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Further Studies on the Feasibility of One-Day *Salmonella* Detection by Enzyme-Linked Immunosorbent Assay

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A model system previously developed for the rapid detection of *Salmonella typhimurium* in foods was improved and extended to many other *Salmonella* serotypes. The original protocol, which consisted of an overnight nonselective culture followed by a specific enzyme-linked immunosorbent assay (ELISA), was modified and improved. A sandwich ELISA which used polyclonal antibodies for the capture stage and a cocktail of monoclonal antibodies for the detector stage was developed. The assay recognized a wide range of *Salmonella* serotypes; *S. enteritidis*, the most important serotype in the United Kingdom had a detection limit in the ELISA of about 4×10^2 cells ml^{-1}. The cultural stage prior to the ELISA was either a single nonselective broth (incubated for 28 h) or a preenrichment broth (incubated for 7 h) plus a selective broth (incubated for 21 h). Antibodies which bind to cells grown in the unfavorable conditions of a selective medium were selected. It was concluded that, in the future, the shortened protocols for the detection of *Salmonella* spp. in foods described here will be of considerable value.

The occurrence of food poisoning caused by *Salmonella* spp. continues at an unacceptably high level in many Western countries. For example, in the United Kingdom in 1991 there were in excess of 20,000 reported cases (12). Although various rapid methods have been described, detection of the pathogen is still mostly by traditional microbiological techniques, which take several days for completion.

In a previous report (7) we introduced the idea of detection of *S. typhimurium* in foods within 24 h by using a nonselective culture combined with a specific enzyme-linked immunosorbent assay (ELISA). This was a model system intended to lay the basis for detection of a much wider spectrum of *Salmonella* serotypes.

Several ELISAs are available commercially (e.g., from Organon-Teknika, Cambridge, United Kingdom; Tecra, Bio-Merieux Ltd., Basingstoke, United Kingdom; and Rhone-Poulenc Diagnostics, Glasgow, United Kingdom), but each of these still uses multistage culture of the food sample and, indeed, uses a postselective enrichment broth as an additional step. Such protocols add to the complexity and length of the procedure, although savings on conventional procedures are still possible.

Here we report the results of extending our single-culture system for *S. typhimurium* to the detection of other *Salmonella* serotypes by use of a mixed-antibody ELISA. Modifications to the original protocol (7) were made as the work progressed in order to increase the sensitivity and reliability of the system, but keeping in mind the practicalities of the system for routine use. The assay was able to detect *S. enteritidis*, currently the most important serotype in the United Kingdom.

MATERIALS AND METHODS

Bacteria. The nomenclature of *Salmonella* strains is likely in the future to take the form, of, for example, *S. enterica* subsp. *enterica* serotype Typhimurium (11). Here we will continue with the older form, e.g., *S. typhimurium*, for ease of use.

A large number of bacterial strains were used in the present study; they were mostly from the National Collection of Type Cultures, London, United Kingdom, or were received as gifts from other workers. When necessary, the exact source is specified in the text. As required, particular phases of *Salmonella* H-antigen expression were selected as described previously (7).

Cell suspensions used for ELISA standards were prepared by centrifugation of cultures grown in either a chemically defined medium (SCDM [4, 7]) or Oxoid heart infusion broth (Unipath Ltd., Basingstoke, United Kingdom). The cells were resuspended in saline (NaCl, 8.5 g $liter^{-1}$) and adjusted either for optical density, by using a known relationship between the A_{650} and the cell number, or for protein concentration, as determined by the method of Lowry et al. (8). All suspensions were heat killed in a water bath (72°C, 15 min) before use in the ELISA.

Preparation of immunogens. Flagellar protein (flagellin) was extracted from dense suspensions of *Salmonella* serotypes that were grown in SCDM by the method of Ibrahim (4). The purity of the preparations was assessed by polyacrylamide gel electrophoresis.

Immunization. Polyclonal antibodies (PAbs) were produced in rabbits and monoclonal antibodies (MAbs) were produced in mice, both as described previously (7). For immunization of rabbits, initial injections were with flagellin (100 μg) in Freund's complete adjuvant; boosters with incomplete adjuvant were then given at appropriate intervals. For mice, 50 μg of protein in adjuvant was used.

ELISA protocols. (i) Screening for antibody production. Screening for antibody production (both polyclonal and monoclonal) was carried out on bacteria-coated microtitration plates. The plate-coating method and antibody-binding assay were performed as described previously (7).

