Optimization of a rapid dot-blot immunoassay for detection of *Salmonella enterica* serovar Enteritidis in poultry products and environmental samples

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Abstract

An immunoassay was developed for the detection of *Salmonella* serovar Enteritidis in poultry and environmental samples. This assay consisted of a two-step procedure that involved an enrichment step using whole egg homogenate (EH) as the enrichment medium and detection by a monoclonal antibody (MAb)-based dot-blot assay. Egg homogenate enriched *Salmonella* Enteritidis was heated to 100°C for 10 min in the presence of cholic acid, a detergent, to liberate the lipopolysaccharide (LPS) antigen in gelled egg matrix. This was subsequently transferred onto a nitrocellulose membrane for detection with MAb 2F11. Several commercially available media were compared with egg homogenate for their relative ability to resuscitate and propagate *Salmonella* Enteritidis to detectable levels. Incubation in EHI, trypicase soy broth (TSB), and lactose broth (LB) resulted in comparable levels of *Salmonella* Enteritidis as demonstrated by viable plate counts. *Salmonella* Enteritidis grown in TSB exhibited the greatest visual intensity showing a positive test when tested by the dot-blot assay. Incubation time necessary to detect one cfu of *Salmonella* Enteritidis was reduced from 20 to 10 h using TSB as the enrichment broth. Addition of ferrous sulphate or ferrioxamine E or cholic acid in the enrichment broth had negligible negative effects on the growth of *Salmonella*. *Salmonella* Enteritidis when incubated with a mixture of naturally contaminated or artificially inoculated competitive micro-organisms in environmental samples at a ratio of 1:10^3, was able to reproduce to detectable numbers for the immunoassay. This method was able to detect all plague types (PT 1, 6, 7, 8, 13, 13a, 14b, 21 and 28) with unique ribopatterns. The results demonstrated that *Salmonella* Enteritidis, when pre-enriched in a medium containing ferrous sulphate or cholic acid, could be readily detected in the presence of 100-fold higher competition of other micro-organisms.

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1. Introduction

The incidence of human illness associated with food contaminated with *Salmonella enterica* serovar Enteritidis has increased over the last 20 years. *Salmonella* Enteritidis is presently the second most frequently reported *Salmonella* serovar isolated from human infections in the United States (Barhart et al., 1991); however, the prevalence of *Salmonella* Enteritidis in Canada is much lower (Popp et al., 1991). Eggs, egg products, poultry and poultry products are considered the major sources of *Salmonella* Enteritidis (Ruzickova, 1994). Isolation of specific species with conventional methods depends largely on biochemical differences between the target organism and the competing microflora (Zee, 1994). Traditional detection of *Salmonella* Enteritidis is labor intensive, difficult and expensive as the culturing and identification of these bacterial isolates requires up to 1 week. These methods may differentiate among *Salmonella* species using specific antiserum at the confirmation stage.
There are few rapid methods for the detection of Salmonella Enteritidis. Several enzyme-linked immunoabsorbent assay (ELISA) systems have been developed for the detection of Salmonella. These assays which require as little as 18 h are much faster than conventional methods (Lee et al., 1989; Huang et al., 1999); however, they suffer from some disadvantages including cross-reaction with other organisms (Lee et al., 1989), and low detection limits which may be related to the low binding capacity of the polystyrene plates for bacteria. In contrast, assays utilizing nitrocellulose as a solid phase are considerably more sensitive than the ELISA as the nitrocellulose has much higher capacity for protein and lipopolysaccharide (LPS) binding than polystyrene plates which are often used in the ELISA (Sarasombat et al., 1988). Wang et al. (1995a, b) developed a related method, designated polymyxin cloth enzyme assay, for the detection of Salmonella Enteritidis from eggs using anti-O:9 antisera. However, this antibody (anti-O:9 antiserum) has a greater potential to display cross-reactions with other Salmonella serovars than the MAb 2F11 (Masi and Zawistowski, 1995) utilized in the current study, which is extremely sensitive and specific.

