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An evaluation of a commercially available enzyme immunoassay test for the rapid detection of salmonellae in food and environmental samples

BY J. P. HARFORD
Public Health Laboratory, Bridle Path, York Road, Leeds

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SUMMARY

A total of 91 food and environmental samples were examined for the presence of salmonellae using a commercially available enzyme immunoassay kit (EIA) and a conventional culture technique. A 78% agreement was obtained, but re-examination of culture-negative, EIA-positive samples gave agreement of 86%. The problem of comparing EIA and culture results is discussed. A partially selective pre-enrichment broth was tested in 37 samples and gave better EIA ratios. Artificially contaminated cooked foods gave 100% agreement.

INTRODUCTION

Standard cultural methods for the detection of salmonellae in food and environmental samples usually involve overnight pre-enrichment in non-selective broth, followed by selective enrichment for a further 2 days. For example, both the Food and Drug Administration's Bacteriological Analytical Manual Technique (Flowers, 1985) and the International Organisation for Standardisation method (Van Leusden, Van Schothorst & Beckers, 1982) require at least 4 days to obtain a negative result. This can be an unacceptable delay in the food industry and in routine food-testing laboratories.

More rapid methods involving the serological analysis of enrichment cultures have been developed. The Enrichment Serology Technique of Sperber & Diebel (1969), consisted of H-agglutination tests being carried out directly on enrichment broths. Results were available within 50 h. While they obtained excellent agreement with traditional culture Mohr, Trenk & Yeterian (1974) found 13–14% false–positive reactions. Immunofluorescence has been widely tested, but again false–positive rates of around 7% have been reported (Mattingly *et al.* 1985) and the technique has not gained wide acceptance.

The advent of enzyme-linked immunoassay (ELISA) technology prompted Krysinski & Heimsch (1977) to investigate the macroscopic visualization of salmonellae on cellulose acetate membranes. Using specially prepared specific anti-*Salmonella typhimurium* flagella antiserum, promising results were obtained. However, cross-reactivity was found when a commercial polyvalent H-antiserum was used.

Minnich, Hartman & Heimsch (1982) employed Spicer Edwards polyvalent

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anti-H (Difco) serum purified by using staphylococcal protein A (SPA) affinity chromatography to remove the predominantly IgM cross-reacting somatic antibodies. Using an indirect enzyme immunoassay (EIA), pure-culture studies indicated good specificity. The sensitivity was such that 10^6 organisms/ml culture fluid were required to elicit a strong response. In this study of 98 naturally contaminated foodstuffs, all culturally confirmed positives were detected by EIA after overnight incubation in buffered peptone water (BPW). An additional seven specimens were positive by EIA but not by culture. Although these might have been considered false positives, the authors presented evidence to indicate that they were true results and signified greater sensitivity of the EIA.

A further development was the introduction of a monoclonal antibody (MOPC 467) which was specific for a flagellar determinant found on many salmonella species. Robison, Pretzman & Mattingly (1983) used this antibody in a direct EIA and found that out of 100 representative salmonella serotypes 94 were reactive in the EIA, whereas none of 27 flagellated non-salmonella organisms was positive. The sensitivity of the assay was of the same order as that found by Minnich, Hartman & Heimsch (1982). Mattingly *et al.* (1985) described a modification of this assay utilizing the antibody 'capture' or 'sandwich' assay. Testing more than 350 strains of salmonella, 97% were positive with MOPC 467. A further monoclonal antibody (6H4) was produced which reacted with the remaining strains.

This present study was undertaken to evaluate an EIA kit and assess the usefulness of EIA for screening food and environmental samples for salmonellae. The kit tested was the ELISA Screening Kit for Salmonella (Common Structural Antigen) produced by Kirkegaard and Perry Laboratories, Maryland, USA. The technique is a sandwich EIA using a polyclonal antibody (CSA-1) which recognizes numerous common structural antigens widely shared by salmonellae. The antibody was affinity-purified for enhanced specificity and sensitivity. Essentially the CSA-1 antibody on the surface of a microtitre plate immobilizes salmonella antigens which are then visualized by overlaying with peroxidase-labelled antibody, followed by enzyme substrate. The assay measures the difference in absorbance between a sample taken at the time of inoculation and a second one after overnight incubation. To obtain the result the day after receipt of the sample the ELISA was usually performed on BPW cultures inoculated 16–20 h previously with pure cultures, naturally contaminated material or artificially contaminated foodstuffs. A partially selective pre-enrichment medium, consisting of BPW supplemented with 0·1% malachite green (BPW/MG), was also tested in an attempt to increase the numbers of salmonellae present relative to the competing organisms.

