A RAPID DOT-BLOT IMMUNOASSAY FOR THE DETECTION OF *SALMONELLA ENTERITIDIS* IN FOODS

BY

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Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
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Political Science
- General 0615
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- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
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BY

MARK A. YOSHIMASU

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

MASTER OF SCIENCE

To my family, Paul, Rosanne
Cathy and Lisa for their
unending love and support.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviation	Definition
A-AP	Avidin-alkaline phosphatase
AP	Alkaline phosphatase
ATCC	American Type Culture Collection
B-GAM	Biotinylated goat anti-mouse IgG
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BSA	Bovine serum albumin
CFU	Colony forming units
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
GAM	Goat anti-mouse IgG
GAM-AP	Goat anti-mouse IgG-alkaline phosphatase
kDa	Kilodalton
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
NBT	p-nitroblue tetrazolium chloride
PAb	Polyclonal antibody
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween 20
PLL	Poly-L-lysine
pNPP	p-nitrophenyl phosphate
PT	Phage type
RIA	Radioimmunoassay
RNA	Ribonucleic acid
S-AP	Streptavidin-alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SC	Selenite cysteine broth
SMP	Skim milk powder
SPC	Standard plate count
TB	Tetrathionate broth
TBS	Tris buffered saline
TTBS	Tris buffered saline containing Tween 20

ABSTRACT

Eggs contaminated with *Salmonella enteritidis* are believed to be an important vehicle for the increase in food-borne human salmonellosis observed in many countries since the late 1980s. This is due to the infection of laying hens with *S. enteritidis* and the resultant transovarian transmission of *S. enteritidis* into the egg. Conventional culture methods used in the detection of *Salmonella* are time consuming and labour intensive.

The objective of this study was to establish and optimize a dot-blot immunoassay for the detection of *S. enteritidis* in fresh shell eggs, poultry and other foods and feed. Homogenized eggs were inoculated with *S. enteritidis*, incubated at 37°C and heated in the presence of sodium cholate. After a short cooling period, egg samples were placed on PBS saturated nitrocellulose membrane strips. Lipopolysaccharide antigens were transferred via diffusional forces from the sample onto nitrocellulose strips. Strips were blocked and probed with an anti-LPS monoclonal antibody specific to the D_1-serogroup of *Salmonella*. Membranes were incubated with biotinylated goat anti-mouse IgG, strepavidin alkaline phosphatase and developed with a substrate solution. Positive results were observed as purple dots on the membrane. Samples of poultry and other foods were incubated in homogenized eggs. After incubation, egg samples were taken, heated and applied to the immunoassay. The addition of heat and sodium cholate were required to release the LPS antigens from the bacterial membrane. Sodium azide and Triton X-100 were found to be efficient in LPS removal. However, they were not as effective when applied to the dot-blot immunoassay. One *S. enteritidis* cell per 25 gm of egg was readily

detected after a 20 h incubation period. Incorporation of ferrous sulfate in 500 gm of egg allowed for the detection of one *S. enteritidis* cell after 20 h of incubation. Detection of *S. enteritidis* was feasible in homogenized egg after a 24 h incubation period even when out competed 1 to 400 with other bacteria . *Salmonella enteritidis* was also detected in artificially inoculated chicken meat, ice cream, skim milk powder and poultry feed. Overall, this method required 24 h for detection of *S. enteritidis* in eggs and 28 h for detection in other foods and feed. All results were confirmed through culture methods indicating a good correlation between both methods.

FOREWORD

This thesis has been written in the manuscript style. The two manuscripts are interrelated but independent of each other and have been prepared according to the instructions by the specific journals to which they are being submitted. Manuscript 1, outlines the development and optimization of a rapid dot-blot immunoassay for the detection of *Salmonella enteritidis* in eggs, poultry and other foods and feed. This paper will be submitted to Applied and Environmental Microbiology. The second manuscript describes the evaluation of various detergents in the preparation of *S. enteritidis* for use in the dot-blot immunoassay. This will be submitted to the International Journal of Food Microbiology.

The authors and titles of the manuscript are:

1. Yoshimasu, M. and Zawistowski, J. 1996. Development and Development and Optimization of a Rapid Dot-blot Immunoassay for the Detection of *Salmonella enteritidis* in Eggs, Poultry and Other Foods and Feed.

2. Yoshimasu, M. and Zawistowski, J. 1996. The effect of Detergents on Extractibility of Lipopolysaccharides from *Salmonella enteritidis.*

I. INTRODUCTION

Salmonella enteritidis is one of the dominant serotypes isolated from cases of food poisoning worldwide. In Canada, isolation of *S. enteritidis* from humans increased from 884 in 1987 to 1,257 in 1994, (Khakhria *et al.*, 1995). Similarly, in the United States, *S. enteritidis* in humans increased from 1,884 in 1980 to 8,572 in 1990, (Mason, 1994). However, the largest increase was observed in Britain where isolation of *S. enteritidis* in humans increased from 12,000 in 1982 to over 31,000 in 1992, (Roberts and Sockett, 1994).

Epidemiological investigations conducted worldwide have identified that fresh shell eggs and egg-associated products are important vehicles of infection by *S. enteritidis*. This was established in 1988 when St. Louis *et al.* (1988) analyzed the results of 65 foodborne outbreaks in northeastern United States. Twenty-seven of the 35 outbreaks with identified food vehicles were associated with the consumption of egg or egg-containing foods. Infection of laying hens with *S. enteritidis* and the resultant contamination of eggs is believed to be important in the marked increase in human salmonellosis.

Conventional culture methods (AOAC, 1995) for detection of *Salmonella* require up to seven days. These procedures are labour-intensive requiring pre-enrichment, selective enrichment and a variety of serological and biochemical tests (van der Zee, 1994). In contrast to conventional methods, two basic ELISA procedures are available, indirect and sandwich (Keller *et al.*, 1993; Lee *et al.*, 1989). These assays utilize antisera

as well as monoclonal antibodies (MAb's) against flagella (van der Zijderveld *et al.*, 1992), lipopolysaccharide (Wang *et al.*, 1995) and fimbriae (Thorns *et al.*, 1994) of *S. enteritidis*. Immunoassays can be completed in two days or less, however, they still demand a pre-enrichment, selective enrichment and often a post enrichment step. Many antibodies produced against *S. enteritidis* have also shown cross-reactivity with other bacteria.

The objective of this study was to establish and optimize a dot-blot immunoassay which was developed in our laboratory. Monoclonal antibodies specific to D_1-serogroup of *Salmonella* (Masi and Zawistowski, 1995) were employed. The optimized test was evaluated for its performance in the detection of *S. enteritidis* in fresh shell eggs. Furthermore, the application of the dot-blot immunoassay for detection of this pathogen in other foods and feed was investigated.

II. LITERATURE REVIEW

A. Introduction

In the past 10 years, many countries have witnessed a dramatic increase in the cases of clinical infection involving *Salmonella enteritidis*. Epidemiological evidence shows the link between gastroenteritis to the consumption of foods containing raw or under cooked eggs, or poultry meat. These studies also indicated that although the same vehicles of transmission were involved, different phage types (PT) of *S. enteritidis* are prevalent in different parts of the world. In the UK, PT4 is the most common (Roberts and Sockett, 1994) while in Canada and the United States, PT8 and PT13a are the most common type of *S. enteritidis* (Poppe, 1994; Mason, 1994).

B. Epidemiology Associated with Eggs

Contamination of chicken meat with *S. enteritidis* has been well established (Rampling *et al.*, 1989), however, epidemiological investigations have quickly identified fresh shell eggs and egg associated products as important vehicles of transmission. This was established in 1988 when St. Louis *et al.* (1988) analyzed the results of 65 foodborne outbreaks in the northeastern United States. Twenty-seven of the 35 outbreaks with identified food vehicles were associated with the consumption of egg or egg-containing foods. Since then, many case-control studies have revealed and reinforced the association of *S. enteritidis* and shell eggs, (Cox, 1995a).

C. Egg Shell Contamination

Association between *Salmonella* and shell eggs was established in the 1960s when cases of salmonellosis were found to be caused by faecal contamination of egg shells, (Blumenthal, 1990). These outbreaks, known as horizontal transmission, were caused through transient intestinal infection of the laying hen leading to shell contamination after laying (Clay and Board, 1991). It has been hypothesized that the cuticle at and for a brief period following oviposition is immature thus acting as a relatively ineffective barrier to bacterial translocation across the shell (Dolman and Board, 1992). Faecal extract has been observed to enhance contamination of eggs at and immediately following oviposition (Clay and Board, 1992). Correlation between egg shell contamination and faeces positive for *S. enteritidis* PT13 in artificially infected hens was established (Gast and Beard, 1990a).

D. Contamination of Egg Contents

There has been some controversy over the route by which *S. enteritidis* gains entry into the egg. There is the possibility that *S. enteritidis* present on the egg shell can migrate through the shell and associated membranes, however, there has been no correlation between contamination of the shell and contents (Cox, 1995a). Haigh and Betts (1991) showed that *Salmonella* was able to penetrate the shell and underlying membrane to contaminate egg contents. Similar findings were reported by Schoeni *et al.* (1995). It has been speculated that contamination of intact eggs is more the result of infected reproductive tissue. *Salmonella enteritidis* can be deposited during egg formation

in the ovary or oviduct before the egg is covered by the shell (Humphrey, 1994). This type of contamination has been termed vertical transmission. Other serotypes have been isolated from egg shells but only *S. enteritidis* has been isolated from the contents of intact eggs (Humphrey, 1994).

In an attempt to understand the mechanism of transovarian transmission, Thiagarajan *et al.* (1994) conducted experiments to examine isolates of *S. enteritidis* from preovulatory follicles of artificially infected hens. It is conceivable that *S. enteritidis* can contaminate the yolk *in vivo* by interacting and invading the hen's ovary granulosa cells. When the follicle ruptures during ovulation, some of the contaminated granulosa cells may slough off resulting in contamination of the egg. It has also been suggested that *S. enteritidis* binds to fibronectin, an adhesive protein, which is synthesized and secreted by the granulosa cells (Novero and Asem, 1993).

E. Prevalence of *Salmonella*-Positive Egg Contents

The prevalence of contaminated eggs from naturally infected flocks has been variable. Recent studies involving either artificially or naturally infected hens have observed a clustering of eggs with *Salmonella*-positive contents. Gast and Beard (1990a) reported that artificially infected hens produced *S. enteritidis* contaminated eggs at a high frequency over the first two weeks of exposure. Other studies (Humphrey *et al.*, 1989b; Timoney *et al.*, 1989 and Shivaprasad *et al.*, 1990) reported similar findings.

The microbiological population present in the egg contents tend to be low. In a survey of 132 fresh laid eggs from experimentally infected hens, four were tested positive

for *S. enteritidis* (Gast and Beard, 1992). The average level of contamination was 5.5 CFU/ml. Similar results were reported by Humphrey *et al.* (1989a), however, they found that naturally contaminated eggs contained less that 10 *S. enteritidis* cells per egg.

F. Growth and Survival in Eggs

1. Growth and survival in albumen

There is conflicting evidence regarding the survival of *S. enteritidis* in the albumen. Albumen contains ovotransferrin which chelates iron inhibiting the growth of Gram-negative bacteria (Clay and Board, 1991). Subsequently, the growth of *S. enteritidis* is reduced in the albumen, although cells remained viable at 20°C or 30°C but declined at 4°C (Lock and Board, 1992). Similar results were obtained by Humphrey and Whitehead (1993). Conversely, earlier results by Humphrey (1990a) indicated that survival of *S. enteritidis* in albumen was best at low temperatures while at optimal temperatures for bacterial growth, cells steadily declined.

Chart and Rowe (1993) reported the expression of three iron regulating outer membrane proteins when *S. enteritidis* was grown in the presence of ovotransferrin. They concluded that the growth of *S. enteritidis* in stored eggs is due to the migration of iron from the yolk into the albumin and that ovotransferrin would not prevent the growth due to expression of these iron sequestering proteins.

2. Migration of *S. enteritidis* from albumen to yolk

Braun and Fehlhaber (1995) studied the migration of *S. enteritidis* from the albumen into the egg yolk. *S. enteritidis* was able to migrate from the albumen into the

egg yolk during storage. When eggs were incubated at 20°C and 30°C, cells were present in the yolk after one to two days. At 7°C, cells were present after 14 days. Similar results were described by Baker (1990).

3. Site of contamination in whole egg

Examination of eggs from artificially and naturally infected hens has revealed that the principal site of infection is either in the albumen or outside the membrane surrounding the yolk (vitelline membrane). A ten-fold increase was observed in the first 10 to 24 hours when *S. enteritidis* was inoculated into the albumen around the vitelline membrane (Humphrey and Whitehead, 1993). Growth ceased for approximately two weeks but in the third week, cells increased to $> 10^6$ per egg. Once the bacterium has depleted its iron reserves, it enters the stationary phase where little or no changes in growth occur. This phase is temperature dependent and at 20°C, it can last for three to four weeks (Humphrey, 1994). During storage, the vitelline membrane degrades to a point where is becomes sufficiently permeable to allow *S. enteritidis* to invade the yolk or allow the migration of iron into the albumen (Humphrey and Whitehead, 1993)

4. Effect of temperature on growth of *S. enteritidis* in eggs

Temperature has a significant effect on the growth of *S. enteritidis* in eggs. Kim *et al.* (1989) observed an increase in bacterial growth with an increase in temperature during egg storage. When inoculated eggs were held at 8°C, no growth was reported, while at 10°C, growth slowly increased with an extended lag phase (Humphrey, 1990a). At 12°C and above, growth was rapid with a short lag phase. Similar findings were reported by Hammack *et al.* (1993) and Saeed and Koons, (1993). Eggs stored under

conditions where temperatures fluctuated between 18 and 30°C also led to rapid growth after 6 to 10 days (Humphrey and Whitehead, 1993).

5. Survival of *S. enteritidis* after cooking

Consumption of eggs contaminated with *S. enteritidis*, whether alone or in the form of egg products, represent a potential threat to public health. Humphrey *et al.* (1989c) demonstrated that *S. enteritidis* present in the yolk can survive in under cooked eggs. When all or part of the yolk remained liquid and where cooked yolks did not exceed temperatures which were lethal for salmonellae, survival was largely unaffected by the level of inoculum. When growth exceeded 10^8 cells/gm of yolk, no standard cooking method completely eliminated the organism. In a later study, Humphrey *et al.* (1990) concluded that the egg yolk is protective and increases the heat resistance of *S. enteritidis*. Exposure to alkaline conditions, similar to those in albumen (pH 9.0), have also been found to increase the heat resistance in *S. enteritidis* PT4 (Humphrey *et al.*, 1991). Conversely, if eggs are initially stored at refrigerated temperatures (4°C to 8°C), *S. enteritidis* is more sensitive to heat (Humphrey, 1990b).

G. Change in the Host-Pathogen Relationship

Salmonella enteritidis is a pathogen of many mammals and fowls. However, the incidence of this organism in poultry has been predominant in many countries (Mason, 1994; Edel, 1994 and Poppe *et al.*, 1991).

1. Route of infection in poultry

Most *Salmonella* infections in poultry are due to ingestion of the bacterium. The organism moves through the alimentary tract where is can adhere to and penetrate the intestinal epithelial cells. Some strains of *Salmonella* can invade, survive and spread into the reticuloendothelial system. If this occurs, *Salmonella* can then disseminate into other tissues causing serious systemic diseases (Barrow *et al.*, 1987). This pathogen has been isolated from the peritoneum (Shivaprasad *et al.*, 1990), caecum (Gast and Beard, 1990b), liver and spleen (Shivaprasad *et al.*, 1990; Gast and Beard, 1990b and Barrow and Lovell, 1991), heart (Barrow and Lovell, 1991), ovaries and oviducts (Barnhart *et al.*, 1991; Gast and Beard, 1990b) of chickens.

2. Epidemiology of *S. enteritidis* in poultry

Acute outbreaks of *S. enteritidis* infections generally occur in young birds and birds under extreme stress. Induced molting has been found to increase the susceptibility and increase the severity of intestinal infection by *S. enteritidis* (Holt and Porter, 1992; Holt, 1995). It seldomly occurs in semi mature and adult birds under normal conditions, (Suzuki, 1994). The infection seldom causes mortality in hens more than one month old and resistance increases with age possibly due to the development of normal flora in the intestine and immune system (Corrier *et al.*, 1991). No clinical signs of disease were observed in 52-week and 18-week old laying hens orally inoculated with *S. enteritidis* PT4 (Humphrey *et al.*, 1989a). Experimental inoculations of hens with *S. enteritidis* exceeding 10^8 cells/ml via oral or intravenous routes resulted in some clinical signs (Cox, 1995a). At these levels of inoculum, faecal shedding occurred predominantly during the

first two week postinoculation (Shivaprasad *et al.*, 1990), intestinal colonization persisted for up to 22 weeks (Gast and Beard, 1990a,b).

3. Effects of different phage types of *S. enteritidis*

Different phage types exhibit considerable variation in virulence. Hinton *et al.* (1990b) found that *S. enteritidis* PT4 was more invasive in young chicks than PT7, 8 and 13a. Eight strains of *S. enteritidis* representing three different phage types were evaluated in experimentally infected chicks and laying hens (Gast and Beard, 1992). Significant differences in mortality rates in chicks, total egg production, frequency of *Salmonella*-positive eggs, and serum antibody response in hens were observed between strains. Mortality ranging from 96% (PT4) to 20% (PT13a) in four phage types of *S. enteritidis* were also observed by Barrow (1991).

H. Control of *S. enteritidis* Infections in Poultry

For several years, the control of *Salmonella* spp. in poultry has been a top priority in many countries (Mason, 1994). In the United States, a nationwide mandatory testing program for *S. enteritidis* in all egg-layer flocks has been proposed (Mason, 1994). Surveillance of all primary breeding flocks, multiplier breeding flocks, hatcheries, replacement pullet flocks, commercial laying hens, feed ingredients and environmental samples has been initiated (Bryant, 1990). Any layer flock considered to be the source of egg-implicated outbreaks would be tested and eggs would be diverted to pasteurization plants.

Research has focused on eradication of salmonellae by competitive exclusion

(Oyarzabal *et al.*, 1995; Behling and Wong, 1994) hygienic processing of feed (Bailey, 1988), administration of antimicrobial agents in drinking water (Goodnough and Johnson, 1991) and a combination of competitive exclusion and antimicrobial agents (Seuna *et al.*, 1980). All these applications have met with limited success.

Barrow *et al.* (1991) vaccinated laying hens with either *S. gallinarum* 9R or a mutant of a *S. enteritidis* PT4 strain. A reduction in the number isolated from organs and eggs was noted, however, *S. enteritidis* was still present.

I. Biology of *S. enteritidis*

1. Relationship between stains

Using multilocus enzyme electrophoresis, Beltran *et al.* (1988, cited in Cox, 1995a) analyzed the relationship among strains of *S. enteritidis* and other serotypes. After analyses of 258 isolates of *S. enteritidis*, more than 90% represented a single clonal line. Olsen *et al.* (1994) used IS200 profiling, pulsed-field gel electrophoresis, restriction fragment length polymorphism typing and ribotyping to characterise 33 phage types of *S. enteritidis*. The study found two clonal lines, one containing PT4 and the other containing PT8 and PT13a.

2. Plasmids

Most strains of *S. enteritidis* possess a 38 MDa plasmid virulence gene (Brown *et al.*, 1993). The plasmid is associated with virulence in BALB/c mice, however, it is not associated with colonization of the interaction mucosa and it is not required for invasion into deeper tissues as strains missing this plasmid have reached the liver and

spleen of day-old chickens (Hinton *et al.*, 1990b). This plasmid does enable *S. enteritidis* to persist within reticuloendothelial cells (Finlay and Falkow, 1988). The 38 MDa plasmid is an important virulence factor for strains of *S. enteritidis* in mice. However, the enhanced virulence of the current strains for poultry are unlikely to be the result of changes in this plasmid (Chart *et al.*, 1989).

3. Lipopolysaccharide

Outer membrane lipopolysaccharides (LPS) are an important factor in the virulence of *S. enteritidis*. Lipopolysaccharide are composed of three components: lipid A, inner core and O-side chain oligosaccharides (Lindberg, 1980). The lipid A is a potent endotoxin (Finlay and Falkow, 1988). Interaction of the endotoxin with macrophage and lymphocytes will activate these cells which in turn cause the release of several factors leading to a wide variety of biological effects (D'Aoust, 1991). The O-side chains are composed of repeating oligosaccharide units which vary between *Salmonella* species. Differences in length and composition significantly alter the virulence. (Finlay and Falkow, 1988). It has also been hypothesized that LPS may be involved in attachment to and invasion of intestinal epithelial cells (Finlay and Falkow, 1988). LPS is also important in resistance to lytic action of the complement cascade (Suzuki, 1994). Resistance of *S. enteritidis* to macrophage phagocytosis is also affected by the composition of the O-side chain (Suzuki, 1994). For *S. enteritidis*, the presence of tyvelose prevents phagocytosis (D'Aoust, 1991).