A novel immunoassay utilizing nitrocellulose has been developed in our laboratory for the detection of Salmonella Enteritidis in eggs, poultry, feed and foods (Yoshimasa and Zawistowski, 2001). This system does not require highly trained technicians or the expensive equipment needed for the conventional ELISA or fluorescent antibody techniques, thus it can be conducted in most laboratories.

The dot-blot based system utilizes a monoclonal antibody specific to the LPS O-9 antigen present in Salmonella Enteritidis (Masi and Zawistowski, 1995). The organism is initially cultured in homogenized whole egg supplemented with ferrous sulfate as the enrichment medium. One Salmonella Enteritidis cell in 500 g of homogenized whole egg was detected after 20 h of enrichment giving a total test time of 1 day (Yoshimasa and Zawistowski, 2001). This paper compares synthetic pre-enrichment media with the homogenized whole egg for its relative ability to detect Salmonella Enteritidis by the immunoassay. Attempts to shorten and optimize the assay are also reported. Salmonella isolates used in this study were further confirmed by ribotyping.

2. Materials and methods

2.1. Materials

RPMI 1640 medium was purchased from Gibco BRL-life Technologies (Grand Island, New York); aerobic plate count agar (APC), selenite cysteine broth, nutrient broth, lactose broth, trypticase soy broth, brilliant green agar with sulphanilamide, triple sugar iron agar and lysine iron agar were from BBL (Cockeysville, Maryland); peptone, Salmonella O antisera factors 9, 4, 5 and poly A-1 and Vi were from Difco (Detroit, Michigan); cholic acid was from Sigma-Aldrich Chemical Co. (St. Louis, Missouri); electrophoresis grade hydroxymethyl aminonitromethane (Tris), NBT (nitroblue tetrazolium chloride), and BCIP (5-bromo-4-chloro-3-indolyphosphate) were from BioRad (Hercules, California); streptavidin alkaline phosphatase conjugate was from Cedarlane Laboratories Ltd. (Hornby, ON); ferrioxamine E was kindly provided by Dr. R. Reissbrodt, Federal Health Office, Wemigerode, Germany. All other chemicals and reagents were of analytical grade.

2.2. Bacteria and culture conditions

Bacteria used in this study were Salmonella Enteritidis (SE) PT1, PT8, PT13, PT13a (Laboratory Center for Disease Control, Ottawa, Canada), SE PT1, PT7, PT14b, PT21, and PT28 (Laboratory for Animal Diseases, Ames, Iowa, USA), Salmonella Berta, Salmonella Pullorum, Salmonella Maarseen, Escherichia coli and Citrobacter freundii were purchased from ATCC. Salmonella Arizona, Salmonella Enteritidis with unknown phage type, Salmonella Heidelberg and Proteus vulgaris were from University of Manitoba (Winnipeg, Canada). The cultures were maintained on APC slants at 4°C until use. A loopful of culture was inoculated into 5 ml of nutrient broth and incubated for 24 h at 37°C. APC slants were inoculated with broth cultures of Salmonella Enteritidis and incubated for 24 h at 37°C. Slants were washed with 0.85% saline, harvested and serially diluted in 0.1% peptone water. The number of viable cells was determined as cfu from APC plates after 24 h of incubation, were prepared as given above. The specific concentration of all serially diluted bacterial suspensions was confirmed by plating onto APC agar.

2.3. Ribotyping

Cultures used in this study were genetically characterized by using an automated RiboPrinter™ following the manufacturer’s instructions (Qualicon, Inc., Wilmington, Delaware). The riboprint patterns were generated by using PvuII restriction enzyme and compared with the database that was provided with the instrument.

2.4. Monoclonal antibody production

The monoclonal antibody 2F11 was produced and characterized by Masi and Zawistowski (1995). Hybridoma culture supernatant was partially purified by ammonium sulfate precipitation (Harlow and Lane,
1988) and used at a 1/25 dilution throughout the entire study or was purified by protein A-sepharose and used at 1:1000 dilution.

