THE OPTIMIZATION OF A RAPID DOT-BLOT IMMUNOASSAY FOR THE DETECTION OF *SALMONELLA ENTERITIDIS.*

A Thesis

Submitted to the Faculty

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Graduate Studies

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by

Joanna H. Bzikot

In Partial Fulfillment of the

Requirements for the Degree

of

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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I dedicate this thesis to Jeff,
and to Mum and Dad.
I would never make it without you!

I hereby declare that I am the sole author of this thesis.

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ABSTRACT

Throughout the last 20 years the incidence of human illness associated with foods contaminated with *Salmonella enteritidis* has been increasing. *S. enteritidis* is presently the second most frequently reported *Salmonella* serovar isolated from human infection in the United States. Conventional methods for the detection of this pathogen are expensive and time consuming. We previously described an Enzyme Linked ImmunoSorbent Assay (ELISA) for detection of this pathogen in foods. This assay comprises a two-step procedure that involves an enrichment step followed by a monoclonal antibody based dot-blot assay. Homogenized egg is used as the enrichment medium. The aim of this study was to optimize the assay. Commercially available media were compared with whole egg homogenate for relative ability to resuscitate and propagate *S. enteritidis* to detectable levels. Incubation in whole egg homogenate, tripticase soy broth (TSB), and lactose broth (LB) resulted in comparable numbers of *S. enteritidis*. Results of the immunoassay found that TSB gave the strongest visual representation showing a positive test for *S. enteritidis*. Incubation time necessary to detect one *S. enteritidis* colony forming unit was reduced from 20 to 10 h using TSB as the enrichment broth.

Addition of detergent before incubation had a negligible negative effect on the growth of this organism. When *S. enteritidis* was incubated with a mixed flora of ($1:10^2$ CFU/mL) competitive microorganisms, in either TSB or in homogenized whole egg supplemented with ferrous sulphate it was able to reproduce to detectable numbers for the immunoassay. When environmental samples were tested, the same ratio of *S. enteritidis* to competitive flora was detected.

The antibody was purified and freeze-dried in the presence of various exipients and its stability over time was tested by ELISA. The antibody was stable over the storage period of 70 days when stored alone and with trehalose or mannitol as cryoprotectant, even at storage conditions of 50 °C. Maltose and sucrose degraded the antibody at elevated temperatures. This is thought do be due to participation in Maillard browning reaction between the reducing sugars and the antibody.

I. INTRODUCTION

The prevalence of *Salmonella enteritidis* as a cause of human illness has increased throughout recent years. The numbers of *S. enteritidis* infections in the North Eastern United States increased about seven-fold between the years 1975 and 1987 (Guthrie, 1992). Growth in incidence of *S. enteritidis* infection has also been observed in Europe and the United Kingdom (Guthrie, 1992). In Canada, reports of *S. enteritidis* infections in humans have only increased slightly in recent years, and its prevalence is not as dramatic as in Europe and the U.S. (Poppe, 1994).

There is speculation that the reason why this pathogen is infecting humans in increasing numbers is due to the spread of *S. enteritidis* in poultry flocks, especially breeding flocks which then permit rodents and other animals to spread the organism (Guthrie, 1992).

Because of its impact on human health and potential economic damage to the poultry industry the control and elimination of this pathogen is important. Conventional methods for the detection of *S. enteritidis* require up to seven days for confirmation. These tests are labour and time consuming, and expensive,

requiring pre-enrichment, selective and isolating media as well as a variety of biochemical tests to differentiate between species (Van der Zee, 1994).

Several alternative assays have been developed in order to reduce the time requirements and increase the sensitivity of detection methods for *Salmonella* (Baumler *et al.*, 1997; Bej *et al.*, 1994; McClelland and Pinder, 1994; Thomason, 1981). Among these assays are several ELISA systems (Holt *et al.*, 1995; Kerr *et al.*, 1992; D'Aoust and Sewell, 1988; Robison *et al.*, 1983). Most of these assays only replace the agar-plating stage of the cultural assay and may introduce a third post-enrichment broth before the sample can be assayed (Lee *et al.*, 1990). These assays have the advantage of great time reduction over the conventional method, taking as little as 18 hours (Lee *et al.*, 1989) but they do suffer from some disadvantages including cross-reaction with other organisms (Lee *et al.*, 1989).

A novel immunoassay has been developed for the detection of *S. enteritidis* in eggs, poultry and other foods and feed. This system does not require highly trained technicians. It does not require the expensive equipment needed for conventional ELISA or fluorescent antibody techniques, so it can be conducted in any laboratory. The ELISA based system utilizes a monoclonal antibody produced and characterized by Masi and Zawistowski (1995). This antibody is specific to the lipopolysaccharide O-9 present in *S. enteritidis*. Using homogenized whole egg as the enrichment medium, and adding ferrous sulphate, the assay is able to

detect one CFU of *S. enteritidis* in 500 g homogenized whole egg after 20 hours incubation at 37 °C. This results in a total test time of just one day (Yoshimasu, 1996).

The objective of this study was to develop and optimize the existing assay in order to simplify the test and prepare it for commercialization. With this purpose in mind we compared complex, commercial pre-enrichment media with homogenized whole egg for relative ability to detect *S. enteritidis* by the immunoassay. Other attempts to shorten and optimize the assay were also examined. The monoclonal antibody was freeze-dried in the presence of cryoprotectants in order to improve its shelf-life and stability.

II. LITERATURE REVIEW

A. Introduction

Salmonella are ubiquitous microorganisms that have been found in most animal species in most geographic areas of the world (Thomason, 1981). The genus *Salmonella* contains more than 2,300 different serovars (Popoff, and Minor, 1992 cited in Pignato *et al.*, 1995), however, only a few species cause the majority of human infection (Pignato *et al.*, 1995). The most common infectious *Salmonella* species are *Salmonella enteritidis* and *Salmonella typhimurium.* Together these two organisms account for three-quarters of all salmonellosis cases each year (McClelland and Pinder, 1994). As all *Salmonella* serovars are primarily pathogenic for animals, the most common source of *Salmonella* infection for humans is contaminated food of animal origin (Pignato *et al.*, 1995).

For many years *Salmonella enteritidis* accounted for about six percent of the *Salmonella* isolates associated with human food-borne illness in the United States (Stephenson *et al.*, 1991). This percentage has increased since 1976. In 1990 *S. enteritidis* became the most frequently reported *Salmonella* serovar isolated in the United States, accounting for 21% of all *Salmonella* isolates (Mishu

et al., 1994). Between 1975 and 1987 the Centres for Disease Control (CDC) in Atlanta determined that the numbers of *S. enteritidis* infections in the North Eastern United States had increased about seven fold (Guthrie, 1992). Expansion in the incidence of *S. enteritidis* infection has also been observed in Europe and the United Kingdom (Guthrie, 1992). In Canada, the incidence of *S. enteritidis* infections in humans has only increased slightly in recent years, its increase in prevalence has not been as dramatic as in Europe and the U.S. (Poppe, 1994). In Europe, most human isolates of *S. enteritidis* belong to phage type 4. Strains isolated in Canada and the United States belong primarily to phage type 8 (Poppe *et al.*, 1991).

B. Distribution of *S. enteritidis*

Although *S. enteritidis* has a wide range of hosts, there is evidence that some strains have a predilection for poultry (Guthrie, 1992). In a Canadian study, 18% of chicken flocks examined contained *S. enteritidis* in the chicken's colon at the time of slaughter (Prescott and Gellner, 1984 cited in Barnhart *et al.*, 1991). There is speculation that the increase in prevalence of *S. enteritidis* among humans could be due to the spread of *S. enteritidis* in poultry flocks, especially breeding flocks which then permit rodents and other animals to spread the organism (Guthrie, 1992).

S. enteritidis may be transferred between poultry via faecal contamination. In one study, layer hens were orally infected with *S. enteritidis*. This organism was found in the birds livers, caeca, oviducts, and ovaries after three weeks (Barnhart *et al*, 1991). Horizontal spread of the organism between birds being the mode of infection.

In over 70 % of *S. enteritidis* infections reported, the source of infection has been traced to grade A table eggs (Stephenson *et al.*, 1991). While improved sanitation procedures have led to a decrease in incidence of outbreaks due to faecally contaminated eggs, outbreaks of salmonellosis continue to occur due to the presence of *Salmonella* within the intact egg transmitted by the hen via transovarian infection. If eggs are fertilized, then transovarian infection will lead to the hatching of infected chicks (Stephenson *et al.*, 1991). Ascending infections may also contaminate the egg prior to laying (Hinton and Bale, 1994). *S. enteritidis* is rarely recovered from the ovules of laying hens but inoculation of birds by placing this organism directly into the cloaca results in birds laying infected eggs (Hinton and Bale, 1994).

C. Detection Methods

Detection of *Salmonella* in foods is difficult because foods naturally contaminated with this organism usually contain very low numbers, one CFU per

25 gram food sample is not unusual (Andrews, 1985). In eggs defiled via vertical transmission, fewer than 10 *S. enteritidis* cells per egg is the usual contamination level (Humphrey *et al.*, 1989). *Salmonella* cells surviving any food processing conditions tend to be debilitated. They will require time in favourable conditions to recover before they are able to reproduce, therefore they may be missed by the conventional detection method.

1. Conventional detection of *Salmonella*

Traditionally, salmonellae have been detected and isolated in foods via a series of microbiological culture media. The various media used follow a series of steps necessary to increase *Salmonella* counts to the point where they are detectable. Conventional methods of detection provide a theoretical level of sensitivity of one *Salmonella* per 25 g food, which is close to lower levels of contamination found in food samples. However, detection may be obstructed by the presence of other microorganisms that compete with *Salmonella* during cultural enrichment (Blackburn, 1993).

Pre-enrichment is the first step of the conventional detection method. This stage allows the recovery of any stressed cells. It is a non-selective stage and allows the growth of all organisms present. The pre-enrichment stage is particularly important in samples where *Salmonella* are potentially damaged. Selective enrichment allows survival and growth of *Salmonella* while reducing the

numbers of non-*Salmonella* in the broth. However, some routinely used selective enrichment broths are inhibitory to *S. enteritidis* (Van der Zee, 1990). Segregation of *Salmonellae* is achieved by using selective agar plates to produce presumptive isolates. At the confirmation stage presumptive isolates are subjected to a number of serological and biochemical tests to confirm the organism is *Salmonella* and to determine its serotype (Van der Zee, 1994).

Using the conventional detection procedure, only after serotyping of suspected colonies and DNA typing can *S. enteritidis* be confirmed. This has two disadvantages; firstly, as some routinely used selective enrichment broths are inhibitory to *S. enteritidis* its prevalence may be underestimated. Secondly, even if the selective enrichment is not inhibitory to *S. enteritidis*, if there is a large population of other *Salmonella* present, the presence of *S. enteritidis* may be underestimated or overlooked (Van der Zee, 1994). Isolation of specific *Salmonella* species with conventional methods depends largely on differences in biochemical reactions and resistance to antibiotics between the target organism and the competing flora. In fact, only in very rare cases do distinct differences exist between individual *Salmonella* serovars. Therefore, conventional procedures for specific isolation of *S. enteritidis* are scarce (Van der Zee, 1994).

Another disadvantage of the conventional method is that presumptive identification of salmonellae on agar media requires a minimum of 4 days with an

additional 2 to 3 days for biochemical screening and serological confirmation of isolates (D'aoust *et al.*, 1983).

2. Rapid Detection Methods

The necessity of rapid methods of *Salmonella* detection for the regulatory and industrial sectors of the food industry is increasingly important. A rapid method should be cost effective, as sensitive as the standard procedure, and simple if it is to gain acceptance and become widely used (Andrews, 1985). Rapid procedures to detect salmonellae in foods have been of two types; biological, based on the biochemical or growth characteristics of the molecule, and immunological, based on the serological or antigenic characteristics (Mattingly *et al.*, 1985).

i. Modification of the Conventional Method

Many researchers attempts at speeding up the detection of *Salmonellae* have been made by modifying the conventional method in order to reduce time requirements and/or increase sensitivity.

To reduce time requirements some have attempted to shorten the length of the pre-enrichment stage. An optimum pre-enrichment incubation period for effective recovery of injured cells has yet to be determined (D'Aoust and Maishment, 1979). However, short pre-enrichment periods such as 6 hours lead to large numbers of false negative results (D'Aoust and Maishment, 1979, D'Aoust

and Sewell, 1988). In addition, injured cells may not have enough time to recover and replicate to sufficient numbers for detection. Even with a pure culture, studies showed that six hours was not sufficient for the resuscitation of injured cells (van Schothorst *et al.*, 1972, cited in D'Aoust, 1984).

Another attempt to shorten the assay time has been to omit the pre-enrichment step and incubate samples directly in the selective enrichment. This method has been found to significantly decrease the sensitivity of the procedure (D'Aoust, 1984). Ideally, selective media is damaging to competing microorganisms and selective for *Salmonella*.. It may, however, also damage *Salmonella*, especially those injured in processed foods and therefore they may have difficulties replicating in this broth.

Selective and pre-enrichment phases also have been incorporated into one broth. Sveum and Kraft, (1981) used non-selective broth for a 4 h incubation that was followed by the addition of selective agents and further incubated for a total of 24 h. This modification has compared well with conventional methods and has reduced total assay time by one day (Sveum and Kraft, 1981). Wax-coated capsules containing selective agents have been added to non-selective basal broths. The capsules gradually release selective agents in order to make the media more selective (Sveum and Hartman, 1977). However, a 48 h incubation was required to reach isolation rates comparable with conventional methods (Blackburn, 1993).

Supplemented TSB pre-enrichment media was incubated for 4 hours followed by the addition of agents to make the medium selective towards *Salmonellae* (Martin and Katz, 1991). Injured *Salmonella bonn* was incubated in this medium in the presence of equal initial numbers of uninjured *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*. After 8 h of incubation there was a 100-fold greater population of *S. bonn* than all other organisms. After 20 hours, the numbers of *S. bonn* were 10^3-fold greater than *E. coli*, and 10^6-fold greater than those of the other two bacteria (Martin and Katz, 1991).

Direct plating of inoculated pools of egg contents onto selective media has been attempted (Gast, 1993b; Gast and Holt, 1995b). However, in order to achieve 100% detection sensitivity by this method, pools containing 10 eggs need be contaminated with 10^7 or more *Salmonella* (Gast and Holt, 1995b). Because of its relatively high detection threshold, culturing of egg pools by direct plating is heavily dependent on the extent of multiplication of *Salmonella* during incubation of the pools (Gast and Holt, 1995a).

Media has been modified in order to increase the sensitivity of the conventional assay. Most modifications of pre-enrichment media have been targeted at the enumeration of *Salmonella* in eggs and egg products (van der Zee, 1994).

Pre-enrichment of egg samples has achieved significant success with addition of iron to the pre-enrichment broth. It has been established with albumen in vitro and in whole shell eggs, that iron chelation by ovotransferrin is the major growth inhibitor of gram-negative bacteria (Clay and Board, 1991). By the addition of iron the growth of these organisms is enhanced (Reissbrodt *et al.*, 1996). For example, addition of 35mg/mL ferrous sulphate to TSB pre-enrichment medium improved the detection of *S. enteritidis* inoculated into whole eggs (Gast, 1993a). When *S. enteritidis* is inoculated into pools of homogenized whole egg containing ferrous sulphate, after incubation at 37 °C for 24 hours, average counts of *S. enteritidis* were one log higher than for inoculated pools of eggs incubated without added iron (Gast and Holt, 1995b; Cudjoe *et al.*, 1994a). The siderophore ferrioxamine E can be used by *Salmonella* in iron restricted environments but not by many other enteric bacteria (Reissbrodt *et al.*, 1996). By incorporation of this compound into pre-enrichment broth, *Salmonella* are more effectively isolated from mixed cultures (Reissbrodt *et al.*, 1996).

Selective enrichment media has also been modified in order to increase specificity towards *Salmonella.* The combined use of Salmosyst broth and Rambach agar, which utilizes the ability of salmonellae to produce acid from propylene glycol for their differentiation from other enteric bacteria, was found to have a 97.9% sensitivity as compared with an 81.2% sensitivity using the

conventional method (Pignato *et al.*, 1995). Using Salmosyst as both pre- and selective enrichment broth, and Rambach agar for selective isolation, the time necessary for the test was reduced from 4-6 days to 48 h and media requirements are reduced significantly (Pignato *et al.*, 1995).