MATERIALS AND METHODS

The 19 serotypes of salmonellae tested were isolates from routine food and environmental samples (Table 1). Fifteen other organisms were selected as representative of the competing flora and are shown in Table 2. The competitors were obtained by plating out overnight BPW cultures on to MacConkey agar (Oxoid, CM7) and selecting the predominant organisms. All organisms were stored on

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Table 1. *Specificity of the EIA (19 serotypes of salmonella)*

Salmonella serotype	EIA ratio (absorbance of BPW cultures before and after overnight incubation)
S. anatum	2·8
S. typhimurium (PT 110)	2·7
S. dublin	4·1
S. infantis	3·2
S. typhimurium (PT 49a)	2·6
S. stanley	3·1
S. eimsbuettel	3·1
S. enteritidis	5·4
S. braenderup	3·3
S. unnamed (O-rough)	4·9
S. unnamed (O-rough)	2·3
S. unnamed (non-motile)	1·4
S. arizonae	2·7
S. arizonae	4·8
S. arizonae	3·7
S. abaetetuba	3·3
S. llandoff	2·6
S. paratyphi B	5·7
S. agona	7·0

Table 2. *Specificity of the EIA (competing organisms)*

Organism	EIA ratio (absorbance of BPW cultures before and after incubation)
Escherichia coli	1·0
Escherichia coli	1·0
Citrobacter freundii	1·1
Citrobacter freundii	1·1
Citrobacter freundii	1·1
Enterobacter intermedium	< 1·0
Enterobacter sp.	1·2
Enterobacter cloacae	< 1·0
Enterobacter cloacae	1·0
Proteus mirabilis	1·1
Proteus mirabilis	1·1
Pseudomonas aeruginosa	1·1
Flavobacterium sp.	1·0
Hafnia alvei	1·0
Aeromonas hydrophila	< 1·0

Dorset's egg agar slopes. For the experiments the organisms were lightly inoculated into 20 ml of BPW and incubated at 37 °C for 16–20 h.

Samples of inoculated BPW cultures were removed before and after incubation (IN and OUT samples) to detect any increase in salmonella antigen. To each 2 ml IN sample was added 55 μl of 37% formaldehyde and the samples were then stored at 4 °C. After incubation of the BPW cultures the pH was adjusted to between 5·0 and 7·0 with sodium hydroxide or acetic acid. A further 2 ml OUT sample was then removed and treated with formaldehyde as above. All IN and OUT samples were placed in a boiling water bath for 15 min and then cooled to room temperature before testing.

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Sensitivity .

S. anatum and *S. enteritidis* were selected for these studies, as the former had given a weak reaction and the latter a strong reaction in the pure-culture studies. Tenfold serial dilutions of overnight BPW cultures were made in BPW from 10^{-1} to 10^{-5}. The EIA was performed on each dilution in the normal manner. A viable count was also performed on each culture by dispensing appropriate dilutions on plate count agar (Oxoid, CM 325) using a Spiral Plate Machine (Don Whitley Scientific).

Naturally contaminated samples

Material was selected that regularly yielded salmonellae on culture. It consisted of chicken neck skins (13), raw liquid egg (33), chicken faeces (2) and sewer swabs (12). Additionally samples of black pepper known to contain *S. abaetetuba*, cooked pork from which *S. derby* had been grown and raw turkey contaminated with *S. newport* were examined. Other samples, hitherto untested for salmonellae, were baby milk powder, pasteurized liquid egg, raw bovine livers, cooked meat, confectionery products and other spices. Approximately 25 g of material was added to 225 ml of BPW and incubated for 16–20 h at 37 °C. Samples were prepared for EIA and also cultured for salmonellae by adding 1 ml of BPW cultures to 10 ml of Rolfe's tetrathionate broth (Rolfe, 1946). After overnight incubation at 42 °C the broth was streaked on to Brilliant Green MacConkey Agar (Harvey & Price, 1979). Plates were incubated for at least 24 h before being examined for typical colonies, which were identified by standard methods (Harvey & Price, 1974).

Comparison of BPW and BPW/MG

Thirty-seven samples were assayed in parallel, with a further 25 g sample being inoculated into BPW containing 0·1 g/l malachite green (BPW/MG) as described by Van Schothorst & Renaud (1985).