2.5. Preparation of egg samples and commercial media

Large grade A eggs were obtained from a local supermarket and were used within 1 week of purchase. The surface of the eggs were washed with 70% ethanol and opened aseptically. Pooled egg contents were placed into stomacher bags and mixed for 30 s using stomacher lab-blender 400 (Seward Laboratory, London, UK). Portions of homogenized egg (25 ml) were transferred into 50 ml polypropylene tubes (Falcon, Oxnard, California). Tryptic soy broth, peptone water and lactose broth were prepared according to the manufactures instructions. Aliquots (25 ml) were placed in 50 ml polypropylene tubes and the contents were sterilized at 121°C prior to use. Tubes containing commercial media or blended eggs were then inoculated with Salmonella Enteritidis and incubated for 20 h at 37°C. Thereafter, 2.5 ml of 15% cholic acid was added to each tube and 0.5 g agar was added to tubes containing synthetic media to solidify the media so that it can be cut in discs for testing. All tubes were heated at 100°C for 10 min in a water-bath. Tubes were vortexed to ensure melting and dispersion of agar throughout the media. Following heating, the samples were placed in a freezer to speed matrix setting.

2.6. Immunoblotting

The immunoblotting was carried out according to Yoshinami and Zawistowski (2001) with modification. Briefly, solidified synthetic media or whole egg media spiked with Salmonella were removed aseptically from the tubes and a sterile borer was used to cut a 2 mm disc from the center of each sample. The disc was placed on a PBS pre-wetted nitrocellulose membrane. A 15 μl drop of heat-inactivated Salmonella Enteritidis PT8 was placed on the membrane as a positive control. After 5 min, the discs were removed from the membrane. The membrane was then washed twice with phosphate buffered saline (PBS). Membrane was then blocked with 5% skim milk powder in PBS for 45 min. The skim milk powder was removed by washing the membrane twice with Tris buffered saline containing 0.05% Tween 20 (TBST) and MAb 2F11 was added and incubated for 1 h at 37°C. The membrane was washed with TBST and biotinylated goat-anti-mouse antibody was added and incubated for 1 h at 37°C. The membranes were developed with streptavidin alkaline phosphatase conjugate and a nitroblue tetrazolium/5-bromo-4-chlor-3-indolyl phosphate substrate. Color development was stopped by rinsing the membrane with distilled water.

2.7. Effect of agar and cholic acid on the growth of Salmonella Enteritidis

Trypt case soy broth and lactose broth were inoculated with Salmonella Enteritidis and cholic acid with or without added agar and incubated for 20 h before dot immunoblotting assay.

2.8. Determination of optimal amount of iron

Trypt case soy broth (25 ml) and homogenized whole egg were each inoculated with about 17 cfu of Salmonella Enteritidis. Varying concentrations of ferrous sulphate ranging from 0 to 80 μg/ml were added to each tube. After incubation for 12 h at 37°C, samples were serially diluted and enumerated using APC agar.

2.9. Detection of Salmonella Enteritidis in the presence of added competing micro-organisms

Trypt case soy broth and egg homogenate were inoculated with Salmonella Enteritidis, P. vulgaris, E. coli, C. freundii and Salmonella Heidelberg, alone and in various combinations and ratios. The samples were incubated for 16 or 20 h at 37°C before proceeding with the Dot-blotting assay.

2.10. Enumeration of Salmonella Enteritidis in naturally contaminated samples with the addition of ferrous sulphate or ferrioxamine E

Samples of wash water (25 ml) obtained from various stages in the production line of a commercial poultry processing plant, were inoculated with 1, 10, 100 and 1000 cfu of Salmonella Enteritidis. Negative controls were not inoculated. The wash water samples were then added to 225 ml of either trypticase soy broth or whole egg homogenate containing either 21 mg ferrous sulfate for the whole sample or 60 ng/ml ferrioxamine E. Samples were incubated for either 16 or 24 h at 37°C and 25 ml were then dispensed into 50 ml conical flasks before proceeding with the Dot-blotting assay.