Semi-solid agar allows the preferential growth of motile organisms and has been used as a selective enrichment medium, resulting in higher isolation rates of *Salmonella* (Van der Zee, 1994). Motility enrichment assumes that the motility of salmonellae is less impaired by the selective medium than the motility of competitive bacteria (Busse, 1995). Semi-solid agar will not detect any non-motile strains of *Salmonella. As S. enteritidis* are motile, this will not limit isolation of this particular species. Rappaport-Vassiliadis media (MSRV) has been modified to be semi-solid (de Smedt and Bolderdijk, 1987). Using this motility agar it was found that 60 cells of *Salmonella*/mL in pre-enrichment media after incubation were necessary for a positive result. When one drop of media is added to the semi-solid plate, the few *Salmonella* present in that one drop are capable of motility and growth on the MSRV medium. Competitors are inhibited by this medium even when they outnumber *Salmonella* by a factor of 10^7 (de Smedt and Bolderdijk, 1987).

ii. Alternative Detection Methods

Before *Salmonella* can be detected by an alternative rapid method, especially if it is to be isolated from a food product, cultural enrichment is almost always necessary in order to bring the population of *Salmonella* up to detectable numbers (Reissbrodt and Rabsch, 1993). Rapid detection systems cannot actually detect the lower limit of natural contamination of one cell/25g sample. They must rely on an enrichment step to furnish detectable concentrations, usually 10^5 or 10^6 CFU/mL of *Salmonella* is necessary. Depending on the method, the enrichment step may also need to be selective for the target organism (McClelland and Pinder, 1994).

a. Fluorescent Antibody Assay

The fluorescent antibody assay (FA) was one of the first immunologically based methods used to detect S*almonellae* (Mattingly *et al.*, 1985). This procedure is based on staining of the O, Vi and H antigens of *Salmonella* by use of specific antibodies labeled with fluorescein. The FA assay reduces the time necessary to detect *Salmonella* by one to three days (Thomason, 1981). This assay is limited by the specificity of the antibodies used, (Thomason, 1981) although by using a combination of antibodies the specificity of the FA technique has been improved (Blackburn, 1993). Products containing high numbers of enteric flora cannot be

tested by the FA assay as enterics will outgrow *Salmonella* leading to large numbers of false positive results (Thomason, 1981). This assay requires post-enrichment to dilute the food product which would otherwise contribute to background fluorescence (Munson *et al.*, 1976). Expensive equipment and well trained analysts are also necessary (D'Aoust, 1984).

b. DNA-DNA Hybridization

Several DNA-DNA hybridization tests for the detection of *Salmonella* have been recently available. Through the systematic screening of *Salmonella* DNA libraries and hybridization studies, sequences have been found that are present in all or most strains of *Salmonella* (Groisman *et al.*, 1993). It has been difficult to isolate a DNA segment specific to *Salmonellae* because *Salmonella* have no unique virulence factors or toxin genes (Tsen *et al.*, 1994). Several suitable segments in *Salmonella* spp. have been found for amplification (Bej *et al.*, 1994; Cano *et al.*, *1993;* Jones *et al.*, 1993; Tsen *et al.*, 1994). Using gene cloning techniques and oligonucleotide synthesis almost any nucleic acid sequence can be prepared in large quantities and used as a probe to identify the specific unique gene codes of the target organism (Walker and Dougan, 1989). The Polymerase Chain Reaction (PCR) is probably the preferred system for the amplification of target DNA. However, under non-stringent conditions hybridization with DNA from non-target members of *Enterobacteriaceae* will occur (Baumler *et al.*, 1997).

The amplification of the wrong sequences could lead to false-positive results (Kwok, 1990; Erlich et al., 1991). For this reason PCR generally shows a major improvement in speed though not necessarily sensitivity compared with viable culture detection methods (Bej et al., 1994). PCR takes 3-5 hours if pre-enrichment is not necessary and between 10-24 hours with pre-enrichment of samples (Cohen *et al.*, 1994b; Cohen *et al.*, 1994a). There are specific problems associated with the use of PCR for detection of food-borne organisms. Many food components inhibit the PCR (Grant and Kroll, 1993; Soumet *et al.*, 1994), therefore the detection sensitivity of the method will vary with the food samples tested (Tsen *et al.*, 1994). Blood, urine, faecal material and bilirubin, a degradation product of haemoglobin, also inhibit PCR (Widjojoatmodjo *et al*, 1992). Amplification of DNA leads to the detection of both viable and non-viable organisms which will lead to false-positive results. This method, especially when combined with PCR, requires expensive equipment.

The DNA amplification procedure by PCR has been combined with immunological methods (Widjojoatmodjo *et al.*, 1992; Luk, 1994). A detection assay for *S. enteritidis* in eggs has been developed which combines modified pre-enrichment with PCR amplification of *iroB*, a gene segment specific to all subspecies of *Salmonella enterica* (Baumler *et al.*, 1997). After pre-enrichment the *Salmonella* was isolated by immunomagnetic separation to remove substances

that may interfere with the PCR (Baumler *et al.*, 1997). PCR amplified products were examined by southern hybridization.

c. Enzyme Linked Immunosorbent Assays

Enzyme immunoassays are attractive alternatives to traditional culture methods. Using a 96 well microtiter plate about 30 triplicate samples can be assayed at one time (Robison *et al.*, 1983). There is also a significant decrease in time requirements compared to the conventional method. Immunoassays using polyclonal or monoclonal antibodies against *Salmonella* have been developed. In order for an EIA to be effective the antibody specificity and affinity for the target antigen must be high (Mattingly *et al.*, 1985). Many antigens are shared by *salmonellae* with other members of *Enterobacteriaceae* leading to the possibilities of false positive results (Kerr *et al.*, 1992). Conversely, some antibodies have been developed that will not detect all strains of *Salmonella* (Robison *et al.*, 1983). Combinations of antibodies have been used in immunoassays to improved the detection of all strains of *Salmonella* (D'Aoust and Sewell, 1988). Antibodies have also been developed that are specific to a particular strain of *Salmonella* (Holt *et al.*, 1995; Lee *et al.*, 1989; Lee *et al.*, 1990; Masi and Zawistowski, 1995)

Immunomagnetic separation, using magnetic beads with anti-*Salmonella* antibodies affixed to their surface have been used to isolate *Salmonella* from mixed culture The beads, when mixed with a *Salmonella* contaminated product,

will bind the organisms to their surface via the antibodies. The *Salmonella*-bead complex can be pulled out of solution by a magnet and the organisms thus concentrated. The beads can then be plated on differential culture media and the organisms can be identified using an immunoassay or DNA probe specific for *Salmonella* (Cudjoe *et al.*, 1994a,b; Holt *et al.*, 1995). The high viscosity of some matrices leads to loss of magnetic beads during washing which affects the results of the assay (Cudjoe *et al.*, 1994a).

Flow cytometry and monoclonal antibodies have been combined to detect *S. typhimurium* in dairy products (McClelland and Pinder, 1994). The sample, containing fluorescently labeled anti-*S. typhimurium* antibodies attached to any *S. typhimurium* present, is injected into a fluid that passes under an objective via a hydrodynamic focusing flow cell. The sample passes through a beam of light that causes the labeled cells to emit fluorescent pulses, and each pulse is detected by a photomultiplier tube (Brailsford and Gatley, 1993). By computer analysis the number of cells present is determined. Using a milk sample clearing procedure, which causes the fat to rise to the surface of the sample for removal by centrifugation, this method was able to detect *S. typhimurium* in concentrations as low as 10^3 cells/mL in an analysis time of 40 minutes. After 6 h of non-selective enrichment the detection limits were 10 cells/mL in milk and 1 cell/mL in eggs,

even when *E. coli* outcompeted the targeted *S. typhimurium* 10,000 fold (McClelland and Pinder, 1994).

d. ELISA based test kits.

Several immunologically based detection kits have been developed for the detection of *Salmonella* spp. and are available commercially.

The PATH-STIK contains all the reagents needed to detect the presence of *Salmonella* in enriched broth media. When the bottom part of the stick is dipped into the enriched broth, the sample is carried into the device by a wick. The sample crosses the reagent pad containing anti-*Salmonella* antibodies and onto the membrane strip, where the end result is visualized as a pink/purple line after ten minutes. A positive control is included, and if two lines appear after ten minutes the sample is considered positive for *Salmonella*. The appearance of only one line after the wait is a negative result (Brinkman *et al.*, 1995). The PATH-STIK test compares well with conventional methods with an overall sensitivity of 93.0% and specificity of 96.4% towards *Salmonella* spp. (Brinkman *et al.*, 1995).

The Salmonella-TEK screen kit incorporates two monoclonal antibodies specific to *Salmonella* spp. bound to the well of a microtiter plate to detect the presence of these organisms (Van Pouke, 1990). Using Salmosyst broth for enrichment followed by detection with Salmonella TEK the total analysis time for detection of *Salmonella* has been reduced to 31 hours (Van Pouke, 1990).

The BioEnzabead enzyme immunoassay technique detects *Salmonella* antigens in heated extracts of M-broth cultures. The extracts are reacted with polyvalent myeloma protein (IgA) and monoclonal (IgG) antibodies iron adsorbed to the surfaces of polycarbonate-coated ferrous metal beads held in microtiter plates. The protein-antigen complex on metal beads is reacted with a peroxidase conjugate. Development of a green colour upon addition of enzyme substrate is indicative of *Salmonella* contamination (D'Aoust and Sewell, 1988). This assay was found to have only 1.6 % false negative rate. Cross-reactivity ranged from 2 to 40 % with members of *Enterobacteriaceae*, and *Pseudomonas* spp. (D'Aoust and Sewell, 1988).

D. Necessity of Iron

Iron is essential to life in practically all living cells (Cheng *et al.*, 1988). Within a cell iron functions as a catalyst. The suitability of iron as a biocatalyst may be due to its two stable valencies and wide range of oxidation-reduction potential (Payne, 1988). The capacity of microorganisms to compete for the iron they need for growth and metabolism is an essential attribute of virulence (Colonna *et al.*, 1985). An important host defense system aimed at microbial starvation appears to be limitation of the free iron supply (Puschmann and Ganzoni, 1977). When bacteria are starved of iron they typically show a reduced

growth rate and exhibit morphological changes suggesting inhibition of DNA synthesis or cell division (Payne, 1988). The absolute amount of iron required for growth is often difficult to determine and is influenced by culture conditions (Payne, 1988).

A correlation between virulence and iron assimilation has been established for a large number of animal pathogens (Visca *et al.*, 1991). A total of 21 strains from different species of *Salmonella* were isolated from environmental waters and examined for the presence of iron uptake systems. All strains were found positive for synthesis of some kind of iron chelating compounds (Aznar *et al.*, 1989).

1. Mechanisms of iron transport

The mechanism of iron transport in enterics depends on the concentration of free iron in the environment. When iron is freely available, at concentrations of at least 5-10 μM iron (Earhart, 1996) it is thought to enter the cell through a low affinity transport system. As iron becomes limiting, higher-affinity systems are required to maintain sufficient intracellular levels of iron (Payne, 1988). The ability of bacteria to avail themselves of bound iron correlates with the biosynthetic ability of the microorganism to excrete iron-binding compounds, or iron transport compounds, when grown in media containing sub-optimal amounts of iron (Garibaldi, 1970). Siderophores are low molecular weight, iron-chelating compounds with very high affinities for iron (Payne, 1988). These compounds are

synthesized and secreted by microbes in response to iron stress. Organisms able to produce siderophores sequester most or all available iron and thus may repress the growth of other organisms in the same environment (Freedman *et al.*, 1989). Strains of bacteria can use not only endogenously produced siderophores, but also siderophores produced by other organisms, due to specific transport systems (Earhart, 1996). Siderophores are derepressed in low iron environments, and have high enough affinities for iron to solubilize it or remove it from lower affinity complexes (Payne, 1988). Enterobactin and aerobactin are well-known siderophores of *Enterobacteriaceae* (Reissbrodt and Rabsch, 1993). In a total of 230 *Salmonella* strains screened for enterobactin and aerobactin production, all isolates were found to produce the siderophore enterobactin. Only 26 isolates, all belonging to *Salmonella wien,* produced the siderophore aerobactin (Visca *et al.*, 1991). Bacteria transport Fe^{3+} siderophores into the cell with special transport systems (Reissbrodt and Rabsch, 1993). *Salmonella* has a transport system for some of the ferrioxamin-type siderophores such as ferrioxamine E (Reissbrodt and Rabsch, 1993). As ferrioxamine E is utilized by few other microorganisms including *Citrobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *Enterobacter* spp. and *Yersinia enterocolitica* (Reissbrodt *et al.*, 1996) the ability to use this siderophore to obtain iron is fairly specific to *Salmonella.*

2. Iron and eggs

Because *S. enteritidis* is a significant pathogen associated with eggs, its ability to survive and/or grow in the albumen has been studied. The albumen contributes both chemical and mechanical defense to the egg. The viscosity of the proteins constituting the albumen hampers the movement of bacteria so that they suffer impeded passage to the yolk. The albumen contains the antimicrobial component lysozyme, and an alkaline pH (9.5) which, as well as being unfavourable to bacterial growth encourages the chelation potential of ovotransferrin (Board and Tranter, 1994). The levels of 'free' iron in egg white are considerably lower than those necessary for bacterial growth (Chart and Rowe, 1993). *S. enteritidis* growth has been found to be significantly retarded when incubated in trypticase soy broth containing ovotransferrin. Exponential growth was delayed by about 2 hours in this medium due to the presence of ovotransferrin (Chart and Rowe, 1993). The lag in growth rate is thought to be caused by *S. enteritidis* adapting to the iron restricted environment, by expressing iron-regulated outer membrane proteins and the siderophore enterobactin (Chart and Rowe, 1993). Enterobactin has a much higher binding affinity for iron than that of ovotransferrin (Chart and Rowe, 1993). However, the principal siderophore of *Salmonella*, enterobactin, is ineffective in a medium containing large amounts of the protein albumen (Reissbrodt and Rabsch, 1993). *Salmonella enteritidis* found

in the whites of intact shell eggs do not generally multiply to any significant extent. Occasionally, large numbers have been found in a single egg, usually after several weeks of storage. This is thought to be due to migration of the yolk towards the microorganism so that its nutrients are accessed (Bradshaw *et al.*, 1990).

In experiments when iron was incorporated into the bacterial inoculum before inoculating an egg, the shell eggs were spoiled by all 10 species tested, these included strains of *S. typhimurium* and *Salmonella pullorum*. Of the ten species, only four were able to spoil the egg if inoculated without iron; *Pseudomonads*, *Aerobacter*, *Alcaligenes* and *Paracolobactrum* (Garibaldi and Bayne, 1961). *S. enteritidis* was not utilized in these experiments. When control eggs were immersed in sterile water containing 10 ppm of iron, the natural inoculum on the nest-clean eggs was able to penetrate through the shell and spoil 2 of the 12 eggs tested (Garibaldi and Bayne, 1961). This demonstrates the significant contribution of iron to the spoilage of shell eggs and indicates that the lack of available iron in albumen plays a major contribution to its antimicrobial defense.

3. High iron concentrations

Inhibition of bacterial growth occurs at high iron concentrations. This has been explained as being due to the precipitation of ferric hydroxide which attaches

to the cell wall of the bacterium and decreases the cell wall's permeability to nutrients and oxygen (Khoury-Doughly *et al.*, 1976).

E. Competition

In mixed microflora, the competition between species may have a considerable effect on the relative rates of population growth (Rhodes *et al.*, 1985). In fresh, raw materials such as poultry and egg products, the major part of the flora consists of *Enterobacteriaceae* and *Pseudomonads* which will readily compete with *Salmonella* (Brinkman *et al.*, 1995). *Pseudomonas* spp. are potent inhibitors of pathogenic bacteria associated with food and plants (Cheng *et al.*, 1995). The antimicrobial activity of pseudomonads is attributed, in part, to production of the extracellular iron-chelating molecules, siderophores which sequester any available iron leaving the pathogens iron starved (Cheng *et al.*, 1995). In some food systems, the dominant flora is comprised of organisms such as lactic acid bacteria. These organisms produce a variety of substances that inhibit growth of pathogenic bacteria. The inhibitors include acids, H_2O_2, bacteriocins and others (Freedman *et al.*, 1989). Increasing the amount of nutrients may favour the growth of competitive microorganisms which may, in turn, have an inhibitory effect on the growth and isolation of salmonellae (van Schothorst and Renaud, 1982). Non-selective enrichment of microorganisms is

thought to consist of two phases. During the first phase, different microorganisms multiply independently, as long as one does not inhibit another, until a maximum level, or molar concentration, is reached by a dominant species, either due to a higher initial level or shorter generation time. During the second stage of non-selective enrichment the microorganisms in minority will continue to multiply and equalize in numbers with the dominant species (van Schothorst and Renaud, 1982).

1. In selective media

In selective media, ideally, the competitive flora are inhibited or reduced, and the *Salmonella* multiply with a mean generation time of about one hour. When there are 'too many' competitive bacteria at the beginning of enrichment, *Salmonella* do not start to multiply until the number of competitors is sufficiently reduced (van Schothorst and Renaud, 1982). With more than 10^7 competing CFU/mL present in the inoculum, the isolation of *Salmonella* on BGA agar becomes virtually impossible because of the heavy growth of competitors, especially *Klebsiella* and *Enterobacter* species (van Schothorst and Renaud, 1982). As far as Gram-positive bacteria are concerned, selective media for salmonella are quite efficient (Busse, 1995). However, even in selective media many Gram-negative bacteria may overgrow *Salmonella* spp..

Studies have shown that the growth rate of *S. typhimurium* from concentrations as low as 10^2 CFU/mL is unaffected by the presence of competitors at concentrations of up to 10^5 CFU/mL. Growth is inhibited when the competitor load exceeded 10^5 CFU/mL and no growth occurs with 10^8CFU/mL of competing organisms (Duffy *et al.*, 1995; Beumer *et al.*, 1991). A study conducted to assess the growth of *S. typhimurium* in a hydroponic nutrient solution in the presence of normal microflora (Riser *et al.*, 1985) found that the normal flora grew exponentially in the presence of *Salmonella* and reached optimum levels by 24 h. Growth of *Salmonella* was generally retarded during this period. Therefore the presence of normal flora restricted multiplication of *Salmonella*. Once the aerobic bacterial counts had reached a saturation level for the nutrient solution, *Salmonella* counts began to decrease, regardless of their initial or final concentration. This was due to competition for nutrients by more aggressive organisms (Riser *et al.*, 1985).

