PRODUCTION, CHARACTERIZATION AND APPLICATION OF A MONOCLONAL ANTIBODY SPECIFIC FOR *SALMONELLA* LIPOPOLYSACCHARIDE O-9 ANTIGEN

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PRODUCTION, CHARACTERIZATION AND APPLICATION OF A MONOCLONCAL ANTIBODY SPECIFIC FOR SALMONELLA LIPOPOLYSACCHARIDE O-9 ANTIGEN

BY

ANDREA PATRICIA MASI

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

MASTER OF SCIENCE

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This Thesis is Dedicated To The Memories of

My Brother Javier and Grandfather Sixto,

Two Individuals Who I truely Miss

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LIST OF ABBREVIATIONS

A-AP	Avidin-alkaline phosphatase
AP	Alkaline phosphatase
ATCC	American Type Culture Collection
B-GAM	Biotinlylated goat-anti-mouse IgG
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
GAM-Ap	Goat-anti-mouse-IgG-alkaline phosphatase
kDa	Kilodalton
KDO	2-Keto-3-deoxy-D-manno-octulosonic acid
LPS	Lipopolysaccharides
MAb	Monoclonal Antibody
NBT	p-nitroblue tetrazolium chloride
PAb	Polyclonal Antibody
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween 20
PEG	Polyethylene glycol
PLL	Poly-L-lysine

pNPP	p-nitrophenyl phosphate
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
S-AP	Streptavidin-alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SC	Selenite cysteine broth
SPC	Standard plate count
TB	Tetrathionate broth
TBS	Tris-buffered saline
TTBS	Tris-buffered saline containing Tween 20

ABSTRACT

In the past decade, there has been an increase in the number of human salmonellosis cases due to infection with *Salmonella enteritidis*. Poultry, eggs and egg-containing foods have been associated with *S. enteritidis* outbreaks. The culture methods for the detection of *S. enteritidis* in food are scarce. They are also time consuming, laborious and expensive if large number of samples are to be tested. The objective of this study was to develop a rapid immunoassay for the detection of *S. enteritidis* in eggs. In order to accomplish this objective, the monoclonal antibody 2F11 (IgG_{2a}) was produced against *S. enteritidis* by the fusion of P3X63-Ag8.653 myeloma cells with spleenocytes of a mouse immunized with heat attenuated (80°C, 20 min) *S. enteritidis* cells. Specificity of this antibody was determined by ELISA and SDS-PAGE followed by immunoblotting. The MAb was reactive with various phage types of *S. enteritidis* (1, 4, 8, 13, 13a) and other D_1-serovars. Both, heat-treated and live *S. enteritidis* were bound by 2F11 with approximately equal avidity. In addition, 2F11 demonstrated high recognition of LPSs isolated from *S. enteritidis* regardless of phage types. The antibody was specific to factor O-9 of LPS from D_1-*Salmonella* serovars. Results of ELISA and immunoblotting show that the epitope recognized by the antibody was partially composed of tyvelose and mannose and also determined by the nature of the glycosidic bonds between monosaccharides in the trisaccharide backbone of the O-specific chain of LPS

from D_1-*Salmonella*. The detection limit of a MAb 2F11-based ELISA employing poly-L-lysine pre-coated microplates was 10^4 *S. enteritidis* PT 4 cells per/ml of buffer. Furthermore, a MAb 2F11-based dot-blot assay for the detection of *S. enteritidis* in eggs was developed. The assay utilized PBS-coated nitrocellulose membrane strips as the solid phase upon which the extracted LPS antigen (egg-containing *S. enteritidis* is heated in the presence of sodium cholate) was applied. A solid disc of cooked egg was placed on the strips and the antigen was transferred to the membrane *via* diffusional forces. After blocking and probing the strips with MAb 2F11, detection was accomplished by incubating with biotinylated antibodies, streptavidin-alkaline phosphatase and substrate solution (NBT/BCIP). Positive results were observed as purple dots on the nitrocellulose. This procedure does not require the use of a pre-enrichment broth to increase the number of *Salmonella* needed for its detection. Egg samples inoculated with one *S. enteritidis* cell per 25 g of egg yielded over 10^6 cells per ml after 20 h of incubation at 37°C, which was readily detected by the assay.

FOREWORD

This thesis has been written is the manuscript style. The two manuscripts are interrelated but independent of each other and have been prepared according to the instructions given by the specific journals to which they are being submitted. Manuscript I, outlines the production and characterization of a monoclonal antibody to factor O-9 of the LPS of *S. enteritidis*. This paper was submitted to Food and Agricultural Immunology. The second manuscript describes the developement of an immunoassay for the detection of *S. enteritidis* in eggs and will be submitted to the Journal of Food Protection.