2.11. Culture confirmation

Following incubation, 1 ml from each sample was added to 10 ml of selenite cysteine broth and incubated for 24 h at 37°C. A loopful of selenite cysteine broth was then streaked onto brilliant green sulphadiazine agar and salmonella–shigella agar and incubated for 24 h at 37°C. Presumptive isolates were then transferred to triple sugar iron and lysine iron agar slants and incubated for 24 h at 37°C. Confirmation of typical isolates was performed serologically using Salmonella O-9 factor antiserum. Salmonella 4, 5, and the poly A-1 factor antiserum was used to confirm presence of
non-D-group Salmonella in cases where samples tested positive by conventional culture for Salmonella Enteritidis.

3. Results and discussions

A dot-blot assay for the detection of Salmonella Enteritidis in foods and feeds was developed by Yoshimasa and Zawistowski (2001) and the assay involved a two-step procedure; a 20 h enrichment step utilizing homogenized whole egg, followed by a dot-blot assay utilizing anti-Salmonella Enteritidis MAb (2F11). The specificity of the MAb 2F11 which reacts with LPS as antigen, was tested against certain Salmonella Enteritidis phage types in ELISA using both homogenized whole eggs or synthetic media (Masi and Zawistowski, 1995); however, the specificity of this antibody against several other Salmonella Enteritidis phage types was not tested using the dot-blot, therefore, we have tested 10 different phage types using this dot-blot assay and the whole egg mixture as the enrichment media. All the phage types gave positive reaction with MAb 2F11, with no apparent differences in the intensity of the reaction. In order to ascertain the similarity among the different Salmonella Enteritidis phage types, and to differentiate them from other non-Enteritidis Salmonella, we have ribotyped them using an automated riboprinter and PstI as a restriction enzyme (Table 1). All phage types showed a characteristic identification pattern similar to DUP 2035 (Qualicon, Inc.); therefore, this system was not suitable for differentiating differences within different phage types (Table 1).

Since all the phage types had similar reaction to antibody, PT8 was chosen for most of the experiments. This study was undertaken to evaluate the efficacy of synthetic media to replace the egg homogenate for the propagation of Salmonella Enteritidis to be effectively detected in the dot-blot assay. Using conventional methodology for the detection of Salmonella it has been found that the choice of a pre-enrichment medium used is not critical for its recovery from raw and processed foods (D'Aoust and Maishment, 1979; Poelma et al., 1981). Highly nutritive broths, detergent containing media and formulations adapted for the selective growth of Enterobacteriaceae do not promote recoveries of Salmonella to levels greater than those obtained with nutrient and lactose broths (D'Aoust and Maishment, 1979). In this study three media were chosen for comparison with homogenized whole egg; lactose broth, peptone water and trypticase soy broth. All three media are commonly used to pre-enrich Salmonella in conventional detection procedures (Reissbrodt, 1995). Fig. 1 shows the immunoblots of LPS obtained from Salmonella Enteritidis grown in the three synthetic media as well as in homogenized whole egg. The results demonstrated that peptone water was far less effective than the other media for the replication of this organism. This is simply explained by the low quality of the nutrients that failed to support high Salmonella growth. In a previous study on the pre-enrichment of Salmonella Enteritidis,

<table>
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<th>Cultures</th>
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<th>Ribopattern DUP-ID with PstI</th>
<th>Identified by RiboPrinter as</th>
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<td>NT</td>
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</table>