4. Fimbriae

Fimbriae are thin organelles expressed on the surface of *S. enteritidis* (Thorns *et*

al., 1994). They carry a major protein that consist of repeating subunits (Thorns *et al.*, 1990). Four types of fimbriae have been associated with *S. enteritidis*; SEF14, SEF17, SEF18 and SEF21 (Cox, 1995b). Of the four, SEF17 has been found to be associated with adherence and pathogenicity due to its binding to fibronectin. As mentioned earlier, part of the mechanism of transovarian transmission involves *S. enteritidis* binding to fibronectin which is secreted by the granulosa cells (Novero and Asem, 1993).

In Australia, several strains of *S. enteritidis* have been shown to be avirulent or exhibit low virulence in day-old chicks (Cox, 1995a). The avirulent strains possess the 38 MDa plasmid and express long chain LPS typical of a virulent *S. enteritidis*, however, it does not appear to synthesize SEF17 (Cox, 1995b). This suggests that the expression of the gene for SEF17 plays a significant role in systemic infections of *S. enteritidis* in poultry.

J. Detection

Although the control of *S. enteritidis* requires action at all levels of transmission, reliable testing with statistically significant sampling and sensitive analytical methods are still important. Methods involving a series of enrichment steps have been developed. However, a need for methods which provide results rapidly with a sensitivity similar or greater than the conventional culture methods are required. These methods should be reliable, specific, simple and cost effective.

1. Conventional culture method

Conventional culture methods for the isolation of *S. enteritidis* can be broken

down into four distinct phases: (1) pre-enrichment, which allows for the recovery of any stressed or injured organisms; (2) selective enrichment, which allows for the growth of *S. enteritidis* while reducing the growth of non-salmonella; (3) isolation, using selective agar plates to produce presumptive isolates and (4) confirmation through a variety of serological and biochemical tests (Flowers *et al.*, 1992).

a) Pre-enrichment

A wide variety of approved pre-enrichment broths are available for resuscitation of *Salmonella* spp (AOAC, 1995). However, only a few methods have been developed or modified for detection of *S. enteritidis* in egg and egg products. The addition of ammonium-iron(III)-citrate to buffered peptone water (Dolzinksy and Kruse, 1992, cited in van der Zee, 1994), ferrous sulfate to trypticase soy broth (Gast, 1993b; Gast and Beard, 1992) and ferrioxamine-type siderophores to buffered peptone water (Reissbrodt and Rabsch, 1993) have increased the isolation of *S. enteritidis*. For intact eggs, Humphrey and Whitehead (1992) added three volumes of buffered peptone water to the contents of one egg and incubated for 24 h at 37°C. For four or more eggs, incubation was increased to 48 h. Good results were obtained by addition of novobiocin and cefsoludin following incubation. However, egg samples used for this study were only from flocks artificially infected with *S. enteritidis*.

b) Selective enrichment

For selective enrichment, the use of semi-solid agar has been proved to increase the isolation rate of *S. enteritidis* (Perales and Erkiaga, 1991). Semi-solid media which appear to favour the isolation of *S. enteritidis* are: (1) Semi-solid Rappaport-Vassiliadis

(SRV) according to Goosens *et al.* (1984); (2) Modified Semi-solid Rappaport-Vassiliadis (MSRV), according to De Smedt and Bolderdijk (1987); (3) Diagnostic Semi-solid Salmonella Agar (DIASALM) according to van der Zee (1991, cited in van der Zee, 1994). The efficiency of semi-solid agars is due to the ability of *S. enteritidis* to migrate through the selective medium forming motility zones (van der Zee, 1994). Addition of nitrofurantoin to the media has also shown to increase isolation of *Salmonella* (Rampling *et al.*, 1990). Nitrofurantoin, an antimicrobial drug, has been used in the poultry industry to prevent and control *Salmonella* infections. Rampling and co-workers (1990) found that 98% of *S. enteritidis* PT4 strains were resistant while 64% of other serovars were susceptible to nitrofurantoin. Addition of nitrofurantoin to selective media might increase the chances of isolating *S. enteritidis* from samples which might be contaminated with other serovars.

Direct selective plating versus pre-enrichment for detection of *S. enteritidis* in eggs has been investigated. Stephenson and co-workers (1991) concluded that pre-enrichment was required for recovery of *S. enteritidis* from egg albumen, although no significant differences were observed for recovery in whole egg and egg yolk. Direct plating of this pathogen from egg pools was also evaluated (Gast, 1993a). Using pre-enrichment and selective enrichment allowed for detection of a significantly higher percentage of contaminated egg pools than by direct plating. Gast (1993a) concluded that direct plating can provide a relatively rapid and inexpensive method for detecting *S. enteritidis* in egg pools although this method is not particularly reliable because it can yield false positives.

c) Confirmation

To confirm the presence of *S. enteritidis* in food samples, many laboratories applied serological techniques. This is achieved through the use of antisera that recognize somatic (O) and flagellar (H) antigens (Flowers *et al.*, 1992). Because these antisera are specific to D1-serogroup, a few other *Salmonella* species may be also detected. Once positive results are obtained from serological testing, cultures can be sent to a reference centre for complete identification through biotyping, phage typing and plasmid DNA profiling.

d) Advantages and disadvantages of culture method

The conventional culture method is an official and approved AOAC (1995) method used for detection of *S. enteritidis* in foods. Methods are labour-intensive, time consuming and expensive. For specific isolation of *S. enteritidis*, culture methods cannot differentiate between *S. enteritidis* and other serovars without the use serological followed by biochemical tests and DNA finger-printing. A presumptive negative requires four to five days while complete identification requires up to seven days or longer (Andrews, 1985). It has also been found that some of the routinely used selective enrichment broths are inhibitory to *S. enteritidis* (van der Zee, 1994).

2. Hydrophobic grid membrane filter

Rapid detection of *Salmonella* based on the hydrophobic grid membrane filter (HGMF) was first introduced by Entis *et al.* (1982). Filtration of a selective enrichment broth through the HGMF reduced this enrichment step from 24 to 6 h. Subsequently, 18 to 24 h was required for pre-enrichment, 6 to 8 h for selective enrichment followed by 18 to 24 h on a selective agar. Biochemical and serological testing is still required from

presumptive positives. Although initially, this technique was limited to six food categories (AOAC, 1990), the procedure has been expanded to include all foods (AOAC, 1995). The protocol was also modified to include detection of *Salmonella* spp. in egg products (Entis, 1996). An initial inoculum of one to two cells per gm of product was detected by this method. Presumptive results were obtained in as little as 42 h with confirmation requiring an additional 24 h. The only drawback to this method is the use of a single selective enrichment and plating agar. This may prevent the detection of some strains of *Salmonella* which are more readily detected by other selective broths and/or selective agars.

3. Immunoassays

a) Fluorescent antibodies immunoassays

The fluorescent antibody (FA) immunoassay was the first officially recognized rapid method for detection of *Salmonella* (AOAC, 1995). After a 52 hour enrichment (AOAC, 1995), samples are stained with fluorescent-labelled antibodies against *Salmonella* antigens and detected using fluorescent microscopy. Cells which fluoresce and have the same typical morphology of *Salmonella* constitute a presumptive positive. Results must be confirmed by cultural methods. Haglund and co-workers (1964, cited in Blackburn, 1993) and Silliker *et al.* (1966) used this technique to detect *Salmonella* in eggs and egg products using H antisera.

Cross-reactivity with non-*Salmonella*, expensive equipment, training in FA methodology, labour intensive and subjective interpretation of results made FA difficult for the food industry to accept this method.

b) Enzyme immunoassays

Although radioimmunoassays have also been used for detection of salmonellae in foods, the disadvantage of handling radioisotopes led to the development of enzyme immunoassays (EIA) (Blackburn, 1993). Antibodies used in EIA can be either polyclonal or monoclonal and the enzyme activity of the labelled antibody can be determined by fluorometric, luminometric or colorimetric (Porstmann and Kiessig, 1992).

Recently, a number of monoclonal antibodies (MAb) against a variety of antigenic factors of the D-serogroup of *Salmonella* have been developed. These antibodies have potential use in EIA, however, their effectiveness has not been fully evaluated through interlaboratory testing.

Two monoclonal antibodies against *S. enteritidis* were developed for use in an enzyme-linked immunosorbent assay (ELISA) and agglutination assay (Keller *et al.*, 1993). Both MAb's exhibited cross-reactivity with other serogroups that yielded false negatives in eggs. Furthermore, assays employing these antibodies require at least 48 h for completion. Wen-Chuan Lin *et al.* (1992) also produced three MAbs against *S. enteritidis*. While all three MAb's appeared to be specific for *S. enteritidis*, evaluation against non-*Salmonella* was not attempted. Lee *et al.* (1989) developed a rapid ELISA for detection of *Salmonella* in eggs. A minimum of 10 *S. enteritidis* cells per egg was detected in less than 24 h, although the MAb cross-reacted with some strains from B-serogroup *Salmonella.* Thorns *et al.* (1994) produced a MAb specific to a fimbrial polypeptide of *S. enteritidis*. Also, this antibody yielded false positives and in one case and in others, *S. enteritidis* PT2 was not recognized. Brigmon *et al.* (1995) designed a

sandwich ELISA for the detection of *S. enteritidis* in poultry and eggs. Cross-reactivity was not exhibited with 35 non-*Salmonella* and 32 *Salmonella* species and *S. enteritidis* was detected in chicken meat, skin and whole eggs in 24 h. The sensitivity of the ELISA for detection of this pathogen in chicken meat and skin was similar to results reported by Keller *et al.* (1993). However, a decrease in sensitivity occurred when eggs were tested. A level of 10^7 *S. enteritidis* cells/ml was required for detection.

4. Alternative solid phase surfaces for enzyme immunoassays

Although microtitre plates are the most common solid phase surfaces for EIA, Blais and Yamazaki (1989) used a polyester cloth for capturing *Salmonella* antigens. The polyester cloth was soaked with either PAbs (Blais and Yamazaki, 1989) or polymyxin B (Blais and Yamazaki, 1990a) to capture salmonellae antigens. Using anti-serogroup D_1 rabbit antiserum, Wang *et al.* (1995) were able to detect *S. enteritidis* in eggs using the polymyxin-cloth. Egg contaminated samples heated in the presence of a 15% sodium cholate solution facilitated the release of LPS from the bacterial membrane which in turn was captured by the polymyxin-cloth. The assay detected 10^6 cells per ml of egg in less than 24 h. However, PAbs exhibited cross-reactivity with other enteric bacteria.

Holt *et al.* (1995) used magnetic beads coated with anti-*Salmonella* antibodies to capture *S. enteritidis* in contaminated egg pools. Bound *S. enteritidis* were concentrated and detected through an ELISA employing a MAb conjugated with horseradish peroxidase. The assay detected 6×10^5 cells per ml in 24 h. Cross-reactivity was not observed but only 8 *Salmonella* strains representing 7 serogroups and 3 non-*Salmonella* species were tested.

5. Immunomagnetic separation

To reduce the time used for enrichment, immunomagnetic separation using Dynabeads anti-Salmonella (Dynal, Oslo, Norway) has been employed (Cudjoe *et al.*, 1994b). Contaminated raw eggs were diluted and mixed with Dynabeads and incubated for 10 min. After incubation, magnetic beads were concentrated and streaked onto selective agar. After 24 h enrichment, *S. enteritidis* was detected from an initial inoculum of one to two cells in 500 ml of raw homogenized egg supplemented with ferrous sulphate. Similar results were reported by Holt *et al.* (1995), although ferrous sulphate was not incorporated and longer incubation was required.

6. Egg pooling

Gast (1993a) pooled the contents of more than one egg which permitted a statistically meaningful sample size without creating excessive demands for laboratory time. Pooled eggs were incubated at 37°C for four days and transferred to culture media for isolation and identification. Culturing egg pools from experimentally infected hens yielded presumptive positive results after eight days (Gast, 1993b). The addition of ferrous sulphate to medium reduced time required for presumptive positive to two days (Gast and Holt, 1995b). However, about five days was needed to confirm the presence of *Salmonella* in pooled eggs by direct plating.

III. MANUSCRIPT 1

DEVELOPMENT AND OPTIMIZATION OF A RAPID DOT-BLOT IMMUNOASSAY FOR THE DETECTION OF *SALMONELLA ENTERITIDIS* IN EGGS, POULTRY AND OTHER FOODS AND FEED

A. ABSTRACT

A simple, rapid and economical method for the detection of *Salmonella enteritidis* in eggs, poultry and other products was developed and optimized. The method employs a two step procedure comprising an enrichment step followed by a monoclonal antibody-based dot-blot assay. Eggs were homogenized, inoculated with *S. enteritidis* (1-500 cells/25 gm) and incubated for 20 h at 37°C. The mixture was heated in the presence of sodium cholate to release the lipopolysaccharide (LPS) antigen from the bacterial membrane. The antigen, uniformly dispersed within the gelled egg matrix, was transferred onto a solid support through diffusional forces and detected by MAb 2F11 specific to the LPS O-9 antigen of *S. enteritidis*. *Salmonella enteritidis* was detected in the presence of six other bacteria when out competed 1 to 400 in homogenized egg after incubation. Incorporation of ferrous sulfate allowed the detection of one *S. enteritidis* cell/500 gm of homogenized egg after 20 h at 37°C. This pathogen was also detected when out competed 1 to 300 other bacteria after incubation. To assess the feasibility of detection of *S. enteritidis* in other matrices, poultry, ice cream and chicken feed samples were inoculated with 1-10 cells/25 gm homogenized liquid egg and incubated for 24 h at 37°C. A 25 gm sample was heated in the presence of sodium cholate and the antigen detected using the immunoassay. Skim milk powder was inoculated with 1-10 cells/25 gm, homogenized in brilliant green water, equilibrated for 1 h at room temperature and incubated for 6 h at 37°C. Subsequently, one ml was transferred to 25 gm homogenized eggs and incubated for an additional 17 h at 37°C. Samples were heated with sodium cholate and antigens detected via the dot-blot assay. All results were confirmed by

culture method indicating a good correlation between both tests.

B. INTRODUCTION

In the last decade, there has been a dramatic increase in human *Salmonella enteritidis* infections in many countries across Europe as well as in North and South America (Cox, 1995a). Fresh shell eggs, egg associated products and poultry meat have been recognized as important vehicles of infection by *S. enteritidis*.

Conventional methods for the detection of *Salmonella* in foods are labour intensive, time-consuming and expensive particularly for testing a large number of samples. Some of the routinely used selective enrichment broths have been found to be inhibitory to *S. enteritidis* (van der Zee, 1994). Rapid methods have been developed for the detection of *S. enteritidis*. Gast and Holt (1995b) incorporated iron into egg pools prior to incubation followed by direct plating onto selective agar. Presumptive results were obtained in two days. Entis (1996) developed the ISO-GRID 2-day rapid screening method for detection of *Salmonella* spp. in egg products. Other rapid methods based on different principles, such as latex agglutination (Thorn *et al.*, 1994), enzyme immunoassays (Wang *et al.*, 1995; Brigmon *et al.*, 1995; Lee *et al.*, 1989) and immunomagnetic separation (Cudjoe *et al.*, 1994b) have been developed for the detection of *S. enteritidis*.

All immunological-based rapid methods which are specific for the detection of *S. enteritidis* suffer from the same drawbacks; one or more enrichments broths or in some cases, post-enrichment broths are required in the test protocal. Cross-reactivity has been

observed with most monoclonal antibodies produced against *S. enteritidis* (Thorn *et al.*, 1994; Keller *et al.*, 1993 and Lee *et al.*, 1989). Polyclonal antibodies also exhibit non-specific reactions with other enteric bacteria.

This paper reports on the development and optimization of a rapid dot-blot immunoassay for the detection of *S. enteritidis* in eggs, poultry and other products. The method utilizes a D_1-serospecific anti-LPS O-9 monoclonal antibody.

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). Nutrient broth, standard methods agar, tetrathionate broth, selenite cysteine broth, XLD agar, bismuth sulfite agar, triple sugar iron agar, lysine iron agar, peptone, Salmonella O antiserum factor 9 and Salmonella O antiserum factor 4,5 were purchased from Difco, (Detroit, MI). Brilliant green agar with sulfadiazine was purchased from BBL (Cockeysville, MD). Electrophoresis grade hydroxymethyl aminomethane (Tris), NBT (nitroblue tetrazolium chloride), BCIP (5-bromo-4-chloro-3-indolyphosphate) and streptavidin were purchased from BioRad Laboratories (Hercules, CA). Streptavidin alkaline phosphatase was purchased from Cedarlane Laboratories Ltd (Hornby, ON). 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\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). Ammonium sulphate purified tissue culture supernatant was used at a dilution of 1/25.

3. Bacteria and culture conditions

Salmonella enteritidis phage type (PT) 8 was obtained from the Laboratory Center for Disease Control, Ottawa, Canada. The culture was maintained on a standard plate count (SPC) agar slant. A loopful was inoculated into 5 ml of nutrient broth and incubated for 24 h at 37°C. An SPC slant was swabbed with *S. enteritidis* PT8 and incubated for 24 h at 37°C. Slants were washed with 1 ml of 0.85% saline. Cells were harvested and serially diluted in 0.1% peptone water. Viable counts were estimated by plating with SPC agar and incubating for 24 h at 37°C.

Salmonella heidelberg, *Proteus vulgaris*, *Pseudomonas fluorescens* (University of Manitoba, Microbiology Department), *Escherichia coli*, *Citrobacter freundii* and *Alcaligenes faecalis* (ATCC) were prepared as above. Incubation temperatures for *A. feacalis* and *P. fluorescens* were 30°C.

All bacterial suspensions, serially diluted to specific concentrations for inoculation, were confirmed by subsequent plate counts using SPC agar (quadruplet).

4. Preparation of membranes

Nitrocellulose (BioRad Laboratories, Hercules, CA), Immobilin (Millipore,

Mississauga, ON), Hybond-N (Amersham Life Sciences, Oakville, ON), BioTrace HP and Ultrabind (Gelman Sciences Ltd., Montreal, QU) were cut into 8.5 x 2.5 cm or 5.0 x 2.5 cm strips and incubated in either phosphate buffered saline (PBS; pH 7.2), poly-L-lysine in PBS (PLL; 1 µl/ml) or polymyxin B in PBS (5 µg/ml).

5. Preparation of egg samples

Large grade A eggs were obtained from the local supermarket and used within one week of purchase. Eggs were scrubbed with 70% ethanol and opened aseptically. The contents were placed into stomacher bags (Seward Medical, London, UK), mixed for 30 sec using a stomacher lab-blender 400 (Seward Laboratory, London, UK) and 25 gm portions placed into 50 ml polypropylene tubes (Falcon, Oxnard, CA). Each tube was inoculated with approximately 10^6, 10^7, 10^8 or 10^9 cells/ml of egg. The negative control was inoculated with 1 ml of 0.85% saline. Tubes were vortexed and 2.5 ml (0.1 volume) of 15% sodium cholate in PBS (pH 7.2) was added. After mixing, the mixture was heated for 10 min at 100°C and cooled for 30 min at 4°C. Using a sterile core borer (10 mm dia), egg core samples were taken and cut into discs 2 mm thick using a sterile surgical blade.

To determine the detection limit of the assay, the contents of two eggs (100 gm) were aseptically pooled, mixed as above and a 4 ml inoculum of either 1, 5, 10, 50 or 500 cells/ml of live *S. enteritidis* PT8 was added. The negative control was inoculated with 4 ml of 0.85% saline. All samples were incubated for 20 h at 37°C. Following incubation, 25 gm samples from each inoculum were distributed into polypropylene tubes and prepared as above.

To examine the specificity of the assay, the contents of two eggs (100 gm) were aseptically pooled, mixed as above and a 2 ml inoculum of 2 cells/ml of live *S. enteritidis* PT8 was added. In addition, a 2 ml inoculum of either 33, 50, 67, 100 or 133 cells/ml of each bacterium (*S. heilderberg*, *E. coli*, *P. vulgaris*, *C. frundii*, *A. faecalis* and *P. fluorescens*) was added. At these bacterial concentrations, the ratio of *S. enteritidis* PT8 to competing bacteria were 1:100, 150, 200, 300 and 400 per 50 gm of egg. The negative control contained either 33, 50, 67, 100 or 133 cells/ml for each competing bacterium but did not contain *S. enteritidis*. All samples were incubated for 24 h at 37°C. Following incubation, all samples were treated as above.

To determine the detection limit in a large sample size, ten eggs (500 gm) were aseptically open, mixed as above and a 1 ml inoculum of either 1, 5 or 10 cells/ml of live *S. enteritidis* PT8 was added. The negative control contained 1 ml of 0.85% saline. Prior to mixing, 17.5 mg of ferrous sulfate was added to each sample. All samples were incubated for 20 h at 37°C. Following incubation, 25 gm samples from each inoculum were placed into polypropylene tubes and prepared as above.

The specificity in a large sample size was determined using ten eggs (500 gm). Eggs were aseptically opened, mixed as above and a 1 ml inoculum of 2 cells/ml of live *S. enteritidis* PT8 was added. A 1 ml inoculum of either 100 or 133 cells/ml of each competing bacteria was also added. The ratio of *S. enteritidis* PT8 to competing bacteria were 1:300 and 1:400 per 500 gm of egg. *Salmonella enteritidis* PT8 was excluded from the negative control but a 1 ml inoculum of either 100 or 133 cells/ml for each bacterium was added. Prior to mixing, 17.5 mg of ferrous sulfate was added to each sample. All

samples were incubated for 24 h at 37°C. Following incubation, 25 gm samples were distribuated into polypropylene tubes and prepared as above.

