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Detection of *Salmonella* with the BioEnzabead™ Enzyme Immunoassay Technique

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ABSTRACT

The performance of the double-antibody (MOPC 467 and 6H4) BioEnzabead™ enzyme-linked immunosorbent assay was evaluated with pure cultures and naturally contaminated foods. The immunoassay detected all but four (1.6%) of the 250 *Salmonella* test strains and showed high levels of cross-reactivity (30.7%) among the 75 strains of non-salmonellae examined. *Citrobacter freundii* figured prominently as a source of erroneous results. Although the BioEnzabead™ system identified the 41 foods found to be contaminated by a standard cultural procedure, a high rate of false-positive reactions (46%) was nevertheless encountered with high moisture foods. Attempt at method brevity through application of the immunoassay at the preenrichment level was unsuccessful resulting in the detection of only 24 (58.5%) of the 41 contaminated foods. Greater recoveries were obtained with tetrathionate brilliant green enrichment broth incubated at 43°C than at 35°C.

Development of rapid methods for the detection of *Salmonella* in foods has met with limited success. Standard cultural procedures involving enrichment of food samples in a non-selective medium (preenrichment) require a minimum of four days to obtain presumptive evidence of *Salmonella* contamination. Most attempts at method brevity have focused on selective enrichment cultures as test material and have generally succeeded in reducing the analytical time by a single day (*6*). DNA probes and immunological techniques utilizing monoclonal and polyclonal antibodies are recent adjuncts to the active research field of diagnostic biotechnology and their potential impact on the effective and reliable detection of *Salmonella* in foods is most promising (*6,12,19,22*).

The original BioEnzabead™ system (formerly Litton Bionetics, Charleston, SC) detected *Salmonella* antigens in M-broth postenrichment cultures by a sandwich enzyme-linked immunosorbent technique (EIA). *Salmonella* antigens in heated extracts of M-broth cultures were reacted with polyvalent MOPC 467 myeloma protein (IgA) iron adsorbed to the surface of polycarbonate-coated ferrous metal beads held in microtiter plates. The "MOPC 467-antigen" complex (positive sample) on the metal bead was then reacted with a peroxidase conjugate of the MOPC 467 antibody. Development of a green color (OD_{410}) of threshold intensity upon addition of enzyme substrate was indicative of *Salmonella* contamination (*8,20*). Although initial studies of the MOPC 467 BioEnzabead™ system produced favorable results (*10,20*), a subsequent evaluation reported unacceptably high numbers of false-negative reactions with pure *Salmonella* cultures and lack of specificity towards non-salmonellae, notably *Citrobacter* and *Morganella* spp. (*8*). A high incidence of false-positive reactions with naturally contaminated foods was equally notable in this study. Subsequent modification of the BioEnzabead™ system to carry both the MOPC 467 myeloma protein and a 6H4 monoclonal antibody (IgG) on individual metal beads (Organon Teknika, Charleston, SC) led to the present evaluation of the two-antibody system with pure cultures and naturally-contaminated foods.

MATERIALS AND METHODS

Test cultures

A total of 222 *Salmonella* cultures previously tested with the MOPC 467 BioEnzabead™ system (*8*) were included in this study with particular emphasis on strains that had formerly given false-negative reactions. An additional 28 *Salmonella* cultures not previously subjected to BioEnzabead™ testing were also included; 19 of the 28 strains consisted of nonmotile *S. pullorum* and *S. gallinarum*. A total of 250 *Salmonella* strains representing 28 somatic groups and 75 non-salmonellae strains (12 genera, 20 species) were therefore examined in this study.

Cultural detection of Salmonella in foods

In the standard cultural assay, food samples (100 g) were preenriched for 16 to 18 h at 35°C in 9 volumes of lactose broth (Fig. 1). Replicate portions (1 ml) of each preenrichment culture were selectively enriched in tetrathionate brilliant green broth incubated for 16 to 18h at 43°C (TBG_{43}) and 35°C (TBG_{35}), and in selenite cystine incubated at 35°C (SC_{35}). Each enrichment culture was streaked on bismuth sulfite (BS) and brilliant green sulfa (BGS) agar plates and incubated overnight at 35°C. Suspect colonies were screened biochemically on triple sugar (TSI) and lysine iron (LI) agars, and confirmed serologically with polyvalent and single grouping antisera (*17*).