aLaboratory for Disease Control, Ottawa, Canada.
bLaboratory for Animal Diseases, Ames Iowa, USA.
cUniversity of Manitoba, Winnipeg, Canada.
dAmerican Type Culture Collection.
Stephenson et al. (1991) compared five media; lactose broth, peptone water, trypticase soy broth, nutrient broth, and brain heart infusion broth. Similar results were obtained with all media. Consequently, trypticase soy broth was arbitrarily recommended as the enrichment medium of choice for the recovery of *Salmonella Enteritidis* from egg yolk (Stephenson et al., 1991). In another study, Reisbrodt (1995) found peptone-water to be the medium of choice for general-purpose pre-enrichment of *Salmonella*. However, peptone water was found to be least effective in our assay and therefore, this medium was omitted from subsequent tests. Fig. 2 shows the immunoblots for LPS obtained from *Salmonella Enteritidis* grown in lactose broth, trypticase soy broth, and egg homogenate. Equal numbers of *Salmonella Enteritidis* were produced in both lactose and trypticase soy broth as assessed by APC after 20 h of incubation. A positive immunoblot for LPS from *Salmonella Enteritidis* was obtained when the culture was grown in all above-mentioned media. However, the immunoblots employing inocula produced in lactose broth and egg homogenate were less intense than that for trypticase soy broth. In both cases uninoculated media (negative control) gave no reaction. It was necessary to solidify the media before applying it to the nitrocellulose membrane. This was effectively achieved by adding 2% granulated agar to the same media. This modification retained the conditions of the immunoblot assay as closely as possible to the original assay. The solid matrix yielded superior dot-blot images to that of the liquid media as the liquid medium tended to disperse on the membrane.

The addition of iron has been shown to greatly improve the recovery of *Salmonella Enteritidis* from eggs (Gast, 1993; Gast and Holt, 1995a, b; Yoshimasu and Zawistowski, 2001). *Salmonella Enteritidis* has also been found to grow rapidly following the addition of ferric ammonium citrate to albumin (Lock and Board, 1992). Therefore, the effect of iron addition on *Salmonella Enteritidis* growth in homogenized whole egg, lactose broth and trypticase soy broth was assessed. Results of the immunoblot APC counts showed that iron addition either in ferrous or ferric form had no effect on growth of *Salmonella Enteritidis* in the TSB or LB (data not shown). Aerobic plate counts for these media reached $5 \times 10^{11}$ cfu when *Salmonella Enteritidis* was incubated in the presence of both forms of iron and without iron. These media are probably not iron deficient therefore the addition of this element to the media may have not enhanced the growth of bacteria. In contrast, when iron was added to homogenized egg, both ferrous and ferric forms were found to equally enhance growth. Counts on APC agar reached $2.7 \times 10^{11}$ cfu/ml after *Salmonella Enteritidis* was incubated for 20 h in egg homogenate with either form of iron whereas without the iron *Salmonella Enteritidis* level was $5.8 \times 10^{10}$ cfu/ml. Ovotransferrin and other iron-chelating compounds reduce the amounts of available iron in the albumin. Therefore, the addition of iron to the egg homogenate enhanced *Salmonella* growth (Chart and Rowe, 1993). In the present study, we determined the amount of added iron for maximum growth of *Salmonella Enteritidis* in homogenized egg to be about 85 μg/ml as ferrous after 12 h incubation.

LPS immunoblots obtained when *Salmonella Enteritidis* grown in lactose broth were consistently less intense than those obtained for the organism grown in trypticase soy broth, although both media equally support *Salmonella* growth as shown by APC. This might be related to the presence of some nutrients in the LB that interfere with the binding of LPS to NC membranes thus, decreasing the blot intensity. For this
reason trypcase soy broth was chosen as the most promising synthetic media for comparison with homogenized whole egg in further studies.

Conventional detection of Salmonella requires a 24 h pre-enrichment incubation. Also in previous studies using homogenized whole egg as an enrichment medium, it was found that 20 h incubation at 37°C was necessary to bring the population of Salmonella Enteritidis up to $10^6$ cfu/ml which is the minimum population needed for detection by the immunoblot (Yoshimatsu and Zawistowski, 2001). Since a decrease in incubation time is beneficial to the overall efficiency and convenience of the assay, we assessed the minimum time necessary to detect Salmonella Enteritidis when incubated in trypcase soy broth. Fig. 3 shows that a $10^4$ cfu inoculum of Salmonella Enteritidis in 25 ml of trypcase soy broth can be detected by the immunoblot assay after 10 h of incubation. In this regard, studies have shown that Salmonella Enteritidis at 37°C commences exponential growth at about 6 h after inoculation into trypcase soy broth (Chart and Rowe, 1993). In this study, the cholic acid and agar added to the media prior to the enrichment step had no adverse effects on the growth of Salmonella Enteritidis.