2. Competition within eggs

Gram-positive microorganisms tend to dominate contamination of outer surfaces of egg shells while Gram-negative bacteria cause the rotting of the contents. Factors in the egg, including low levels of readily available non-protein nitrogenous substances in the albumen, select for the fast growing but non-fastidious Gram-negative bacteria (Dolman and Board, 1992). A study was

conducted to examine the relative growth of five different microorganisms in the air sac of hens eggs. The five organisms; *Pseudomonas putida, S. enteritidis* (both isolated from eggs), *E. coli, Enterococcus faecalis* and *Staphylococcus xylosus* (isolated from chicken faeces) were inoculated into the air sacs of chicken eggs and stored at various temperatures. All five organisms were isolated from the inner membrane of the air cell of eggs incubated at 4°C. Only *S. xylosus, S. enteritidis*, and on one occasion, *E. faecalis* were recovered from the membrane in eggs stored at 37°C. The pseudomonad attained numerical dominance in both inner membrane of air cell and in the albumen of eggs stored at 15 or 20°C. *S. enteritidis* failed to compete with pseudomonads in eggs incubated below 30°C, but out-competed *E. coli* in eggs incubated at 37°C (Dolman and Board, 1992).

F. Stability of biologically active proteins.

Enzymes and antibodies are becoming increasingly important to industry, for the production of food as well as for incorporation into assay kits. Antibodies are convenient and valuable tools for various immunochemical and biochemical analyses. The stability of antibodies over the short term does not necessarily warrant much attention regarding their use as reagents for immunochemical analyses where they are generally stored and used under mild conditions (Shimizu *et al.*, 1993). However, the variability of the immunological activity during

storage is an important factor when ELISAs are to be carried out over extended periods of time (Montoya and Castell, 1987) or when an immunoassay detection kit is to be stored for periodic use. Monoclonal antibodies are usually stored as small aliquots at -20 °C or -70 °C, or they may be stored at 2-4 °C with added preservatives to retard microbial growth (Draber *et al.*, 1995). Frozen or refrigerated storage becomes difficult in areas where there is limited access to refrigeration (Blakeley *et al.*, 1990) and inconsistent transport or storage temperatures become a problem as freeze-thaw cycles are potentially damaging to monoclonal antibodies (Draber *et al.*, 1995).

The technical, therapeutic, or commercial value of a protein is measured by its activity (Franks *et al.*, 1991). For isolated and purified solutions of proteins to be viable commercial products capable of being shipped and stored, they have to be stable for weeks, months, or even years (Franks *et al.*, 1991). For this reason, methods to increase the long term stability of antibodies have been examined.

1. Natural Stability of globular proteins

Globular proteins are naturally stabilized by a combination of hydrogen bonding, electrostatic interactions, and hydrophobic interactions as well as cross-linking, metal complexing and specific binding of ions and cofactors. The amino acid composition and sequence in the protein define its functionality in specific

environments. The ordered, functional structures of proteins reflect two tendencies that are often opposed. On the one hand, proteins fold to minimize their free energy. Minimizing free energy leads to tightly packed hydrophobic interiors and hydrophilic exteriors. On the other hand, proteins organize themselves to recognize a ligand (Shoicet *et al.*, 1995). Maximum function leads to active-site clefts where charged and polar groups are isolated from water and where hydrophobic areas are exposed to the solvent (Shoicet *et al.*, 1995). Protein residues that contribute to catalysis or ligand binding are not optimal for protein stability. There is a balance between stability and function. The folded configuration of globular proteins is only 5-10 kcal/mol more stable than unfolded even in ideal environmental conditions (Pace *et al.*, 1988). Most proteins are readily inactivated by even mild chemical or physical treatment. In solution, most globular proteins will lose activity over time even under optimal environmental conditions due to degradative processes such as oxidation and deamination (Franks *et al.*, 1991). Because of the inherent low stability of globular proteins and the importance of maintaining their stability, numerous approaches to increase protein stability have been applied.

2. Solute Addition

i. In aqueous solution.

Addition of stabilizing solutes to labile proteins is a common means of protecting them during preparation and storage (Carpenter *et al.*, 1990). In aqueous solution a diverse number of solutes have been shown to be effective at minimizing protein denaturation due to environmental stress (Carpenter *et al.*, 1990). The only common characteristic shared by these solutes is preferential exclusion from the protein's surface. This preferential exclusion causes the native structure of monomers and the polymerized form of oligomeric proteins to be stabilized because denaturation or dissociation, respectively, would lead to a greater contact surface between the protein and the solvent (Carpenter and Crowe, 1988b). Conversely, preferential binding of co-solvent would induce depolymerization since there is greater cosolvent binding to monomers than to the polymer (Carpenter *et al.*, 1990).

The sugars, such as sucrose and invert sugar have been found to protect hen egg yolk immunoglobulin (IgY) from heat, acid and pressure in a concentration dependent manner (Shimizu *et al.*, 1994). Unprotected IgY loses activity at temperature of 75°C or higher. The addition of 30-50% of either sugar to the IgY solution suppresses denaturation up to temperatures of 80°C (Shimizu

et al., 1994). Greater than 30% of either sugar also protects IgY to acid conditions of pH 3.0 and pressure up to 5000 psi (Shimizu *et al.*, 1994).

For a solute to provide protection to a given protein in solution it must be present in relatively high concentration in order for preferential exclusion to occur (Carpenter *et al.*, 1990). High concentration of solute will ensure that there is a deficiency of the solute in the immediate domain of the protein relative to the solute concentration in the bulk solution.

ii. in dehydrated systems

Dried solid proteins are generally more stable than the corresponding aqueous solutions (Pikal *et al.*, 1991). Conventional drying techniques, using high process temperatures often result in altered or decreased biological activity of the protein (Adams, 1991). Freeze-drying was developed to avoid the damage that may occur upon drying. Freezing reduces the rate of denaturing chemical reactions and concentrates the product in a low-temperature viscous phase between crystals of pure water ice. Water is thought to be indispensable for the maintenance of the structure and function of a protein since it is intimately involved in the folding that is mandatory for a polypeptide to become a protein (Carpenter *et al.*, 1987b). Removal of the residual water that functions to 'screen' charged groups on the protein surface can lead to distortions of protein conformation as the charged groups satisfy their bond requirements with other

charged residues. Addition of protective agents while removing residual water, can result in stable freeze-dried products with very long shelf lives at ambient temperatures.

Only certain carbohydrates such as disaccharides, can preserve enzyme activity during desiccation (Carpenter *et al.*, 1990). The addition of 100 mM sucrose to myosin ATPase leads to retention of 92 % of its original activity, addition of 30 mM sucrose to catalase prior to freeze-drying results in recovery of 85 % of the original activity (Crowe *et al.*, 1987). Trehalose and maltose have been found to be the most effective organic solutes for the protection of phosphofructokinase, one of the most labile enzymes known. Glucose was found to be much less effective than disaccharides; trehalose and maltose, suggesting that the subunit orientation of the sugar moiety is critical for enzyme stabilization (Carpenter *et al.*, 1987a). The ability of sugars to protect proteins during desiccation can be attributed to their ability to hydrogen bond to proteins and thereby substitute for structural water (Roser, 1991). The importance of hydrogen bond formation between trehalose and dehydrated lysozyme has been demonstrated. Freeze-dried lysozyme was stabilized when trehalose formed hydrogen bonds with the enzyme upon dehydration. When trehalose did not bind via hydrogen bonding to the protein during desiccation, no protein stabilization occurred (Carpenter *et al.*, 1990).

In the case of desiccation, the protection given by the solute is not strictly concentration dependent. At higher concentrations the disaccharide may crystallize leaving less sugar available to hydrogen bond with the protein (Carpenter *et al.*, 1990). The maintenance of amorphism by the solute is thought to be an essential property for stabilization (Izutsu *et al.*, 1993).

Draber and coworkers (1995) studied the stability of IgM antibodies freeze-dried in the presence of trehalose. They found that the binding activities of the freeze-dried antibodies were retained, without any observable decrease, for two years of storage at 4°C or at ambient temperature. After 14 days of storage at 50°C trehalose still stabilized the freeze-dried antibody (Draber *et al.*, 1995).

3. Structural modification

Many proteins contain cross-links, the function of which is presumably to stabilize the native conformation. This is particularly true of intra-chain disulfide cross-links (Johnson *et al.*, 1978). The folded conformation of a protein can generally be stabilized through the addition of a cross-link either by chemical modification or by the addition of a disulfide bond (Pace *et al.*, 1988). Disulfide bonds make a substantial contribution to the maintenance of the tertiary structure of proteins. Attempts have been made to introduce disulfide bonds to proteins to improve stability (Matsumara *et al.*, 1989a). The disulfide bridge is thought to

reduce the configurational backbone chain entropy in the denatured protein, thus increasing the stability of the folded protein. Matsumara and coworkers (1989b) designed and constructed four different disulfide mutants of the naturally disulfide-free enzyme T4 lysozyme. They found that mutants with double disulfides outside of the active site cleft of the enzyme had activity identical to the activity of the wild-type enzyme, both in oxidized and reduced forms. The double disulfide mutant was more stable than the wild-type enzyme, retaining activity up to 10°C higher temperature than the wild type. The double mutants were more heat stable than the single mutants, and the triple mutant had a melting temperature 23.4°C higher than the wild-type enzyme. However, mutants with disulfide linking residues which spanned the active site cleft of the enzyme showed virtually no enzymatic activity when oxidized (Matsumara *et al.*, 1989b).

Perry and Wetzel (1984) also introduced a disulfide bond to phage T4 lysozyme. They then tested the protein stability by heating both the wild type and the mutant enzyme to 65°C and removing portions at various times to test for residual activity. The disulfide mutant never lost more than 50% of its initial activity while the wild-type only had 0.2% of starting activity remaining after 2 hours at 65 °C (Perry and Wetzel, 1984).

The thermostability of enzymes has also been improved by replacing internal amino acids. In one study, the polar amino acid, asparagine, located in the

internal apolar environment of a bacterial protease was replaced with a non-polar leucine by site-directed mutagenesis (Eijsink *et al.*, 1991). The thermostability of the mutant was 0.7 +/- 0.1°C higher than that of the wild-type enzyme.

III. MANUSCRIPT 1

OPTIMIZATION OF THE DOT-BLOT IMMUNOASSAY FOR THE DETECTION OF *SALMONELLA ENTERITIDIS.*

A. ABSTRACT

An immunoassay was developed for the detection of *Salmonella enteritidis* in eggs, poultry, and other foods and feeds. This assay comprises a two-step procedure that involves an enrichment step followed by a monoclonal antibody based dot-blot assay. Homogenized whole egg was used as the enrichment medium. After inoculation with 25 mL of sample and incubation for 20 hours at 37 °C the egg medium is heated to 100 °C for 10 min in the presence of cholic acid to liberate the lipopolysaccharide (LPS) antigen from *S. enteritidis*. The LPS was uniformly dispersed throughout the gelled egg matrix and subsequently transferred onto a solid support by diffusional forces. It was detected by Mab 2F11 which is specific to the O-9 antigen of *S. enteritidis*. This test does not require expensive equipment or trained personnel. Commercially available media were compared with egg homogenate for relative ability to resuscitate and propagate *S. enteritidis* to detectable levels. All other experimental conditions remained constant. Incubation in egg, tripticase soy broth (TSB), and lactose broth (LB) resulted in comparable levels of *S. enteritidis* as demonstrated by viable plate counts. *S. enteritidis* grown in TSB exhibited the strongest visual representation showing a

positive test for *S. enteritidis*. Incubation time necessary to detect one *S. enteritidis* cell was reduced from 20 to 10 h using TSB as the enrichment broth. Addition of a detergent, cholic acid, before incubation had negligible negative effects on the growth of *S. enteritidis*. When incubated with a mixed flora of competitive microorganisms, $1{:}10^2$, *S. enteritidis* was able to reproduce to detectable numbers for the immunoassay when out competed $1{:}10^2$ in the initial inoculum. This was true for samples incubated in TSB and in homogenized whole egg supplemented with ferrous sulphate (85 μg/mL). When environmental samples were tested, the same ratio of *S. enteritidis* to competitive flora was detected.

B. INTRODUCTION

The incidence of human illness associated with foods contaminated with *S. enteritidis* has increased throughout the last 20 years. *S. enteritidis* is presently the second most frequently reported *Salmonella* serovar isolated from human infection in the United States (Barnhart *et al.*, 1991). The prevalence of *S. enteritidis* in Canada is much lower (Poppe, 1991). The detection and irradication of this organism are important, especially as the reasons for its recent increase in

prevalence throughout the world are not known. Eggs, egg products, poultry and poultry products are considered the major sources of *S. enteritidis* (Ruzickova, 1994).

Detection of *S. enteritidis* is difficult. Isolation of specific species with conventional methods depends largely on differences in biochemical reactions, resistance to antibiotics etc., between the target organism and the competing microflora (Van der Zee, 1994). Conventional detection methods only differentiate between *Salmonella* spps. at the confirmation stage. As the culturing and identification of these bacterial isolates requires up to one week, traditional detection of *S. enteritidis* is also labour intensive and expensive.

Rapid methods for the specific detection of *S. enteritidis* are scarce. Several ELISA systems have been developed for the detection of *Salmonella.* Most of these assays only replace the agar-plating stage of the cultural assay and may introduce a third post-enrichment broth before the sample can be assayed (Lee *et al.*, 1990). These assays have the advantage of great time reduction over the conventional method, taking as little as 18 hours (Lee *et al.*, 1989) but they do suffer from some disadvantages including cross-reaction with other organisms (Lee *et al.*, 1989).

A novel immunoassay has been developed for the detection of *S. enteritidis* in eggs, poultry and other foods and feed. This system does not require highly

trained technicians or the expensive equipment needed for conventional ELISA or fluorescent antibody techniques, so it can be conducted in any laboratory. The ELISA based system utilizes a monoclonal antibody produced and characterized by Masi and Zawistowski (1995). This antibody is specific to the lipopolysaccharide O-9 present in *S. enteritidis*. Using homogenized, whole egg as the enrichment medium, and adding ferrous sulphate, the assay is able to detect one *S. enteritidis* cell in 500g homogenized whole egg after 20 hours incubation at 37 °C giving a total test time of one day (Yoshimasu, 1996). This paper compares synthetic pre-enrichment media with the homogenized whole egg for relative ability to detect *S. enteritidis* by the immunoassay. Attempts to shorten and optimize the assay are also examined.

C. MATERIALS AND METHODS

1. Reagents and chemicals

RPMI Medium 1640 with L-glutamine was purchased from Gibco BRL-Life Technologies (Grand Island, NY). Magnesium chloride was purchased from Fisher Scientific (Fairlawn, NJ). Standard methods agar, selenite cysteine broth, nutrient broth, lactose broth, trypticase soy broth, brilliant green agar with sulphadiazine, triple sugar iron agar and lysine iron agar were purchased from

BBL (Cockeysville, MD). Peptone, Salmonella O antiserum factor 9, Salmonella O antiserum factor 4,5 and Salmonella O antiserum Poly A-I & Vi were purchased from Difco. Agar was purchased from Metheson Coleman and Bell (Rutherford, NJ). Cholic acid was purchased from Sigma Specialty Chemicals Co. (Paris, KY). Electrophoresis grade hydroxymethyl aminomethane (Tris), NBT (nitroblue tetrazolium chloride), and BCIP (5-bromo-4-chloro-3-indolyphosphate) were purchased from BioRad Laboratories (Hercules, CA). Streptavidine alkaline phosphatase was purchased from Cedarlane Laboratories Ltd (Hornby ON). Ferrioxamine E was kindly provided as a gift by Dr. R. Reissbrodt, Federal Health Office, Wernigerode, Germany. All other chemicals were analytical grade or better and purchased from Mallinckrodt Specialty Chemicals Co. (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Distilled deionized water (Barnstead NANOpure, series 550, ULTRApure water system D4754 115 VAC: Barnstead\Thermolyne Corp., Dubuque, IA) was used in all experiments.

2. Monoclonal antibody

Monoclonal antibody 2F11 (ATCC HB-11891) was produced and characterized by Masi and Zawistowski (1995). Tissue culture supernatant that was purified using ammonium sulphate was dialyzed against PBS (pH 7.2) before using at a 1/25 dilution in all experiments.

3. Bacteria and culture conditions

Salmonella enteritidis phage type (PT) 8 was obtained from the Laboratory Centre for Disease Control, Ottawa, Canada. The culture was maintained on standard methods agar. A loopful of culture was inoculated into 5 mL of nutrient broth and incubated for 24 hours at 37 °C. SPC slants inoculated with broth cultures of *S. enteritidis* PT8 were incubated for 24 hours at 37 °C. Slants were washed with 0.85 % saline, harvested and serially diluted in 0.1% peptone water. Viable counts were estimated by plating with standard methods agar and incubating for 24 hours at 37 °C.

Salmonella heidelberg, *Proteus vulgaris*, (University of Manitoba, Microbiology Department), *Escherichia coli*, *Citrobacter freundii* (ATCC) were prepared as above. All bacterial suspensions, serially diluted to specific concentration for inoculation were confirmed by plating onto standard plate count agar.

4. Preparation of membrane

Nitrocellulose (BioRad Laboratories, Hercules, CA) was cut into appropriately sized strips and incubated in phosphate buffered saline (PBS; pH 7.2) for 30 minutes before immunoblot use.