TABLE 1. *Detection of Salmonella in foods.*

Food	Number of samples Tested	Positive[a]	Serovar
HIGH MOISTURE			
Poultry			
Carcasses/cut-up	15	11	*S. typhimurium* (2); *S. schwarzengrund* (2); *S. infantis* (2); *S. hadar* (2); *S. haardt* (1); *S. heidelberg* (1); *S. senftenberg* (1)
Giblets	14	7	*S. hadar* (3); *S. typhimurium* (1); *S. schwarzengrund* (1); *S. infantis* (1); *S. untypable* (1)
Others[b]	5	2	*S. saint-paul* (1); *S. infantis* (1)
Pork			
Sausages (raw)	10	1	*S. schwarzengrund* (1)
(fermented)	3	3	*S. brandenburg* (1); *S. infantis* (1); *S. bredeney* (1)
Minced meat	4	0	NA[c]
Giblets	3	0	NA
Hocks	1	1	*S. mbandaka* (1)
Beef[d]	4	0	NA
Fish/shellfish[e]	4	1	*S. brandenburg* (1)
Others[f]	3	0	NA
Subtotal	66	26	
LOW MOISTURE			
Spices	9	8	*S. glostrup* (2); *S. abaetetuba* (1) *S. morehead* (1); *S. oranienburg* (1); *S. anatum* (1); *S. untypable* (2)
Pasta	4	2	*S. infantis* (1); *S. mbandaka* (1)
Chocolate	4	2	*S. napoli* (1); *S. typhimurium* (1)
Animal feeds	6	3	*S. schwarzengrund* (1); *S. amager* (1); *S. montevideo* (1)
Egg powder	1	0	NA
Cocoa beans	1	0	NA
Subtotal	25	15	
TOTALS	91	41	

[a]Based on combined EIA and cultural test results.
[b]Positive chicken nugget (1/1) and chicken sausages (1/4).
[c]NA = not applicable.
[d]Includes minced meat (3) and roast (1).
[e]Positive snails (1/1) and negative lobster (1), eels (1) and cuttlefish (1).

EIA test

Bacterial cultures stored at room temperature on semi-solid agar slopes (*8*) were subcultured twice in nutrient broth incubated for 24 h at 35°C. A portion (1 ml) of each nutrient broth culture was then inoculated into 9 ml of M-broth, and incubated for 24 h at 35°C. The entire M-broth culture was sedimented at 2500 x *g* for 20 min, and the pellet suspended in 1 ml phosphate-buffered saline (pH 7.5). The bacterial suspension was heated for 20 min in a boiling water bath and stored at room temperature pending EIA analysis (*8*).

In the BioEnzabead™ detection of *Salmonella* in foods (Table 1), equal portions (0.5 ml) of TBG_{35} and SC_{35} enrichment cultures were inoculated into a single tube containing 10 ml of M-broth (Fig. 1). After incubation for 6 h at 35°C, the culture was streaked on BS and BGS, and the remaining portion sedimented and heated in a boiling water bath as described above. Heated extracts (0.2 ml) were assayed according to instructions from the manufacturer. The intensity of green color (positive reaction) was measured colorimetrically at 410 nm using a Minireader II (Dynatech Laboratories Inc.). Identification of isolates responsible for false-positive EIA reactions in foods was undertaken on a limited basis by inoculating representative colonies on corresponding BS and BGS plates into fresh tubes of M-broth. The resulting culture was subjected to EIA assay and non-salmonellae isolates producing a positive EIA reaction were identified to the genus or species level with the API 20E system (Analytab Products Inc., Plainview, NJ).

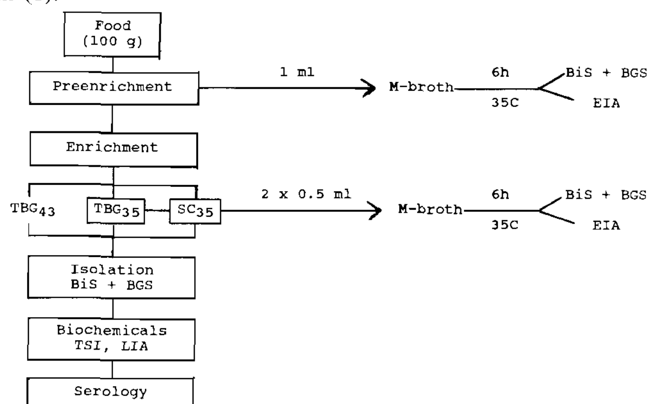

Figure 1. *Detection of* Salmonella *in foods by cultural and immunoassay technique.*

To investigate the potential for method brevity through application to the BioEnzabead™ test after preenrichment, a 1-ml portion of each preenrichment culture arising from standard cultural analyses was inoculated into a tube of M-broth (10 ml) and incubated for 6 h at 35°C. The culture was then streaked onto BS and BGS and the remaining portion analyzed by EIA as described above.