Salmonella Enteritidis has been shown to be present in mixed populations of fecal and enteric bacteria. In order for Salmonella Enteritidis to be detected in a mixed flora, it must reach a sufficient concentration. In addition, the assay to be used should have minimum cross-reactivity. When Salmonella Enteritidis was incubated in the presence of a mixed microflora consisting of C. freundii, P. vulgaris, Salmonella Heidelberg, and E. coli, it was detected at ratios of 1:10$^0$, 1:10$^1$, and 1:10$^2$ (Salmonella competitor) in the initial inoculum using egg homogenate supplemented with ferrous sulfate as enrichment medium. In contrast, Salmonella Enteritidis was detected only at a ratio of 1:10$^0$ and 1:10$^1$ when TSB was the enrichment medium (Fig. 4). In previous work using homogenized whole egg it was found that one Salmonella Enteritidis cfu could be detected by the dot-blot assay after incubation with up to 400 cfu of competing bacteria consisting of various genera and species commonly found in poultry (Yoshimatsu and Zawistowski, 2001). Dolman and Board (1992) inoculated the inner membrane of the air cell of shell eggs with a mixed flora of bacteria to determine their ability to compete at different storage temperatures. The organisms used in their study included Pseudomonas putida, Salmonella Enteritidis, E. coli, Enterococcus faecalis and Staphylococcus xylosus, which were isolated from either eggs or poultry feces. Dolman and Board (1992) isolated all five organisms from the inner membrane of the eggs that were stored at 4°C. Storage at 37°C for 15 days led to recovery of only S. xylosus, Salmonella Enteritidis and E. faecalis. Further, Salmonella Enteritidis attained populations of $10^5$ cfu/ml (Dolman and Board, 1992). The authors also noticed that Salmonella Enteritidis out-competed E. coli when the eggs were incubated at 37°C but failed to compete with Pseudomonas species in eggs incubated below 30°C. These studies indicated that Salmonella Enteritidis has the ability to competitively exclude other species and it is well adapted to competitors than some other species that are generally encountered in food such as E. coli.

The feasibility of using the immunoblot assay to detect Salmonella Enteritidis in the presence of other bacteria was also assessed in the current study by examining wash water samples from a local poultry plant. A sample of water that had been used to wash the crates in which chicken arrived at the plant was found to have an initial load of $10^5$ cfu/ml of organisms. This sample did not give conclusive results when inoculated with $10^2$ Salmonella Enteritidis per 25 ml, and incubated for 16 or 24 h in trypcase soy broth or the egg medium and were tested by the dot-blot assay. It is probable that in this test the competitor ratio was too high and as such Salmonella Enteritidis did not multiply sufficiently to be detected. In addition, Salmonella Enteritidis was not
detected using a conventional method. Crate wash water is contaminated primarily by feces and associated organisms, and it has been found that direct contact with sewage organisms is more detrimental to *Salmonella* than contact with diffusible by-products of the sewage organisms (Riser et al., 1985).

The next experiment was performed using scaler over-run water, containing 10^2 cfu/ml. The use of trypsinase soy broth, enabled detection of *Salmonella Enteritidis* as early as 16 h of incubation at a ratio of 1:10^2 *Salmonella Enteritidis* to other organisms (Fig. 5). The ferrous supplemented egg medium also enabled the detection of *Salmonella Enteritidis* in the presence of other organisms at a ratio of 1:10^5. However, with the other samples tested (wash water), the supplemented egg medium gave better results than trypsinase soy broth, both after 16 and 24 h. In carcass rinse water containing a microflora load of 10^6 cfu/ml, *Salmonella Enteritidis* was also detected at a ratio of 1:10^5 using the egg as an enrichment medium.

In complex mixtures of micro-organisms competition between species may have a considerable effect on the relative rates of population growth (Rhodes et al., 1985). In fresh, raw food such as poultry and egg products, the major part of the flora consists of *Enterobacteriaceae* and *Pseudomonas*, which will readily compete with *Salmonella* (Brinkman et al., 1995). *Pseudomonas* species are potent inhibitors of the pathogenic bacteria associated with food and plants (Cheng et al., 1995).