6. Preparation of poultry samples

Fresh poultry was obtained from the local supermarket and used within 2 days of purchase. Twenty-five gram samples were aseptically inoculated with approximately 1, 5 or 10 cells/ml and stored for 24 h at 4°C to simulate normal storage conditions. The negative control was inoculated with 1 ml of 0.85% saline. Samples were mixed in 225 gm of homogenized egg for two min and incubated for 24 h at 37°C. After incubation, 25 gm samples were taken and prepared as above.

7. Preparation of skim milk powder samples

Skim milk powder was obtained from the University of Manitoba, Food Science Department. Samples (25 gm) were aseptically inoculated with approximately 1, 5 or 10 cells/ml and stored for 24 h at room temperature to mimic normal storage conditions. The negative control was inoculated with 1 ml of 0.85% saline. Samples were equilibrated for one h in 225 ml of brilliant green water at room temperature. After equilibration, samples were mixed for two min and incubated for 7 h at 37°C. After incubation, 1 ml was transferred into 25 gm of homogenized egg and incubated for 17 h at 37°C. After incubation, 25 gm samples were taken and prepared as above.

8. Preparation of ice cream samples

Ice-cream was obtained from the local supermarket and used within 2 weeks of purchase. Twenty-five gram samples were aseptically inoculated with approximately 1, 5 or 10 cells/ml and stored for 24 h at -16°C. The negative control was inoculated with

1 ml of 0.85% saline. Samples were mixed in 225 gm of homogenized egg for two min and incubated for 24 h at 37°C. After incubation, 25 gm samples were taken and prepared as above.

9. Preparation of poultry feed samples

Poultry feed was obtained from the University of Manitoba, Animal Science Department. Twenty-five gram samples were aseptically taken, inoculated with approximately 1, 5 or 10 cells/ml and stored for 24 h at room temperature. The negative control was inoculated with 1 ml of 0.85% saline. Samples were equilibrated for one h in 225 gm of homogenized egg at room temperature, mixed for two min and incubated for 23 h at 37°C. After incubation, 25 gm samples were taken and prepared as above.

10. Optimized dot-blot immunoassay

All steps throughout the immunoassay were performed at room temperature. Egg discs were placed on PBS soaked 0.45 µm nitrocellulose strips for 5 min. Samples were removed and membranes washed in PBS (pH 7.2) for 2 min. Strips were blocked for 45 min in 5% skim milk powder in Tris buffered saline (TBS, pH 7.5). After blocking, membranes were incubated with MAb 2F11 for 45 min and then incubated for 1 h in biotinylated goat anti-mouse IgG. Subsequently, strips were washed in TTBS and incubated with streptavidin-alkaline phosphatase for 1 h. After each step, strips were washed twice with TTBS (TBS with 0.05% Tween 20) for two min at room temperature. After a final wash, strips were developed with BCIP (5-bromo-4-chloro-3-indolylphosphate)/ NBT (nitroblue tetrazolium chloride) substrate solution. Color development was terminated by rinsing strips in distilled water.

11. Culture method

Following incubation, one ml of egg mixture was transferred to 0.1% peptone water and serially diluted. Viable counts were estimated by plating on SPC agar (quadruplet) and incubating for 24 h at 37°C. In addition, one ml of egg or one ml of egg containing either poultry, ice cream, SMP or feed from each sample (inoculated and control) was transferred to 9 ml of selenite cysteine and tetrathionate broth and incubated for 24 h at 37°C. Recovery of *S. enteritidis* was confirmed by streaking onto XLD agar, brilliant green sulfadiazine agar and bismuth sulfite agar and incubated for 24 h at 37°C. Isolated *S. enteritidis*-suspect colonies were then transferred to triple sugar iron and iron agar slants and incubated for 24 h at 37°C. Serology confirmation was performed using Salmonella O-9 factor antiserum. For artificially inoculated egg samples containing mixed population of bacteria, Salmonella 4,5 factor antiserum was also used to differentiate between B and D_1-serogroups.

D. RESULTS AND DISCUSSION

Detection of *Salmonella enteritidis* in this study utilized a two-step procedure comprising an enrichment step followed by a monoclonal antibody-based dot-blot immunoassay. Monoclonal antibody 2F11, which is specific to the D_1-serogroup of *Salmonella* was previous produced and characterized by Masi and Zawistowski (1995).

Nitrocellulose, Immobilin (polyvinylidene difluoride), BioTrace HP (positively charged nylon) and Hybond-N (neutral nylon) were evaluated as solid supports for the dot-blot immunoassay. All membranes with the exception of Hybond-N (Northern

blotting) are currently used for Southern blotting.

To increase the antigen binding capacity of the membranes, two pre-coating agents were evaluated; poly-L-lysine and polymyxin B. Poly-L-lysine has been reported to enhance the detectability of free LPS from various gram-negative bacteria (Takahashi *et al.*, 1992) while polymyxin B, an antibiotic, has an affinity for bacterial LPS (Wang *et al.*, 1995).

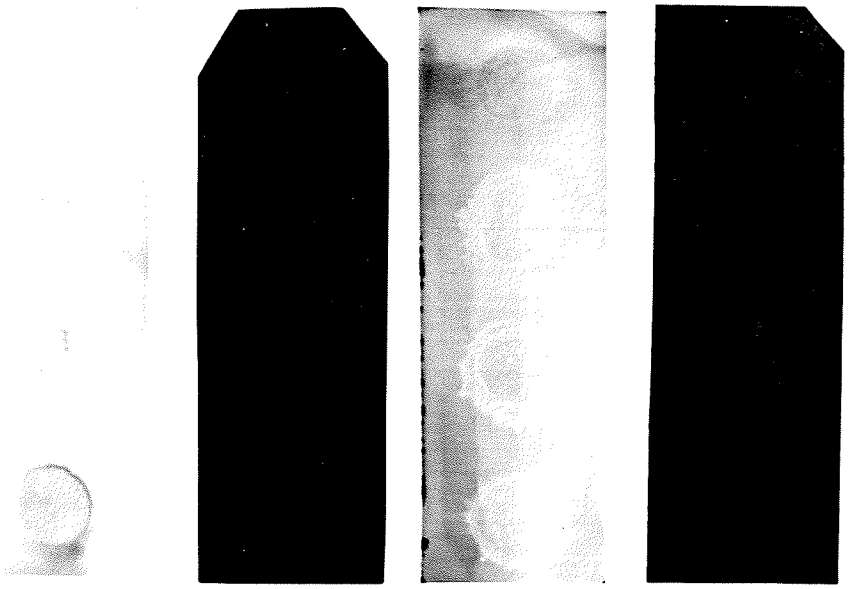
Four blocking agents were evaluated; 5% skim milk powder in TBS (pH 7.5), 3% gelatin in TBS (pH 7.5), 3% bovine serum albumin in PBS (pH 7.2) and 0.3% Tween 20 in PBS (pH 7.2). All are common blocking agents employed in immunoblotting (Harlow and Lane, 1988).

Suitable blotting membranes for the dot-blot immunoassay consisted of PBS-soaked nitrocellulose strips (Fig. 3.1a) blocked with either 3% gelatin, 5% SMP or 3% BSA. Skim milk powder was chosen as a blocking agent since it is inexpensive and readily available. Immobilin membrane produced a low background with all blocking and pre-coating agents, however, all bacterial concentrations including the negative control were detected (Figure 3.1c). No differences in color intensity were observed between the targeted bacteria and negative control. BioTrace and Hybond-N produced a high background regardless of the type of blocking and pre-coating solutions used (Figure 3.1b,d). Either LPS was unable to bind to the membrane or non-specific binding of MAb 2F11 or biotinylated IgG led to background coloration.

Furthermore, the effect of incubation times on each precedure step was evaluated. Nitrocellulose was incubated in PBS (pH 7.2) for 24, 0.75, 0.50 and 0.25 h (Figure

Figure 3.1 The effect of blotting membranes on the dot-blot immunoassay.
a: Nitrocellulose, b: BioTrace HP, c: Immobilin and d: Hybond-N.

S. enteritidis PT8
(CFU/ml of egg)



3.2). At 0.25 h incubation, results were not clear as compared to those for the other times. No differences were observed among other incubation times. Membranes were blocked with 5% SMP for 2.0, 1.5, 1.0 and 0.5 h (Figure 3.3). Color intensity was slightly reduced at 2.0 h incubation, however, no differences were noticed between other times. MAb 2F11 was incubated at 24, 2.0, 1.5, 1.0 and 0.5 h (Figure 3.4). Color intensity was weak at incubation times at 0.5 and 1.0 h and strong at 2.0 and 24 h. Best results were obtained at 1.5 h of incubation. Biotinylated goat anti-mouse IgG was incubated for 2.0, 1.5, 1.0 and 0.5 h (Figure 3.5). Incubation between 0.5 to 1.0 h provided strong coloration at each concentration. Streptavidin alkaline phosphatase was incubated for 2.0, 1.5, 1.0 and 0.5 h (Figure 3.6). Incubation for 1.0 h produced strong, well defined results. In order to minimize the time required to perform the assay, nitrocellulose was incubated for 0.5 h in PBS, blocked for 0.75 h, probed with MAb 2F11 for 1.0 h and incubated with B-GAM and S-AP for 1.0 h each (Figure 3.8).

Studies on feasibility of use of an enhanced dot-blot immunoassay were also conducted. In this respect, a variety of systems were investigated; i) an immunoassay employing MAb 2F11, biotinylated IgG, streptavidin and biotinylated alkaline phosphatase conjugate, ii) an immunoassay system comprising biotinylated monoclonal antibodies and streptavidin alkaline phosphatase conjugate and iii) biotinylated MAbs in a sandwich dot-blot immunoassay.

To increase the sensitivity of the assay, streptavidin was used in conjunction with biotinylated alkaline phosphatase. Streptavidin, which contains four binding sites

Figure 3.2 The effect of pre-coating time on the dot-blot immunoassay. Nitrocellulose strips were incubated in PBS at room temperature.

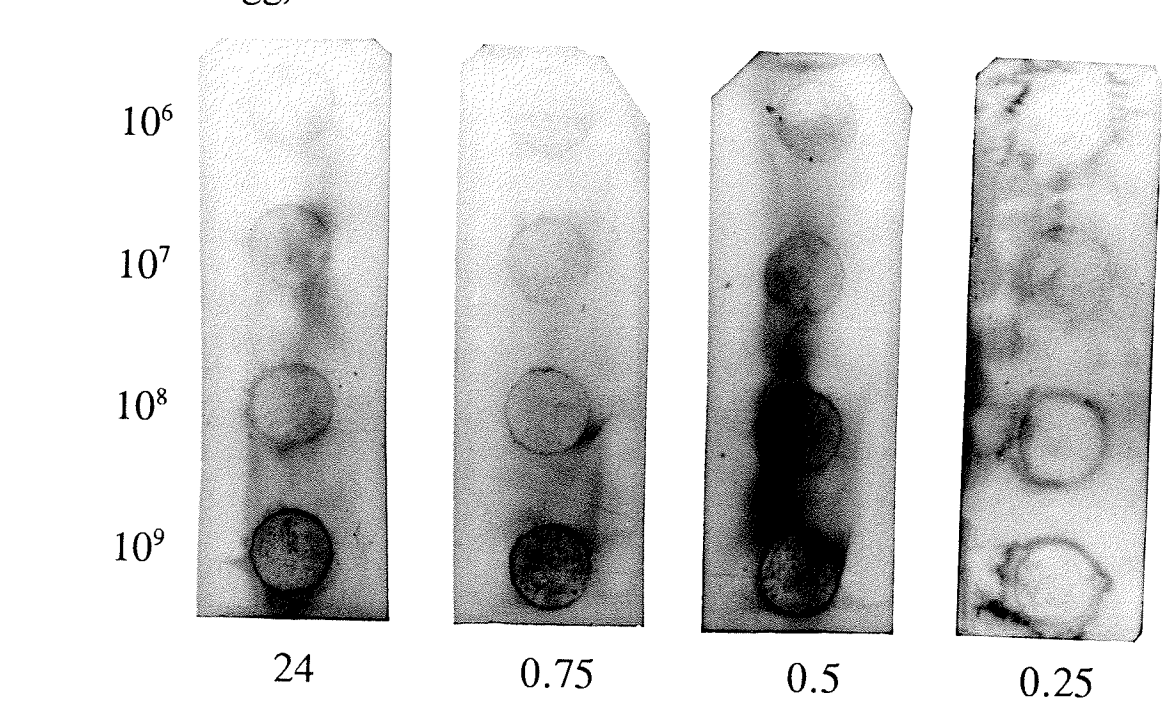

S. enteritidis PT8
(CFU/ml of egg)
10^6
10^7
10^8
10^9
24
0.75
0.5
0.25
Incubation time (h)

Figure 3.3 The effect of blocking time on the dot-blot immunoassay. Nitrocellulose strips were incubated with 5% skim milk powder in PBS (pH 7.2) at room temperature.

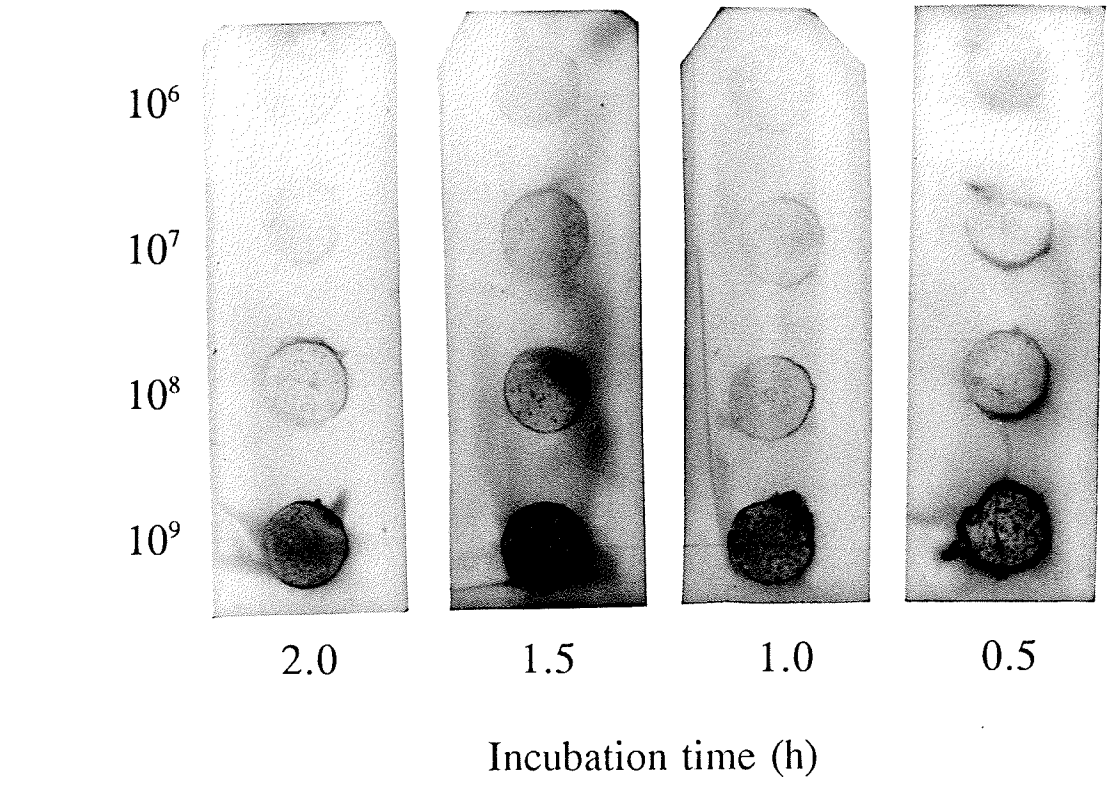

S. enteritidis PT8
(CFU/ml of egg)
10^6
10^7
10^8
10^9
2.0
1.5
1.0
0.5
Incubation time (h)

Figure 3.4 The effect of incubation time with monoclonal antibodies on the dot-blot immunoassay. Nitrocellulose strips were incubated with MAb 2F11 at room temperature.

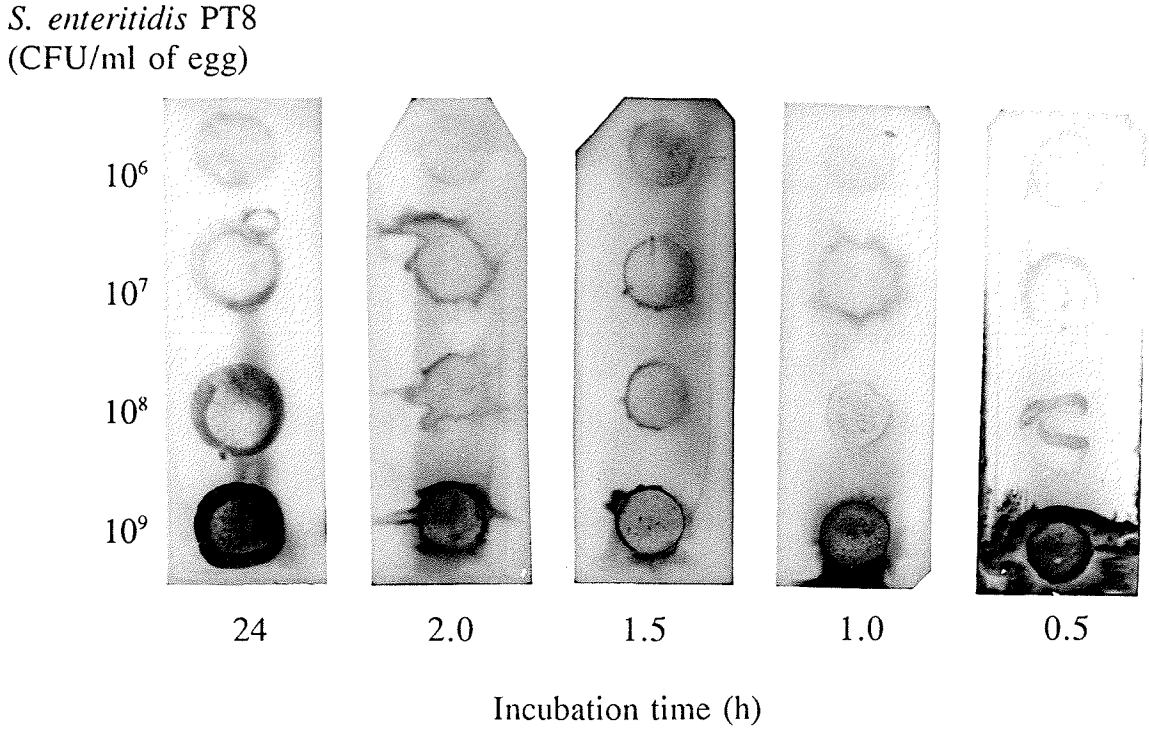

S. enteritidis PT8
(CFU/ml of egg)
10^6
10^7
10^8
10^9
24
2.0
1.5
1.0
0.5
Incubation time (h)

Figure 3.5 The effect of incubation time with biotinylated IgG (B-GAM) on the enhanced dot-blot immunoassay. Nitrocellulose strips were incubated with B-GAM for various times followed by streptavidin alkaline phosphatase for 1 h at room temperature.

S. enteritidis PT8
(CFU/ml of egg)

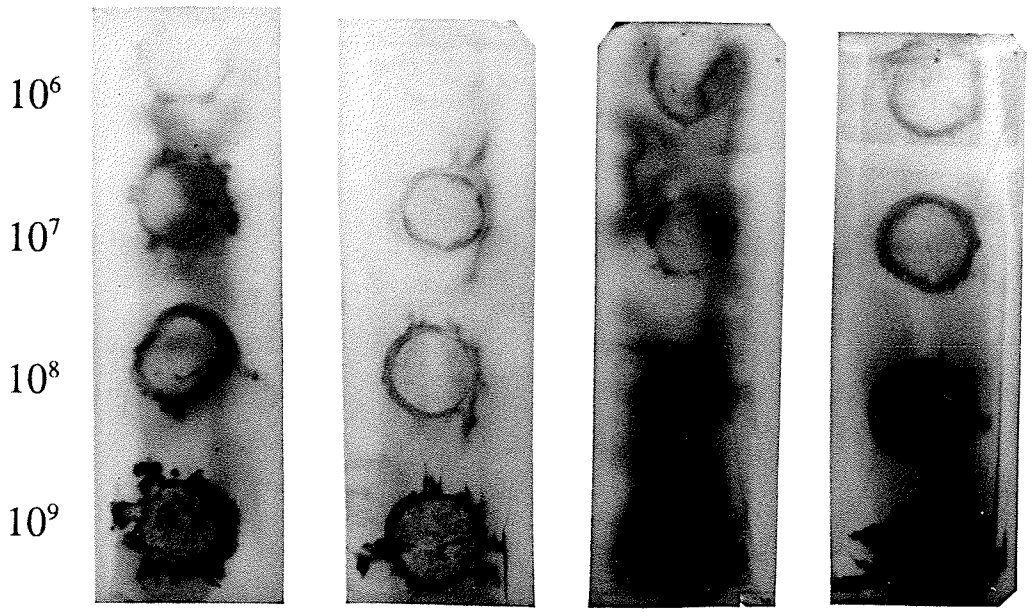


Figure 3.6 The effect of incubation time with streptavidin alkaline phosphatase (S-AP) on the enhanced dot-blot immunoassay. Nitrocellulose strips were incubated with B-GAM for 1 h followed by incubation with S-AP for various times at room temperature.