(ii) Identification and quantification of responses. For identification and quantification of the responses of *Salmonella* serotypes and potential cross-reacting bacteria, both in standard curves and from food samples, an excess-reagent

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TABLE 1. Mixtures of PAbs (capture) used in the ELISAs

Immunogen used to raise PAb	Presence of PAb in the following antibody mixture:		
	A	B	C
S. hadar phase 1 flagellin	+	+	
S. virchow phase 1 flagellin	+	+	
S. agona flagellin	+		
S. typhimurium phase 1 flagellin			+
KPL CSA-1[a]			+

[a] Kirkegaard and Perry Ltd., Dynatech Laboratories, Billinghurst, United Kingdom.

sandwich ELISA was devised. Three mixtures of PAb preparations (Table 1) were used at different times for the capture stage. Microtitration plates were coated with the chosen PAb mixture, which was prepared as described previously (7). Samples (200 μl) of cell suspensions or culture fluid, diluted as appropriate in phosphate-buffered saline containing Tween (PBST [14]), were incubated in the plates at 37°C for various times (1 to 2 h). After washing five times with PBST in an automated plate washer, detector antibodies (200 μl; a mixture of MAbs) were added. The MAbs and their specificities were as follows: MAb IFRN 0107, *S. enteritidis* and five other *Salmonella* serotypes; MAb IFRN 0111, a broad spectrum of *Salmonella* serotypes; MAb IFRN 0402, *S. typhimurium* plus *S. agona* (minor specificity only); and MAb IFRN 0960, *S. montevideo* plus three other *Salmonella* serotypes. The MAb cocktail was prepared by diluting the hybridoma culture supernatant in PBST to give a final dilution of 1/5 for each MAb. Incubation was again done at 37°C for 1 to 2 h. After further washing, binding of the MAbs to the captured bacteria was quantified by the addition of anti-mouse immunoglobulin G horseradish peroxidase-labelled antibody (diluted 1/2,000 in PBST; Sigma Chemical Co.) that was incubated for 30 min at 37°C. The presence of enzyme-labelled antibody was revealed with a substrate that was based on tetramethylbenzidine (200 μl; Cambridge Veterinary Sciences, Cambridge, United Kingdom). After color development the reaction was stopped by the addition of sulfuric acid (2 M; 50 μl) and the A_{450} was read with a dedicated plate reader. All assays were performed at least in duplicate.

Development of cultural stage. (i) Single-culture system. In the protocol for the single-culture system, samples (foods, food slurry, etc.) were incubated in SCDM at a food:medium ratio of 1:9 (wt/vol); either 25 g of sample in 225 ml of medium or 10 g of sample plus 90 ml of medium was used (see below). The medium was incubated for 16 to 18 h at 37°C. After incubation, culture fluid was streaked onto XLD agar (Oxoid; Unipath Ltd.) for the isolation of *Salmonella* spp. and was heat treated as mentioned above for use in the sandwich ELISA. Presumptive *Salmonella* colonies on XLD agar were purified and confirmed by biochemical testing by using the API 20E system (BioMerieux Ltd.).

(ii) Application of single-culture system in comparative trials. Two trials to compare the single-culture ELISA system with conventional isolation methods were carried out by the methodology described previously (6). The trials examined artificially inoculated poultry feed (10-g samples [6]) and naturally contaminated chicken portions (25-g samples) obtained from a poultry processing plant.

(iii) Use of *Salmonella* reference capsules. Reference capsules (1) containing *S. typhimurium* physiologically stressed by spray drying in milk were used to assess the performance of the methods. The capsules each contained approximately five *S. typhimurium* cells and were a gift from P. H. in't Veld, Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands. Capsules were added to 100 ml of SCDM and were incubated at 37°C for up to 48 h, with samples taken for viable counts and ELISA at intervals.

(iv) Selective enrichment culture system. Adaptation of the standard cultural methods for isolation of *Salmonella* spp. (5) was investigated. Appropriate dilutions of suspensions of *S. enteritidis* and *S. typhimurium* were inoculated into pre-enrichment broth to give a series of inocula in the range 2.0 ml^{-1} to 2×10^4 ml^{-1}. Pre-enrichment was in buffered peptone water for various periods of time for up to 18 h at 37°C. After pre-enrichment, selective enrichment was in Oxoid Rappaport-Vasilliadis broth (RV broth; 9.9 ml; transfer volume, 0.1 ml; Unipath Ltd.) and Oxoid Muller-Kaufmann tetrathionate broth (MK broth; 9.0 ml; transfer volume, 1.0 ml; Unipath Ltd.). The selective broths were incubated at 42°C (RV broth) or 37°C (MK broth) for various periods of time for up to 24 h before streaking onto XLD agar for isolation of *Salmonella* strains and treating with heat for ELISA as described above.