Artificially contaminated foods

The various foodstuffs shown in Table 5 were added in 25 g amounts to 225 ml BPW. An overnight broth culture of a routine isolate of *S. typhimurium* was diluted in quarter-strength Ringer's solution, and 0·1 of a suitable dilution (usually 10^{-7}) was added to BPW culture. This produced between 0·4 and 2·4 colony-forming units (c.f.u.) of salmonella per gram of food. Salmonella counts were made by spreading 0·1 ml on MacConkey agar. The cultures were incubated for 16–20 h and the EIA performed.

EIA procedure

The manufacturer's instructions were followed throughout.

Sensitization

Linbro EIA Microtitration plate wells (Flow Laboratories) were sensitized with 100 μl of CSA-1 antibody diluted 1/100 in coating solution (0·001 M dibasic sodium phosphate, 0·015 M sodium chloride) incubated at room temperature (RT) for 1 h in a moist chamber. The plate was then emptied and the residual fluid tapped out on to paper towels.

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Blocking

To each well was then added 300 μl of the diluent/block solution, the composition of which was not specified in the instruction information. After 10 min at room temperature the plate was emptied and the residue tapped out.

Addition of test samples and controls

To appropriate wells were added 100 μl of test culture IN and OUT samples in duplicate. Additionally 100 μl of diluent/block solution was added to 4 wells constituting 2 conjugate control wells and 2 substrate control wells. The plate was incubated as above, after which the well contents were again tapped out. Two manual wash cycles were then performed.

Addition of conjugate

Some 100 μl of a 1/100 dilution of peroxidase conjugated CSA-1 in diluent/block solution was added to all wells except the substrate control, to which 100 μl of diluent/block solution only was added. Incubation was as above, followed by three wash cycles with a final 5 min soak in wash fluid and thorough tapping out.

Addition of substrate

Immediately before use equal amounts of substrate solution 1 (2·2′-azinobis [3-ethylbenzthiazolinesulphonic acid]) and substrate solution 2 (hydrogen peroxide) were mixed together and 100 μl dispensed to each well. After 20 min at RT the absorbance of each well was determined at 405 nm on an EIA plate reader (model 210, Artek Systems Corporation).

Controls

In addition to the conjugate and substrate controls already described, uninoculated BPW was also processed with each run to exclude the presence of any salmonella antigen. A positive control of *S. anatum* was also included in each run to ensure adequate colour development, and to assess the reproducibility of the assay.

Interpretation

As the intensity of colour is generally proportional to the amount of antigen present in the sample, cultures where the colour in the OUT sample is more intense than the IN sample should be regarded as positive for salmonellae though very small increases in the absorbance of the OUT sample over the IN sample were not considered significant. In this study the results are expressed as the ratio of the IN and OUT absorbance. It was observed that a positive threshold of equal to or greater than 1·6 gave a reasonable discrimination between positive and negative samples.

RESULTS

Specificity, sensitivity and reproducibility

Tables 1 and 2 show the EIA ratios of the salmonellae and the competing organisms respectively. The competing organisms showed no significant cross-

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Table 3. *Comparison of the EIA and conventional culture procedures for the detection of salmonellae in naturally contaminated food and environmental samples*

Culture	EIA	No. of samples (percentage)	Interpretation
+	+	35 (38)	Total agreement between methods
−	−	36 (40)	
+	−	7 (8)	False EIA negatives
−	+	*13 (14)	Possible false EIA positives or false culture negatives
Total samples		91	

* Retesting subsamples gave eight more culture-positive results, increasing the total agreement to 86%.

reactivity with the antibodies employed. The salmonellae, with one exception, gave ratios of greater than two. Included were two O-rough strains, three *S. arizonae*, an isolate of *S. paratyphi B* from water, various common isolates and some isolates rarely encountered in this laboratory. The exception was a non-motile variant (06, 7:–:–) which gave a ratio of 1·4. This was slightly raised in comparison with EIA ratios obtained with organisms other than salmonellae.

The sensitivity of the assay was determined by diluting overnight BPW cultures of salmonellae and obtaining the EIA ratio of each dilution. *S. anatum* gave a positive reaction, i.e. a ratio equal to or greater than 1·6, at a level of $3{\cdot}0 \times 10^8$ c.f.u./ml and *S. enteritidis* at $4{\cdot}5 \times 10^5$ c.f.u./ml.

The reproducibility of the assay was such that the same strain of *S. anatum* assayed each run gave a mean value of 3·1, a standard deviation of 0·45 and a coefficient of variation of 14%.

The control, consisting of uninoculated BPW, included in each run showed no activity.