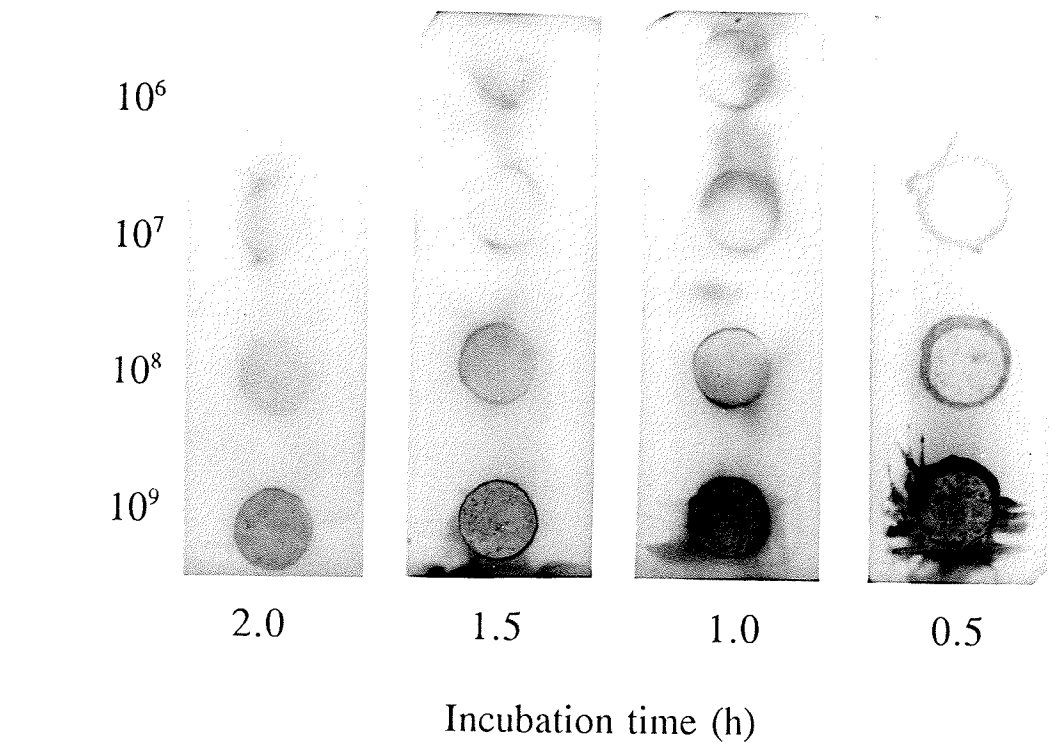
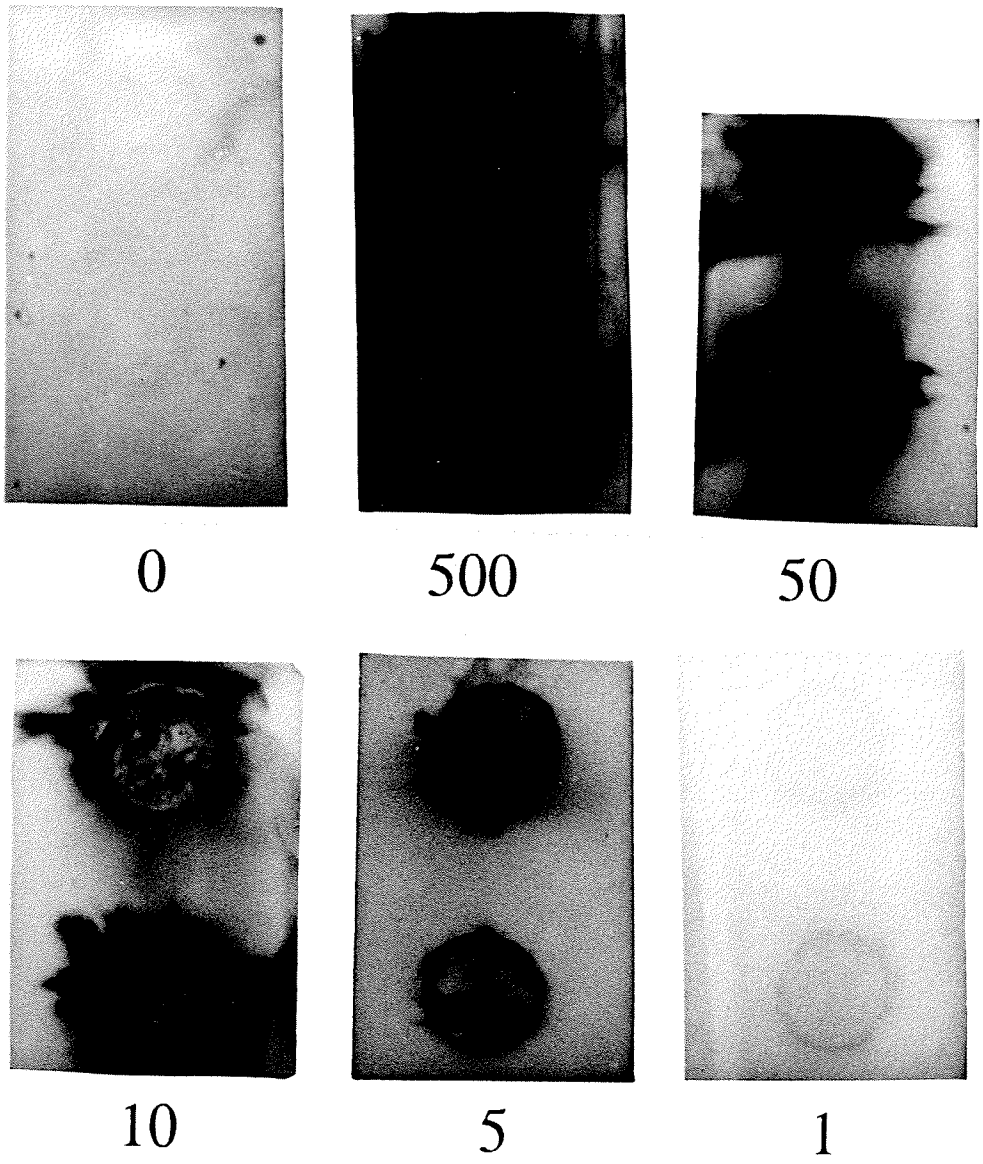

S. enteritidis PT8
(CFU/ml of egg)
10^6
10^7
10^8
10^9
2.0
1.5
1.0
0.5
Incubation time (h)

Figure 3.8 Detection of *S. enteritidis* in artificially inoculated eggs by the dot-blot immunoassay.



S. enteritidis PT8
(initial inoculum/25 gm of egg)

for biotin, could potentially increase the amount of biotinylated-AP by 3-fold. Nitrocellulose strips were probed with MAb 2F11 and incubated with biotinylated IgG followed by streptavidin. All samples including the negative control were detected (Figure 3.7b).

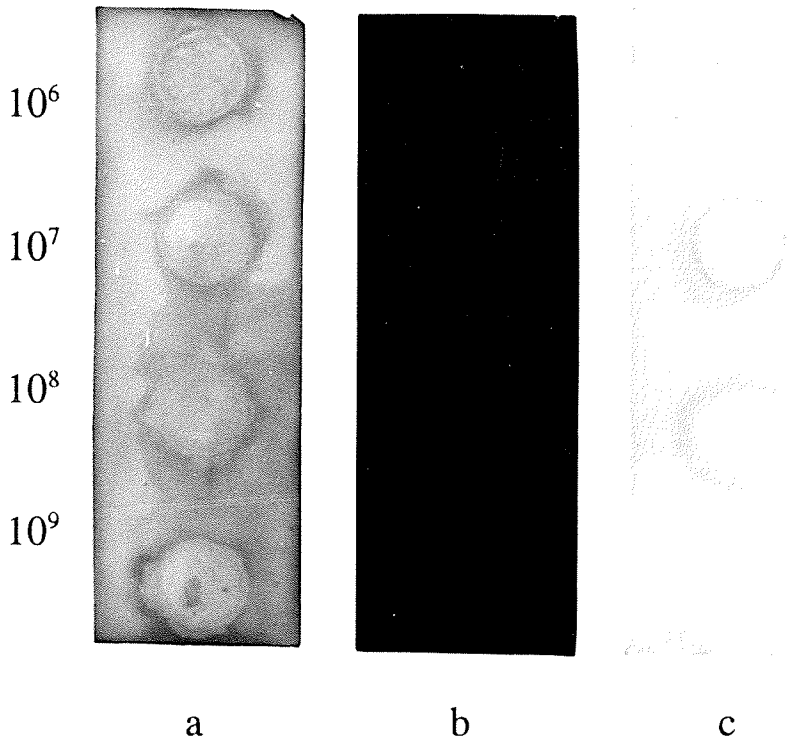
In order to reduce the time required to perform the assay, MAb 2F11 was biotinylated. This would eliminate the need for biotinylated IgG. Membranes were probed with biotinylated MAbs and incubated with streptavidin alkaline phosphatase. All samples including the negative control were positive (Figure 3.7a). Although non-specific binding was not exhibited with MAb 2F11, once biotinylated, non-specific binding due to the biotin may have occured. A sandwich dot-blot immunoassay was also attempted using the biotinylated MAbs and Ultrabind membrane. This membrane contains active aldehyde groups which have a high affinity for covalent immobilization of monoclonal antibodies. Membranes were incubated with MAb 2F11 and blocked prior to the addition of egg samples. All bacterial concentrations including the negative control were detected (Figure 3.7c). Since Ultrabind has an affinity for antibodies, blocking may have not been adequate to prevent biotinylated MAbs from binding to the membrane.

Based on the obtained results, the use of biotinylated MAb 2F11 and streptavidin in the assay were unsuccessful in detecting *S. enteritidis* in contaminated eggs. Best results were obtained when membranes were probed with MAb 2F11 followed by B-GAM and S-AP. This approach was then applied in the detection of *S. enteritidis* in eggs, poultry and other foods and feed.

In the detection of *S. enteritidis* in eggs, cells were grown directly in homogenized

Figure 3.7 Detection of *S. enteritidis* in artificially inoculated eggs by the dot-blot immunoassay. a: biotinylated monoclonal antibody 2F11 followed by streptavidin alkaline phosphatase, b: sandwich immunoassay using Ultrabind, c: biotinylated IgG followed by strepavidin and biotinylated alkaline phosphatase.

S. enteritidis PT8
(CFU/ml of egg)



egg without the need of pre-enrichment and selective broths and media. Our method demonstrated that only 20 h of incubation was needed for *S. enteritidis* to reach detectable levels without the need to isolate the organism (Table 3.1). After incubation for 24 h at 37°C, an initial inoculum of 1, 5, 10, 50 or 500 cells per 25 gm of homogenized egg increased to approximately 10^6, 10^7, 10^8, 10^8 and 10^9 cells/ml. These bacterial concentrations were readily detected by the dot-blot immunoassay (Figure 3.8). For detection of 500 cells per 25 gm, a distinct circular pattern was not observed. Due to the high bacterial concentration after incubation, LPS antigens saturated the entire strip thus coloration of the strip occurred. *Salmonella enteritidis* was not detected from negative control samples.

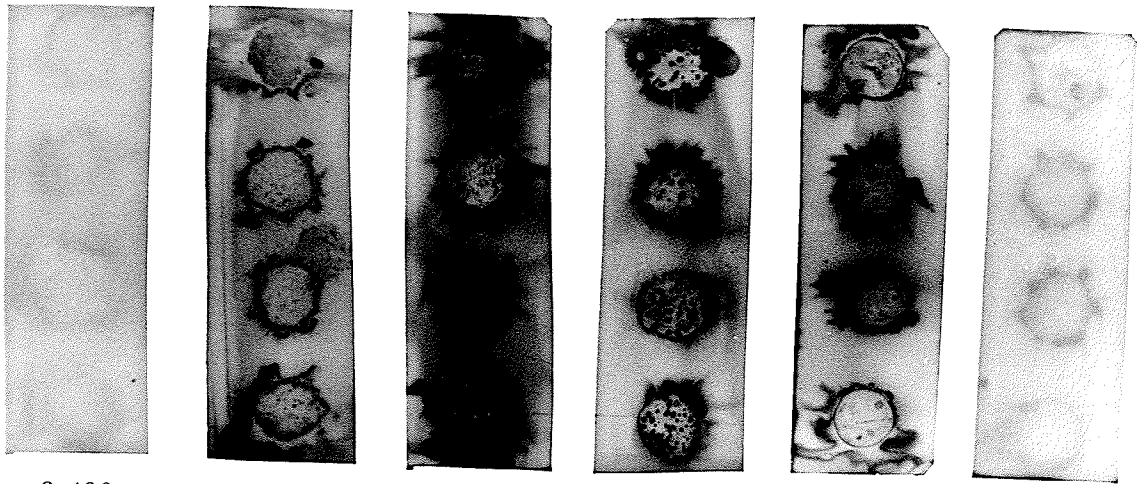
Contamination by trans-shell transmission frequently involves a mixed infection which has been found to be dominated by gram-negative bacteria (Board, 1966). In order to assess the ability of the dot-blot to detect *S. enteritidis* among a mixed population of bacteria, six species were selected. *Salmonella heidelberg* has been frequently isolated from poultry and egg shells (Jones *et al.*, 1995; Waltman *et al.*, 1992; Poppe *et al.*, 1991; Barnhart, 1991) while *E. coli*, *P. vulgaris*, *C. freundii*, *A. faecalis* and *P. fluorescens* are common spoilage organisms associated with eggs (Board, 1966). The results of these studies are presented in Figure 3.9. When *S. enteritidis* was out competed 1 to 100, 150, 200, 300 and 400, the immunoassay was able to detect *S. enteritidis* in eggs.

Eggs contain an adequate amount of nutrients to support the growth of *S. enteritidis* (Baker *et al.*, 1983). Although antimicrobial agents are present in the albumen

Table 3.1 - Enumeration and detection of *S. enteritidis* from artificially inoculated eggs by the dot-blot immunoassay and conventional culture method

Initial inoculum (CFU/25 gm of egg)	CFU/ml after incubation at 37°C for 20 h	Detection	
		dot-blot assay	culture method
500	1.3×10^9	Yes	Yes
50	4.0×10^8	Yes	Yes
10	1.6×10^8	Yes	Yes
5	1.8×10^7	Yes	Yes
1	2.0×10^6	Yes	Yes
0	0	No	No

Figure 3.9 Detection of S. enteritidis in artificially inoculated eggs in the presence of the mixed population of bacteria (*S. heidelberg*, *Citrobacter freundii*, *Proteus vulgaris*, *Escherichia coli*, *Alcaligenes faecalis* and *Pseudomonas fluorescens*) by the dot-blot immunoassay.



Ratio of *S. enteritidis* PT8 to competitors
(per 50 gm of egg)

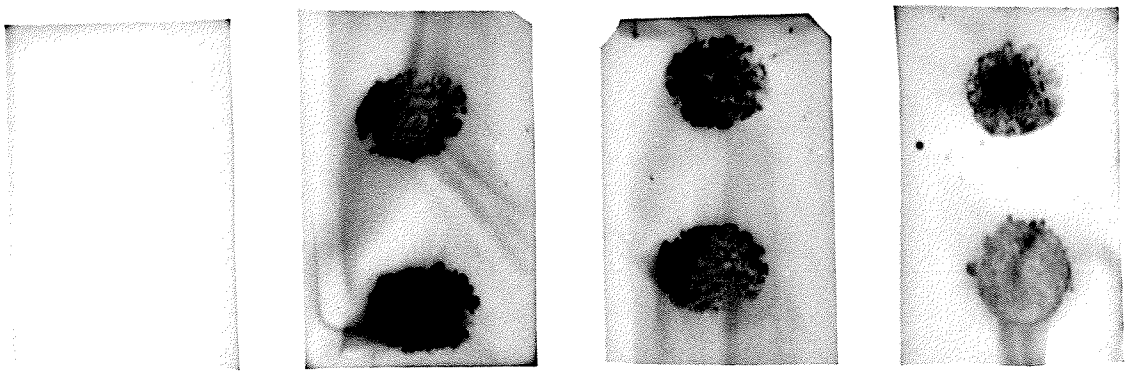
such as conalbumen and lysozyme, both are neutralized when the yolk and white are homogenized thus allowing for microbial growth (Brooks, 1960; Galyean and Cotterill, 1972). However, Cudjoe and co-workers (1994a) found that yolk/albumen mixtures still yielded inhibitory effects on the growth of bacteria. Gast and Holt (1995a) suggested that the ability of *S. enteritidis* to grow rapidly in liquid whole eggs is a characteristic among various strains. This could explain why *S. enteritidis* is able to rapidly multiply in homogenized eggs despite the inhibitory effects that still remain after mixing the yolk and albumen. This could also explain why *S. enteritidis* was able to survive and proliferate when it was out competed 1 to 400. Growth of competing bacteria may have been supressed from the remaining inhibitory effects of the yolk/albumen mixture while *S. enteritidis* was able to propagate. It has also been reported that *S. enteritidis* PT4 was able to out compete other gram negative bacteria at incubation temperatures near 37°C in eggs (Dolman and Board, 1992).

Since the number of *Salmonella*-positive eggs laid by infected hens are low with small numbers of *S. enteritidis* present, a test must be highly sensitive. Gast (1993a) pooled the contents of eggs which would permit a statistically meaningful sample size, however, some form of pre-enrichment was required. Pooled raw eggs supplemented with ferrous sulfate allowed the detection of *S. enteritidis* at low inoculum levels (Gast and Holt, 1995a; Cudjoe *et al.*, 1994a). Incubation of 1, 5 or 10 cells/ml of *S. enteritidis* per 500 gm homogenized egg (10 eggs) supplemented with ferrous sulfate allowed cells to multiply to detectable levels (Table 3.2). After incubation for 20 h at 37°C using the dot-blot immunoassay, *S. enteritidis* was readily detected (Figure 3.10). Sixteen pools of 10

Table 3.2 - Enumeration and detection of *S. enteritidis* from artificially inoculated egg pools supplemented with ferrous sulphate by the dot-blot immunoassay and conventional culture methods

Initial Inoculum (CFU/500 gm of egg)	CFU/ml after incubation at 37°C for 20 h	Detection	
		dot-blot assay	culture method
10	2.0×10^8	Yes	Yes
5	1.6×10^7	Yes	Yes
1	9.2×10^6	Yes	Yes
0	0	No	No

Figure 3.10 Detection of *S. enteritidis* in artificially inoculated eggs supplemented with ferrous sulphate by the dot-blot immunoassay.



0 10 5 1

S. enteritidis PT8
(initial inoculum/500 gm of egg)

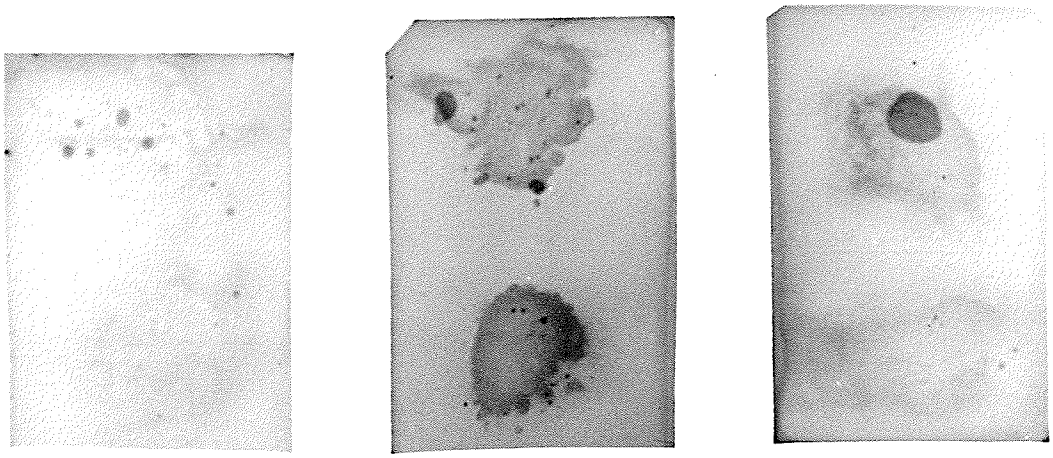
eggs inoculated with approximately one cell of *S. enteritidis* per ml were each detected by the dot-blot after the incubation period. An additional four pools of 10 eggs each retained as uninoculated controls were negative by the dot-blot. The specificity was also examined in egg pools supplemented with ferrous sulfate. Samples were inoculated with the same six competing bacteria and incubated for 24 h at 37°C. When *S. enteritidis* was initially out-competed 1 to 300, detection of *S. enteritidis* was still feasible (Figure 3.11).