In one experiment, selective enrichment was followed by postenrichment in SCDM (9 ml; 1.0-ml transfer volume), and incubation was carried out for up to 6 h at 37°C. The samples were then streaked onto XLD agar and treated with heat for ELISA as described above.

(v) Detection of *Salmonella* in chicken samples using modified systems. In two experiments, the natural microbial flora from a fresh oven-ready chicken was extracted by macerating the skin (250 g) with sterile saline (NaCl, 8.5 g $liter^{-1}$; 300 ml) in a Stomacher 400 (Seward Medical, London). After coarse filtration, this suspension was heat stressed as described below and was used as a diluent to prepare a series of inocula of unheated or heat-stressed *Salmonella* cells. The inocula (1 ml) were then added to SCDM (9 ml) for extended incubation of a single culture (28 h at 37°C) or as pre-enrichment (7 h at 37°C) for a subsequent selective enrichment culture in RV broth (21 h at 42°C). After incubation, SCDM and RV broths were heat treated for ELISA as described above. The viable counts of the *Salmonella* inocula were determined from a parallel dilution series plated onto XLD agar. The total viable counts of the flora from chicken samples were determined by plating suitable dilutions onto Oxoid heart infusion agar (Unipath Ltd.). The use of unstressed inocula of *Salmonella* isolates in a stressed flora from chickens was intended to approximate cross-contamination to heated food.

Stressed bacteria. *Salmonella* cultures were grown in Oxoid heart infusion broth at 37°C overnight, and the A_{650} was adjusted to give 10^7 cells ml^{-1}. The cell suspension was heated at 52°C for 60 min (10), and the degree of damage was assessed by comparing a most probable number (MPN) count in SCDM with an MPN in RV broth. These counts were determined in microtitration plates by using 100-μl inocula from a suitable dilution series (eight replicates per dilution) with 200-μl volumes of broth.

RESULTS

Antibody production. The antibodies identified in the present study varied in their specificities, depending on the immunogen used and, in the case of MAbs, on the selection procedure. Because three different mixtures of antibodies were used for the capture stage in the ELISAs described

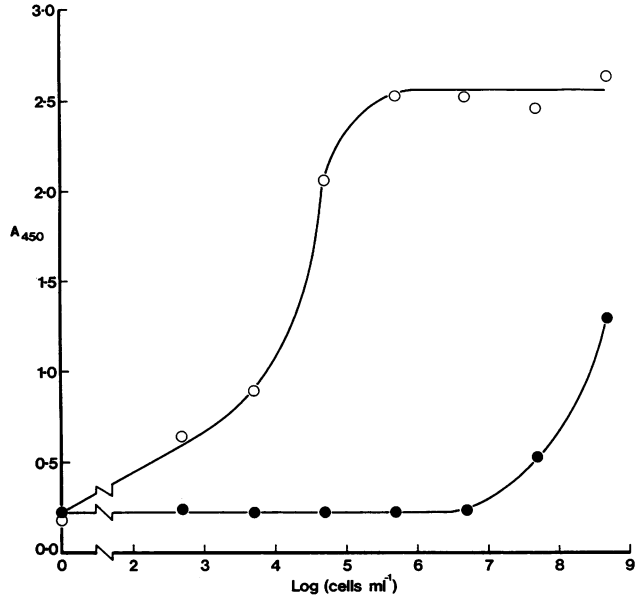

FIG. 1. Standard curves for *S. enteritidis* (grown in Oxoid heart infusion broth) in ELISA by using PAb mixture A (●) or PAb mixture C (○) as capture antibodies. In both cases, detection was with the MAb cocktail and anti-mouse immunoglobulin G horseradish peroxidase-labelled antibody.

here, assay cross-reactivities are given for the mixture under study.