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TABLE 2. *Performance of BioEnzabead™ with pure cultures.*

Bacteria	Number of strains Tested	Positive	Negative	Erroneous results
Salmonella spp.	250	246	4	*S. bareilly* (1) *S. hvittingfoss* (2) *S. muenchen* (1)
Enterobacteriaceae	60	21	39	*C. freundii* (15) *E. coli* (3) *Morganella* sp. (1) *Yersinia enterocolitica* (2)
Others[a]	15	2	13	*Pseudomonas putrefaciens* (2)

[a]*Aeromonas, Campylobacter* and *Pseudomonas* spp.

TABLE 3. *BioEnzabead™ detection of* Salmonella *in foods.*

Food	No. samples Tested	Positive[a]	Recovery patterns Cult+ / EIA+	Cult+ / EIA-	Cult- / EIA+	Cult- / EIA-
High moisture[b]	66	26	26	0	31	9
Low moisture[c]	25	15	15	0	4	6
TOTAL	91	41	41	0	35	15

[a]By standard cultural procedure.
[b]Raw meat, cheese, and liquid eggs.
[c]Chocolate, spices, pasta, egg powder, and animal feeds.

RESULTS AND DISCUSSION

Coupling of the 6H4 and MOPC 467 antibodies on individual metal beads markedly increased the sensitivity of BioEnzabead™ with pure *Salmonella* cultures. In contrast to the 7.2% rate of false-negative reactions obtained with the original single-antibody (MOPC 467) system (*8*), modification to include a second antibody (6H4) on each coated bead provided for the detection of all but 4 (1.6%) of 250 *Salmonella* test cultures (Table 2). Repeated testing of these false-negative cultures yielded OD_{410} values ranging from 0.09 to 0.18, well below the threshold absorbance value of 0.20 for positive reactions. The observation that all 19 strains of non-motile *S. pullorum* and *S. gallinarum* were detected by immunoassay is of interest because it challenges the assumption that the MOPC 467 and 6H4 antibodies recognize non-serotypic flagellar antigens (*19*). Lack of BioEnzabead™ specificity with non-salmonellae (30.7%) was notable whereby 15 of 17 test cultures of *Citrobacter freundii* yielded positive reactions (Table 2). An earlier report of similarly high rates of false-positive reactions with the single MOPC 467 antibody system underlines the non-specificity of this myeloma antibody (*8*). Although the contribution of the 6H4 antibody to the problem of cross-reactivity could not be assessed in the present study, earlier work failed to show reaction of this antibody with non-salmonellae (*19*). Several related studies using the BioEnzabead™ system reported conspicuous levels of non-specific reactions with non-salmonellae ranging from 2% to 40% (*9,13,14,23*). BioEnzabead™ recognition of non-members of *Enterobacteriaceae* such as *Pseudomonas* spp. is equally noteworthy (Table 2).

Performance of BioEnzabead™ with naturally and artificially contaminated foods has generated considerable interest. In the present study involving 91 test foods (Table 1), all of the 41 naturally-contaminated foods identified by a standard cultural technique were successfully detected by immunoassay (Table 3). High moisture foods were primarily responsible for the high rate of false-positive results. Limited investigations into the bacterial species implicated in 16 of the 35 false-positive results (Table 3) identified *C. freundii* (4), *Aeromonas hydrophila* (4), *Proteus* spp. (3), *Citrobacter amalonaticus* (2), *Pseudomonas aeruginosa* (2) and *Enterobacter cloacae* (1). Earlier studies had also identified high moisture foods as an important source of erroneous results. In the examination of naturally and artificially contaminated foods and animal feeds, raw meats accounted for 45% (*13*), 50% (*23*), and 58% (*9*) of reported false-positive reactions by immunoassay. In other studies, the threshold value (OD_{410} $\geq$0.20) for positive reactions was arbitrarily raised to reduce the number of false-positive reactions (*3,24*). Appreciable numbers of cross reactions were also uncovered in a major collaborative analysis of inoculated foods involving 25 laboratories. Of 331 contaminated samples identified by a standard cultural procedure, 321 (97%) were detected by the BioEnzabead™ system, and all but 7 of the 27 (7.8%) false-positive results originated from raw turkey meat (*14*). This collaborative study led to adoption of the BioEnzabead™ technique at the level of Official First Action (AOAC) for the rapid detection of *Salmonella* in foods (*2*). Further improvement of the system was recently introduced through replacement of the laborious sedimentation of M-broth cultures with increased incubation of the M-broth from 6 h (original protocol) to 14 h, with a concomitant decrease in the period of incubation of