Naturally contaminated samples

Table 3 shows the results of 91 samples tested in the EIA and by conventional culture. There was 78% agreement between the two tests; 8% were false EIA negatives and 14% false EIA positives or false culture negative results. However, eight of the latter samples were shown to be positive for salmonellae when further samples were retested in BPW or in BPW/MG. Taking these results into consideration, 86% agreement was obtained. The 31 samples that did not usually yield salmonella gave 97% agreement, while the 60 heavily contaminated samples gave 68% agreement between the two methods.

Nineteen different serotypes of salmonella were recovered by the conventional culture technique.

Comparison of BPW and BPW/MG

Table 4 shows the culture and EIA results for 37 samples inoculated into both BPW and BPW/MG. The total agreement for BPW/MG was slightly superior, being 73% compared to 65%. False EIA negatives were similar for both treatments, but potential false EIA positives were lower with BPW/MG (16%) than with BPW (27%). Three more salmonellae were isolated by the BPW/MG system

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Table 4. *Comparison of the EIA and conventional culture procedures for the detection of salmonellae using samples incubated overnight in either BPW or BPW supplemented with 0·1 g/l of malachite green (BPW/MG)*

		No. of samples (percentage)		
Culture	EIA	BPW	BPW/MG	Interpretation
+	+	17 (46)	19 (51)	Total agreement between methods
−	−	7 (19)	8 (22)	
+	−	3 (8)	4 (11)	False EIA negatives
−	+	10 (27)	6 (16)	Possible false EIA positives or false culture negatives
Total samples		37	37	

Table 5. *Results of the 10 foods artificially inoculated with* S. typhimurium

Sample type	C.f.u. of salmonella added to BPW culture	EIA ratio	Culture result
Cream	11	4·8	+
Curry sauce	11	1·9	+
Cooked pie	11	6·1	+
Vanilla slice	11	1·7	+
Cooked ham	11	7·2	+
Potted meat	11	5·3	+
Milk powder	60	5·6	+
Spice 1	17	4·5	+
Spice 2	17	2·0	+
Spice 3	17	1·0	−

than with BPW. Out of 25 samples from which salmonellae were isolated from either broth, 17 (68%) of the BPW/MG cultures gave higher ratios than BPW cultures. Five samples were 2–3 times greater.

Artificially contaminated foods

Table 5 shows the results obtained using 10 foods artificially contaminated with *S. typhimurium*. Apart from a sample of spice, all gave ratios greater than the positive threshold of 1·6 and the *S. typhimurium* was recovered by conventional culture. The spice which failed was Tandoori Masala, which may have exerted an inhibitory effect on the salmonella.

DISCUSSION

The labour-intensive and time-consuming culture technique for detecting the presence of salmonellae in foods has prompted the investigation of more rapid methods. With EIA there is the potential for a rapid, sensitive and specific test that is amenable to automation (D'Aoust, 1984). However, the quality of the antibody employed is critical, to ensure that cross-reactions do not occur, and detection of most serotypes of salmonella is feasible. Minnich, Hartman & Heimsch (1982) employed purified polyclonal antibody, while others have used monoclonal antibody (Robison, Pretzman & Mattingly, 1983; Smith & Jones, 1983).

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The EIA employed in this study utilized affinity-purified polyclonal antibody against an unspecified common structural antigen. Specificity studies with pure cultures indicated that cross-reactions would be uncommon and that a good range of serotypes would be detected. The only strain of salmonella that failed to elicit a good response was a non-motile variant, perhaps indicating the antibody is raised against a flagellar component of salmonella which would be in common with the antibodies employed by other workers (Minnich, Hartman & Heimsch, 1982; Robison, Pretzman & Mattingly, 1983).

Sensitivity studies showed that for *S. enteritidis* at least 10^5 organisms/ml of BPW were required to give a strong response in the EIA, while *S. anatum* required more than 10^8 organisms/ml, thus demonstrating the differing sensitivity found among serotypes. These findings broadly agree with the sensitivity reported in other studies (Robison, Pretzman & Mattingly, 1983; Minnich, Hartman & Heimsch, 1982; Flowers, 1985). Interestingly, salmonellae bearing the G-complex H-antigens all seemed to react particularly well with the CSA-1 antibody.