ELISA. A broad-specificity ELISA was produced by using PAb mixture A with the MAb cocktail. The ELISA was cross-reactive with the following pure cultures: *S. aberdeen*, *S. agona*, *S. anatum*, *S. bareilly*, *S. bergen*, *S. berta*, *S. bovis-morbificans*, *S. bracknell*, *S. braenderup*, *S. bredeney*, *S. dublin*, *S. enteritidis*, *S. give*, *S. goerlitz*, *S. greenside*, *S. hadar*, *S. heidelberg*, *S. huttingfoss*, *S. ibadan*, *S. indiana*, *S. infantis*, *S. kampemba*, *S. kedougou*, *S. livingstone*, *S. manchester*, *S. meunchen*, *S. meunster*, *S. montevideo*, *S. moscow*, *S. newington*, *S. newport*, *S. orion*, *S. panama*, *S. poona*, *S. ribandaka*, *S. stanley*, *S. st paul*, *S. thompson*, *S. typhimurium*, *S. virchow*, and *S. weltevreden*. However, a more sensitive ELISA for the major serotype, *S. enteritidis*, was produced by using PAb mixture C as the capture antibody with the same detector antibodies. Comparative standard curves for *S. enteritidis* with these two ELISAs are shown in Fig. 1. PAb mixture C gave a very sensitive ELISA result (Fig. 1), with a detection limit of about 4×10^2 cells ml^{-1}.

The following non-*Salmonella* species were not detected in the ELISAs (several strains of each): *Escherichia coli*, *Enterobacter agglomerans*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Citrobacter koseri*, *Citrobacter freundii* (three strains, including the type strain NCTC 9750), *Citrobacter ballerupensis*, and *Listeria monocytogenes*. However, two further strains of *C. freundii* (NCTC 6271 and NCTC 6272) were detected, particularly when PAb mixture C was used as the capture antibody.

Development of cultural stage. (i) Application of single-culture system in comparative trails. The single-culture system with an ELISA with PAb mixture A was used in two collaborative trials. In a study whose results were partially reported elsewhere (6, 15), poultry feed was artificially contaminated at several levels with an array of *Salmonella*

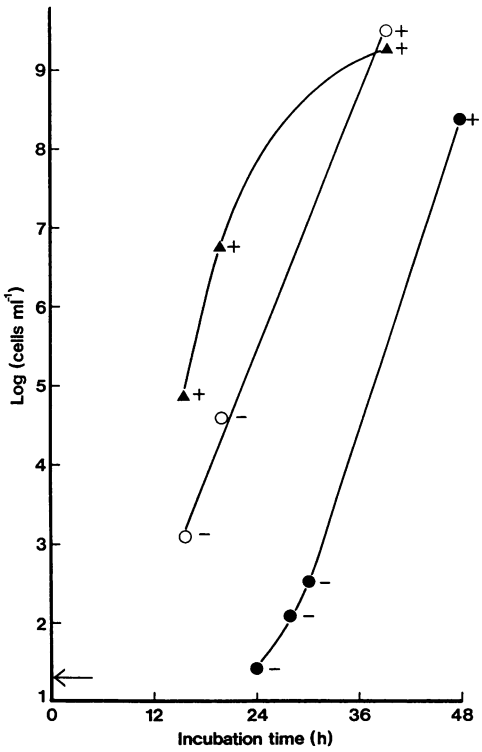

FIG. 2. Growth curves in SCDM broth for *S. typhimurium* inoculated as reference capsules. For each time point, viable count and an ELISA result are shown. ●, ○, and ▲, viable counts for individual capsules; + and −, ELISA result at the indicated time point. The inoculum was a nominal 5×10^{-2} cells ml^{-1} (one capsule in 100 ml of medium). The arrow indicates the limit of detection of viable count.

serotypes and was successfully analyzed (15). However, further results from that study indicated that the protocol used, although rapid, was not always sufficiently sensitive. Fifty samples were artificially contaminated with 0.3 to 43 CFU g^{-1} by the method of Hinton (3) using a total of 16 serotypes. Forty-eight samples were found to be positive by culture (standard cultural method), but only 22 samples were found to be positive by ELISA (ELISA with PAb mixture A as the capture antibody). Another study, with the same protocol, on naturally contaminated chicken portions confirmed this finding. Although the false-positive rate (compared with detection by the standard cultural method) was very low, the false-negative rate was unacceptable (data not shown).

(ii) Use of *Salmonella* reference capsules. A possible reason for the lack of sensitivity described above was indicated by the experiment with the *Salmonella* reference capsules, which showed that, in some cases, the lag times for the recovery of stressed bacteria can be longer than the 4 to 6 h, which has been suggested previously (13). Figure 2 shows that while for two capsules the lag time before the exponential phase of growth was probably on the order of 6 to 8 h, for a third capsule a lag time of nearer 16 h was indicated. In the latter case, detection by ELISA (PAb mixture A) was considerably delayed. Further experiments with the reference capsules (data not shown) confirmed that modification of the cultural stage was required.