Detection of *S. enteritidis* from poultry, ice cream and poultry feed was conducted by direct enrichment in homogenized eggs followed by the immunoassay. After incubation of 1, 5 or 10 cells/ml for 24 h at 37°C, concentrations of *S. enteritidis* were sufficient to be detected with the developed immunoassay (Figures 3.12, 3.13 and 3.14).

Detection of *S. enteritidis* in skim milk powder required cells to be enriched prior to incubation in homogenized eggs. Incubation of approximately 1, 5 or 10 cells/ml for 7 hours at 37°C in brilliant green water was sufficient to ensure that a 1 ml sample contained enough cells for transfer to homogenized eggs. An additional incubation of 17 hours at 37°C was sufficient for *S. enteritidis* to be detected (Figure 3.15).

One of the important features of the developed dot-blot immunoassay is based on the distribution of LPS in the gelled egg matrix which was formed upon heating. Through the addition of sodium cholate and the application of heat, LPS antigens of *S. enteritidis* are released from the bacterial membrane. This allowed the antigen to be transferred through diffusional forces from the egg sample onto the solid support and subsequent detection by MAb 2F11. Formation of a purple colour is considered to be an indication of the presence of *S. enteritidis*. Althoug other detergents have been

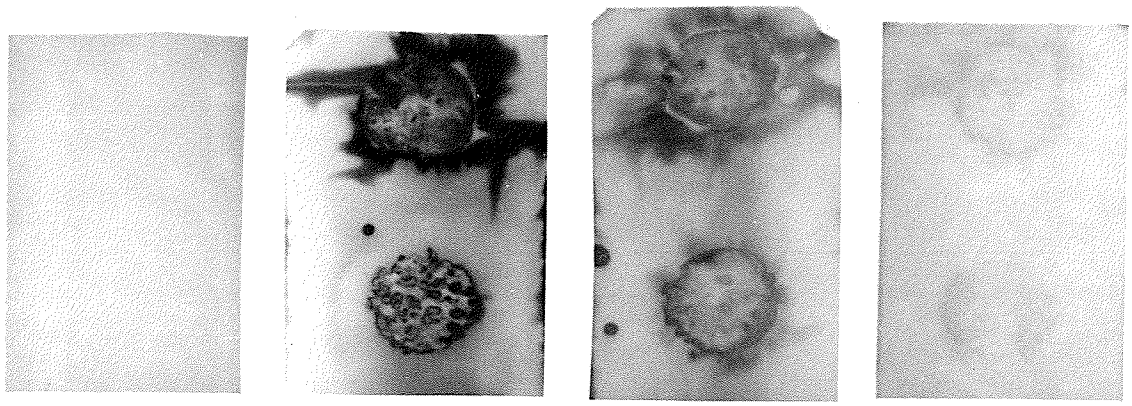
Figure 3.11 Detection of *S. enteritidis* in artificially inoculated eggs supplemented with ferrous sulphate in the presence of the mixed population of bacteria (*S. heidelberg*, *Citrobacter freundii*, *Proteus vulgaris*, *Escherichia coli*, *Alcaligenes faecalis* and *Pseudomonas fluorescens*) by the dot-blot immunoassay.



0:400 1:300 1:400

Ratio of *S. enteritidis* PT8 to Competitors
(per 500 gm of egg)

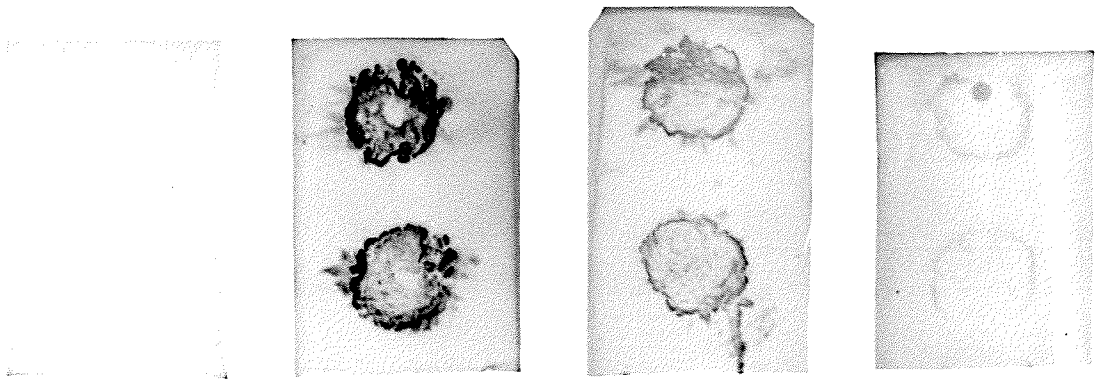
Figure 3.12 Detection of *S. enteritidis* in artificially inoculated poultry by the dot-blot immunoassay.



0 10 5 1

S. enteritidis PT8
(initial inoculum/25 gm of poultry)

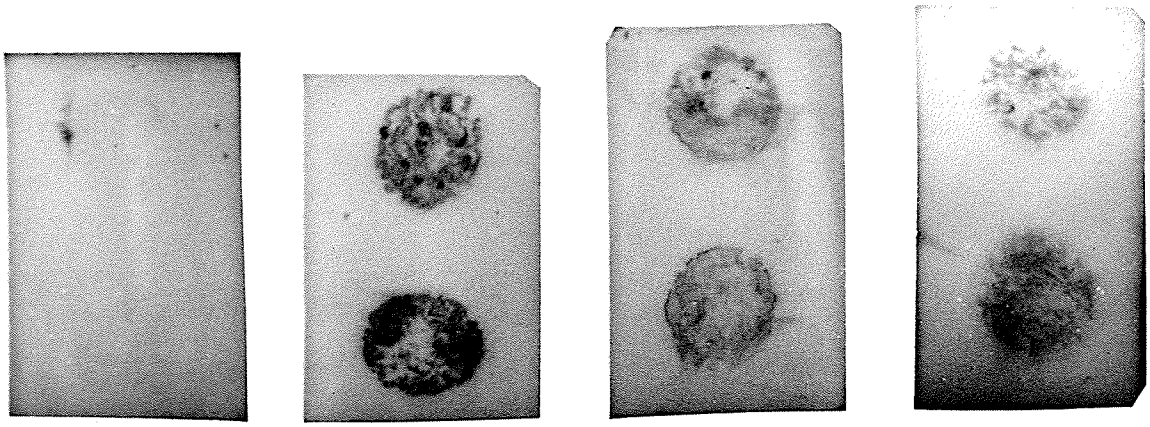
Figure 3.13 Detection of *S. enteritidis* in artificially inoculated ice cream by the dot-blot immunoassay.



0 10 5 1

S. enteritidis PT8
(initial inoculum/25 gm of ice cream)

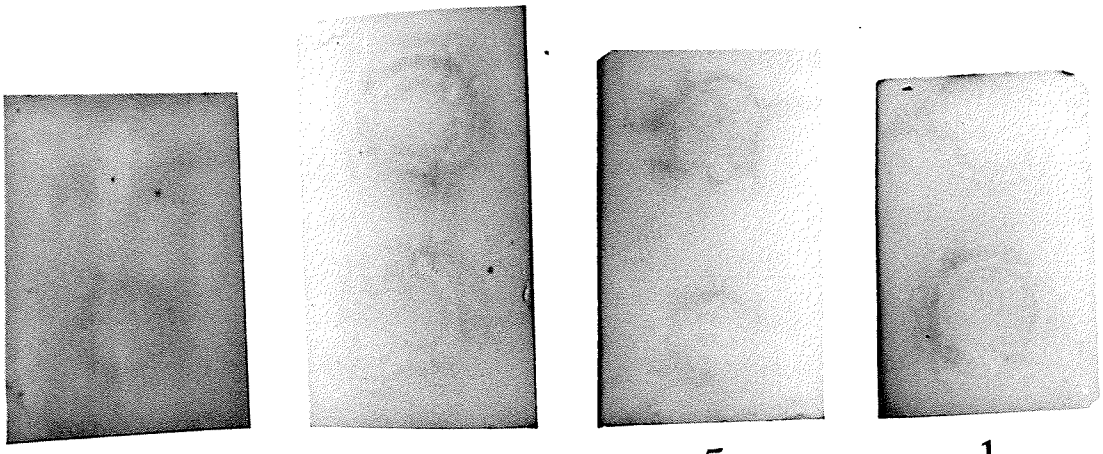
Figure 3.14 Detection of *S. enteritidis* in artificially inoculated poultry feed by the dot-blot immunoassay.



0 10 5 1

S. enteritidis PT8
(initial inoculum/25 gm of poultry feed)

Figure 3.15 Detection of *S. enteritidis* in artificially inoculated skim milk powder by the dot-blot immunoassay.



S. enteritidis PT8
(initial inoculum/25 gm of skim milk powder)

used for extraction of LPS antigens (Blais and Yamazaki, 1990a), Wang and co-workers (1995) found that a 15% sodium cholate solution was the most efficient in LPS removal.

E. CONCLUSION

The developed dot-blot immunoassay is a simple and rapid test requiring 24 h for detection of *S. enteritidis* in shell eggs. From an inoculum of one cell/25 gm of egg, *S. enteritidis* was detected. Incorporation of ferrous sulfate allowed for the detection of one *S. enteritidis* cell/500 gm of egg. Detection of this pathogen in eggs contaminated with high numbers of competing organisms was possible. Detection of *S. enteritidis* in poultry and other foods and feed required less than 30 h. All results were confirmed by culture method indicating a good correlation between both methods.

IV. MANUSCRIPT 2

THE EFFECT OF DETERGENTS ON EXTRACTIBILITY OF LIPOPOLYSACCHARIDES FROM *SALMONELLA ENTERITIDIS*

A. ABSTRACT

The ability of various detergents to release lipopolysaccharide antigens from the bacterial membrane of *Salmonella enteritidis* was evaluated. As determined by an enzyme-linked immunosorbent assay using poly-L-lysine coated microplates, heat treatment of *S. enteritidis* cells was insufficient for removal of LPS antigens from the bacterial membrane. In the presence of iminodiacetic acid, ethylenediaminetetraacetate, and sodium cholate, the sensitivity of the ELISA increased. However, collodial gold staining followed by transmission electron microscopy revealed that iminodiacetic acid and ethylenediaminetetraacetate were not as effective. Sodium cholate, sodium azide and Triton X-100 were more effective. Of all tested detergents for the use in the dot-blot immunoassay, sodium cholate was the only detergent able to release LPS antigens when mixed with artificially contaminated eggs.

B. INTRODUCTION

Lipopolysaccharides (LPS) are essential components of the cell wall of gram negative bacteria (Schnaitman and Klena, 1993). They interact with the environment exhibiting a varity of biological activites, such as pyrogenicity, complement activation and lethal toxicity (Kotina and Takada, 1990). Lipopolysaccharides consist of three components; lipid A, inner core and O-side chain oligosaccharides (Nikaido and Vaara, 1985).

Lipid A is composed of four hydroxy fatty acids and a glucosamine disaccharide (Schnaitman and Klena, 1993). It is the endotoxic portion of the LPS and is used to

anchor the LPS molecule to the bacterial membrane.

The polysaccharide portion of the LPS consists of the core and the O-specific side chain. The core consists of an inner and outer core while the O-specific chain consists of a repeating oligosaccharide unit which contains up to six sugar residues and may be 70 units long (Peterson and McGroarty, 1985). Since the ring form, type of linkage and substitution of the monosaccharide residues are characterisitic and unique to a bacterial species, the O-chain is species specific (Rietschel and Brade, 1992). In this respect, tyvelose is the unique sugar of O-antigen factor 9 of *Salmonella* serogroup D.

Since the O-chain is specific to the *Salmonella* serogroup, it is used as a molecular marker to develop monoclonal antibodies and subsequently immunoassays. Rapid methodology available for detection of *S. enteritidis* is represented by two basic enzyme-linked immunosorbent assay (ELISA) procedures, indirect and sandwich ELISA. These methods employ antisera and monoclonal antibodies produced against LPS antigens (Lu *et al.*, 1991; Brigmon *et al.*, 1992 and Torensma *et al.*, 1992; Masi and Zawistowski, 1995).

Using an antibody-coated polyester cloth, Blais and Yamazaki (1989) detected *Salmonella* LPS antigens from sample extract. Using heat treatment and ethylenediaminetetraacetate (EDTA), LPS antigens were dissociated into a non-sedimentable form. The sensitivity of this method was improved by heating *Salmonella* cells in the presence of sodium deoxycholate or Triton X-100 (Blais and Yamazaki, 1990). Subsequently, the method was modifed by replacing sodium deoxycholate with sodium cholate. The method was used for extraction of *Salmonella* LPS antigens from

contaminated eggs (Wang *et al.*, 1995).

This paper reports on the reactivity of a D_1-serospecific anti-LPS monoclonal antibody with LPS of *S. enteritidis* as assessed by ELISA and transmission electron microscopy. The effect of various detergents on the ability to extract LPS antigens from the bacterial membrane of *S. enteritidis* and subsequent reactivity of MAbs with bound and free LPS was studied. The application of these detergents in a rapid dot-blot immunoassay for the detection of *S. enteritidis* in contaminated egg samples was also investigated.

C. MATERIALS AND METHODS

1. Reagents and chemicals

RPMI Medium 1640 with L-glutamine was purchased from Gibco BRL-Life Technologies (Grand Island, NY). Tween 80 was purchased from BDH, Inc. (Toronto, ON). Sodium deoxycholate, magnesium chloride and ethylenediaminetetraacetate were purchased from Fisher Scientific (Fairlawn, NJ). Nutrient broth, standard methods agar and peptone were purchased from Difco, (Detroit, MI). Goat anti-mouse IgG (H+L) human IgG adsorbed gold conjugate, goat anti-mouse IgG (H+L) alkaline phosphatase conjugate, electrophoresis grade hydroxymethyl aminomethane (Tris) and sodium dodecyl sulfate (SDS) were purchased from BioRad Laboratories (Hercules, CA). Phosphotungstic acid was obtained from the Department of Botany, University of Manitoba, (Winnipeg, MB). 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\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). MAb 2F11 was specific to the D_1-serogroup of *Salmonella* and exhibited the highest reactivity with phage types 1, 4, 8, 13 and 13a of *S. enteritidis.*

Monoclonal antibody was produced as ascitic fluid according to Harlow and Lane (1988). Briefly, male BALB/c mice (6-8 weeks old) were injected with 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid) into the peritoneum. After 7 days, 5×10^5 to 5×10^6 hybridoma cells were injected into the peritoneum. Within two to three weeks following injection, ascitic fluid was removed, incubated for 1 h at 37°C and transferred to 4°C overnight. Ascitic fluid was centrifuged at 3000xg for 10 min, supernatant harvested and a titre performed in an ELISA.

3. Bacteria and culture conditions

Salmonella enteritidis phage type (PT) 1, 4, 8, 13 and 13a were obtained from the Laboratory Centre for Disease Control, Ottawa, Canada. A loopful of bacterial culture maintained on a standard plate count (SPC) agar slant was inoculated into 5 ml of nutrient broth and incubated for 16 h at 37°C. Following incubation, SPC slants were swabbed and incubated for 16 h at 37°C. Slants were washed with 1 ml of saline (0.85%), cells harvested and serially diluted in 0.1% peptone water. Counts were estimated by colony

forming units on SPC plates.

Cell suspensions were diluted to specific concentrations and heated for 10 min at 100°C or attenuated for 10 min in the presence of either 15% sodium cholate, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 0.1% ethylenediaminetetraacetate, 0.1% Tween 80 or 0.1% Triton X-100. After heat or detergent treatment, cell suspensions were used for an indirect ELISA or collodial gold staining as described below. In order to remove free LPS from bacterial cells, suspensions were centrifuged for 10 min at 2500xg. The supernatant and the resuspended bacterial pallet was then tested by an indirect ELISA. To remove traces of detergents, cell suspensions were dialyzed for two days at 4°C with six changes in deionized water and tested using an ELISA. Samples were also centrifuged and the supernatant and resuspended bacterial pellet tested using an indirect ELISA.

4. ELISA protocol

Polyvinyl chloride microplates (Falcon, Oxnard, CA) were pre-coated with poly-L-lysine. Briefly, 100 µl/well of poly-L-lysine (20 µg/ml in phosphate buffered saline (PBS), pH 7.2) were used to coat 96-well plates for 30 min at 37°C. Plates were washed three times with deionized water and used in an indirect ELISA as described below.

Bacterial suspensions (10^7 cells in 100 µl/well) or LPS (100 µl/ well) were applied onto pre-coated poly-L-lysine microplates and incubated for 1 h at 37°C. After the plates were washed six times with PBST (PBS with 0.5% Tween 20), 200 µl of blocking solution (PBST with 5% (w/v) skim milk powder) was added to each well and incubated for 1 h at 37°C. Plates were washed and incubated with 100 µl/well of monoclonal

antibody (purified culture supernatant or ascitic fluid) diluted in PBST, containing 0.1% (w/v) skim milk for 1 h at 37°C. Purified supernatant was diluted 1:16 while ascitic fluid was diluted 1:500 prior to use. Plates were washed and incubated with 100 µl/well of goat anti-mouse IgG alkaline phosphatase conjugate (diluted 1:3000 in PBST containing 0.1% skim milk) for 1 h at 37°C and developed with ρ-nitrophenol phosphate in 0.1 M diethanolamine buffer, pH 9.8 (100 µl/well). Plates were incubated overnight in the dark at room temperature and read at λ 405 nm using a Titretek Multiscan ELISA plate reader (Flow Laboratories, McLean, VA).

5. Colloidal gold labelling

Colloidal gold staining was performed according to a modified method of Cloeckaert and co-workers (1990). *Salmonella enteritidis* PT8 culture was diluted to approximately 5.1 x 10^8 cells/ml in autoclaved deionized water (pH 5.9, 17.8 MΩ). Five µl of the cell suspension was applied to formvar coated 200-mesh nickel grids (Department of Botany, University of Manitoba, Winnipeg, MB) and air dried for 2 h at room temperature. Grids were blocked with PBS containing 3% (w/v) bovine serum albumin (BSA) and incubated for 30 min at 37°C. Grids were washed four times with wash buffer (150 mM NaCl, 10 mM Tris and 0.5% tween 20) and incubated with ascitic fluid for 2 h at 37°C. Ascitic fluid was diluted 1:200 in PBST (PBS with 0.5% Tween 20) prior to use. Grids were washed and stained with goat anti-mouse IgG conjugated gold conjugate for 20 h at room temperature. Colloidal gold was diluted 1:25 in antibody buffer (150 mM NaCl, 20 mM Tris with 0.1% BSA, 0.005% Tween 20 and 0.4% gelatin) prior to use. Grids were washed four times with wash buffer, deionized water and air

dried. Next, grids were viewed through a Hatachi H-7000 transmission electron microscope (Hitachi Scientific Instruments, Mountain View, CA). The amount of gold bound to the first 100 cells examined on the grid was reported.

For negative staining, bacterial solutions were diluted to approximately 5.1 x 10^8 cells/ml in autoclaved deionized water (pH 5.92, 17.8 MΩ). Five µl of cell suspension was applied to a formvar coated 200-mesh nickel grid for 15 min and removed. Grids were then stained with 5 µl of 1% phosphotungstic acid for 40 sec. Grids were air dried and viewed through the transmission electron microscope.

6. Preparation of eggs

Large grade A eggs were purchased from the local supermarket and used immediately or stored at 4°C and used within one week. Eggs were scrubbed with 70% ethanol and opened aseptically. Contents were placed into stomacher bags (Seward Medical, London, England) and mixed for 30 sec using a stomacher lab-blender 400 (Seward Laboratory, London England). Twenty-five gm portions were distributed into 50 ml polypropylene tubes (Falcon, Oxnard, CA) and inoculated with 10^9 cells of *S. enteritidis* per ml of egg. Each tube was then heated for 10 min at 100°C in the presence of each tested detergent and cooled for 30 min at 4°C. Using a sterile core borer (10 mm in diameter), egg core samples were taken and cut into 2 mm thick discs.

7. Dot-blot immunoassay

Egg samples were placed on pre-wet nitrocellulose (BioRad, Hercules, CA) strips and incubated for 5 min at room temperature. Samples were removed, membranes washed in phosphate buffered saline (PBS, pH 7.2) and blocked with 5% skim milk

powder in tris buffered saline (TBS, pH 7.5) at room temperature. After blocking, membranes were washed for 2 min with TTBS (TBS with 0.05% Tween 20) and incubated with MAb 2F11 for 45 min. Strips were washed with TTBS and incubated for 1 h in biotinylated goat anti-mouse IgG followed by streptavidin-alkaline phosphatase for 1 h at room temperature. After a final wash in TTBS, strips were developed with BCIP (5-bromo-4-chloro-3-indolylphosphate)/ NBT (nitroblue tetrazolium chloride) substrate solution. Development was stopped by rinsing membranes with distilled water.

D. RESULTS AND DISCUSSION

The outer membrane of gram negative bacteria is strongly asymmetric, with phospholipids in the inner leaflet and lipopolysaccharide in the outer leaflet. The core portion of the LPS molecule contains a number of negatively charged groups thus LPS molecules adjacent to one another would result in strong electrostatic repulsions (Sukupolvi and Vaara, 1989). Cations which neutralize these charges have a critical role in preserving the molecular organization of the outer membrane. Since LPS are linked to the cell wall primarily through hydrophobic or ionic interactions, hydrophobic compounds may affect the structure (Sukupolvi and Vaara, 1989).