Minnich, Hartman & Heimsch (1982) had shown that the use of pre-enrichment cultures was feasible, and might even be superior to the protracted method whereby selective broth is inoculated from pre-enrichment cultures. Flowers (1985), however, disagreed and presented evidence that that method may not be suitable. The EIA on all samples tested gave agreement with cultures in 71 samples (78%). In seven samples (8%) the EIA failed to detect culture-positive specimens. However, the ratios in four were 1·5, 1·5, 1·4 and 1·4, which is just below the positive threshold of 1·6, and it is likely that the numbers of salmonellae in the culture just failed to reach the required level for detection. Studies had already demonstrated that these serotypes would be detected in EIA. Thirteen (14%) were EIA-positive but culture-negative. This apparently high level of either false-positive EIA or false-negative culture may be misleading when traditional culture is used as the yardstick by which to judge the EIA. It is recognized that the isolation rate of salmonellae from heavily contaminated material is strongly influenced by factors such as the amount of the competing flora and cultural procedure used (Beckers *et al.* 1985; Van Schothorst & Renaud, 1985) and culture will inevitably fail from time to time. Consequently, some apparent EIA false positives will be due to failure to isolate salmonellae. Assuming salmonellae have reached detectable levels in the EIA they should be less affected by the number of competitors. In a number of samples where an apparent false EIA positive had occurred the predominant competitors were tested as pure cultures and no activity was found. Also, out of the 13 samples, initially EIA-positive but culture-negative, 8 subsamples subsequently grew salmonellae, giving a total agreement of 86%. Therefore the evidence for them being true positives is fairly convincing.

Out of the 31 samples not usually yielding salmonellae, agreement was found in 30 (97%). The one aberrant result was a sample of pasteurized liquid egg which was marginally positive in the EIA but was culture-negative. The other group of samples from which salmonellae were frequently isolated gave total agreement in 41 out of 60 samples (68%). By their nature the chicken neck skins, chicken faeces, sewer swabs and raw egg samples contain high numbers of competing organisms, and work by Van Schothorst & Renaud (1983), which has been supported by this laboratory (unpublished), suggests that in such samples salmonellae may not consistently reach 10^5 organisms/ml in pre-enrichment cultures. Thus in terms of

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sensitivity and specificity the EIA was rigorously tested with this group of samples.

Given that it seemed pertinent to obtain the highest possible numbers of salmonellae in BPW cultures, a trial using BPW supplemented with 0·1 g/l of malachite green was carried out. This combination had been tested by Van Schothorst & Renaud (1985) and found to enhance the possibility of isolating salmonellae from heavily contaminated material even when stressed salmonellae were present. A further three positive cultures were obtained with BPW/MG, giving a possible false-positive EIA rate of 16%, compared with a rate of 27% for BPW. The BPW/MG, therefore, does appear to perform as well as BPW. The rate of false EIA negatives was not improved by the use of BPW/MG. However, 17 out of 25 samples from which salmonellae were isolated in this trial gave higher EIA ratios, 5 being over twice as great. It seems this broth allows the salmonellae to reach higher numbers, giving stronger reactions and thus greater definition of positivity. The combined total agreement for BPW and BPW/MG was 92% suggesting that with a larger sample size better agreement would be found.

Artificially contaminated foods with low numbers of *S. typhimurium* gave 100% agreement with the culture results. The one negative EIA result which was also culture-negative was a sample of Tandoori Masala barbecue powder which was presumably inhibitory to the salmonella. Three samples did give low ratios, albeit positive, and interestingly on routine culture gave aerobic counts of around 1000 organisms/g food. All the other samples gave counts of < 200. These data provide further weight to the concept of the negative influence of competing flora on the numbers of salmonellae in pre-enrichment cultures (Beckers *et al.* 1985).

This kit is capable of detecting salmonellae in food and environmental samples. A problem exists in assessing the rate of false EIA positives, as conventional culture usually performs sub-optimally in heavily contaminated material. The evidence from naturally contaminated samples that do not usually yield salmonellae and artificially contaminated food suggests that the EIA would perform very satisfactorily in a wide range of cooked products. Using overnight BPW cultures does provide a much more rapid result, but the numbers of salmonellae may not be consistently high enough to elicit a strong response. Therefore ways to enhance the growth of salmonellae in BPW cultures should be sought, and the use of a partially selective broth requires further investigation.

Approximately 7% of food samples examined in this laboratory yield salmonellae on culture. Samples giving a positive EIA result would have to be cultured in a conventional manner if the serotype of the salmonellae was required for epidemiological purposes.

Kits used in this study cost £318.55 plus VAT. This provided 200 tests a kit at £1.45 a test. With additional labour and material costs the final EIA cost is two and a half times greater than that of our conventional culture procedure. However, the EIA result would be available the day after receipt of the sample, and savings in technical time could generate more resources to investigate potential positives with a wide range of media which would undoubtedly increase the isolation rate of salmonellae.

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