(iii) Use of a selective enrichment stage. An ELISA with

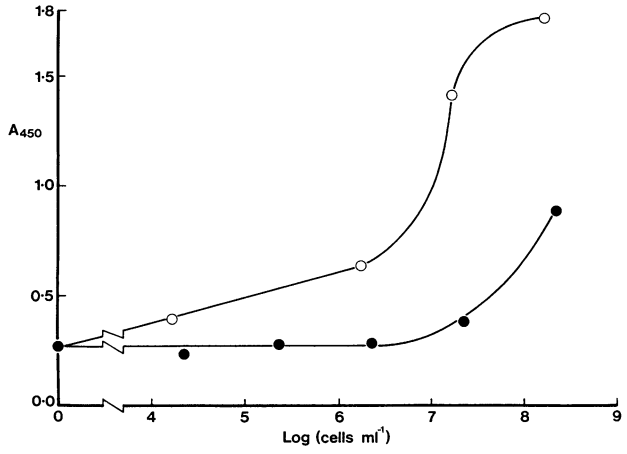

FIG. 3. Standard curves for cells of *S. typhimurium* grown in SCDM (○) or RV broth (●). Capture antibodies were PAb mixture B; detection was as described in the legend to Fig. 1.

PAb mixture A was carried out on the RV and MK culture fluids. It was found that even with the longest incubation times (18 h of pre-enrichment and 24 h of selective enrichment), some samples were still negative by this ELISA, even though they were positive by plating onto XLD agar. Although the introduction of a postenrichment stage greatly improved the ELISA signal (data not shown), this added to the complexity and length of the procedure.

To investigate the reason for the poor ELISA signal in the selective broths, cells grown either in RV broth or in SCDM were compared in the ELISA (PAb mixture B). Figure 3 shows that surface antigen expression in *S. typhimurium* (as detected by the antibodies used in the present study) is very much poorer in RV broth than in SCDM. However, further work indicated that an ELISA with PAb mixture C as the capture antibody would show binding with *S. enteritidis* and *S. typhimurium* grown in RV broth. A comparison of the ELISA results for *S. enteritidis* (heat stressed or unstressed) grown in pure culture in SCDM (24 h at 37°C) or RV broth (19 h at 42°C) obtained by using two different capture antibody formulations is given in Table 2. Culture in RV broth was preceded by pre-enrichment in SCDM for 5 h, but that amount of time was insufficient for recovery of heat-stressed cells prior to culture in selective medium. It is interesting that even for unstressed cells, detection (by plating or ELISA) was poorer by the use of selective enrichment than by use of our single-culture system. No RV cultures were positive by ELISA with PAb mixture B, but three of nine cultures were positive with PAb mixture C; the same three of nine cultures were positive by plating onto XLD agar. Incubation of SCDM for a further 4 h gave one more positive ELISA result with PAb mixture C.

(iv) Detection of *Salmonella* in chicken samples using modified systems. Two experiments in which incubation times were modified were carried out. *Salmonella* cells that were artificially inoculated into chicken skin flora were assessed by using a single culture in SCDM (28 h at 37°C) or selective culture in RV (7 h of pre-enrichment in SCDM at 37°C plus 21 h of selective enrichment at 42°C) and then ELISA with PAb mixture C. The results of these two experiments were complicated by the presence of natural *Salmonella* contamination in the chicken carcasses that we used. In the first experiment (Table 3), the single-culture SCDM and ELISA system with extended incubation worked well for many samples. However, when the level of the added inoculum of *Salmonella* was very low (and was probably zero in several instances), then the RV culture and ELISA system gave more positive results, presumably because the natural contamination in the chicken was detected. In an attempt to avoid this problem in the second experiment, four portions of a chicken extract were enriched for *Salmonella* spp. in two selective media prior to setting up the experiment and they were found to be negative. This extract was then used for the experiment. However, two uninoculated control samples were run in the experiment itself (Table 4), and one was positive for *Salmonella* spp. Many samples in the experiment were negative by all tests, indicating that only a very low level of natural contamination by *Salmonella* spp. was present and that interference because of this low level of contamination was probably of little significance. In the experiment (Table 4), the 28-h single-culture SCDM and ELISA system was much superior (20 of 32 samples were

TABLE 2. Detection of *S. enteritidis* in pure culture by ELISA from selective or nonselective broth[a]