It has been previously shown that *Salmonella* LPS antigens can be extracted by heating *Salmonella* cells in the presence of EDTA, sodium deoxycholate and sodium cholate (Wang *et al.*, 1995; Blais and Yamazaki, 1990b). This procedure for antigen preparation was successfully employed in the detection of *Salmonella* LPS antigens in an enzyme immunoassay utilizing a polymxyin-cloth.

The objective of this study was to evaluate the efficacy of various detergents on extractibility of LPS antigens antigens from the bacterial membrane of *S. enteritidis*.

In this respect, cell suspensions of *S. enteritidis* phage type (PT) 1, 4, 8, 13 and 13a were diluted in peptone water to a specific concentrations and mixed with one of the following detergents dissolved in PBS (pH 7.2). The detergents tested were sodium cholate, sodium deoxycholate, sodium dodecyl sulphate (SDS), Tween 80, Triton X-100, sodium azide, ethylenediaminetetraacetate (EDTA) and iminodiacetic acid.

Table 4.1 shows the reactivity of MAb 2F11 with various *S. enteritidis* phage types that were either heat treated, treated with detergents or live. From these results, it was observed that heat treatment alone was moderately effective in LPS removal as can be judged by about 30% increase in absorbance value compared to the live control. Antibodies prominently reacted with LPS antigens extracted with sodium cholate, EDTA and iminodiacetic acid treatments yielding up to 110% increase in absorbance value. Triton X-100 and sodium azide treated samples appeared to have no affect on LPS extraction. Absorbance values for Tween 80, sodium deoxycholate and SDS were lower than the live control indicating possible interference by these detergents. The above results reflect overall MAb reactivity with free and cell-bound LPS. In order to assess the efficacy of detergents on extractibility of LPS, detergent treated bacterial suspensions were centrifuged and supernatant and pellets were used for ELISA to determine reactivity of MAb 2F11 with free and cell-bound LPS, respectively. Table 4.2 shows the effect of detergents on the reactivity of MAb 2F11 with free LPS. The decrease in absorbance values with live samples indicated that Triton X-100, sodium azide and Tween 80 had

Table 4.1 - Effect of detergents on reactivity of MAb 2F11 with *S. enteritidis* as assessed by ELISA[a]

Treatment	Absorbance (405nm)[b]				
	SE PT1	SE PT4	SE PT8	SE PT13	SE PT13a
Live (Control 1)	0.936	0.860	0.800	1.048	1.033
Heated (Control 2)	1.492	1.448	1.488	1.415	1.588
Ethylenediaminetetraacetate	1.970	1.874	1.780	1.682	1.968
Sodium cholate	1.889	1.791	1.794	1.558	1.935
Iminodiacetic acid	1.436	1.220	1.797	1.799	1.861
Triton X-100	1.475	1.220	1.088	1.905	1.201
Sodium azide	0.717	0.823	0.905	1.345	0.785
Tween 80	0.614	0.468	0.586	0.441	0.745
Sodium deoxycholate	0.401	0.492	0.388	0.788	0.637
Sodium dodecyl sulfate	0.411	0.296	0.390	0.278	0.439

[a] Using microplates pre-coated with poly-L-lysine
[b] Average of 4 trials

Table 4.2 - Effect of detergents on reactivity of MAb 2F11 with free LPSs of *S. enteritidis* cells as assessed by ELISA[a]

Treatment[c]	Absorbance (405nm)[b]				
	SE PT1	SE PT4	SE PT8	SE PT13	SE PT13a
Live (Control 1)	0.354	0.378	0.301	0.339	0.350
Heated (Control 2)	1.545	1.563	1.552	1.469	1.641
Ethylenediaminetetraacetate	1.832	1.864	1.864	1.610	1.985
Sodium cholate	1.630	1.873	1.873	1.543	2.002
Iminodiacetic acid	1.448	1.014	1.811	1.783	1.830
Triton X-100	1.232	1.379	1.077	0.902	1.338
Sodium azide	0.728	0.798	0.746	1.291	1.683
Tween 80	0.808	0.630	0.659	0.472	0.813
Sodium deoxycholate	0.546	0.397	0.397	0.405	0.531
Sodium dodecyl sulfate	0.214	0.280	0.280	0.173	0.365

[a] Using microplates pre-coated with poly-L-lysine
[b] Average of 4 trials
[c] After treatment, samples were centrifuged at 3500xg for 10 min

some effect on LPS removal. No differences were observed with the remaining detergent treatments. Effect of detergents on the reactivity of MAb 2F11 with cell-bound LPS are shown in Table 4.3. A decrease in reactivity with cell-bound LPS was observed with sodium cholate. However, reactivity remained unchanged with EDTA, iminodiacetic acid, SDS, sodium deoxycholate and sodium azide. Absorbance values slightly decreased for Triton X-100 indicating a lower reactivity with bound LPS while a slight increase was observed with Tween 80 indicating a higher reactivity with bound LPS. In order to assess potential interference of detergents with the ELISA protocol, cell suspensions were dialyzed. Table 4.4 shows the reactivity of MAb 2F11 after dialysis of detergent treated suspensions. Absorbance values for sodium cholate and EDTA were similar before and after dialysis. A 2-fold increase was observed with SDS and a 3-fold with sodium deoxycholate. A slight increase was noted with sodium azide and Triton X-100 while a slight decrease occured with iminodiacetic acid and Tween 80. Reactivity of MAb 2F11 with free LPS from dialyzed and centrifuged treated samples are shown in Table 4.5. No differences were observed for most detergents with the exception of sodium azide and SDS. A slight increase in absorbance values was noted for SDS while a slight decrease was observed with sodium azide. Table 4.6 demonstrates the reactivity of MAb 2F11 with cell-bound LPS in the bacterial pellet after dialysis and centrifugation. Although a decrease in reactivity with cell-bound LPS was observed with sodium cholate, no differences were noted with EDTA and Triton X-100. Slight increases in absorbance were observed with Tween 80, iminodiacetic acid and sodium deoxycholate while a decrease was seen with sodium azide and SDS. Above results indicate that sodium

Table 4.3 - Effect of detergents on reactivity of MAb 2F11 with cell-bound LPSs of *S. enteritidis* as assessed by ELISA[a]

	Absorbance (405nm)[b]				
Treatment[c]	SE PT1	SE PT4	SE PT8	SE PT13	SE PT13a
Live (Control 1)	0.847	0.904	0.742	1.092	0.906
Heated (Control 2)	1.574	1.455	1.647	1.572	1.732
Ethylenediaminetetraacetate	1.961	1.862	1.786	1.912	1.986
Iminodiacetic acid	1.640	1.446	1.864	1.825	1.520
Tween 80	0.919	1.044	0.951	1.265	0.951
Triton X-100	0.776	0.572	0.782	0.317	0.845
Sodium azide	0.563	0.692	0.712	0.998	0.733
Sodium deoxycholate	0.413	0.613	0.478	0.219	0.589
Sodium dodecyl sulfate	0.382	0.563	0.476	0.630	0.502
Sodium cholate	0.407	0.428	0.315	0.478	0.422

[a] Using microplates pre-coated with poly-L-lysine

[b] Average of 4 trials

[c] After treatment, samples were centrifuged at 3500xg for 10 min and pellet resuspended in PBS (pH 7.2)

Table 4.4 - Effect of detergents on reactivity of MAb 2F11 with *S. enteritidis* as assessed by ELISA. Detergents were removed by dialysis prior to immunoassay

	Absorbance (405nm)[b]				
Treatment[c]	SE PT1	SE PT4	SE PT8	SE PT13	SE PT13a
Ethylenediaminetetraacetate	1.898	1.905	1.929	1.944	1.936
Sodium cholate	1.717	1.552	1.955	1.886	1.937
Triton X-100	1.394	1.310	1.515	1.306	1.195
Sodium deoxycholate	1.261	0.992	1.225	0.887	1.286
Sodium azide	0.923	1.094	1.249	0.853	0.989
Sodium dodecyl sulfate	0.931	1.023	1.131	0.662	0.911
Iminodiacetic acid	0.963	0.881	0.951	0.957	1.068
Tween 80	0.619	0.350	0.578	0.470	0.498

[a] Using microplates pre-coated with poly-L-lysine
[b] Average of 4 trials
[c] After treatment, samples were dialyzed for 2 days in deionized water

Table 4.5 - Effect of detergents on reactivity of MAb 2F11 with free LPSs of *S. entertidis* as assessed by ELISA. Detergents were removed by dialysis prior to immuoassay

	Absorbance (405nm)[b]				
Treatment[c]	SE PT1	SE PT4	SE PT8	SE PT13	SE PT13a
Ethylenediaminetetraacetate	1.840	1.885	1.920	1.939	1.949
Sodium cholate	1.786	1.662	1.955	1.887	1.761
Triton X-100	1.392	1.209	1.504	1.273	1.029
Sodium deoxycholate	1.297	1.073	1.189	1.047	1.469
Sodium dodecyl sulfate	1.017	1.023	1.137	0.823	0.948
Sodium azide	0.836	0.888	1.254	0.842	0.850
Iminodiacetic acid	0.936	0.829	0.879	0.789	0.852
Tween 80	0.679	0.934	0.379	0.391	0.403

[a] Using microplates pre-coated with poly-L-lysine
[b] Average of 4 trials
[c] After treatment, samples were dialyzed, centrifuged at 3500xg for 10 min

Table 4.6 - Effect of detergents on reactivity of MAb 2F11 with cell-bound LPSs of *S. enteritidis* as assessed by ELISA. Detergents were removed by dialysis prior to immuoassay

	Absorbance (405nm)[b]				
Treatment[c]	SE PT1	SE PT4	SE PT8	SE PT13	SE PT13a
Ethylenediaminetetraacetate	1.906	1.555	1.018	1.898	1.822
Triton X-100	1.357	1.231	1.326	1.340	1.221
Iminodiacetic acid	1.113	1.112	1.336	1.104	1.254
Sodium dodecyl sulfate	1.019	1.157	1.336	1.295	1.280
Tween 80	1.118	0.977	1.425	1.020	1.017
Sodium deoxycholate	0.966	1.142	1.214	1.093	1.076
Sodium azide	0.946	1.019	1.276	1.147	1.149
Sodium cholate	0.822	0.690	0.901	0.876	1.084

[a] Using microplates pre-coated with poly-L-lysine
[b] Average of 4 trials
[c] After treatment, samples were dialyzed, centrifuged at 3500xg for 10 min and pellet resuspended in PBS (pH 7.2)

cholate was the only detergent which did hinder the ELISA results. Results were alsoconsistant for each *S. enteritidis* phage type tested.

The efficacy of the detergent treatment on extractibility of LPS from bacterial cells were also assessed by transmission electron microscopy. Figure 4.1 shows a negative stain of an intact *S. enteritidis* cell, while labelling of *S. enteritidis* live cells with MAb 2F11 and goat anti-mouse IgG conjugated with collodial gold is shown in Figure 4.2. A dense layer of gold particles covered the bacterial cell indicating that the LPS leaflet is well attached to the bacterial outer membrane and is expressed in a high frequency in the intact cell. Other workers have reported that bacterial LPS may account for 50 to 70% of the outer monolayer (Lugtenberg and van Alphen, 1983). Heat-treatment of bacterial cells, which is the most common method used to release LPS (Masi and Zawistowski 1995; Ewing, 1986), proved only partially effective in our experiments (Figure 4.3 and 4.4). Half of the cells viewed were covered with gold particles indicating that LPS was not completely extracted. The addition of sodium cholate led to approximately 95% of the cells lacking gold particles on the bacterial surface (Figure 4.5). Triton X-100 and sodium azide had similar effects extracting LPS from approximately 90% of the *S. enteritidis* cells viewed. Results with the remaining detergents indicated that all cells viewed had gold particles present on the cell surface although not as dense as the live control (Figure 4.6).

Sodium dodecyl sulfate, sodium deoxycholate and Tween 80, which are hydrophobic compounds (Nikaido and Vaara, 1985), were not effective for LPS removal. Due to the hydrophobic properties of the outer membrane (Schnaitman, 1970), these

Figure 4.1 Electron micrograph of *S. enteritidis* PT8 negatively stained. Magnification 17,000x.

Figure 4.2 Electron micrograph of *S. enteritidis* PT8 probed with MAb 2F11 ascites and goat anti-mouse IgG conjugated to 20 nm gold spheres. Magnification 17,000x.

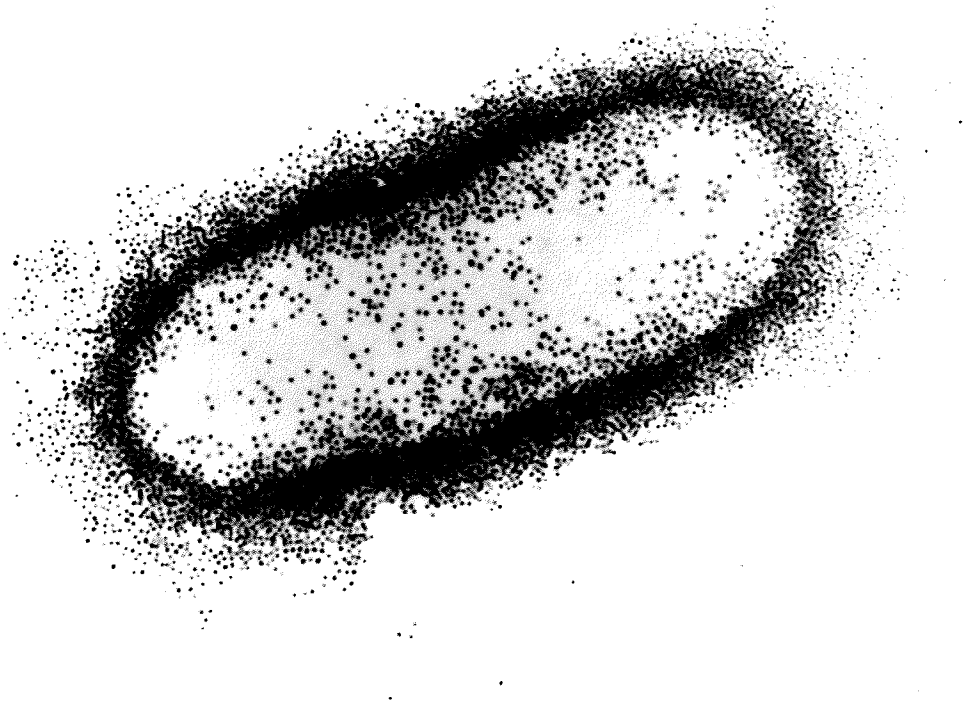


Figure 4.3 Electron micrograph of *S. enteritidis* PT8 probed with MAb 2F11 ascites and goat anti-mouse IgG conjugated to 20 nm gold spheres following heating for 10 min at 100 °C, 50% of cells viewed. Magnification 17,000x.

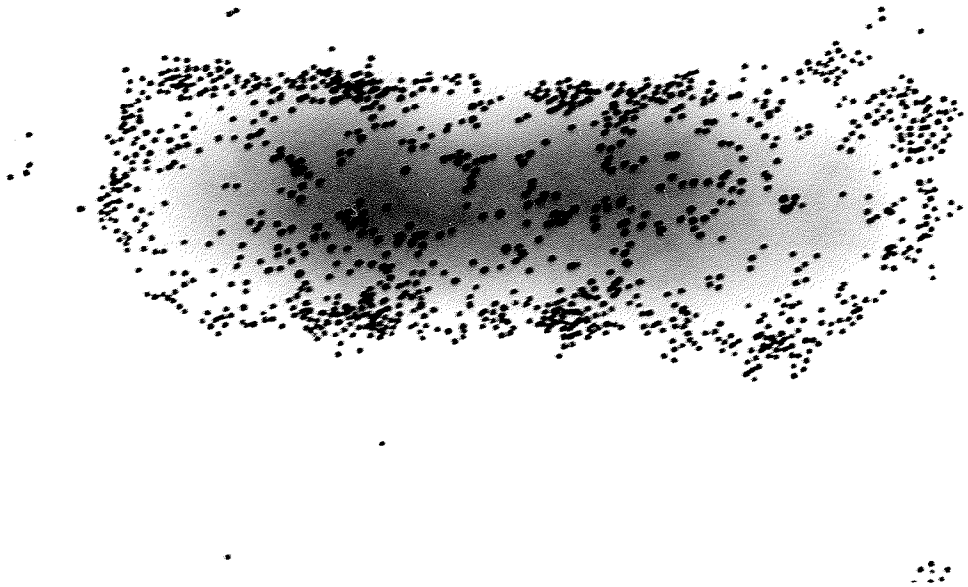


Figure 4.4 Electron micrograph of *S. enteritidis* PT8 probed with MAb 2F11 ascites and goat anti-mouse IgG conjugated to 20 nm gold spheres following heating for 10 min at 100 °C, 50% of cells viewed. Magnification 17,000x.

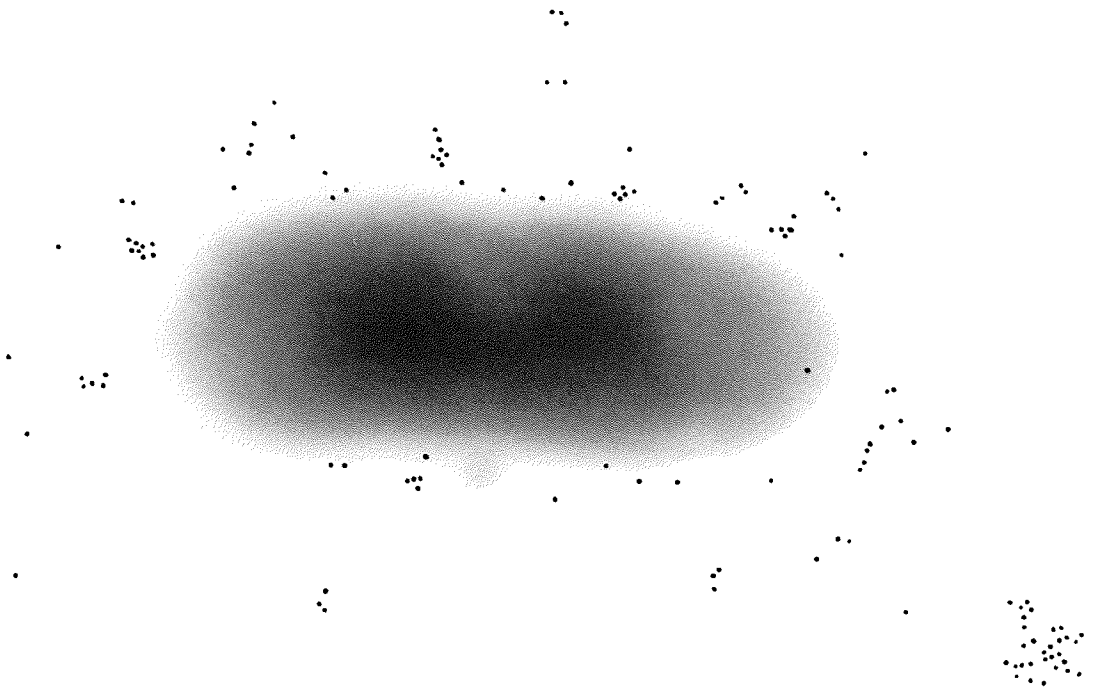


Figure 4.5 Electron micrograph of *S. enteritidis* PT8 probed with MAb 2F11 ascites and goat anti-mouse IgG conjugated to 20 nm gold spheres following addition of sodium cholate, 95% of cells viewed. Magnification 17,000x.