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TABLE 4. *BioEnzabead™ detection of* Salmonella *in preenrichment cultures.*

Food	No. samples		Recovery patterns[b]			
	Tested	Positive[a]	Cult+ / pEIA+	Cult+ / pEIA-	Cult- / pEIA+	Cult- / pEIA-
High moisture	66	26	19	7	27	13
Low moisture	25	15	5	10	2	8
TOTAL	91	41	24	17	29	21

[a]By standard cultural procedure.
[b]pEIA = EIA detection of *Salmonella* from preenrichment cultures.

TABLE 5. *Productivity of enrichment-plating conditions.*

Food	Number positive samples[a]	Salmonella positive samples					
		TBG_{43}		SC_{35}		TBG_{35}	
		BS	BGS	BS	BGS	BS	BGS
High moisture	26	26	26	23	18	24	23
Low moisture	15	14	13	11	9	14	8
TOTALS	41	40	39	34	27	38	31
PERCENT	100	98	96	83	66	93	76

[a]Based on combined cultural and EIA results.

enrichment broths from 18 h to 6 h (*16*). The novel approach which applies to dry foods only, allows the immunoassay to be performed in the morning of a workday. Collaborative evaluation of the abbreviated technique demonstrated near equivalence with a standard cultural method and low incidence of false-positive reactions (*16*). Although use of short (6 h) selective enrichment finds support in the literature (*11,15*), recent studies based on 127 naturally contaminated foods showed 9% and 28% incidence of false-negative results with high and low moisture foods, respectively (D'Aoust, unpublished results). Other studies have also underlined the limited sensitivity of short selective enrichment (*21,27*).

Method brevity through application of the BioEnzabead™ assay at the preenrichment stage is contraindicated because the approach failed to identify 17 (42%) of the 41 positive food samples (Table 4). These data support earlier findings with meat and poultry products where immunoassay detection of *Salmonella* in preenrichment cultures only showed a 50% correct identification of contaminated samples (*10*). It was also demonstrated earlier that omission of M-broth postenrichment or non-centrifugation of the M-broth culture adversely affected the sensitivity of the BioEnzabead™ procedure (*8*).

The productivity of TBG_{43} enrichment in our study exceeded that obtained with the same medium incubated at 35°C (Table 5). Although several standard methods of analysis and research papers have already recognized the benefit of selective enrichment at elevated temperature through increased repression of competing microflora and facilitated recovery of salmonellae on plating media (*5,17,18*), other reference procedures remain obstinate in their continued enrichment of foods at a permissive (35°C) temperature (*25,26*). The previously noted low selectivity of SC (*5,7*) likely played in the comparatively poor performance of this medium (Table 5). A more recent comparison of different enrichment regimens for detection of salmonellae in 251 naturally contaminated foods further confirmed the value of enrichment at elevated temperature as evidenced by a 10% lower recovery of salmonellae from high moisture foods with TBG_{35} than with TBG_{43} (D'Aoust, unpublished data). BS consistently yielded greater isolations of *Salmonella* than BGS (Table 5). Although agar media such as Hektoen, xylose-lysine desoxycholate, and brilliant green agars have found wide application in food analysis, the high selectivity and saccharide-independent response of BS makes this a medium of choice for the isolation of typical and atypical *Salmonella* biotypes (*1,4,7*).

The present study has underlined the ability of the double-antibody BioEnzabead™ system to reliably detect *Salmonella* in foods. However, the inherent lack of specificity of the MOPC 467 antibody and attendant high numbers of false-positive reactions with high moisture foods places certain limitations on the efficacy of this novel technology. Replacement of the MOPC 467 with a more highly specific antibody should be entertained if future application of the BioEnzabead™ immunoassay is not to be restricted to low moisture foods (*16*). Attempts to eliminate the laborious sedimentation step in the original technique through use of short (6 h) selective enrichment is of concern because of definite indications in the literature that short enrichment adversely affects the sensitivity of cultural methods.

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these indicators are accepted by United States animal health authorities for monitoring the cooking of meats for export in countries where the FMD virus is endemic. Periodic inspections in the processing plants by food inspectors from local animal health services, are the routine way to minimize this kind of fraud.

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