Cell type and inoculum (CFU ml^{-1})	SCDM culture			RV broth culture		
	Detection on XLD agar	ELISA (PAb mixture B) (A_{450})	ELISA (PAb mixture C) (A_{450})	Detection on XLD agar	ELISA (PAb mixture B) (A_{450})	ELISA (PAb mixture C) (A_{450})
Unstressed cells						
170	+	2.15 (+)[b]	2.39 (+)	+	0.23 (−)	2.06 (+)
17	+	1.87 (+)	2.41 (+)	+	0.18 (−)	1.68 (+)
1.7	+	1.89 (+)	2.35 (+)	−	0.14 (−)	0.19 (−)
0.17	+	2.10 (+)	2.32 (+)	−	0.14 (−)	0.19 (−)
Heat-stressed cells[c]						
39	+	1.64 (+)	2.35 (+)	−	0.13 (−)	0.23 (−)
3.9	+	0.56 (+)	2.30 (+)	−	0.18 (−)	0.17 (−)
0.39	−	0.18 (−)	0.19 (−)	−	0.14 (−)	0.16 (−)
0.039	−	0.14 (−)	0.24 (−)	−	0.14 (−)	0.16 (−)
Medium blank		0.14	0.20		0.16	0.18

[a] SCDM was incubated for 24 h at 37°C; RV broth was incubated for 19 h at 42°C after pre-enrichment for 5 h at 37°C.
[b] Results in parentheses are based on an absorbance that was twice the absorbance of the medium blank.
[c] Viable count after heating (88.5% of survivors were sublethally damaged; see text).

TABLE 3. Detection of four *Salmonella* serotypes in a heat-stressed mixed flora derived from chicken skin

Cell type, serotype, and inoculum (CFU ml^{-1})[a]	SCDM culture[b]		RV broth culture[b]	
	Detection of XLD agar	ELISA A_{450}	Detection on XLD agar	ELISA A_{450}
Unstressed *Salmonella* cells				
S. enteritidis				
170	−	0.51 (+)[c]	+	1.53 (+)
17	+	0.76 (+)	+	2.39 (+)
1.7	+	0.35 (+)	+	2.21 (+)
0.17	+	0.62 (+)	+	2.28 (+)
S. typhimurium				
86	+	2.36 (+)	+	2.36 (+)
8.6	+	2.34 (+)	+	2.40 (+)
0.86	+	2.10 (+)	+	2.42 (+)
0.086	+	0.60 (+)	+	2.21 (+)
S. newport				
67	+	0.71 (+)	+	0.56 (+)
6.7	+	0.51 (+)	+	1.85 (+)
0.67	+	0.33 (+)	+	2.31 (+)
0.067	+	1.82 (+)	+	2.24 (+)
S. virchow				
74	+	1.44 (+)	+	2.38 (+)
7.4	+	1.29 (+)	+	2.30 (+)
0.74	+	0.85 (+)	+	2.22 (+)
0.074	−	0.15 (−)	−	0.15 (−)
Heat-stressed *Salmonella* cells[d]				
S. enteritidis				
27	+	0.72 (+)	+	2.14 (+)
2.7	−	0.20 (−)	+	2.00 (+)
0.27	+	0.21 (−)	+	2.09 (+)
0.027	−	0.21 (−)	+	2.08 (+)
S. typhimurium				
12	+	2.19 (+)	+	2.31 (+)
1.2	+	0.50 (+)	+	2.20 (+)
0.12	+	0.48 (+)	+	2.08 (+)
0.012	+	0.65 (+)	+	2.21 (+)
S. newport				
14	+	1.39 (+)	+	2.19 (+)
1.4	+	0.17 (−)	+	2.18 (+)
0.14	+	0.55 (+)	+	2.15 (+)
0.014	+	1.06 (+)	+	2.27 (+)
S. virchow				
1.0	+	0.84 (+)	+	2.20 (+)
0.1	+	0.23 (−)	+	2.20 (+)
0.01	+	0.17 (−)	−	0.15 (−)
0.001	+	0.35 (+)	+	2.10 (+)
No added *Salmonella* cells	+	0.49 (+)	+	2.24 (+)
Medium blank		0.12		0.16

[a] CFU in chicken extract as determined by extrapolation from a viable count; for each sample, the total background flora was constant at 7.7×10^3 ml^{-1}, as determined after heat stress.
[b] SCDM was incubated for 28 h at 37°C; RV broth was incubated for 21 h at 42°C after pre-enrichment in SCDM for 7 h at 37°C.
[c] PAb mixture C; results in parentheses are based on an absorbance that was twice the absorbance of the medium blank.
[d] From viable counts after heating.