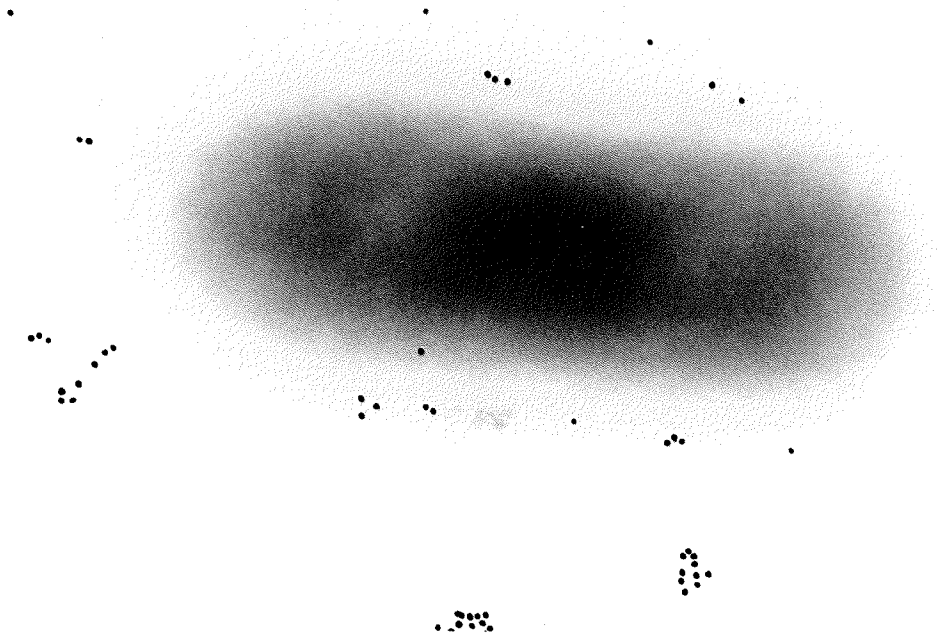
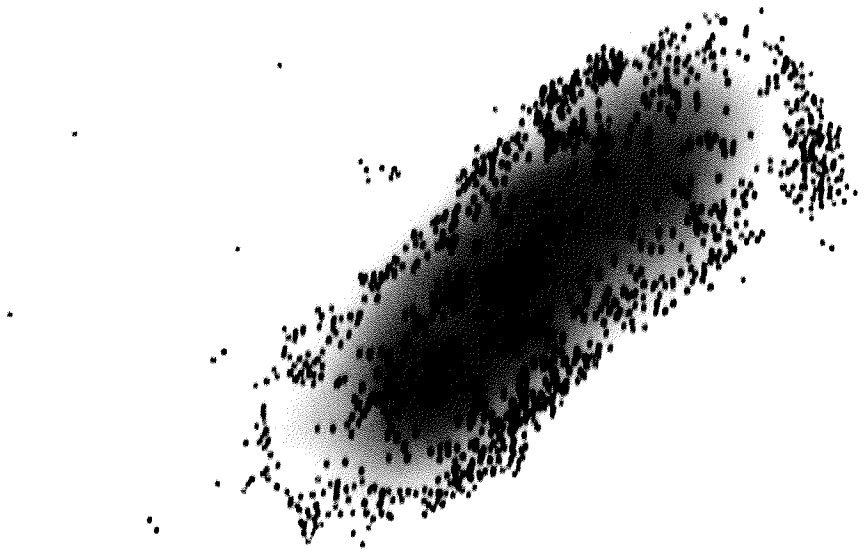


Figure 4.6 Electron micrograph of *S. enteritidis* PT8 probed with MAb 2F11 ascites and goat anti-mouse IgG conjugated to 20 nm gold spheres following addition of sodium deoxycholate. Similar results were obtained with SDS, Tween 80, iminodiacetic acid and ethylenediaminetetraacetate. Magnification 17,000x.



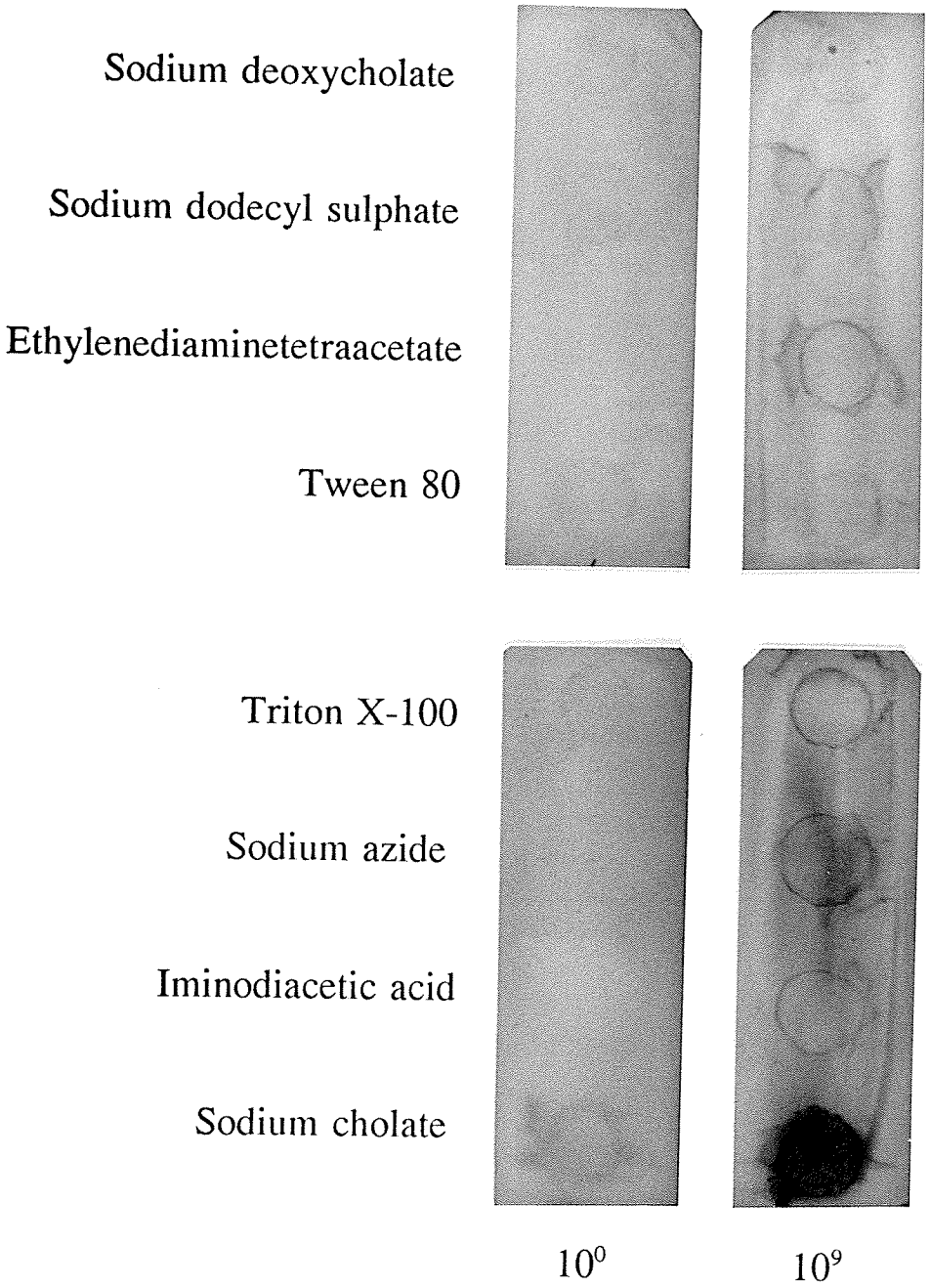
detergents may have been unable to disrupt and release the LPS from the bacterial membrane. Electron microscopy had shown that EDTA and iminodiacetic acid (polycation) provided inaccurate results in the ELISA. Although EDTA has been reported to release LPS from bacterial membranes, only about one third of the LPS was removed (Hammond *et al.*, 1984) due to weakening of LPS-LPS interactions by chelation of divalent cations. In the contrary, sodium cholate, sodium azide and Triton X-100 were successful in extracting LPS antigens from *S. enteritidis*. Triton X-100 has been reported by Collins and Salton (1979) to be effective in LPS extraction. Schnaitman (1970) concluded that Triton interacts with the cell wall phospholipid which disrupts the outer membrane including LPS. Wang and co-workers (1995) successfully employed sodium cholate to extract LPS from eggs contaminated with *S. enteritidis*.

The application of all eight detergents in a rapid dot-blot immunoassay was also studied. Figure 4.7 shows that only sodium cholate was applicable to this type of immunoassay. This detergent was capable of releasing LPS antigens when *S. enteritidis* was inoculated into homogenized eggs at a concentration of 10^9 cells/ml of egg. Slight coloration occurred with the remaining detergents, however, sodium cholate provided the best results. The egg matrix may have interfered in the action of the detergents on the extraction of LPS from *S. enteritidis*.

E. CONCLUSION

Heat treatment alone is not efficient in releasing the LPS antigens from the bacterial membrane. Results of the indirect ELISA indicated that sodium cholate, EDTA

Figure 4.7 The dot-blot immunoassay of eggs inoculated with 10^8 CFU of *S. enteritidis* PT8/ml in the presence of either sodium deoxycholate, sodium dodecyl sulfate, ethylenediaminetetraacetate, Tween 80, Triton X-100, sodium azide, iminodiacetic acid or sodium cholate respectively. Magnification 17,000x


Sodium deoxycholate
Sodium dodecyl sulphate
Ethylenediaminetetraacetate
Tween 80
Triton X-100
Sodium azide
Iminodiacetic acid
Sodium cholate
10^0
10^9
S. enteritidis PT8
(per ml of egg)

and iminodiacetic acid were the most effective in LPS extraction. However, transmission electron microscopy revealed that not all LPS was extracted when EDTA and imunodieacetic acid were used. Sodium azide, Triton X-100 and sodium cholate appeared to be the most effective. Once these detergents were applied in the rapid bot-blot immunoassay, sodium cholate was the only detergent able to extract the LPS antigen from *S. enteritidis* in homogenized eggs and proved to be the most applicable for this type of immunoassay.

V. GENERAL DISCUSSION

Culture methods for the detection of *S. enteritidis* are time consuming, labour intensive and expensive if many samples are analyzed. Furthermore, some commonly used selective enrichment broths have been found to be inhibitory towards *S. enteritidis* (van der Zee, 1994). Methods which provide rapid results with a sensitivity equal or greater than conventional culture methods are required. These methods should be simple, specific, reliable and cost effective. Modified culture methods such as egg pooling followed by selective plating (Gast and Holt, 1995a) and the ISO-GRID 2-day rapid screening method (Entis, 1996) have been developed. These methods have been successful in the detection of *Salmonella* in eggs, however, they are not specific for *S. enteritidis*. Many researchers have turned to immunoassays for rapid detection. Latex agglutination (Thorn *et al.*, 1994), enzyme immunoassays (Wang *et al.*, 1995; Brigmon *et al.*, 1995; Lee *et al.*, 1989) and immunomagnetic separation (Holt *et al.*, 1995; Cudjoe *et al.*, 1994) have been developed for detection of *S. enteritidis*. One of the problems associated with immunological-based methods is cross-reactivity of monoclonal or polyclonal antibodies with other microorganisms.

The dot-blot immunoassay, which was developed and optimized in our laboratory, employs a two-step procedure. Contaminated samples are enriched and then *Salmonella enteritidis* is detected using a monoclonal antibody-based dot-blot assay. The monoclonal antibody (MAb 2F11) utilized in the assay was previously produced and characterized by Masi and Zawistowski (1995). This MAb is specific to the LPS O-9

factor of the D_1-serogroup of *Salmonella.*

A variety of membranes, coating agents and blocking agents were assessed for use in the assay. The optimal system for the immunoassay utilized nitrocellulose strips incubated in PBS and blocked with 5% skim milk powder. Neutral and positively charged nylon membranes produced high backgrounds while polyvinylidene difluoride, which yielded low background, was unable to discriminate between positive and negative control samples. Poly-L-lysine and polymyxin B were examined as pre-coating agents to increase the sensitivity of the assay. Although both agents have been used successfully as coating agents for LPS antigens (Masi and Zawistowski, 1995; Wang *et al.*, 1995), high background was observed with all membranes.

Once the immunoassay components such as nitrocellulose membrane, skim milk powder and PBS were selected for the dot-blot, incubation times for each step in the assay were studied. Best results were obtained when nitrocellulose strips were incubated in PBS for 30 to 45 min, blocked for 45 min, probed with MAb 2F11 for 60 min and incubated with biotinylated goat anti-mouse IgG and streptavidin alkaline phosphatase for 60 min. Development with a BCIP/NBT substrate solution required between 2 to 30 min.

Furthermore, studies were conducted to enhance the optimized immunoassay. Monoclonal antibody 2F11 was biotinylated and used in conjunction with streptavidin alkaline phosphatase. This although would eliminated B-GAM and reduced the assay time by one h, the results were ambigious and we were unable to differentiate between positive and negative control samples. A sandwich dot-blot immunoassay using a high affinity membrane towards monoclonal antibodies was also examined. Membranes were

coated with MAb 2F11, blocked and egg samples applied to the membrane strips. Strips were then probed with biotinylated MAb 2F11. All bacterial concentrations including the negative control were detected. Since the membrane has a high affinity towards antibodies, non-specific binding of biotinylated MAb 2F11 may have occurred due to insufficient blocking.

Streptavidin followed by biotinylated alkaline phosphatase was employed to increase the sensitivity of the immunoassay. Streptavidin contains three binding sites for biotin once biotinylated IgG has been bound. All samples including the negative control were detected.

Based on the results from these studies, MAb 2F11 followed by biotinylated IgG and streptavidin alkaline phosphatase was selected for the detection of *S. enteritidis* in eggs, poultry and other foods and feed.

Salmonella enteritidis was directly enriched in homogenized egg without the need of enrichment and selective steps for detection in homogenized liquid egg. Eggs contain enough nutrients to support microbial growth. Baker and co-workers (1983) found that *S. enteritidis* can easily survive and propagate in yolk/albumen egg mixtures. Thus, a small concentration of *S. enteritidis* cells were able to proliferate to detectable levels without the need to isolate the organism. Homogenized egg samples inoculated with one *S. enteritidis* cell per 25 gm yielded 10^6 cells/ml after 20 h incubation at 37 °C. This concentration was detected by the dot-blot immunoassay.

Prior to detection, contaminated homogenized eggs were heated in the presence of sodium cholate. The combination of heat and detergent initiated the release of LPS

antigens from the bacterial cell membrane which in turn were uniformly distributed within the gelled egg matrix. Circular egg samples were taken and placed on PBS soaked nitrocellulose strips. Through diffusional forces, LPS antigens were transferred onto the membrane and detected by MAb 2F11. Purple dots were considered as positive results.

A variety of detergents were evaluated using ELISA and transmission electron microscopy in conjunction with collodial gold staining for the ability to remove LPS antigens from *S. enteritidis*. Preliminary results from the ELISA indicated that sodium cholate, EDTA and iminodiacetic acid were the most effective in releasing LPS antigens. However, transmission electron microscopy indicated that EDTA and iminodiacetic acid were ineffective. On the contrary, sodium cholate, sodium azide and Triton X-100 were more efficient releasing almost all LPS from the bacteria membrane. All eight detergents were then applied in the dot-blot immunoassay. Sodium cholate was the only detergent able to release LPS when *S. enteritidis* was present in homogenized eggs. With respect to the other detergents, the egg matrix may have interfered in their ability to interact and remove the LPS from the cell membrane.

Whole shell eggs contaminated by horizontal transmission frequently involve a mixed infection usually dominated by gram negative bacteria (Board, 1966). Due to this type of transmission, the specificity of the assay was assessed. Eggs were inoculated with *S. enteritidis*, *S. heidelberg*, *C. freundii*, *P. vulgaris*, *E. coli*, *A. faecalis* and *P. fluorescens* and incubated for 24 h at 37°C. In the mixed microflora, *S. enteritidis* was readily detected by the developed dot-blot even when this pathogen was initially out competed 1 to 400 by other bacteria. Cudjoe and co-workers (1994) found that the

antimicrobial agents in albumen were not exhausted when the yolk and albumen are mixed. If some strains of *S. enteritidis* have the ability to rapidly multiply in egg mixtures (Gast and Holt, 1995b) and the mixture still possess antimicrobial effects, this could explain why *S. enteritidis* was able to survive and multiply to detectable levels in the presence of high numbers of competing bacteria. Dolman and Board (1992) also reported that *S. enteritidis* are able to out compete other gram negative bacteria at incubation temperatures near 37°C.

Studies involving egg pools (9 or 10 eggs) have shown that incorporation of iron enhances the growth of *S. enteritidis* (Gast and Beard, 1995; Cujoe *et al.*, 1994a). Incubation of 1, 5 or 10 *S. enteritidis* cells/500 gm of egg supplemented with ferrous sulfate (17.5 mg) allowed cells to multiply to detectable levels. After 20 h at 37°C, the dot-blot readily detected *S. enteritidis*. *Salmonella enteritidis* was detected when out competed 1 to 300 after incubation of 24 h at 37°C using the same six species of bacteria.

The application of this method was expanded to included other foods and feed. Artificially inoculated poultry meat, ice cream and poultry feed were directly enriched in homogenized egg. Incubation of 1, 5 or 10 cells/25 gm of sample were readily detected by the dot-blot assay. Skim milk powder was also tested, however, samples were enriched in brilliant green water for 7 h at 37°C prior to incubation in homogenized eggs. Samples inoculated with 1, 5 or 10 cells/ml were detected however strips required more time to develop.

VI. CONCLUSION AND RECOMMENDATIONS

A simple, rapid and economical method for the detection of *S. enteritidis* was established and optimized. The dot-blot immunoassay, which utilized an anti-LPS monoclonal antibody specific to the D_1-serogroup of *Salmonella*, was successfully employed in the detection of *S. enteritidis* in fresh shell eggs, poultry and other foods and feed.

Artificially contaminated eggs incubated for 20 h at 37°C allowed for the detection of one *S. enteritidis* cell per 25 gm of food/feed samples. The dot-blot immunoassay also exhibited a detection limit of 10^6 *S. enteritidis* cells/ml. With the addition of ferrous sulfate, incubation of one *S. enteritidis* cell/500 gm of homogenized egg was detected. Even when *S. enteritidis* was initially out competed 1 to 400 by other bacteria, the pathogen was detected by the assay.

The method was also applied to chicken meat, ice cream, skim milk powder and poultry feed. Incubation of 1, 5 or 10 cells of *S. enteritidis* in homogenized egg were detected by the dot-blot immunoassay. Nevertheless, a wider range of products should be analyzed with the assay and the method should be validated via interlaboratory trials.

From the eight detergents examined, sodium cholate, sodium azide and Triton X-100 were the most effective in LPS removal from the bacterial cell membrane. However, once applied in the immunoassay, sodium cholate was the only detergent able to remove LPS when added to contaminated eggs.

The use of enzyme conjugated antibodies should be investigated. Conjugation of MAb 2F11 to enzymes such as alkaline phosphatase or horseradish peroxidase would

eliminate two steps and shorten the assay by approximately two hours.

Currently, a simplified format for this assay is being investigated. A membrane coated dip stick which can be inserted into the heated sample has been proposed. Once the stick has been inserted, it is blocked, probed with MAb 2F11 and incubated with the various immunoreagents. Coloration of the membrane would indicate a positive while a clear stick would be considered a negative. Each step would have its own chamber to incubate the dip stick.

If this assay is to be commercialized, the stability of the MAb must also be assessed. The reactivity of MAb 2F11 over a storage period should be examined. The effect of various buffers, salts, sugars and antimicrobial agents used for storage of antibodies should be studied with MAb 2F11. Also, if MAb 2F11 is lyophilized, studies should be conducted to assess its stability after rehydration.

Detection of *S. enteritidis* required 24 h for contaminated eggs and 28 h for poultry and other products when analyzed through the dot-blot immunoassay.

VII. REFERENCES

Andrews, W. H. 1985. A review of culture methods and their relation to rapid methods for the detection of *Salmonella* in foods. Food Technol. 39:77-82.

AOAC. 1990. "Official Methods of Analysis," 15th ed. Assn. Off. Anal. Chem. Arlington, VA. pp.457-492.

AOAC. 1995. "Official Methods of Analysis," 16th ed. Assn. Off. Anal. Chem. Arlington, VA. pp.55-94.

Baily, J. S. 1988. Integrated colonization control of *Salmonella* in poultry. Poul. Sci. 67:928-932.

Baker, R. C., Hogarty, S., Poon, W. and Vedehra, D. V. 1983. Survival of *Salmonella typhimurium* and *Staphylococcus aureus* in eggs cooked by different methods. Poul. Sci. 62:1211-1216.

Baker, R. C. 1990. Survival of *Salmonella enteritidis* on and in shelled eggs, liquid eggs, and cooked egg products. Dairy Food Environ. Sanit. 10(5):273-275.

Barnhart, H. M., Dreesen, D. W., Bastien, R. and Pancorbo, O. C. 1991. Prevalence of *Salmonella enteritidis* and other serovars in ovaries of layer hens at time of slaughter. J. Food Prot. 54(7):488-491.

Barrow, P. A., Huggins, M. B., Lovell, M. A. and Simpson, J. M. 1987. Observations on the pathogenesis of experimental *Salmonella typhimurium* infection in chickens. Res. Vet. Sci. 42:194-199.

Barrow, P. A., Lovell, M. A. and Berchieri, A. 1991. The use of two live attenuated vaccines to immunize egg-laying hens against *Salmonella enteritidis* phage type 4. Avian Path. 20:681-692.

Barrow, P. A. and Lovell, M. A. 1991. Experimental infection of egg-laying hens with *Salmonella enteritidis* phage type 4. Avian Path. 20:335-348.

Barrow, P. A. 1991. Experimental infection of chickens with *Salmonella enteritidis*. Avian Path. 120:145-153.

Behling, R. G. and Wong, A. C. L. 1994. Competitive exclusion of *Salmonella enteritidis* in chicks by treatment with a single culture plus dietary lactose. Inter. J. Food Microbiol. 22:1-9.

Blackburn, C. W. 1993. Rapid and alternative methods for the detection of salmonellas in foods. J. Appl. Bacteriol. 75:199-214.

Blais, B. W. and Yamazaki, H. 1989. Extraction of *Salmonella* antigens in non-sedimentable forms for enzyme immunoassay. Inter. J. Food Microbiol. 9:63-71.

Blais, B. W. and Yamazaki, H. 1990a. Use of polymyxin-coated polyester cloth in the enzyme immunoassay of *Salmonella* lipopolysaccharide antigens. Inter. J. Food Microbiol. 11:195-204.

