacterial load; therefore, the 41 samples were tested by the T6 capture ELISA and conventional bacteriological methods using only overnight preenrichment cultures. All except one of the milk samples gave negative results by the T6 capture ELISA, with an average OD of 0.10 ± 0.01. The positive sample (in liquid form) gave an average OD of 1.40 with the T6 conjugate from duplicate determinations but showed no bacterial growth on MacConkey, xylose-lysine-deoxycholate, deoxycholate-citrate, and blood agars. It was further tested by the standard enrichment method, with negative results. Testing with the O-

TABLE 6. Detection and serogroup differentiation of *Salmonella* spp. in fresh ground pork samples by the T6 capture ELISA

Organism(s) isolated and identified by conventional bacteriological methods (retrospective isolate)[a]	Mean OD[b] with detecting enzyme conjugate of:	
	T6 MAb	O-factor-specific MAb
S. london and serogroup E_1 (*S. othmarschen*)	1.84	0.44 (MO7.1)
S. london and *S. senftenberg*	0.78	Negative
S. thompson and *S. london*	0.38	Negative
S. muenster	0.70	Negative
S. derby	0.90	0.79 (MO4)
S. meleagridis	0.82	0.69 (MO15)
Serogroup C_1	0.99	Negative
S. derby and serogroup E_1	2.72	Negative
S. anatum	0.64	Negative
S. anatum (serogroup B)	1.01	0.64 (MO4)
S. anatum and serogroup C_1	1.08	Negative
Serogroup C_1	1.71	1.96 (MO7.1)
Serogroup B (serogroup C_1)	1.45	0.69 (MO7.1)
Serogroup B	0.91	>3.0 (MO4)
S. thompson (*S. kingston*)	2.68	1.24 (MO4)
S. isangi (*S. derby*)	1.78	1.91 (MO4)
S. london	0.39	0.91 (MO10)
S. okefoko (*S. thompson*)	0.68	0.95 (MO7.1)
S. fulda (*S. agona*)	0.78	0.76 (MO4)
Non-*Salmonella* sp.	0.07	Negative

[a] Retrospective isolation was carried out on samples which indicated the presence of other-serogroup salmonellae by the O-factor-specific detecting MAb of the T6 capture ELISA but not by conventional methods.
[b] Mean of duplicate determinations.

factor-specific MAb conjugates was also negative, with an average OD of 0.02.

DISCUSSION

A previous study by Choi et al. (4) showed that the T6 capture assay is not sufficiently sensitive for detection of *Salmonella* spp. from enrichment cultures of stool specimens. We believe that inhibitors present in commonly used enrichment media may have prevented the number of *Salmonella* organisms from reaching a level detectable by the immunoassay. We have demonstrated that this difficulty could largely be overcome by introducing an additional step, in which a nonselective medium was inoculated with samples of the enrichment cultures and further cultured for 4 h before being tested by the T6 capture ELISA. The introduction of this additional postenrichment step markedly improved the detection of *Salmonella* spp. from the simulated-food samples (Table 5). We have also shown that the results obtained with the postenrichment cultures of these samples entirely agreed with the bacterial contents of the samples.

The different samples we tested represented different test requirements. Hence, they afford an opportunity to test various modifications of the standard enrichment culture in order to substantially shorten the time required for enrichment-immunoassay. We considered that the simulated-food samples probably represented the most demanding test requirement, because they contained a relatively low number of salmonellae and a large excess of a mixture of other bacteria. The modified enrichment cultures used for these samples were subjected to a nonselective preenrichment step to resuscitate the pathogens contained in the samples. This was followed by a selective enrichment step to selectively expand the number of *Salmonella* organisms and a postenrichment step to increase the final number of *Salmonella* organisms present. As summarized in Table 7, the entire process is completed within 30 h, and the results showed a complete concordance with those of the conventional bacteriological method used.

TABLE 7. Schedule of *Salmonella* detection by enrichment-immunoassay and conventional bacteriological method

Enrichment-immunoassay				Conventional method	
Process	Time required (h)			Process	All samples
	Infant formula	Eggs	Simulated food/pork		
Preenrichment	14–16	6	6	Preenrichment	24
Enrichment	0	0	14–16	Enrichment	24
Postenrichment	0	14–16	4	Plating	24
T6 capture ELISA	3	3	3	Identification	24
Total time (h)	17–19	23–25	27–29		96

Similar to the simulated-food sample, the pork sample obtained from wet markets also had a large and mixed bacterial content. Therefore, the same modified enrichment culture method was applied for the examination of these samples. On the other hand, the eggs and the formula milk samples represent a less demanding test requirement, because the level of contamination is lower and their bacterial contents were less mixed. Hence, it was considered possible to detect *Salmonella* organisms in these samples without having to subject them to selective enrichment. This was confirmed by the results of our study using the shortened nonselective enrichment procedure, which further reduces the test times to 19 and 25 h.