5. Preparation of egg samples

Large table grade A eggs were obtained from a local supermarket and were used within one week of purchase. The surface of the eggs were washed with 70% ethanol and opened aseptically. Pooled egg contents were placed into stomacher bags (Seward Medical, London, UK) and massaged for 30 seconds using a stomacher lab-blender 400 (Seward Laboratory, London, UK). Alternatively they were blended using sterile containers (1L). Portions of homogenized egg (25 mL) were transferred into 50 mL polypropylene tubes (Falcon, Oxnard, CA).

6. Preparation of Media

Trypticase soy broth, peptone water and lactose broth were prepared according to the manufacturers instructions. Aliquots (25 mL) were placed in 50 mL polypropylene tubes (Falcon, Oxnard, CA) and the contents were sterilized at 121 °C prior to use.

7. Determination of effectiveness of media for the immunoblot

Sterilized tubes containing TSB, PW, LB or blended egg were inoculated with *Salmonella enteritidis* and incubated for 20 hours at 37 °C. Thereafter, 2.5 mL of 15 % cholic acid was added to each tube and 0.5 g agar (2%) was added to tubes containing synthetic media. All tubes were heated at 100 °C for 10 min in a water bath. Tubes were vortexed after 5, and 10 minutes to ensure uniform

melting and dispersion of agar throughout the media. Following heating all samples were placed in a freezer to speed matrix setting.

The immunoblot was carried out according to Yoshimasu (1996). Briefly, solidified complex media and homogenized whole egg were removed aseptically from the tubes and a through the centre of each sample was taken using a sterile borer. A 2 mm thick disc was sliced from the centre of each core and placed on a pre-wetted nitrocellulose membrane. A 15 uL drop of heat-attenuated *S. 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), 2 x 2 min. The membrane was then blocked with 5% skim milk powder (SMP) in PBS for 45 minutes. The SMP was removed and the membrane was washed (2X2min) with Tris buffered saline containing tween-20 (TTBS) before adding the antibody and letting it sit for 1 hour. The antibody solution was removed, the membrane washed once again with TTBS and biotinylated Goat-anti-mouse antibody (B-GAM) was added to the membrane. After an hour, the B-GAM was removed, the membrane washed and a solution of streptavidin alkaline phosphatase (S-AP) was added. After the final hour of incubation, the S-AP was removed, the membrane washed, and the colour developer was added. After colour development with BCIP/NBT the reaction was stopped by addition of distilled water.

8. The effect of agar and cholic acid on the growth of *Salmonella enteritidis*.

Trypticase soy broth and lactose broth were inoculated with *S. enteritidis* and cholic acid, agar, both, or neither and incubated for 20 hours before proceeding as before.

9. Determination of the optimal amount of iron

Trypticase soy broth (25mL) and homogenized whole egg were each inoculated with about 17 CFU of *S. enteritidis*. To each tube a different concentration of ferrous sulphate, containing from 0.0 to 80 ug/mL iron was added. After incubation (12 h; 37°C) samples were serially diluted and evaluated using standard plate count agar.

10. Enumeration of *S. enteritidis* in the presence of added competing microorganisms

Trypticase soy broth and egg homogenate were inoculated with *S. enteritidis* and *Proteus vulgaris, Esherichia coli, Citrobacter freundii* and *Salmonella heidelberg,* alone and in various combinations and ratios. The media were incubated for 16 and 20 hours before proceeding with the assay.

11. Enumeration of *S. enteritidis* in naturally contaminated samples

Samples (25 mL) of wash water obtained from various stages in the production line of a commercial poultry processing plant, were inoculated with 10^0, 10^1, 10^2 and 10^3 CFU of *S. enteritidis*. Negative controls consisted of

uninoculated samples. The wash water samples were then added to 225 mL of either trypticase soy broth or homogenized whole egg containing 21.26 mg $FeSO_4$ and incubated for either 16 or 24 hours at 37 °C. Samples of 25 mL were then dispersed into 50 mL conical flasks before proceeding with the assay.

12. Enumeration of *S. enteritidis* in naturally contaminated samples using ferrioxamine E

Samples of wash water (25mL) obtained from a commercial poultry processing plant, were inoculated with 10^1, 10^2 and 10^3 CFU *S. enteritidis*. Negative controls consisted of uninoculated samples. The wash water samples were then added to trypticase soy broth (225 mL) containing 60 ng/mL ferrioxamine E or 225 mL homogenized whole egg with 60 ng/mL ferrioxamine E. Samples were incubated for either 16 or 24 hours at 37 °C . Samples of 25 mL were then dispersed into 50 mL conical flasks before proceeding as before.

13. Culture confirmation

Following incubation, one mL from each sample was added to selenite cysteine broth (10 mL) and incubated for 24 hours 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 hours at 37 °C. Presumptive isolates were transferred to triple sugar iron and lysine iron agar slants and incubated for 24 hours at 37 °C. Confirmation of typical isolates was performed serologically

using Salmonella 0-9 factor antiserum. Salmonella 4,5 factor antiserum and Salmonella poly A-I were used to confirm presence of non D group *Salmonella* in cases where samples taken from the wash water were positive by conventional culture but negative for *S. enteritidis*.

D. RESULTS AND DISCUSSION

The immunoassay for the detection of *Salmonella enteritidis* in foods and feeds involves a two step procedure as outlined by Yoshimasu (1996). A 20 hour enrichment step utilizing homogenized whole egg, followed by a monoclonal antibody based dot-blot assay (Yoshimasu, 1996). The purpose of this study was to determine whether homogenized whole egg was the best enrichment medium for the propagation of *S. enteritidis* for our immunoassay, or whether other synthetic media could be equally effective.

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). Therefore, in this study three media were chosen for comparison with homogenized whole egg; lactose broth, peptone water and trypticase soy broth. All three are commonly used as pre-enrichment media in conventional *Salmonella* detection procedures (Reissbrodt, 1995).

Figure 1 shows the immunoblots of LPS obtained from *S. enteritidis* grown in the three synthetic media as well as in homogenized whole egg. After inoculation of the media with *S. enteritidis*, incubation, and developing the immunoassay, it was immediately evident that peptone water was far less effective than the other media for the replication of this organism for the assay. In a previous study on the pre-enrichment of *S. enteritidis*, Stephenson and coworkers (1991) compared five media such as lactose broth, peptone water, trypticase soy broth, nutrient broth, and brain heart infusion broth. No one media was found to be significantly more effective for recovering *S. enteritidis* from liquid egg yolk. Consequently, trypticase soy broth was arbitrarily recommended as the enrichment medium of choice for the recovery of *S. enteritidis* from egg yolk (Stephenson *et al.*, 1991). In another study, Reissbrodt (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.

Figure 1: Immunoblot of LPS obtained from SE grown in synthetic media and egg. +SE - media inoculated with *S. enteritidis*. - SE - media without *S. enteritidis* (negative control). TSB trypticase soy broth; LB - lactose broth; PW -peptone water.

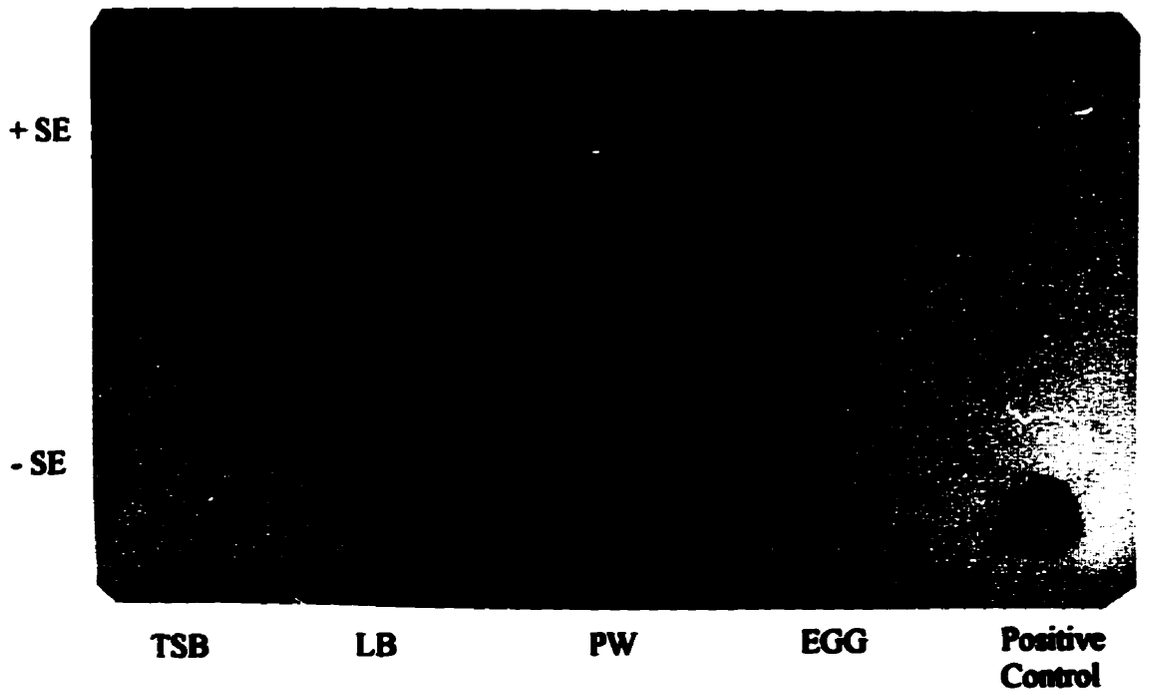
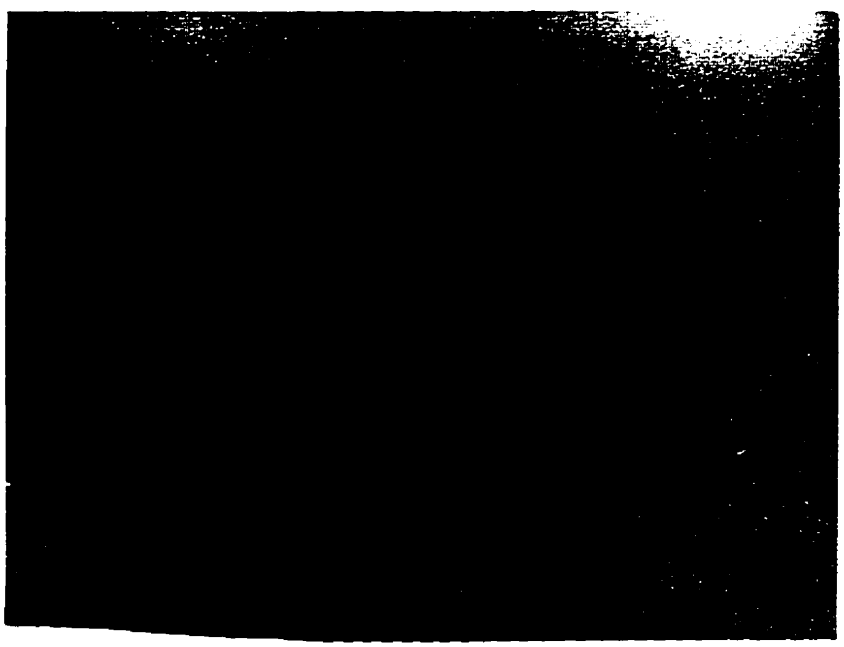

+ SE
- SE
TSB
LB
PW
EGG
Positive Control

Figure 2 shows the immunoblots for LPS obtained from *S. enteritidis* grown in lactose broth and trypticase soy broth. Equal numbers of *S. enteritidis* were inoculated into both lactose and trypticase soy broth as assessed by performing a SPC after 20 hours of incubation. Immunoassays performed using LPS obtained from *S. enteritidis* grown on all media indicated a positive reaction for *S. enteritidis.* However, the immunoblot employing inocula produced in lactose broth was less intense than that for trypticase soy broth. In both cases uninoculated media (negative control) gave no reaction.

In order to retain the conditions of the immunoassay as closely as possible to the original assay, which used homogenized whole egg, it was necessary to solidify the media before applying it to the nitrocellulose membrane. This was effectively achieved by adding two percent granulated agar to the media. The ten minute boiling stage necessary for the heat and cholic acid to extract *S. enteritidis* LPS was also sufficient to melt the agar. Subsequent vortexing before cooling dispersed the agar throughout the media and the resultant cooled matrix was solidified. The solid matrix was found superior to liquid media in forming a blot image on the nitrocellulose as liquid media tended to run and disperse on the membrane.

The addition of iron has been shown to greatly improve the recovery of *S. enteritidis* from eggs (Gast and Holt, 1995a,b,. Yoshimasu, 1996). Therefore, we assessed the effect of iron addition on *S. enteritidis* growth in homogenized whole

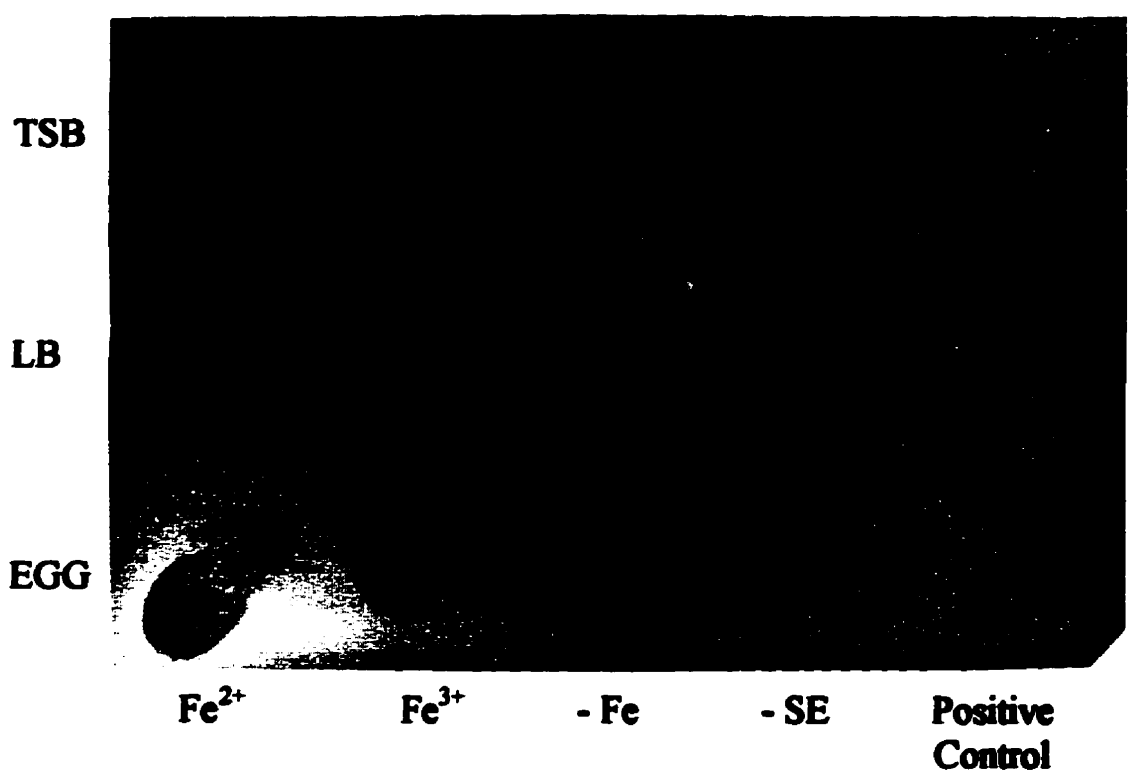
Figure 2. The effect of enrichment medium on immunoblot intensity. TSB - trypticase soy broth; LB - lactose broth.



TSB LB Egg Positive Control

egg, lactose broth and trypticase soy broth. *S. enteritidis* has been found to grow rapidly following the addition of ferric ammonium citrate to albumen (Lock and Board, 1992). Results of the immunoblot (Figure 3) and standard plate counts showed that iron addition either in ferrous or ferric form had no effect on growth of *S. enteritidis* in the TSB or LB. SPC counts for these media reached $5.1x10^{11}$ when *S. enteritidis* was incubated in the presence of both forms of iron and without iron. Because these media are not iron restrictive, addition of this element does not enhance growth of the bacteria. When iron was added to homogenized egg, both ferrous and ferric forms were found to equally enhance growth as compared to egg without added iron. Counts on SPC agar reached 2.7×10^{11} CFU/mL after *S. enteritidis* was incubated in egg homogenate with either form of iron incorporated, without iron addition the final SPC for *S. enteritidis* was 5.8×10^{10} CFU/mL after the 20 h incubation. Ovotransferrin and other iron chelating compounds reduce the amounts of available iron in the albumen. Since iron is essential to the growth of *S. enteritidis*, the addition of iron to the egg must overcome the restrictions due to limited iron availability to this organism in egg contents. The level of 'free' iron in egg white is considerably lower than the amount necessary for bacterial growth (Chart and Rowe, 1993). In the present study we determined the amount of added iron for maximum growth of *S. enteritidis* in homogenized egg to be about 85 ug/mL as $FeSO_4$ (ferrous). After

Figure 3. The effect of iron addition to growth media for SE with respect to immunoblot intensity. TSB - trypticase soy broth; LB - lactose broth.


TSB
LB
EGG
Fe^{2+}
Fe^{3+}
- Fe
- SE
Positive Control

12 h incubation of *S. enteritidis* in egg homogenate containing increasing concentrations of ferrous sulphate counts as determined by SPC reached a plateau at this concentration of iron.