TABLE 4. Detection of four *Salmonella* serotypes in a heat-stressed mixed flora derived from chicken skin

Cell type, serotype, and inoculum (CFU ml^{-1})[a]	SCDM culture[b]		RV Culture[b]	
	Detection on XLD agar	ELISA A_{450}	Detection on XLD agar	ELISA A_{450}
Unstressed *Salmonella* cells				
S. enteritidis				
65	+	2.32 (+)[c]	+	2.31 (+)
6.5	+	2.43 (+)	+	2.21 (+)
0.65	+	2.38 (+)	−	0.28 (+)
0.065	−	0.15 (−)	−	0.14 (−)
S. typhimurium				
29	+	2.29 (+)	+	2.27 (+)
2.9	+	2.34 (+)	+	2.27 (+)
0.29	+	2.31 (+)	+	1.06 (+)
0.029	−	0.78 (+)	−	0.12 (−)
S. newport				
50	+	1.18 (+)	+	0.22 (−)
5.0	+	0.96 (+)	+	0.14 (−)
0.5	+	0.76 (+)	−	0.14 (−)
0.05	+	0.16 (−)	−	0.11 (−)
S. virchow				
40	+	1.89 (+)	+	0.15 (−)
4.0	+	1.59 (+)	+	0.12 (−)
0.4	+	1.55 (+)	+	0.11 (−)
0.04	+	0.25 (−)	−	0.13 (−)
Heat-stressed *Salmonella* cells[d]				
S. enteritidis				
28	+	2.31 (+)	+	2.25 (+)
2.8	−	0.87 (+)	−	0.12 (−)
0.28	−	0.12 (−)	−	0.12 (−)
0.028	−	0.15 (−)	−	0.13 (−)
S. typhimurium				
14	+	2.33 (+)	+	1.37 (+)
1.4	+	2.29 (+)	−	0.21 (−)
0.14	+	0.12 (−)	−	0.11 (−)
0.014	+	1.84 (+)	−	0.12 (−)
S. newport				
44	+	0.99 (+)	+	0.31 (+)
4.4	+	1.05 (+)	+	0.13 (−)
0.44	−	0.13 (−)	−	0.15 (−)
0.044	−	0.14 (−)	−	0.12 (−)
S. virchow				
2.5	−	0.26 (−)	−	0.12 (−)
0.25	−	0.17 (−)	−	0.13 (−)
0.025	−	0.16 (−)	−	0.12 (−)
0.0025	−	0.26 (−)	−	0.12 (−)
No added *Salmonella* cells				
Replicate 1	−	0.33 (+)	−	0.12 (−)
Replicate 2	+	1.16 (+)	+	0.12 (−)
Medium blank		0.13		0.12

[a] CFU in chicken extract as determined by extrapolation from a viable count; for each sample, the total background flora was constant at 5.9×10^3 ml^{-1}, as determined after heat stress.
[b] SCDM incubated for 28 h at 37°C; RV broth incubated for 21 h at 42°C after pre-enrichment in SCDM for 7 h at 37°C.
[c] PAb mixture C; results in parentheses are based on an absorbance that was twice the absorbance of the medium blank.
[d] From viable counts after heating.

ELISA positive; 21 of 32 were XLD agar positive) to the RV broth and ELISA system (8 of 32 samples were ELISA positive; 14 of 32 samples were XLD agar positive).

A number of the cultures in RV broth from samples (Table 4) inoculated with *S. newport* or *S. virchow* were negative by ELISA but positive by plating. When these serotypes were grown in pure culture in RV broth, the ELISA detection limit was no better than 10^8 cells ml^{-1}; it is unlikely that this level was reached in culture of the inoculated samples in RV broth. For the results shown in Table 3, this effect could have been masked by the naturally occurring *Salmonella* serotype which gave a strong ELISA response after culture of the uninoculated sample in RV broth. Further investigation is needed to ascertain whether a different antibody might allow these serotypes to be detected in RV broth.

DISCUSSION

The present work was undertaken to develop a rapid method of detecting *Salmonella* spp. in foods by using a shorter, nonselective cultural procedure prior to a specific ELISA. We reported previously (7) a model system for the detection of *S. typhimurium* and wished to extend this system to cover a large range of *Salmonella* serotypes.

The conventional cultural method (5) takes in excess of 4 days for a confirmed positive result. For an alternative system to be both worthwhile and acceptable, a real improvement in at least one of labor or equipment costs, total detection time, or reliability of detection must be achieved. Currently available commercial ELISAs use a proportion of the conventional method (pre-enrichment and selective enrichment culture) but introduce a further cultural stage, the postenrichment broth. A result is produced on the third day with little reduction in the complexity of the system. The work reported here offers an improvement in time with no additional complexity.