The antigens captured by the immobilized T6 antibodies are free or cell-bound LPS molecules, which may be expected to additionally present O factor located on the branch polysaccharides. Taking advantage of this, we showed that the scope of this assay can be extended to serogroup differentiation of *Salmonella* strains by using conjugates of O-factor-specific MAbs and also to further confirm the presence of salmonellae detected by the T6 conjugate. We showed that 136 strains (88%) of *Salmonella* serovars and the *Salmonella* spp. present in the stimulated-food and the egg samples were correctly differentiated according to their serogroups, while the false-positive result obtained with one milk sample when the T6 conjugate was used could not be confirmed by using the O-factor-specific MAb conjugates. The O-factor-specific MAbs also correctly differentiated salmonellae present in five pork samples which yielded single isolates of *Salmonella* serovars, but discrepancies were seen with the remaining 14 pork samples. Seven of these samples each yielded two or three *Salmonella* serovars, but only one serovar of each sample was correctly identified with the O-factor-specific MAb conjugates. In such instances, it is possible that only one of the serovars present was selectively enriched. The other seven samples were not reactive with the panel of O-factor-specific MAb conjugates, although salmonellae were detected by the T6 conjugate. In these instances, it is possible that the free or cell-bound LPS molecules captured by the immobilized T6 antibodies may not contain sufficient polysaccharide side chains for them to be detected also by the O-factor-specific MAb conjugates. When these isolates have been subcultured, all of them can be correctly differentiated by conjugates of the same panel of O-factor-specific MAbs (unpublished observations). On the basis of these findings, we concluded that for samples which are contaminated with more than one *Salmonella* sp., it is neces-

TABLE 8. Detection rate of the T6 capture ELISA for food samples

Sample type	No. of results			
	True positive	False positive	True negative	False negative
FMEQAS	6	0	5	0
Pork	19	0	1	0
Eggs	1	0	54	0
Formula milk	0	1	40	0
Total[a]	26	1	100	0

[a] Overall specificity and sensitivity for food samples are 99 and 100%, respectively.

sary to first isolate the individual serovars for reliable serogroup differentiation.

In sum, we showed that the T6 capture ELISA is comparable to other similar immunoassays (3, 8, 9) in terms of its detection limit for *Salmonella* spp., but this assay has the advantages that it is highly specific for *Salmonella* spp. and that it is capable of detecting an estimated 98% of all salmonellae known to naturally affect humans, even in the presence of a large excess of other bacteria. We further showed that this assay can be used in combination with appropriate enrichment culture methods to effect rapid and reliable detection of these pathogens in a variety of food samples representing different testing requirements. Samples which contain a high bacterial load, such as the simulated-food and fresh pork samples, are first cultured for 6 h in a nonselective medium, then further cultured for 12 to 16 h in an appropriate selective medium, and finally subjected to 4 h of postenrichment in a nonselective medium. This allows *Salmonella* spp. to be detected in 27 to 29 h (Table 7). We showed that the selective enrichment and the postenrichment steps can be omitted for samples such as eggs and milk, which contain a low bacterial load. Instead, these samples were cultured for 20 and 16 h, respectively, in nonselective culture media and directly tested for *Salmonella* spp. by the T6 capture ELISA. This further reduced the test time to between 17 and 25 h (Table 7). Compared with results obtained concurrently by a conventional method, which requires 96 h, we showed that all 26 samples which had been contaminated with *Salmonella* spp. gave positive results by this combined enrichment culture-immunoassay method, while all but 1 of 101 samples which were not contaminated by *Salmonella* spp. gave negative results by this method (Table 8). It is especially significant that all the samples which gave a negative result for the T6 conjugate were indeed not contaminated by *Salmonella* spp. The high level of negative prediction that this approach affords is an outstanding feature which makes this method especially suited for rapid screening for *Salmonella* spp. in food samples.