In order to further simplify the test, the addition of cholic acid and agar to the liquid media before inoculation and incubation was studied. Figure 4a shows the effect of cholic acid and granulated agar addition prior to incubation on the detection of *S. enteritidis* by the immunoassay. Both standard plate counts and the immunoassay showed that the agar had no effect on the growth of *S. enteritidis* in either lactose broth of trypticase soy broth. SPC counts in trypticase soy broth were 1.0×10^{10} CFU/mL when cholic acid was added to the medium prior to incubation as compared to 2.6×10^{10} CFU/mL when the acid was added just prior to the heating step. Similarly, for lactose broth with 2.8×10^{10} CFU/mL when acid was added before incubation as compared to 1.5×10^{10} CFU/mL when added just prior to heating. It is quite feasible to add the detergent before incubation to reduce the number of reaction steps. Similar results were found when cholic acid was added to the egg before incubation (Figure 4b).

Immunoblots obtained from the LPS of *S. enteritidis* grown in lactose broth were consistently less intense than those obtained for the organism grown in trypticase soy broth. However, by SPC the inoculated and post enrichment counts of *S. enteritidis* were found to be equivalent when grown in both media. For this

Figure 4: The effect of cholic acid (CA) and agar (A) on the immunoblot. TSB - trypticase soy broth; LB - lactose broth. a - + SE + A; b - + SE + CA; c - + SE + CA + A; d - + SE; e - + CA + A; f - Positive control.

a

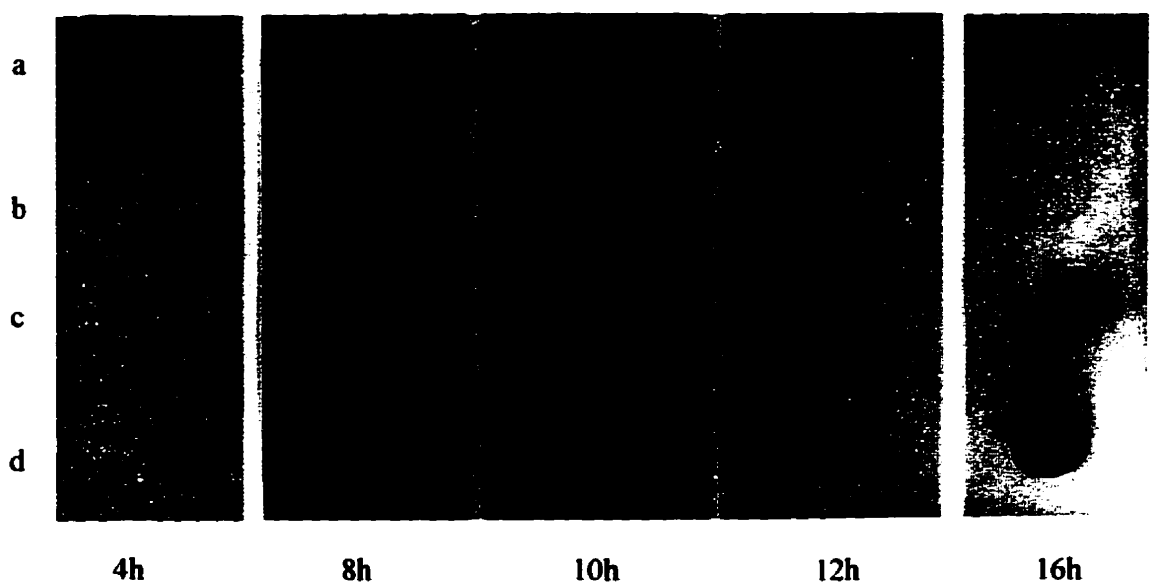
b

reason trypticase 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 hour pre-enrichment incubation. Previous studies using homogenized whole egg as an enrichment medium found that 20 hours incubation at 37 °C was necessary to bring the population of *S. enteritidis* up to 10^6/mL which is the minimum population needed for detection by the immunoblot (Yoshimasu, 1996). 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 *S. enteritidis* when incubated in trypticase soy broth. Figure 5 shows that a 10^1 CFU inoculum of *S. enteritidis* in 25 mL of trypticase soy broth can be detected by the immunoassay after 10 hours of incubation both with and without cholic acid and agar addition. In this regard, studies have shown that *S. enteritidis* commences exponential growth at about 6 hours after inoculation into trypticase soy broth and incubation at 37 °C (Chart and Rowe, 1993).

In horizontal contamination of eggs, and in contaminated poultry and poultry products, *S. enteritidis* has been shown to be present in mixed populations of faecal and enteric bacteria. In order for *S. enteritidis* to be detected in a mixed flora it must reach a sufficient concentration. Large numbers of competitive bacteria in the food sample may affect the growth dynamics of *Salmonella* during

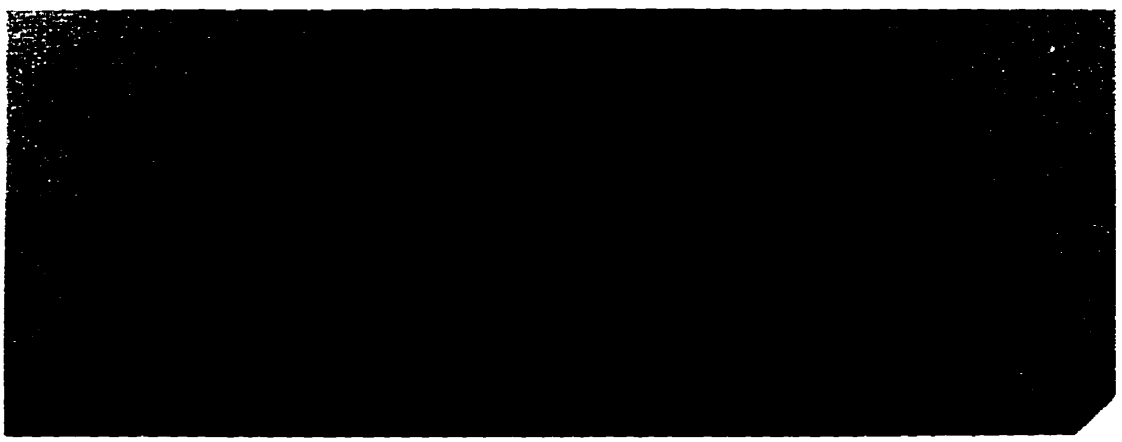
Figure 5. The effect of time of incubation of SE in trypticase soy broth on detection of this pathogen by the immunoblot. a - positive control; b - + cholic acid + agar; c - + cholic acid + agar + SE; d - + SE.


a
b
c
d
4h
8h
10h
12h
16h

pre-enrichment such that very low numbers of *Salmonella* cells may be present (de Smedt and Bolderdijk, 1987; Riser *et al.*, 1985).

When *S. enteritidis* was incubated in the presence of a mixed microflora consisting of *Citrobacter freundii*, *Proteus vulgaris*, *Salmonella heidelberg* and *Escherichia coli*, we found that *S. enteritidis* was detected at ratios of $1:10^0$, $1:10^1$ and $1:10^2$ in the initial inoculum, in both trypticase soy broth and egg homogenate supplemented with ferrous sulphate (Figure 6). In previous work using homogenized whole egg it was found that one *S. enteritidis* CFU could still be detected by immunoassay after incubation with up to 400 CFU of competing bacteria consisting of various genera and species commonly found in poultry (Yoshimasu, 1996). 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. These organisms which were isolated from either eggs or poultry faeces included *Pseudomonas putida*, *Salmonella enteritidis*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus xylosus*. Dolman and Board (1992) found that all five organisms inoculated into the inner membrane of the eggs and stored at 4 °C were subsequently isolated. Storage at 37 °C led to recovery of only *Staphylococcus xylosus*, *Salmonella enteritidis* and *Enterococcus faecalis*. *S. enteritidis* attained populations of 10^5/mL after 15 days storage at 37 °C (Dolman and Board, 1992). The authors (1992) also noticed that

Figure 6. Detection of *S. enteritidis* by the immunoblot assay in the presence of other bacterial competitors. *S. enteritidis* was own in the presence of *C. freundii, P. vulgaris, S. heidelberg* and *E. coli* in the following ratios: a & e - $1:10^0$; b & f - $1:10^1$; c & g $1:10^2$; d & h - $0:10^2$. a-d - trypticase soy broth; e-h - egg i - positive control. All rows represent triplicate results.



a b c d e f g h i

S. enteritidis outcompeted *E. coli* when the eggs were incubated at 37 °C, but failed to compete with *Pseudomonas* spp. in eggs incubated below 30 °C. Riser and coworkers (1985) inoculated *S. typhimurium* into a hydroponic nutrient solution with a natural biological load of 10^3 CFU/mL. They found that *Salmonella* counts never exceeded 10^4 CFU/mL, and always began to decrease after 24 hours (Riser *et al.*, 1985).

The commercial feasibility of the immunoassay to detect *S. enteritidis* in the presence of other bacteria was assessed by examining wash water samples from a local poultry plant. Samples were taken at various stages in the production line and were assessed for the presence of *S. enteritidis*. A sample of water that had been used to was the crates in which chicken arrived at the plant was found to have an initial load of 10^6 CFU/mL of organisms. This sample did not give conclusive results when inoculated with 10^2 *S. enteritidis*/25 mL and incubated for 16 or 24 hours in trypticase soy broth or the egg medium. It is probable that the competitor ratio was too high and as such outcompeted *S. enteritidis.* Consequently, *S. enteritidis* was not detected either by immunoblot or by conventional method. Crate wash water is contaminated primarily by faeces 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 ability of *S.*

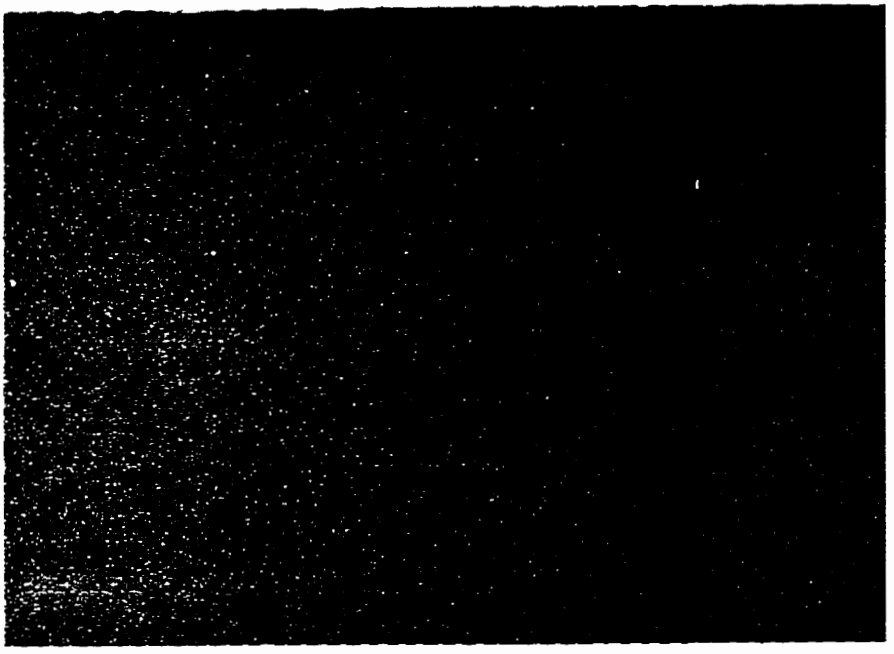
typhimurium to compete effectively in mixed flora has been assessed. In this study chemically defined medium, SCDM, was inoculated with *S. typhimurium*, *Enterobacter aerogenes* and *Escherichia coli* at ratios of *S. typhimurium* to competitors of 1:1, $1:10^1$, $1:10^2$, $1:10^4$ and $1:10^6$. It was found that after 16 hours incubation at 37 °C the final counts for *S. typhimurium* at the two highest ratios of competitors failed to reach more than 2×10^6 CFU/mL (Lee et al., 1990). At this final cell count *S. enteritidis* would not have been detected by our assay as this final population is close to the lower detection limits of the assay.

The next experiment was performed using scalder run-off water, containing a microflora of 10^5 CFU/mL. After inoculation with 10^3 and 10^4 *S. enteritidis*/25mL scalder run-off water, a competitor ratio of $1:10^2$ and $1:10^3$ was established. The use of trypticase soy broth, enabled detection of *S. enteritidis* as early as 16 hours of incubation (Figure 7). The ferrous supplemented egg medium (Figure 8) was less effective than trypticase soy broth and we were only able to detect ($1:10^2$). With the other samples tested, the supplemented egg medium gave better results than trypticase soy broth, both after 16 and 24 hours. In carcass rinse

Figure 7. Detection of *S. enteritidis* in the presence of bacterial competitors (various ratios) in scalder run-off water. Trypticase soy broth was used as enrichment medium. All rows represent triplicate results.

16 h

24 h



$0:10^5$ $1:10^4$ $1:10^3$ $1:10^2$ Positive Control

Figure 8. Detection of *S. enteritidis* in the presence of bacterial competitors (various ratios) in scalder run-off water. Homogenized whole egg + $FeSO_4$ (85 μg/mL) used as enrichment medium. All rows represent triplicate results.

16 h

24 h

0:10^5 1:10^4 1:10^3 1:10^2 Positive Control

water containing a microflora load of 10^4 CFU/mL, we were able to detect an initial inoculum of 10^3 *S. enteritidis*/25mL, a ratio of $1:10^2$ (Figures 9,10). In the defeathering water containing a microflora of 10^6 CFU/mL, we were able to detect *S. enteritidis* which was initially out-competed at a ratio of $1:10^3$ (Figure 11). The initial inoculum consisted of 10^4 CFU *S. enteritidis*/25mL. Gast (1993a) noticed that the frequency of recovery of *S. enteritidis* from inoculated egg pools was significantly affected by the size of the inoculum. The greater the initial inoculum the greater the chance of detection, even when the volume of medium was increased to keep the concentration constant (Gast, 1993a). Shoeni and coworkers (1995) found that lower inoculum levels of *Salmonella* failed to reach as high counts as higher inoculum levels even after extended incubation periods in egg samples (Shoeni *et al.*, 1995).

In complex mixtures of microorganisms competition between species may have a considerable effect on the relative rates of population growth (Rhodes *et al.*, 1985). In fresh, raw materials such as poultry and egg products, the major part of the flora consists of Enterobacteriaceae and Pseudomonads which will readily compete with *Salmonella* (Brinkman *et al.*, 1995). *Pseudomonas* spp. are potent inhibitors of pathogenic bacteria associated with food and plants (Cheng *et al.*, 1995). In three of the samples taken from the poultry plant, the immunoblot was stronger for *S. enteritidis* incubated in supplemented egg medium than trypticase soy broth. Trypticase soy broth is a general purpose resuscitation medium.

Figure 9. Detection of *S. enteritidis* in the presence of bacterial competitors (various ratios) in carcass rinse water. Trypticase soy broth was used as enrichment medium. All rows represent triplicate results.

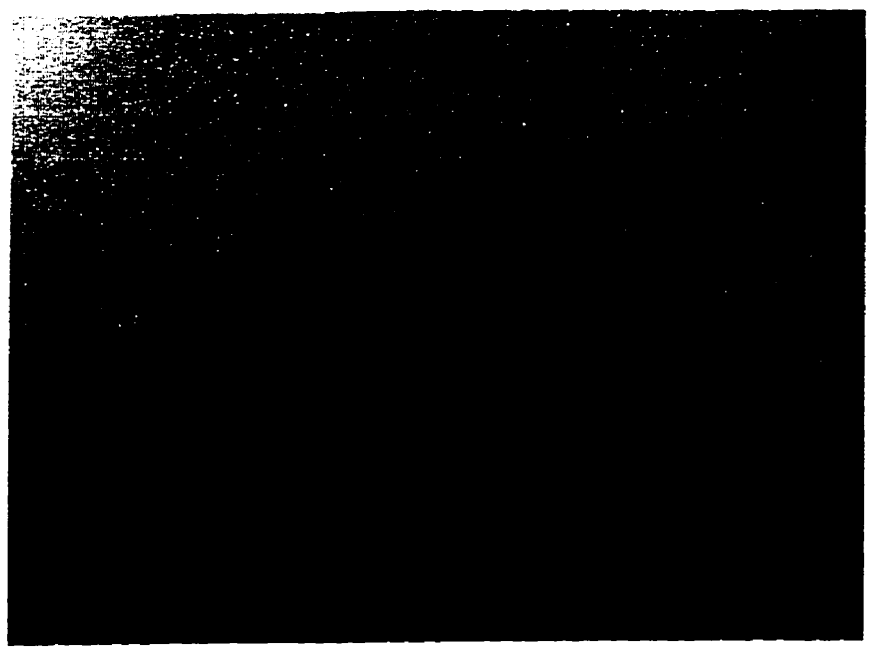
16 h

24 h

$0:10^4$ $1:10^4$ $1:10^3$ $1:10^2$ Positive Control

Figure 10. Detection of *S. enteritidis* in the presence of bacterial competitors (various ratios) in carcass rinse water. Homogenized whole egg + $FeSO_4$ (85 μg/mL) was used as enrichment medium. All rows represent triplicate results.