The use of selective culture introduces several problems into the conventional methodology. First, the compounds used in the selective media tend to be toxic to *Salmonella* cells sublethally damaged by food processing, and thus, a pre-enrichment (resuscitation) stage must be included in the detection protocol. In some cases, even growth of unstressed cells can be inhibited by these media (Table 2). Further evidence of this toxicity was seen in an experiment (data not shown) in which the growth of *S. enteritidis* from different inoculum levels in chicken extract in pre-enrichment and RV selective enrichment was monitored. After subculture from pre-enrichment to RV broth, one sample showed a constant ELISA signal when sampled hourly over an 8-h period. Initially, *Salmonella* cells could be recovered by plating them onto agar, but from 5 h onward, they could no longer be recovered, indicating death of the cells in the selective broth.

Second, the inhibitory effect varies in degree according to the serotype, and thus, it is usually recommended (5) that more than one selective broth be used, preferably one that is based on different selective principles. It would be beneficial to avoid the use of chemically or physically selective agents.

In a nonselective broth, *Salmonella* spp. must compete with the remainder of the flora and do not always grow to the levels achieved in pure culture (Table 3) (7). Thus, an ELISA needs to be sensitive, as well as specific, in order to detect the low number of *Salmonella* cells present. The ELISA (PAb mixture C) reported here is very sensitive for *S. enteritidis*, the most important serotype in the United Kingdom, with a detection limit for pure cultures approaching 10^2 cells ml^{-1} (Fig. 1). This is useful for improving *Salmonella* detection in food samples, since *S. enteritidis* does not always appear to compete well in a mixed flora (SCDM culture, ELISA absorbances; Table 3). We previously reported (7) detection of *S. typhimurium* by ELISA in the single-culture system in 19 h, even when the salmonellae were heavily outnumbered by nonsalmonellae. The present study suggests that extension of the SCDM incubation time to 28 h can, in many cases, give good results for other *Salmonella* serotypes (Table 4) when an appropriate ELISA is used. In some situations (Table 3), selective culture gives better results, but still within a total incubation time of 28 h. It may be necessary to adopt different protocols, depending on the sample type, but the exact basis for this choice remains to be determined.

It should also be noted that antigen expression in at least some *Salmonella* serotypes is affected by selective culture media (Fig. 3), and consequently, suitable antibodies must be used in immunological techniques when these media are used. Furthermore, in some instances, lag times for recovery from injury can be long (Fig. 2). However, recovery from injury is a complex process, and the time for complete repair and thus growth of cells can be longer than the time needed to regain resistance to selective media (9). Shortened pre-enrichment times have been assessed previously (2) by subsequent detection on agar plate medium but were thought to be of limited use. Overgrowth by non-*Salmonella* colonies can make it very difficult to observe presumptive *Salmonella* isolates on these plates; however, ELISA does not suffer from this disadvantage, because it is capable of producing a signal from a target even when the matrix is very unfavorable.

The panel of MAbs used in the present work bound to a wide range of *Salmonella* serotypes (see Results), and additional MAbs are under evaluation. It is believed that the MAbs are largely responsible for the specificity of the ELISAs described here but that sensitivity can, to a considerable extent, be affected by the capture antibody that is used (Fig. 1). The known cross-reactivities of the MAbs are limited to certain strains of *C. freundii* (NCTC 6271 and NCTC 6272). These were culture collection strains, originally isolated in Kentucky in 1941, which possess certain *Salmonella* antigens. The relevance of these strains to current food microbiology is not known. The type strain of *C. freundii* did not react in the ELISA.

When considering a protocol for *Salmonella* detection, the practicalities of the system are highly relevant, as noted earlier in this discussion. To achieve a result on the second day, a total of, for example, 33 h of available analytical time (from 0900 h on day 1 to 1800 h on day 2) is necessary. The culture times outlined in here total 28 h (either single culture or pre-enrichment plus selective enrichment). Our ELISA, using correctly chosen antibodies, can be completed in 3 h, giving a total analytical time within the period of time described above. The conclusion from the present work is that in the future the shortened protocols for *Salmonella* detection described here will offer considerable improvement over current methodologies. The protocols are being validated in interlaboratory comparative trials against the standard culture method by using retail food samples. It is hoped that this information will enable the appropriateness and sensitivity of each system for routine analysis to be determined.

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