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REFERENCES

1. **Andrews, W. H., V. R. Bruce, G. June, F. Satchell, and P. Sherrod.** 1992. *Salmonella*, p. 51–68. *In* FDA bacteriological analytical manual. Association of Official Analytical Chemists, Arlington, Va.
2. **Avrameas, A., and T. Ternynck.** 1971. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry **8:**1175–1179.
3. **Blackburn, C. W.** 1993. Rapid and alternative methods for the detection of Salmonellas in foods. J. Appl. Bacteriol. **75:**199–214.
4. **Choi, D., R. S. W. Tsang, and M. H. Ng.** 1992. Sandwich capture ELISA by a murine monoclonal antibody against a genus-specific LPS epitope for the detection of different common serotypes of *Salmonellas*. J. Appl. Bacteriol. **72:**134–138.
5. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
6. **Kelly, M. T., D. J. Brenner, and J. J. Farmer III.** 1985. *Enterobacteriaceae*, p. 263–277. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
7. **Kohler, G., and C. Milstein.** 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature (London) **256:**495–497.
8. **Lee, H. A., G. M. Wyatt, S. Bramham, and M. R. Morgan.** 1989. Rapid enzyme-linked immunosorbent assay for the detection of *Salmonella enteritidis* in eggs. Food Agric. Immunol. **1:**89–98.
9. **Lee, H. A., G. M. Wyatt, S. Bramham, and M. R. Morgan.** 1990. Enzyme-linked immunosorbent assay for *Salmonella typhimurium* in food: feasibility of 1-day *Salmonella* detection. Appl. Environ. Microbiol. **56:**1541–1546.
10. **LeMinor, L., and M. Y. Popoff.** 1987. Antigenic formulas of the Salmonella serovars. Institut Pasteur, Paris.
11. **Lindberg, A. A., and L. LeMinor.** 1984. Serology of *Salmonella*. Methods Microbiol. **15:**1–144.
12. **Lu, G. Z., R. S. W. Tsang, P. Y. Chau, D. Choi, D. Law, and M. H. Ng.** 1991. Characterization and application of murine monoclonal antibodies that react specifically with the serogroup D_1 *Salmonella*. FEMS Microbiol. Lett. **80:** 135–140.
13. **Luk, J. M. C., K. H. Chan, R. S. W. Tsang, and M. H. Ng.** 1988. Characterization and application of a murine monoclonal antibody specific for the serogroup C_2 *Salmonella*. J. Med. Microbiol. **25:**115–119.
14. **Luk, M. C., R. S. W. Tsang, and M. H. Ng.** 1987. Murine monoclonal antibodies specific for lipopolysaccharide of *Salmonella* serogroup A. J. Clin. Microbiol. **25:**1165–1168.
15. **Nielsen, K. H., R. S. W. Tsang, M. M. Garcia, O. Surujballi, and M. Nemec.** 1993. Competitive enzyme immunoassay for the detection of *Salmonella* lipopolysaccharide. J. Rapid Methods Automation Microbiol. **1:**305–314.
16. **Tsang, R. S. W., K. H. Chan, P. Y. Chau, K. C. Wan, M. H. Ng, and S. Schlecht.** 1987. A murine monoclonal antibody specific for the outer core oligosaccharide of *Salmonella* lipopolysaccharide. Infect. Immun. **55:**211–216.
17. **Tsang, R. S. W., K. H. Chan, N. W. H. Lau, D. K. W. Choi, D. K. S. Law, and M. H. Ng.** 1991. Characterization of murine monoclonal antibodies against serogroup B salmonellae and application of serotyping reagents. J. Clin. Microbiol. **29:**1899–1903.
18. **Tsang, R. S. W., K. H. Chan, N. W. H. Lau, and M. H. Ng.** 1990. Production and characterization of murine monoclonal antibodies specific for serogroups E_1 and E_4 *Salmonella*. Diagn. Microbiol. Infect. Dis. **13:**453–460.
19. **Tsang, R. S. W., K. H. Nielsen, S. Balsevicius, L. Kelly, R. Khakhria, and W. M. Johnson.** 1995. Competitive enzyme immunoassay for the rapid identification of *Salmonella*. J. Rapid Methods Automation Microbiol. **3:**203–214.
20. **Tsang, R. S. W., K. Nielsen, M. D. Henning, S. Schlecht, and S. Aleksic.** 1991. A murine monoclonal antibody that recognizes a genus-specific epitope in the *Salmonella* lipopolysaccharide outer core. Zentralbl. Bakteriol. **274:**446–455.
21. **Wong, S. S. Y., K. Y. Yuen, W. C. Yam, T. Y. Lee, and P. Y. Chau.** 1994. Changing epidemiology of human salmonellosis in Hong Kong, 1982–93. Epidemiol. Infect. **113:**425–434.