16 h



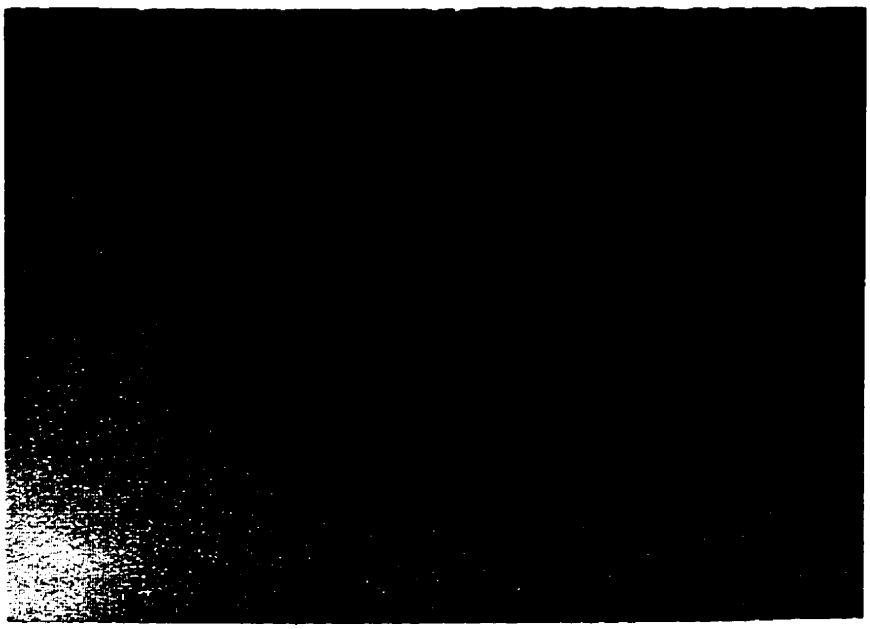
24 h

$0:10^4$ $1:10^4$ $1:10^3$ $1:10^2$ Positive Control

Figure 11. Detection of *S. enteritidis* in the presence of bacterial competitors (various ratios) in defeatherer water after 24 hs incubation.
a - trypticase soy broth used as enrichment medium;
b - homogenized whole egg + $FeSO_4$ used as enrichment medium.
Rows represent triplicate results.

a.

b.



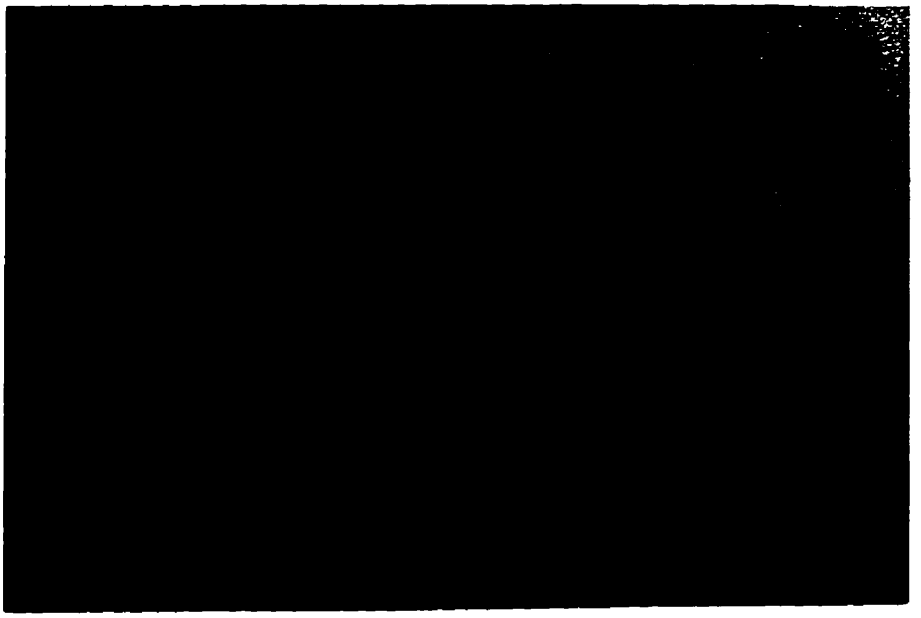
$0:10^6$ $1:10^5$ $1:10^4$ $1:10^3$ **Positive Control**

Increasing the amount of nutrients may favour the growth of competitive microorganisms which may, in turn, have an inhibitory effect on the growth and isolation of salmonellae (van Schothorst and Renaud, 1983). Factors in the egg, such as low levels of non-protein nitrogen in the albumen, select for Gram-negative bacteria because they tend to be less fastidious than Gram-positive organisms (Dolman and Board, 1992). Most of the organisms present in the poultry wash samples would be expected to be Gram-negative, predominantly *Enterobacteriaceae* and *Pseudomonads*. Studies have shown that *S. 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 *S. enteritidis* that it allows it to grow to slightly higher numbers for better detection by the immunoblot than is seen in the non-selective trypticase soy broth.

Figures 12 to 15 show immunoblots of *S. enteritidis* when grown in the presence of microflora from chicken chiller water. Chicken chiller water contained an initial bacterial load of 10^3 CFU/mL. The detection of *S. enteritidis* in the presence of bacterial competitors in the ratio of $1:10^2$ was feasible using both trypticase soy broth (Fig 12) and egg enriched with ferric salts (Fig 13). No significant differences in detection were found as early as 16 h incubation (Figures 12,13). Detection of *S. enteritidis* in the presence of competing bacteria was improved using media modified by the addition of ferrioxamine E (Figures 14,15). *Salmonella* has an almost specific transport system for the siderophore

Figure 12. Detection of *S. enteritidis* in the presence of bacterial competitors (various ratios) in chicken chiller water. Trypticase soy broth was used as enrichment medium. All rows are triplicate results.

16 h

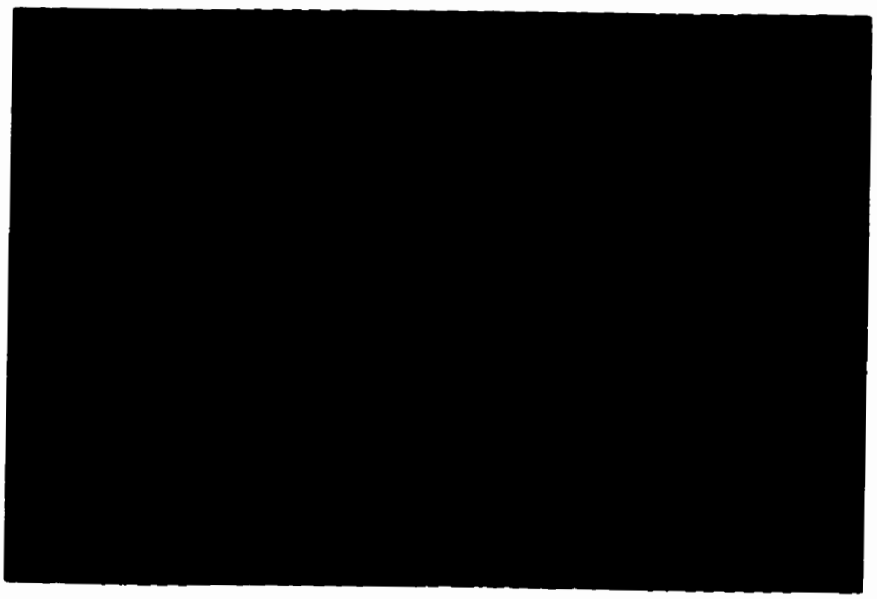


24 h

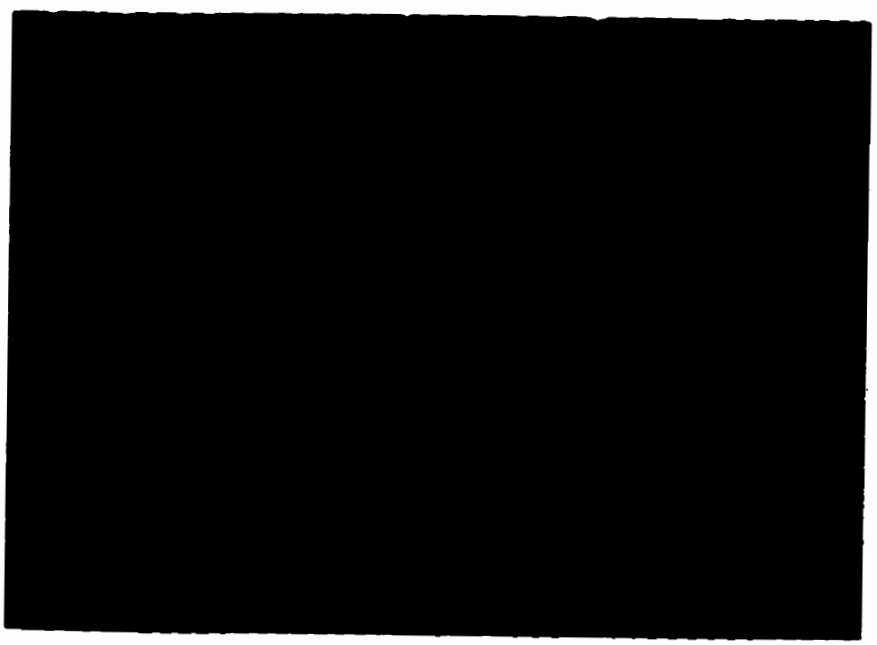
$0:10^4$ $1:10^4$ $1:10^3$ $1:10^2$ **Positive Control**

Figure 13. Detection of *S. enteritidis* in chicken chiller water. Homogenized whole egg + $FeSO_4$ (85 μg/mL) used as enrichment medium. All rows represent triplicate results.

16 h



24 h



$0:10^4$ $1:10^4$ $1:10^3$ $1:10^2$ Positive Control

Figure 14. Detection of *S. enteritidis* in chicken chiller water. Homogenized whole egg + ferrioxamine E (60ng/mL) used as enrichment medium. All rows represent triplicate results.

16 h

24 h

$0:10^3$ $1:10^3$ $1:10^2$ $1:10^1$ Positive Control

Figure 15. Detection of *S. enteritidis* in chicken chiller water. Trypticase soy broth + ferrioxamine E (60ng/mL) used as enrichment medium. All rows represent triplicate results.

16 h

24 h

$0:10^3$ $1:10^3$ $1:10^2$ $1:10^1$ Positive Control

ferrioxamine E (Reissbrodt and Rabsch, 1993). Reissbrodt and coworkers (1996) found that ferrioxamine E supplementation of buffered peptone water allowed for the detection of *Salmonella* initially outcompeted by 10^3-10^4 CFU/mL in egg albumen after only 6 hours of incubation with shaking (Reissbrodt *et al.*, 1996). When homogenized whole egg and trypticase soy broth was supplemented with 60 ng/mL ferrioxamine E before addition of chicken chiller water containing 10^3 CFU/mL. The subsequent immunoblots (Figure 14) showed detection of *S. enteritidis* at all initial inoculum levels, even when initially outcompeted by $1:10^3$ using the egg medium. Less conclusive results were obtained when trypticase soy broth was used (Figure 15). Although detection of *S. enteritidis* was improved at an initial ratio of $1:10^1$, it was not detected when outcompeted by higher numbers of competitors (Figure 15). The lack of success when trypticase soy broth was supplemented with ferrioxamine E may be because this medium is not iron restrictive. Bacteria only synthesize specific iron transport systems in conditions where iron concentrations are limited. In trypticase soy broth *S. enteritidis* would not be expected to synthesize any of these systems. Therefore, *S. enteritidis* would not be able to transport the siderophore into the cell. Ferrioxamine E supplemented Rappaport-Vassiliadis medium has been found to be less effective than other *Salmonella* enrichment media for this reason (Reissbrodt *et al.*, 1996).

In samples taken from a poultry production plant we were able to detect *S.*

enteritidis in the presence of large numbers of natural bacterial competitors. Using the immunoblot we were able to detect *S. enteritidis* even when outcompeted $1:10^2$ in samples taken from the poultry plant using either TSB or whole egg supplemented with ferrous sulphate after incubation for 16 hours. 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 hours, making this method much faster and more convenient than the culture procedure. Incorporation of agar and cholic acid to the media before incubation simplified the method and had no negative effects on the detection of *S. enteritidis*. When ferrioxamine E was incorporated into the homogenized whole egg medium instead of ferrous sulphate, *S. enteritidis* was detected when outcompeted by naturally present bacteria in the ratio of $1:10^3$. In order to improve the detection limit of *S. enteritidis* in the presence of high numbers of competitive organisms, selective agents such as ferrioxamine E, specific for *S. enteritidis* should be further investigated.

IV. MANUSCRIPT 2

STORAGE STABILITY OF AN IgG MONOCLONAL ANTIBODY FREEZE-DRIED IN THE PRESENCE OF CRYOPROTECTANTS.

A. ABSTRACT

The usefulness of antibodies for diagnostic and therapeutic techniques is limited by their stability. Freeze-drying has become the preferred method for protein stabilization. In addition, freeze-dried products can be shipped at ambient temperatures and can be rapidly reconstituted when needed. In the production and distribution of a commercial assay kit, the advantages of a package that does not require refrigerated transport and that can be stored at room temperature are significant. Certain sugars are able to protect biologically active proteins such as antibodies from dehydration induced stress. In this study we examined the stability of a purified monoclonal antibody after freeze-drying in the presence of various cryoprotective agents and storage at different temperatures. Trehalose, mannitol, sucrose and maltose all had equivalent stabilizing effects on the freeze-dried antibody when they were stored at temperatures of - 20 °C, 4 °C or 20 °C for up to 70 days. Storage at 50 °C led to antibody degradation stored in the presence of maltose, and to a lesser extent sucrose. This presumably was due to Maillard reaction occurring between the amino groups of the protein and reducing sugars.

B. INTRODUCTION

Antibodies form the core of many important analytical and preparative biochemical procedures due to their high specificity and simple method of preparation. In recent years many diagnostic and therapeutic techniques employing antibodies have been developed. The usefulness of these assay proteins, however, is limited by their low stability. In the development of any test kit utilizing antibodies, satisfactory stability over a prescribed storage time is essential. Even under optimized environmental conditions, antibodies in solution will gradually lose biological activity over time (Franks *et al.*, 1990) and repeated freeze-thaw cycles are damaging to antibodies (Hazen *et al.*, 1988).

Monoclonal antibodies (MAbs), in the form of spent tissue culture supernatants, ascitic fluids, or purified antibodies are usually stored in small aliquots at - 20 °C or - 80 °C, at 2-4 °C with a preservative, or are freeze-dried (Draber *et al.*, 1995). Freeze-drying has become the preferred method for protein stabilization. Freeze-dried products weigh little, can be shipped at ambient temperatures and can be rapidly reconstituted by rehydration at the point of use (Franks *et al.*, 1991). In the production and distribution of a commercial assay kit, the advantages of a package that does not require refrigerated transport and that can be stored at room temperature are significant.

Several studies have shown that certain sugars have the capability to protect biologically active proteins from stress due to dehydration (Carpenter and Crowe, 1988; Carpenter *et al.*, 1987b; Leslie *et al.*, 1995). The ability of sugars to protect proteins during desiccation can be attributed to their ability to hydrogen bond to proteins and thereby substitute for structural water (Roser, 1991). In this study we examined the stability of a purified monoclonal antibody after freeze-drying in the presence of various cryoprotective agents and storage at different temperatures.

C. MATERIALS AND METHODS

1. Chemicals

RPMI Medium 1640 with L-glutamine was purchased from Gibco BRL-Life Technologies (Grand Island, NY). Trehalose was purchased from BDH Chemicals Ltd, (Poole, Eng). Maltose and sucrose were purchased from Malinckrodt Specialty Chemicals Co. (Paris, KY). Mannitol was purchased from BBL Becton Dickinson Microbiology systems (Cockeysville, MD). Goat anti-mouse IgG (H + L) alkaline phosphatase conjugate was purchased from BioRad Laboratories (Hercules, CA). Protein A , binding buffer and elution buffer for

affinity chromatography were purchased from BioRad Laboratories (Hercules, CA).

All other chemicals were of analytical reagent grade or better and purchased from Mallinckrodt Specialty Chemicals Co. (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Distilled deionized water (Barnstead NANOpure, series 550, ULTRApure water system D4754 115 VAC; Barnstead\Thermodyne Corp., Dubuque, IA) was used in all experiments.

2. Monoclonal antibody

Monoclonal antibody 2F11 (ATCC HB-11891) was produced and characterized by Masi and Zawistowski (1995). Ammonium sulphate purified tissue culture supernatant was dialyzed against phosphate buffered saline (PBS; pH 7.2) before passing it through a Protein A affinity chromatography column. Purified Mab was then concentrated using a Centriplus concentrator (Amicon Inc., Beverly MA) before freeze-drying.

3. Freeze-drying

Aliquots (5 μL) of the affinity purified antibody solution were diluted in 500 ul of sodium phosphate buffer (5mM) containing 0, 0.1, 0.25 or 1.0 M

trehalose, maltose, mannitol, or sucrose. Samples were rapidly frozen and then placed in a VirTis freeze-drier model 12525 (The Virtis Co. Gardiner, NY). All samples were dried under vacuum for 24 hours.

4. Assessment of residual antibody activity

Freeze-dried samples were tested immediately for antibody activity by Enzyme Linked Immunososrbent Assay (ELISA), or stored at 50, 20, 4, or -20 °C in a desiccator for 5 and 10 weeks before testing for antibody activity. All results are averages of triplicate analyses.

D. RESULTS AND DISCUSSION

It is important to assess the long-term stability of antibodies that are to be used in immunologically based assays. Freeze-drying is generally thought to be more disruptive to enzyme function than freeze-thawing or thermally-induced perturbations (Carpenter *et al.*, 1987). However, if freeze-drying can be accomplished effectively, the resulting desiccated product holds many advantages over products stored in aqueous form. For this reason we examined the stability of a freeze-dried purified monoclonal IgG.