In samples taken from the poultry plant, the immunoblot was stronger for *Salmonella Enteritidis* incubated in supplemented egg medium than trypsinase soy broth. Increasing the amount of nutrients in trypsinase soy broth may favor the growth of competitive micro-organisms which may, in turn, have an inhibitory effect on the growth and isolation of *Salmonella* (Schothorst and Renaud, 1982). Factors in the egg, such as low levels of non-protein albumin nitrogen, select for Gram-negative bacteria because they tend to be less fastidious than Gram-positive organisms (Dolman and Board, 1992). Studies have shown that *Salmonella Enteritidis* is capable of out-competing *E. coli* in eggs incubated at 37°C (Dolman and Board, 1992). Perhaps the egg medium is selective enough towards *Salmonella Enteritidis* that it allows it to grow to slightly higher numbers for better detection by the immunoblot than is seen in the non-selective trypsinase soy broth.

Detection of *Salmonella Enteritidis* in the presence of competing bacteria was improved using media modified by the addition of ferrioxamine E (Fig. 6). *Salmonella* has an almost specific transport system for the siderophore ferrioxamine E (Reissbrodt and Rabarch, 1993). Reissbrodt et al. (1996) found that ferrioxamine E supplementation of buffered peptone water allowed for the detection of *Salmonella*. It was initially out competed by 10^3–10^4 cfu/ml in egg albumin after only 6 h of incubation with shaking (Reissbrodt et al., 1996). In the current study homogenized whole egg and

![Fig. 5. Detection of *Salmonella Enteritidis* grown in TSB for 16 or 24 h in the presence of bacterial competitors present in scaler run-off water obtained from poultry plant at ratios of 1:10^5, 1:10^4, 1:10^2, and 0:10^5 (No *Salmonella Enteritidis* added). All rows represent triplicate results.](image)

![Fig. 6. Detection of *Salmonella Enteritidis* by the immunoblot assay in chicken chiller water in the presence of naturally present competitors at a ratio of 1:10^1, 1:10^2, 1:10^3, and 0:10^5 (No *Salmonella Enteritidis* added) *Salmonella Enteritidis* to competitors after 16 or 24 h. Egg homogenate alone + homogenate + ferrioxamine E (A) or TSB alone + TSB + ferrioxamine E (B) was used as the enrichment medium. All rows represent triplicate results.](image)
trypticase soy broth were supplemented with 60 ng/ml ferrocyanochrome E before addition of chicken chiller water containing 10^4 cfu/ml. The immunoblots (Fig. 6) allow the detection of Salmonella Enteritidis in the egg medium at all initial inoculum levels, even when the ratio of Salmonella Enteritidis to other organisms was 1:10^5. However, less conclusive results were obtained when trypticase soy broth was used (Fig. 6). The lack of success when trypticase soy broth was supplemented with ferrocyanochrome E may be due to its high iron concentration. Bacteria only synthesize specific iron transport systems when iron concentrations are limited. Ferrocyanochrome E supplemented Rappaport–Vassiliadis medium has also been found to be less effective than other Salmonella enrichment media for this reason (Reissbrodt et al., 1996).

The results obtained by the immunoblot were comparable with those obtained using the conventional culture method. However, the immunoassay was completed in less than 24 h whereas the conventional procedure requires 7 days for the final confirmation. Therefore, the modified method is much faster and more convenient than the conventional culture procedure. Incorporation of agar and cholic acid into the media before incubation gave superior dot-blot results. When ferrocyanochrome E was incorporated into the homogenized whole egg medium instead of ferrous sulphate, Salmonella Enteritidis was detected when the ratio of Salmonella Enteritidis to other bacteria was 1:10^4. In conclusion the commercial trypticase soy broth proved to be compatible to the egg homogenate for the detection of Salmonella Enteritidis using the dot-blot assay, while the lactose broth and peptone water, appeared to be unsuitable enrichment media. Further, it appeared that egg homogenate is a better medium when working with non-defined mixture of bacteria to which Salmonella Enteritidis was added.

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References