Blais, B. W. and Yamazaki, H. 1990b. Use of detergents in the preparation of *Salmonella* samples for enzyme immunoassay on polymyxin-coated polyester cloth. Inter. J. Food Microbiol. 11:329-336.

Blumenthal, D. 1990. *Salmonella enteritidis*, from the chicken to the egg. FDA Consumer. 24:6-10.

Board, R. G. 1966. Review article: the course of microbial infection of the hen's egg. J. Appl. Bacteriol. 29(2):319-341.

Braun, P. and Fehlhaber, K. 1995. Migration of *Salmonella enteritidis* from the albumin into the egg yolk. Inter. J. Food Microbiol. 25:95-99.

Brigmon, R. L., Zam, S. G., Bitton, G. and Farrah, S. R. 1992. Detection of *Salmonella enteritidis* in environmental samples by monoclonal antibody-based ELISA. J. Immunol. Meth. 152:135-142.

Brigmon, R. L., Zam, S. G. and Wilson, H. R. 1995. Detection of *Salmonella enteritidis* eggs and chicken with enzyme-linked immunosorbent assay. Poul. Sci. 74:1232-1236.

Brooks, J. 1960. Mechanism of the multipication of *Pseudomonas* in the hen's egg. J. Appl. Bacteriol. 23:499-509.

Brown, D. J., Threlfall, E. J., Hampton, M. D. and Rowe, B. 1993. Molecular characterization of plasmids in *Salmonella enteritidis* phage types. Epidemiol. Infect. 110:209-216.

Bryant, E. S. 1990. *Salmonella enteritidis* control. Dairy, Food and Environmental Sanitation. 10(5):271-272.

Chart, H., Threlfall, E. J. and Rowe, B. 1989. Virulence of *Salmonella enteritidis* phage type 4 is related to the possession of a 38 MDa plasmid. FEMS Microbiol. Lett. 58:299-304.

Chart, H. and Rowe, B. 1993. Iron restriction and the growth of *Salmonella enteritidis*. Epidemiol. Infect. 110:41-47.

Clay, C. E. and Board, R. G. 1991. Growth of *Salmonella enteritidis* in artificially contaminated hens' shell eggs. Epidemiol. Infect. 106:271-281.

Clay, C. E. and Board, R. G. 1992. Effect of faecal extract on the growth of *Salmonella enteritidis* in artificially contaminated hens' eggs. British Poul. Sci. 33:755-760.

Cloeckaert, A., de Wergifosse, P., Dubray, G. and Limet, J. N. 1990. Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labelling for electron microscopy and enzyme linked immunosorbent assay. Infect. Immunol. 58:3980-3987.

Collins, M. L. P. and Salton, M. R. J. 1979. Solubility characteristics of *Micrococcus lysodeikticus* membrane components in detergents and chaotroptic salts analyzed by immunoelectrophoresis. Biochem. Biophys. Acta. 533:40-53.

Corrier, D. E., Hargis, B., Hinton, A. Jr., Lindsey, D., Caldwell, D., Manning, J. and Deloach, J. 1991. Effect of anaerobic cecal microfloran and dietary lactose on colonization resistance of layer chicks to invasive *Salmonella enteritidis*. Avian Dis. 35:337-343.

Cox, J. M. 1995a. *Salmonella enteritidis*: the egg and I. Aust. Vet. J. 72(3):108-115.

Cox, J. M. 1995b. *Salmonella enteritidis*: virulence factors in invasive infections in poultry. Trends Food Sci. Tech. 6:407-410.

Cudjoe, K. S., Krona, R., Grϕn, B. and Olsen, E. 1994a. Use of ferrous sulphate and immunomagnetic separation to recover *Salmonella enteritidis* from raw eggs. Inter. J. Food Microbiol. 23:149-158.

Cudjoe, K. S., Krona, R. and Olsen, E. 1994b. IMS: a new selective enrichment technique for detection of *Salmonella* in foods. Inter. J. Food Microbiol. 23:159-165.

D'Aoust, J. 1991. Pathogenicity of foodborne *Salmonella.* Inter. J. Food Microbiol. 12(1):17-40.

De Smedt, J. M. and Bolderdijk, R. F. 1987. Dynamics of *Salmonella* isolation with modified semi-solid rappaport-vassiliadis medium. J. Food Prot. 50:658-561.

Dolman, J. and Board, R. G. 1992. The influence of temperature on the behaviour of mixed bacterial contamination of the shell membrane on the hen's egg. Epidemiol. Infect. 108:115-121.

Edel, W. 1994. *Salmonella enteritidis* eradication programme in poultry breeder flocks in the netherlands. Inter. J. Food Microbiol. 21(1+2):171-178.

Entis, P., Brodsky, M. H., Sharpe, A. N. and Jarvis, G. A. 1982. Rapid detection of *Salmonella* spp. in food by use of the ISO-GRID hydrophobic grid membrane filter. Appl. Environ. Microbiol. 43:261-266.

Entis, P. 1996. Validation of the ISO-GRID 2-day rapid screening method for detection of *Salmonella* spp. in egg products. J. Food Prot. 59(5):555-558.

Ewing, W. H. 1986. Edwards and Ewing's Identification of Enterobacteriaceae, 4th edn., Elsevier, New York, NY.

Finlay, B. B. and Falkow, S. 1988. Virulence factors associated with *Salmonella* species. Microbiol. Sci. 5(11):324-327.

Flowers, R. S., D'Aoust, J. Y., Andrews, W. H. and Bailey, J. S. 1992. *Salmonella.* In "Compendium of methods for the microbiological examination of food. 3rd edition". Edward Brothers, Ann Arbor, MI. pp. 371-422.

Galyean, R. D. and Cotterill, O. J. 1972. Yolk inhibition of lysozyme activity in egg white. Poul. Sci. 51:1346-1353.

Gast, R. K. and Beard, C. W. 1990a. Production of *Salmonella enteritidis*-contaminated eggs by experimentally infected hens. Avian Dis. 34:438-446.

Gast, R. K. and Beard, C. W. 1990b. Isolation of *Salmonella enteritidis* from internal organs of experimentally infected hens. Avian Dis. 34:991-993.

Gast, R. K. and Beard, C. W. 1992. Detection and enumeration of *Salmonella enteritidis* in fresh and stored eggs laid by experimentally infected hens. J. Food Prot. 55(3):152-156.

Gast, R. K. 1993a. Detection of *Salmonella enteritidis* in experimentally infected laying hens by culturing pools of egg contents. Poul. Sci. 72:267-274.

Gast, R. K. 1993b. Recovery of *Salmonella enteritidis* from inoculated pools of egg contents. J. Food Prot. 56(1):21-24.

Gast, R. K. 1993c. Evaluation of direct plating for detecting *Salmonella enteritidis* in pools of egg contents. Poul. Sci. 72:1611-1614.

Gast, R. K and Holt, P. S. 1995a. Differences in the multiplication of *Salmonella enteritidis* strains in liquid whole egg: implications for detecting contaminated eggs from commercial laying flocks. Poultry Science. 74:893-897.

Gast, R. K. and Holt, P. S. 1995b. Iron supplementation to enhance the recovery of *Salmonella enteritidis* from pools of egg contents. J. Food Sci. 58(3):268-272.

Goodnough, M. C. and Johnson, E. A. 1991. Control of *Salmonella enteritidis* infections in poultry by polymyxin B and trimethoprim. Appl. Environ. Microbiol. 57(3):785-788.

Goossens, H., Wauters, G., de Broeck, M., Janssens, M. and Butzler, J. P. 1984. Semi-solid selective motility enrichment medium for isolation of salmonellae from faecal species. J. Clin. Microbiol. 19:941-941.

Haigh, T. and Betts, W. B. 1991. Microbial barrier properties of hen egg shells. Microbios. 68:137-146.

Hammack, T. S., Sherrod, P. S., Bruce, V. R., June, G. A., Satchell, F. B. and Andrews, W. H. 1993. Growth of *Salmonella enteritidis* in grade A eggs during prolonged storage. Poul. Sci. 72:373-377.

Harlow, E. and Lane, D. 1988. "Antibodies: A laboratory manual". Cold Spring Harbour Laboratory. New York, NY.

Hinton, M., Threlfall, E. J. and Rowe, B. 1990a. The invasive potential of *Salmonella enteritidis* phage types for young chickens. J. Appl. Bacteriol. 10:237-239.

Hinton, M., Threlfall, E. J. and Rowe, B. 1990b. The invasiveness of different strains of *Salmonella enteritidis* phage type 4 in young chickens. FEMS Microbiol. Lett. 70:193-196.

Holt, P. S. and Porter, Jr, R. E. 1992. Effect of induced molting on the course of infection and transmission of *Salmonella enteritidis* in white leghorn hens of different ages. Poul. Sci. 71(11):1842-1848.

Holt, P. S., Gast, R. K. and Greene, C. R. 1995. Rapid detection of *Salmonella enteritidis* in pooled liquid egg samples using a magnetic bead-ELISA system. J. Food Prot. 58(9):967-972.

Holt, P. S. 1995. Horizontal transmission of *Salmonella enteritidis* in molted and unmolted laying chickens. Avian Dis. 39:239-249.

Humphrey, T. J., Baskerville, A., Chart, H. and Rowe, B. 1989a. Infection of egg-laying hens with *Salmonella enteritidis* PT 4 by oral inoculation. Vet. Rec. 125:531-532.

Humphrey, T. J., Baskerville, A., Mawer, S., Rowe, B. and Hopper, S. 1989b. *Salmonella enteritidis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol. Infect. 103:415-423.

Humphrey, T. J., Greenwood, M., Gilbert, R. J., Rowe, B. and Chapman, P. A. 1989c. The survival of salmonellae in shell eggs cooked under simulated domestic conditions. Epidemiol. Infect. 103:35-45.

Humphrey, T. J., Chapman, P. A., Rowe, B. and Gilbert, R. J. 1990. A comparative study of the heat resistance of salmonellas in homogenized whole egg, egg yolk or albumin. Epidemiol. Infect. 104:237-241.

Humphrey, T. J. 1990a. Growth of salmonellas in intact shell eggs: influence of storage temperature. Vet. Rec. 126:292.

Humphrey, T. J. 1990b. Heat resistance in *Salmonella enteritidis* phage type 4: the influence of storage temperatures before heating. J. Appl. Bacteriol. 69:493-497.

Humphrey, T. J., Richardson, N. P., Gawler, A. H. L. and Allen, M. J. 1991. Heat resistance of *Salmonella enteritidis* PT4: the influence of prior exposure to alkaline conditions. Lett. Appl. Microbiol. 12:258-260.

Humphrey, T. J. and Whitehead, A. 1992. Techniques for the isolation of *Salmonellas* from eggs. Br. Poult. Sci. 33:761-768.

Humphrey, T. J. and Whitehead, A. 1993. Egg age and the growth of *Salmonella enteritidis* PT4 in egg contents. Epidemiol. Infect. 111:209-219.

Humphrey, T. J. 1994. Contamination of egg shell and contents with *Salmonella enteritidis*: a review. International J. Food Microbiol. 21(1+2):31-40.

Jones, F. T., Rives, D. V. and Carey, J. B. 1995. *Salmonella* contamination in commercial eggs and an egg production facility. Poul. Sci. 74:753-757.

Keller, L. H., Benson, C. E., Garcia, V., Nocks, E., Battenfelder, P. and Eckroade, R. J. 1993. Monoclonal antibody-based detection system for *Salmonella enteritidis*. Avian Dis. 37:501-507.

Khakhria, R., Woodward, D. and Johnson, W. 1995. Top 10 human *Salmonella* serotypes reported in canada during 1994. Safety Watch. 37:4.

Kim, C. J., Emery, D. A., Rinke, H., Nagaraja, K. V. and Halvorson, D. A. 1989. Effect of time and temperature on growth of *Salmonella enteritidis* in experimentally inoculated eggs. Avi. Dis. 33:735-742.

Kotina, S. and Takada, H. 1990. Structural requirements of lipid A for endotoxicity and other biological activities - an overview. In "Endotoxin", Plenum Press, New York, NY.

Lee, H. A., Wyatt, G. M., Bramham, S. and Morgan, M. R. A. 1989. Rapid enzyme-linked immunosorbent assays for the detection of *Salmonella enteritidis* in eggs. Food Agric. Immunol. 1:89-99.

Lindberg, A. A. 1980. Bacterial virulence factors - with particular reference to *Salmonella* bacteria. Scand. J. Infect. Dis. Supplement. 24:86-92.

Lock, J. L. and Board, R. G. 1992. Persistence of contamination of hens' egg albumen in vitro with *Salmonella* serotypes. Epidemiol. Infect. 108:389-396.

Lu, G. Z., Tsang, R. S. W., Chau, P. Y., Choi, D., Law, D. and Ng, M. H. 1991. Characterization and application of a murine monoclonal antibody that reacts specifically with the serogroup D_1 *Salmonella*. FEMS Microbiol. Lett. 80:135-140.

Lugtenberg, B. and van Alphen, L. 198. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram negative bacteria. Biochi. Biophysi. Acta. 737:51-115.

Mammond, S. M., Lambert, P. A. and Rycroft, A. N. 1984. The envelope of gram-negative bacteria. In "The Bacterial Cell Surface". Croom Helm Ltd., Sydney, Australia. pp. 57-146.

Masi, A. and Zawistowski, J. 1995. Detection of live and heat-treated *Salmonella enteritidis* by a D_1-serospecific anti-LPS O-9 monoclonal antibody. Food Agric. Immunol. 7:351-363.

Mason, J. 1994. *Salmonella enteritidis* control programs in the united states. Inter. J. Food Microbiol. 21(1+2):155-169.

Nikaido, H. and Vaara, M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1-32.

Novero, R. P. and Asem, E. K. 1993. Follicle-stimulating hormone-enhanced fibronectin production by chicken granulosa cells is influenced by follicular development. Poul. Sci. 72:709-729.

Olsen, J. E., Skov, M. N., Threlfall, E. J. and Brown, D. J. 1994. Clonal lines of *Salmonella enteritidis* serotype *Enteritidis* documented by IS200-, ribo-, pulsed-field gel electrophoresis and RFLP typing. J. Med. Microbiol. 40:15-22.

Oyarzabal, O. A., Conner, D. E. and Blevins, W. T. 1995. Fructooligosaccharide utilization by Salmonellae and potential direct-fed microbial bacteria in poultry. J. Food Sci. 58(11):1192-1196.

Perales, I. and Erkiaga, E. 1991. Comparison between semisolid rappaport and modified semisolid rappaport-vassiliadis media for the isolation of *Salmonella* species from foods and feed. Inter. J. Food Microbiol. 14:51-57.

Peterson, A. A. and McGroarty, E. J. 1985. High molecular-weight components in lipopolysaccharides of *Salmonella typhimurium*, *Salmonella minnesota* and *Escherichia coli*. J. Bacteriol. 162:738-745.

Poppe, C., Irwin, R. J., Forsberg, C. M., Clarke, R. C. and Oggel, J. 1991. The prevalence of *Salmonella enteritidis* and other *Salmonella* spp. among canadian registered commercial layer flocks. Epidemiol. Infect. 106:259-270.

Poppe, C. 1994. *S. enteritidis* in canada. Inter. J. Food Microbiol. 21(1+2):1-5.

Porstmann, T. and Kiessig, S. T. 1992. Enzyme immunoassay techniques. An overview. J. Immunol. Meth. 150:5-21.

Rampling, A., Upson, R., Ward, L. R., Anderson, J. R., Peters, E. and Rowe, B. 1989. *Salmonella enteritidis* phage type 4 infections: association with hens' eggs. Lancet. 335:1295-1296.

Rampling, A., Upson, R. and Brown, D. F. J. 1990. Nitrofurantoin resistance in isolates of *Salmonella enteritidis* phage type 4 from poultry and humans. J. Antimicrob. Chem. 25:285-290.

Rampling, A. 1993. *Salmonella enteritidis* five years on. Lancet. 342:317-318.

Reissbrodt, R. L. and Rabsch, W. 1993. Selective pre-enrichment on *Salmonella* from eggs by siderophore supplement. Zbl. Bakteriol. 279:344-353.

Rietschel, E. T. and Brade, H. 1992. Bacterial endotoxins. Sci. Amer. August 1992. pp. 54-61.

Roberts, J. A. and Sockett, P. N. 1994. The socio-economic impact of human *Salmonella enteritidis* infections. Inter. J. Food Microbiol. 21(1+2):117-129.

Saeed, A. M. and Koons, C. W. 1993. Growth and heat resistance of *Salmonella enteritidis* in refrigerated and abused eggs. J. Food Prot. 56(11):927-931.

Schnaitman, C. A. 1970. Effect of ethylenediaminetetraacetic acid, triton X-100 and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. Am. Soc. Microbiol. 108:553-563.

Schnaitman, C. A. and Klena, J. D. 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. 57:655-682.

Schoeni, J. L., Glass, K. A., McDermott, J. L. and Wong, A. C. L. 1995. Growth and penetration of *Salmonella enteritidis*, *Salmonella heidelberg* and *Salmonella typhimurium* in eggs. Inter. J. Food Microbiol. 24:385-396.

Seuna, E., Schneitz, C., Nurmi, E. and Makela, P. H. 1980. Combined therapy of *Salmonella* infection in chickens by anti-microbial agents followed by cultured cecal bacteria. Poul. Sci. 59:1187-1192.

Shivaprasad, H. L., Timoney, J. F., Morales, S., Lucio, B. and Baker, R. C. 1990. Pathogenesis of *Salmonella enteritidis* infection in laying chickens. I. studies on egg transmission, clinical signs, fecal shedding and serologic responses. Avian Dis. 34:548-557.

Silliker, J. H., Schmall, A. and Chiu, J. Y. 1966. The fluorescent antibody technique as a means of detecting salmonellae in foods. J. Food Sci. 31:240-244.

Stephenson, P., Satchell, F. B., Allen, G. and Andrews, W. H. 1991. Recovery of *Salmonella* from shell eggs. J. Ass. Off. Anal. Chem. 74(5):821- 826.

St. Louis, M. E., Morse, D. L., Potter, M. E., DeMelfi, T. M., Guzewich, J. J., Tauxe, R. V. and Blake, P. A. 1988. The emergence of grade A eggs as a major source of *Salmonella enteritidis* infections. JAMA. 259:2103-2107.

Sukupolvi, S. and Vaara, M. 1989. *Salmonella typhimurium* and *Escherichia coli* mutants with increased outer membrane permeability to hydrophobic compounds. Biochem. Biophys. Acta. 988:377-387.

Suzuki, S. 1994. Pathogenicity of *Salmonella enteritidis* in poultry. Inter. J. Food Microbiol. 21(1+2):89-105.

Takahashi, K., Fukada, M., Kawai, M. and Tokochi, T. 1992. Detection of lipopolysaccharides and identification of its serotype by an enzyme-linked immunosorbent assay using poly-L-lysine. J. Immunol. Meth. 153:356-368.

Thiagarajan, D., Saeed, A. M. and Asem, E. K. 1994. Mechanism of transovarian transmission of *Salmonella enteritidis* in laying hens. Poul. Sci. 73:89-98.

Thorns, C. J., Sojka, M. G. and Chasey, D. 1990. Detection of a novel fimbrial structure on the surface of *Salmonella enteritidis* by using a monoclonal antibody. J. Clin. Microbiol. 28:2409-2414.

Thorns, C. J., McLaren, I. M. and Sojka, M. G. 1994. The use of latex particle agglutination to specifically detect *Salmonella enteritidis*. Inter. J. Food Microbiol. 21(1+2):47-53.

Timoney, J. F., Shivaprasad, H. L., Baker, R. C. and Rowe, B. 1989. Egg transmission after infection of hens with *Salmonella enteritidis* phage type 4. Vet. Rec. 125:600-601.

Toresma, R., Visser, M. J., Aarsman, C. J. M., Poppelier, M. J. J. G., van Beurden, R., Fluit, A. C. and Verhoef, J. 1992. Monoclonal antibodies that detect live *Salmonella.* Appl. Environ. Microbiol. 58:3868-3872.

van der Zee, H. 1994. Conventional methods for the detection and isolation of *Salmonella enteritidis.* Inter. J. Food Microbiol. 21(1+2):41-46.

van der Zijderveld, F. G., van Zijderveld-Van Bemmel, A. M. and Anakotta, J. 1992. Comparison of four different enzyme-linked immunosorbent assays for serological diagnosis of *Salmonella enteritidis* infection in experimentally infected chickens. J. Clin. Microbiol. 30(10):2560-2566.

Waltman, W. D., Horne, A. M., Pirkle, C. and Johnson, D. C. 1992. Prevalence of *Salmonella enteritidis* in spent hens. Avian Dis. 36:251-255.

Wang, H., Blais, B. W. and Yamazaki, H. 1995. Rapid and economical detection of *Salmonella enteritidis* in eggs by the polymyxin-cloth enzyme immunoassay. Inter. J. Food Microbiol. 24:397-406.

Wen-Chuan Lin, A., Goldsby, R. A. and Snoeyenbos, G. H. 1992. *Salmonella enteritidis*-specific monoclonal antibodies. Avian Dis. 36:455-458.