A great degree of protection is provided by sugars during freeze-drying (Crowe *et al.*, 1987) and many sugars have been recommended as excipients (Roser, 1991). For many products the choice is not crucial as good results can be obtained with a variety of substances (Roser, 1991). For this study we chose sucrose, maltose, mannitol and trehalose as they have all been found successful by other workers in the protection of labile proteins (Crowe *et al.*, 1987; Draber *et al.*, 1995; Izutsu *et al.*, 1993).

Cryoprotection by sugars can be enhanced with an increase in concentration (Draber *et al.*, 1995). For this reason we conducted freeze-drying experiments with concentrations of each sugar ranging from 0 to 1.0 M. Initial results using 1.0 M of each sugar showed no difference between the protective effects of this concentration and 0.25 M concentrations so the higher concentration was omitted from further studies (results not shown).

Figures 16 and 17 show the profile titer of the antibody freeze-dried with either maltose (Figure 16) or sucrose (Figure 17) and stored for 70 days. The antibody still appeared stable after 70 days when stored at 20, 4 and -20 °C (Figures 16 and 2 a,b,c). Storage at 50 °C (Figures 16 and 17 d) with both sugars resulted in significantly decreased antibody titers. Reduction in titer appeared greater with maltose than sucrose; also the reduction in titer appeared to increase with increased concentration of the sugar. The decrease in stability was evident after 35 days of storage with 0.25 M maltose (Figure 18d). Many of the samples

Figure 16: Titer of IgG freeze-dried in the presence of maltose and stored for 70 days. a stored at 20 °C; b stored at 4 °C; c stored at - 20 °C; d stored at 50 °C. ●0.1 M maltose; ▼ 0.25 M maltose; ■ no maltose added.

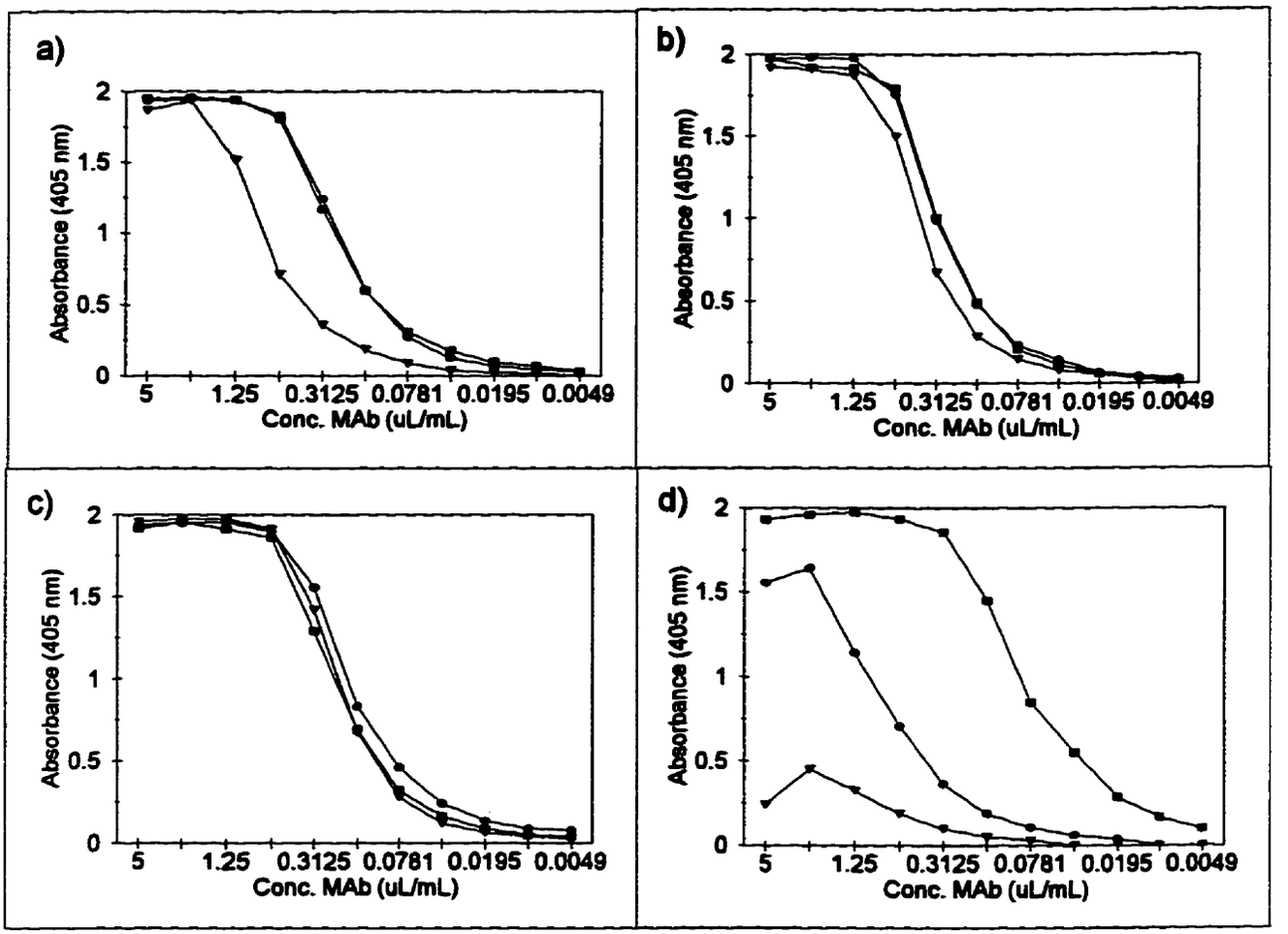

a)
Absorbance (405 nm)
2
1.5
1
0.5
0
5
1.25
0.3125
0.0781
0.0195
0.0049
Conc. MAb (uL/mL)
b)
Absorbance (405 nm)
Conc. MAb (uL/mL)
c)
Absorbance (405 nm)
Conc. MAb (uL/mL)
d)
Absorbance (405 nm)
Conc. MAb (uL/mL)

Figure 17: Titer of IgG freeze-dried in the presence of sucrose and stored for 70 days. a stored at 20 °C; b stored at 4 °C; c stored at - 20 °C; d stored at 50 °C. • 0.1 M sucrose; ▼ 0.25 M sucrose; ■ no sucrose added.

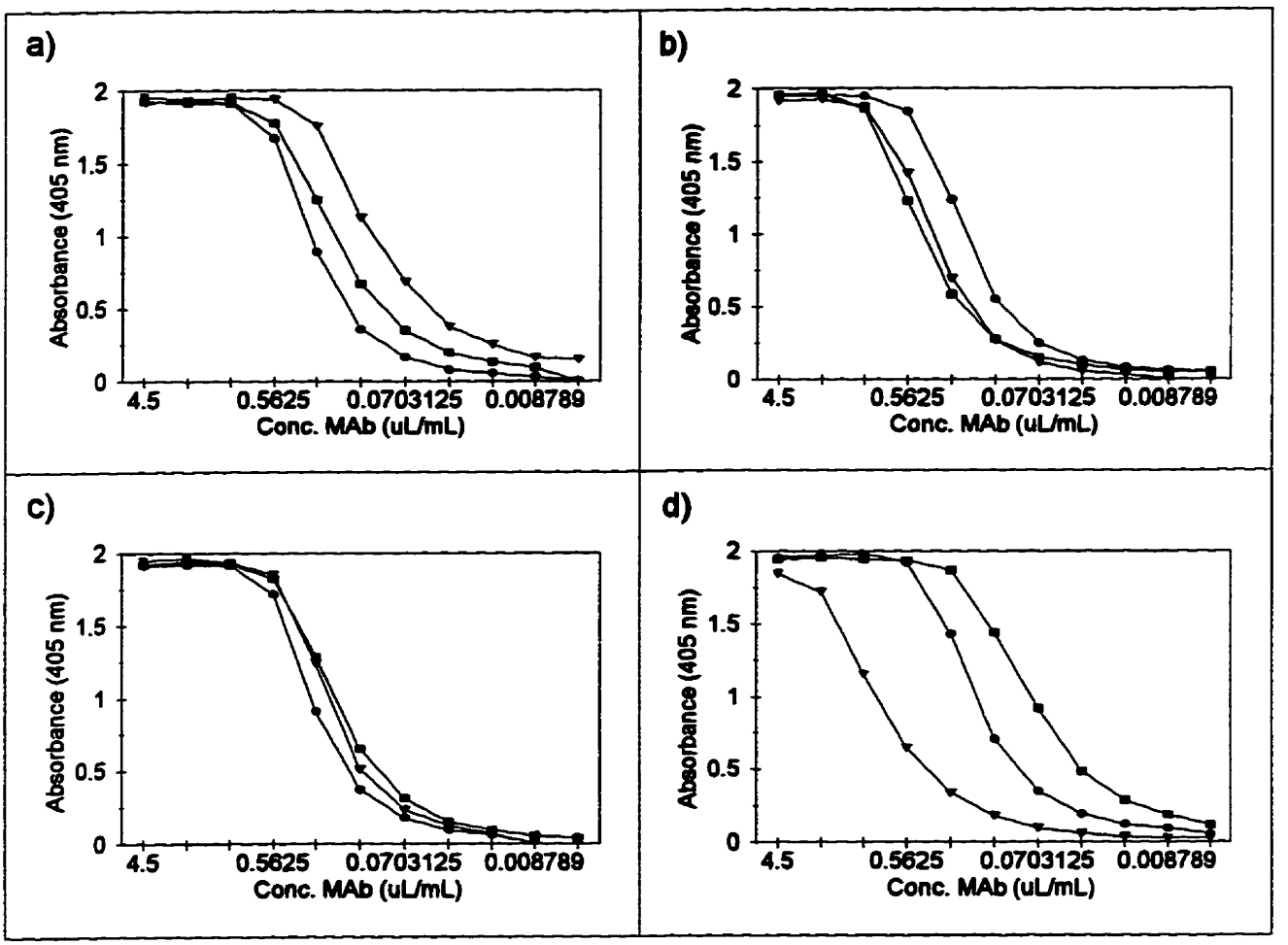
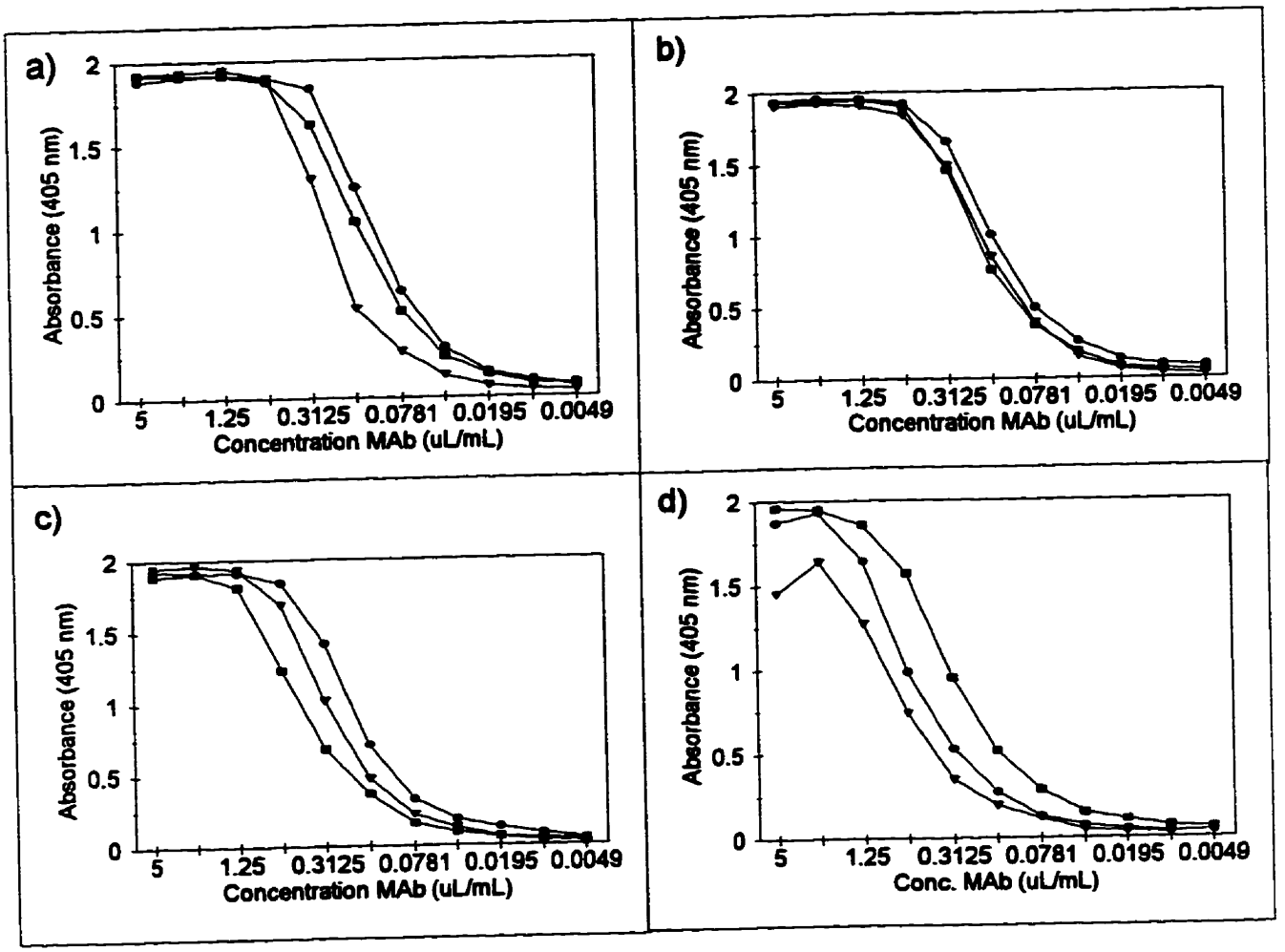

a)
b)
c)
d)
Absorbance (405 nm)
2
1.5
1
0.5
0
4.5
0.5625
0.0703125
0.008789
Conc. MAb (uL/mL)

Figure 18: Titer of IgG freeze-dried in the presence of maltose and stored for 35 days. a stored at 20 °C; b stored at 4 °C; c stored at - 20 °C; d stored at 50 °C. ● 0.1 M maltose; ▼ 0.25 M maltose; ● no maltose added.

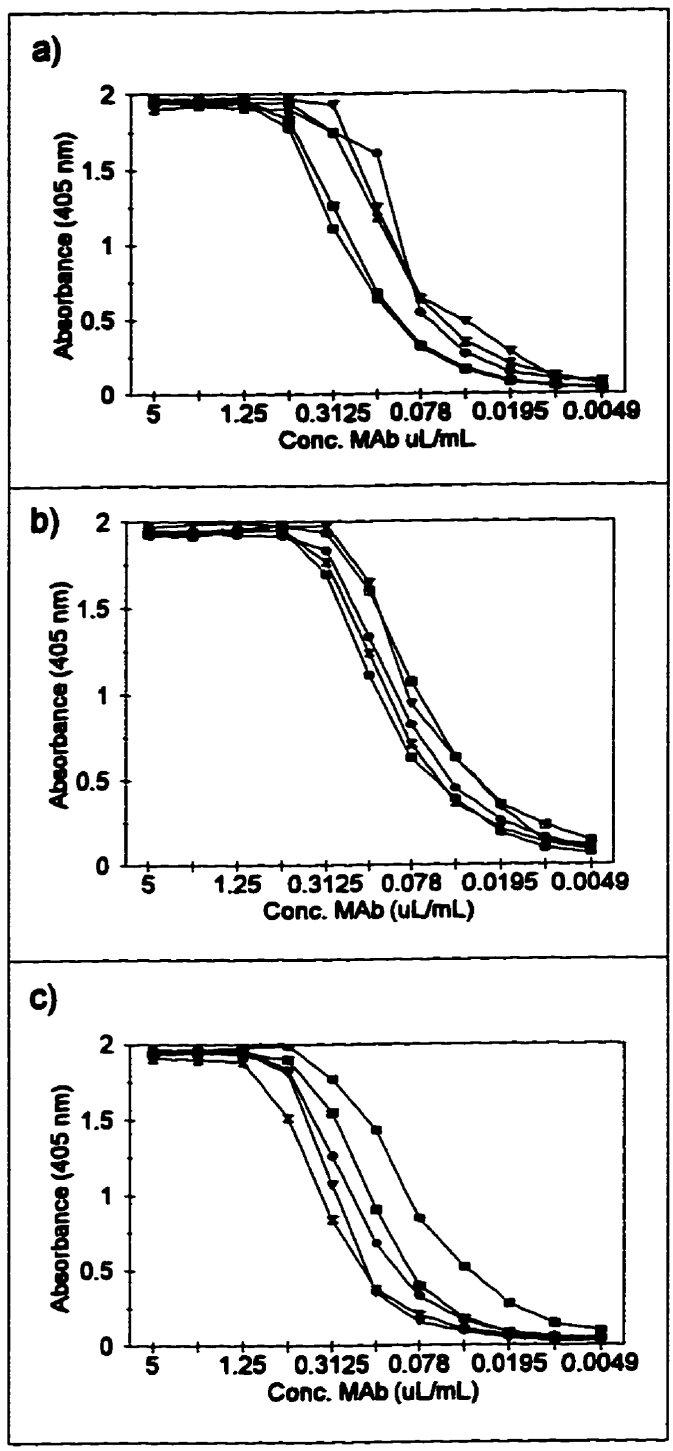

a)
Absorbance (405 nm)
2
1.5
1
0.5
0
5
1.25
0.3125
0.0781
0.0195
0.0049
Concentration MAb (uL/mL)
b)
Absorbance (405 nm)
Concentration MAb (uL/mL)
c)
Absorbance (405 nm)
Concentration MAb (uL/mL)
d)
Absorbance (405 nm)
Conc. MAb (uL/mL)

with reduced titer were also discoloured brown. Prolonged storage of dried proteins in the presence of reducing sugars or sugar alcohols may lead to progressive chemical damage due to Maillard reactions (Roser, 1991). Therefore, reducing sugars should be avoided unless the product will be stored so dry or at such a low temperature that chemical reactivity is impossible (Roser, 1991). The accelerated storage study, at 50 °C, indicated that while the reducing sugar, maltose, showed protective capabilities comparable to the other non-reducing sugars at ambient and lower temperatures, it may be less protective over longer periods of time and at the higher storage temperature of 50 °C due to the initiation of Maillard browning reactions. When Draber and coworkers (1995) studied the protective effects of maltose on freeze-dried IgM, they found that it offered no protection at 50 °C (Draber *et al.*, 1995). Contrary results were obtained by Crowe and coworkers (1987). They examined the ability of various sugars to protect phosphofructokinase purified from rabbit skeletal muscle. The enzyme was completely and irreversibly inactivated during freeze-drying (Crowe *et al.*, 1987). Trehalose and maltose were found to be the most effective excipients for stabilizing this enzyme (Crowe *et al.*, 1987). However, as only short term tests were employed by these authors, it was concluded that protein browning did not contribute to alterations in phosphofructokinase catalytic activity during this brief period (Carpenter *et al.*, 1987). The only structural difference between trehalose

and maltose is that the two α-D-glucose units are joined by a 1,1-glycosidic bond in trehalose and a 1,4-glycosidic bond in maltose (Carpenter *et al.*, 1987) hence their similar stabilizing effects during freeze-drying. IgG freeze-dried with sucrose and stored at 50 °C also showed reduced antibody titer and some Maillard browning. The browning can be accounted for by the fact that while sucrose is a stable non-reducing disaccharide, in the presence of chemically reactive protein amino groups it can split into its constituent monosaccharides glucose and fructose. As these are both reducing monosaccharides, they are susceptible to Maillard Browning, especially at elevated temperatures. Draber and coworkers (1995) also found that sucrose provided substantially lower protection than trehalose when freeze-dried mAbs were stored at 50 °C for only 14 days, probably due to this browning reaction (Draber *et al.*, 1995).

Figure 19 shows the freeze-dried antibody titer with 0.1, .25 M and no added mannitol (control). Figure 19a and 19b show that the antibody stored in 0.1 M and 0.25 M exhibited equivalent titers after 70 days of storage, even at storage conditions of 50 °C. The control antibody, freeze-dried without any protective agent showed variation in titer between storage temperature (Figure 19c). The effectiveness of mannitol as a cryoprotectant at all concentrations, including evaluated under accelerated storage conditions has been shown in other studies. Izutsu and coworkers (1993) freeze-dried the enzyme, β-galactosidase, in the presence of mannitol. They found that mannitol was protective of the enzyme

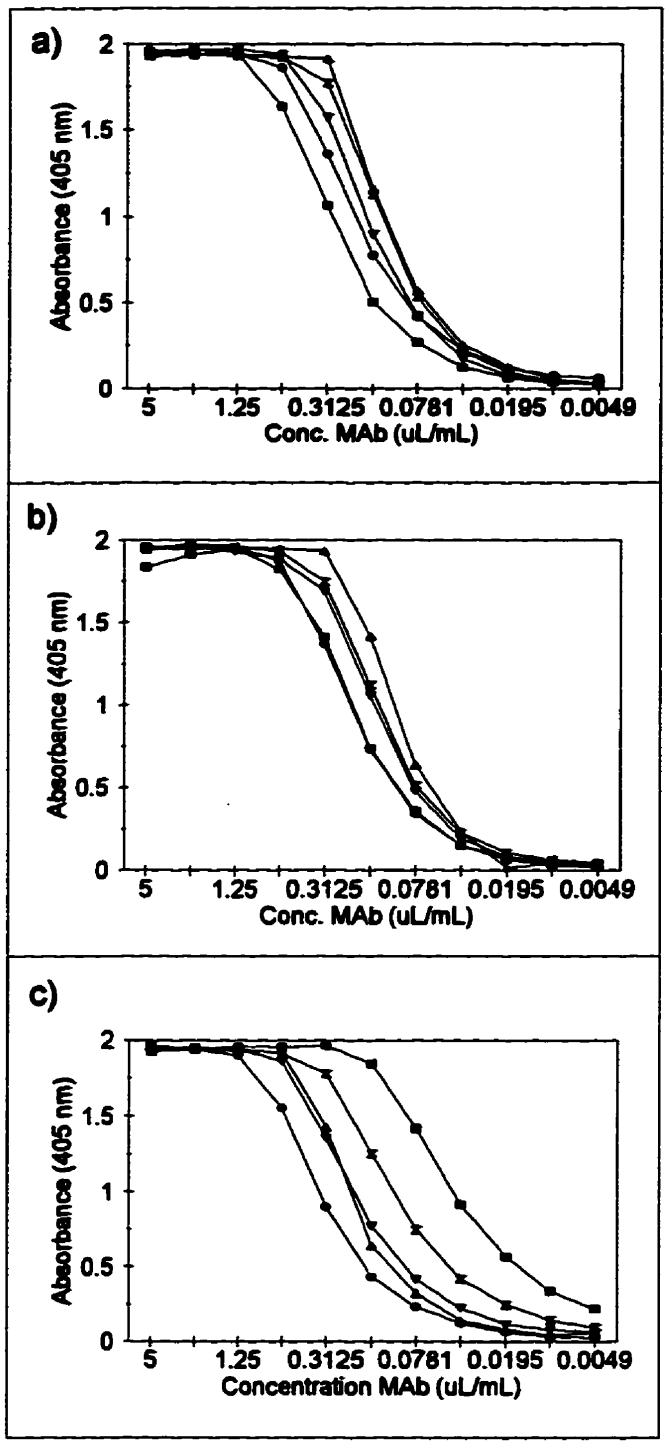
Figure 19: Titer of IgG freeze-dried in the presence of mannitol and stored for 70 days. a stored with 0.1 M mannitol, b stored with 0.25 M mannitol, c control. ▼ not stored; ⧗ stored at 20 °C; ● stored at 4 °C; ⊞ stored at - 20 °C; ■ stored at 50 °C.


a)
Absorbance (405 nm)
2
1.5
1
0.5
0
5
1.25
0.3125
0.078
0.0195
0.0049
Conc. MAb uL/mL
b)
Absorbance (405 nm)
Conc. MAb (uL/mL)
c)
Absorbance (405 nm)
Conc. MAb (uL/mL)

during freeze-drying, and that its effects were dependent upon the concentration and the concentration of the buffer salts. These workers found that mannitol was most effective as a cryoprotectant at 50 - 100 mM in 10 mM phosphate buffer (Izutsu *et al.*, 1993).

Studies using trehalose as an excipient indicated the stability to be very similar to that with mannitol (Figure 20). After freeze-drying in the presence of both concentrations of either trehalose or mannitol, and storage at all temperatures, we found that the antibody titer was essentially unchanged after 70 days as compared with the controls. In our studies trehalose was found to confer equivalent protection of the antibody after freeze-drying and storage as mannitol or the control. Other studies have found trehalose to be extremely effective in protecting labile biological proteins during desiccation (Blakeley *et al.*, 1990; Carpenter and Crowe, 1988b; Draber *et al.*, 1995; Leslie *et al.*, 1995; Roser, 1991). These studies, however, involved highly sensitive proteins, in that they were readily inactivated by freeze-drying without protection. The antibody used for our freeze-drying experiments is of the IgG_{2a} class. This class of antibody contains three disulfide bonds linking the heavy chains and is relatively stable. After freeze-drying and storing at 50 °C for 70 days, it still retained the same titer as the control tested immediately after freeze-drying. During the entire length of the stabilization study the control antibody was as stable as that stored in the

Figure 20: Titer of IgG freeze-dried in the presence of trehalose and stored for 70 days. a stored with 0.1 M trehalose, b stored with 0.25 M trehalose, c control. ▼ not stored; ⧗ stored at 20 °C; ● stored at 4 °C; ⊞ stored at - 20 °C; ■ stored at 50 °C.


a)
Absorbance (405 nm)
2
1.5
1
0.5
0
5
1.25
0.3125
0.0781
0.0195
0.0049
Conc. MAb (uL/mL)
b)
Absorbance (405 nm)
Conc. MAb (uL/mL)
c)
Absorbance (405 nm)
Concentration MAb (uL/mL)

presence of cryoprotectants. It has been reported that IgG can be stored successfully in the frozen state or freeze-dried with no great losses after long-term storage for up to 2 years (Montoya and Castell, 1987). Longer term studies are needed to determine if cryoprotectants are necessary for enhanced preservation of this antibody. However, with high-activity diluted biologicals such as hormones, enzymes and vaccines, it is often necessary to add 'bulking' agents to give added structural strength when freeze-drying (Rey, 1990).

When antibodies were freeze-dried in the presence of 0.1 or 0.25 M cryoprotectant and stored for up to 70 days there was negligible difference between the resulting titer of the antibody with and without cryoprotectant. In fact, antibodies stored without any protective agent still retained titer as strong as that of the antibody tested immediately after freeze-drying. This is consistent with other studies conducted on the stability of antibodies of this class. For example Montoya and Castell (1987) found that freeze-dried peroxidase-labelled IgG suffered very little loss of biological activity during storage at 4°C for (Montoya and Castell, 1987).

In the present study none of the sugars tested appeared to have any affect on the stability of the antibody during the freeze-drying experiment. Figures 19c and 20c show the increased variability between samples freeze-dried without cryoprotectant. Due to the propensity of reducing sugars to participate in Maillard

browning, non-reducing sugars, such as trehalose and mannitol should be used to preserve the protein over long periods of time.

Freeze-drying is a suitable method for the preservation of IgG mAbs. The thermostability of these preparations facilitates their storage at ambient temperature. The need for equipment to maintain low temperatures is eliminated. The use of freeze-dried antibodies could simplify the transport of bioreagents and the standardization of immunoassays.

V. GENERAL DISCUSSION

Culture methods for the detection of *Salmonella enteritidis* are time consuming and labour intensive. The need for rapid methods, which will decrease the time of detection is important to both the food industry and public health. To gain acceptance and become widely used a rapid method should be cost effective, as sensitive as the standard procedure and simple. Numerous rapid methods have been developed for the detection of *Salmonella*. These include immunoassays (Kerr *et al.*, 1992; Holt *et al.*,1995; Lee *et al.*, 1989; Lee *et al.*, 1990), DNA hybridization assays (Bej *et al.*, 1994; Cano *et al., 1993;* Jones *et al.*, 1993; Tsen *et al.*, 1994), flow cytometry (McClelland and Pinder, 1994) and fluorescent antibody assays (Thomason, 1981). None of these methods are without disadvantages. Antibodies used in immunoassays are often apt to cross-reaction with antigens from non-target organisms (Kerr *et al.*, 1992; Robison *et al.*, 1983). DNA hybridization can result in amplification and detection of non-*salmonellae* if hybridization is performed under low stringency conditions (Baumler *et al.*, 1997). The equipment necessary for hybridization assays and flow cytometry is expensive and trained personnel are required.

We have previously described an immunoassay for the detection of *S. enteritidis* in foods and feeds using homogenized whole egg as an enrichment medium, negating a selective enrichment step, and detection via an immunoblot using a monoclonal antibody specific to *S. enteritidis* (Yoshimasu, 1996). This assay was able to detect *S. enteritidis* in 25 hours when outcompeted 1 to 400 (Yoshimasu, 1996). In this study we compared the use of synthetic media with homogenized whole egg for enrichment of *S. enteritidis* prior to detection. We examined other methods to simplify the assay, and examined the stability of the antibody once purified and freeze-dried for incorporation into a shelf-stable detection kit.

Of the three non-selective media which we compared with homogenized whole egg for relative ability to propagate *S. enteritidis* to numbers that could be visualized by the immunoassay, peptone water was found to be inferior. The immunoblots developed from cultures grown in this media were faint. Lactose broth did not give as strong a visual impression on the nitrocellulose membrane as trypticase soy broth. Trypticase soy broth therefore was chosen for subsequent comparisons with the homogenized whole egg medium.

It has been found that addition of iron as ferrous sulphate to homogenized whole egg medium improved detection of *S. enteritidis* (Yoshimasu, 1996). Lactose broth, trypticase soy broth, and homogenized whole egg with added

ferrous sulphate, and ferric chloride were assessed in order to determine if the valence state of the iron would have an effect on growth. Addition of iron had no effect on the growth of *S. enteritidis* in either synthetic media as determined by viable counts on SPC agar. Both forms of iron did improve the growth of the organism in homogenized whole egg and were equivalent in their enhancement. This was also determined by SPC. As albumen is an iron restricted environment the added iron served as a necessary micronutrient supplement. This phenomenon has been observed by other researchers examining the growth of *S. enteritidis* in eggs (Gast and Holt, 1995b; Cudjoe *et al.*, 1994a).

Cholic acid was added to the media before performing the assay in order to release LPS from the bacteria. Agar was added to the TSB in order to produce a solid plug for placement on the nitrocellulose membrane. When we added these two components to the media prior to incubation, a modification to simplify the assay, we found negligible effects of both components on the replication of *S. enteritidis* to numbers sufficient for visualization by the assay as determined by viable counts on SPC agar.

The possibility of shortening the enrichment time was also examined. It was found that even with incorporation of agar and cholic acid before incubation, fewer than 10 cells/25 mL trypticase soy broth could be detected by the immunoblot after 10 hours of incubation.

Growth of *S. enteritidis* to detectable numbers in mixed cultures was compared between TSB and egg supplemented with ferrous sulphate. In tests with artificially introduced competitors both media were comparable, both allowing detection of *S. enteritidis* when outcompeted by $1:10^2$. The same ratio of *S. enteritidis* to competitors was visualized in situations of natural contamination, when *S. enteritidis* was inoculated into wash water samples taken from a poultry processing plant. On one occasion we added ferrioxamine E to both media instead of ferrous sulphate before incubation. We found that this allowed detection of *S. enteritidis* when outcompeted by $1:10^3$, the highest competing ratio tested, when incubated in homogenized whole egg. The ferrioxamine E appeared to have no effect on the detection of this organism in trypticase soy broth however, with the organism only being detected at a ratio of $1:10^2$ after incubation in trypticase soy broth containing ferrioxamine E. This is possibly due to the inability of *S. enteritidis* to use this siderophore in a non iron restrictive environment.

An essential property of any commercial detection kit is the stability of all components. In immunoassays, the least stable component is the monoclonal antibody used. Freeze-drying can be an ideal method for protein stabilization. When properly conducted, freeze-drying yields products that are lightweight, and can be shipped and stored at ambient temperatures. Rehydration is performed when the antibody is needed. We examined the stability of the IgG antibody

freeze-dried in the presence of various excipients and stored for up to 70 days. The monoclonal antibody used in this experiment was of class IgG_{2a}. This class of antibody contains three inter-heavy chain disulfide bonds and is relatively stable. After freeze-drying and storing under accelerated storage conditions of 50 °C for 70 days, it still retained its original activity. During the entire length of the stabilization study the antibody stored alone was as stable as that stored in the presence of cryoprotectants. It has been reported that IgG can be stored successfully in the frozen state or freeze-dried with no great losses after long-term storage (Montoya and Castell, 1987).

Freeze-dried IgG in the presence of maltose and sucrose, when stored at higher temperatures, exhibited reduced antibody titer and brown discolouration. This was probably due to Maillard browning reaction between the reducing sugar maltose, and the reducing constituents of sucrose; glucose and fructose which react with amino groups of the protein. When the antibody was stored in the presence of either of these two exipients at lower temperatures, and trehalose or mannitol at all temperatures, it showed no reduction in titer after 70 days of storage.

VI. CONCLUSION AND RECOMMENDATIONS

A dot-blot immunoassay for the detection of *S. enteritidis* in foods and feeds was shortened and simplified. Synthetic media were compared with iron supplemented homogenized whole egg in order to determine the efficacy of using commercial media for the immunoassay. Trypticase soy broth and ferrous sulphate supplemented homogenized whole egg media were found to be equally effective for enrichment of this organism prior to detection by immunoblotting. Cholic acid and agar were added to the media before incubation in order to reduce reaction steps in performance of the assay. In addition, using trypticase soy broth enrichment, *S. enteritidis* was detected by the immunoblot after only 10 h incubation at 37 °C.

The immunoblot successfully detected *S. enteritidis* in wash water samples taken from a poultry plant. When outcompeted by flora at a ratio of $1:10^2$ *S. enteritidis* was still detected by the immunoassay.

Preliminary results using homogenized whole egg supplemented with ferrioxamine E gave very promising results. In this case the pathogen was detected by the immunoblot when outcompeted $1:10^3$. More studies must be

conducted using homogenized whole egg supplemented with this siderophore in order to confirm the selective advantage conferred upon *Salmonella* in this medium.

The antibody was stable to storage at extreme temperatures after freeze-drying both with and without the protective agents trehalose and mannitol. Further, long term storage studies (over one year) should be conducted on the freeze-dried antibody to determine its stability over greater periods of time.

Using either trypticase soy broth or supplemented whole egg, *S. enteritidis* can be detected in 21 hours by the Dot-Blot immunoassay. The antibody, once purified and freeze-dried is shelf stable for at least 70 days at 50 °C. These results are promising for the production of a commercially available detection kit.

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