The authors and titles of the manuscripts are:

I. 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 (Submitted).

II. Masi, A and Zawistowski, J. 1995. Development of a dot-blot assay for the detection of *Salmonella enteritidis* in eggs (In Preparation).

I. INTRODUCTION

Salmonella are gram-negative facultative anaerobic rods, belonging to the bacterial group *Enterobacteriaceae* (Le Minor and Rohde, 1974). There are over 2300 known serovars of *Salmonella* with their classification being controversial (D'Aoust, 1995). The most widely used and best known classification system is the Kauffmann-White scheme (Lim, 1986). In this scheme salmonellae are distinguished from each other by antigenic structures such as somatic (O), flagellar (H) and capsular (Vi) antigens. *Salmonella* with common somatic antigens are grouped together and designated into 46 serogroups (Tsang *et al*, 1991). Serogroups A, B, C, D and E account for 95% of human salmonellosis (Kerr *et al*, 1992).

Salmonella can cause two food-borne diseases, salmonellosis and enteric fever. Salmonellosis is a global problem persisting as food poisoning. Enteric fever is a more severe illness and is endemic in definite geographical regions of the world, such as Southeast Asia, India, Egypt and Chile (D'Aoust, 1991; Lim, 1986). Enteric fever is caused by human specific serotypes, *S. typhi, S. paratyphi* A , *S. paratyphi* C and *S. sendia* (Tsang et al, 1991).

Salmonellosis occurs as a self-limiting episode of enterocolitis (D'Aoust, 1991). The incubation period can be between eight to 72 h with symptoms including abdominal pains, nausea, diarrhea, anorexia, vomiting, headaches and fever. The elderly, infants and immunocompromised individuals are the most susceptible to infections. Non-typhoid

salmonellosis is restricted to the intestinal tract, with symptoms subsiding within five days of onset of the disease. Diagnosis of this disease relies on the isolation of these agents from stool samples (D'Aoust, 1991). Human salmonellosis is associated with *Salmonella* such as *S. typhimurium, S. enteritidis*, and *S. agona*. Serovars that cause salmonellosis have a wide host range that can include mammals, avians and vertebrates (Tsang *et al*, 1991).

One to seven percent of individuals experiencing *Salmonella* diseases may develop *Salmonella*-triggered reactive arthritis (ReA) (Maki-Ikola and Granfors, 1992). The pathogenesis of this disease is not known. Of the 2300 known serovars of *Salmonella*, 22 are known to trigger ReA. These include *S. typhimurium* and *S. enteritidis* (Maki-Ikola and Granfors, 1992).

In the past decade, the number of human salmonellosis cases due to *S. enteritidis* infection have increased dramatically worldwide (Rodrigue et al, 1989). Poultry are the main source of these bacteria (Poppe et al, 1992). *S. enteritidis* has been isolated from the intestinal tract, ovary, oviduct and spleen of naturally infected hens (Poppe *et al*, 1992). A study analyzing the results of 65 *S. enteritidis* food-borne outbreaks in the United States, found that for the 35 outbreaks which food vehicle of infections were identified, 27 were associated with the consumption of eggs or egg-containing foods (St. Louis *et al*, 1988). Furthermore *S. enteritidis* has been isolated from the contents of clean, intact shell eggs (Humphrey, 1994). There are two possible routes of *S. enteritidis* contamination of eggs, trans-shell (horizontal) or trans-ovarian (vertical)

transmission.

Horizontal transmission refers to egg shell contamination with *Salmonella* as a result of fecal contamination and subsequent translocation of the bacteria into the egg *via* the egg pores or through cracks on the egg shell (Lock *et al*, 1992). Vertical transmission occurs as a result of infections of the reproductive tissue and contamination of eggs prior to the development of the egg shell (Humphrey, 1994). For *S. enteritidis*, it appears that vertical transmission is of more importance (Humphrey, 1994).

The conventional culture method for detection and isolation of *S. enteritidis* in poultry, eggs and other foods are scarce, time consuming, labour intensive and non-specific (Van Der Zee, 1994). These methods involve isolation of the organisms using pre-enrichment as well as selective procedures. In addition, a serological confirmation test has to be performed, since no biochemical assay can give any indication whether the isolated organism is *S. enteritidis*.

Described rapid methods for the serological detection of *S. enteritidis*, are represented by two basic ELISA procedures, the sandwich and indirect ELISA. Both types employ polyclonal (PAb) as well as monoclonal antibodies (MAb) against flagella, fimbriae and lipopolysaccharide (LPS) of *S. enteritidis* (Lee *et al*, 1989; Wang *et al*, 1995; Thorns *et al*, 1994). In contrast to the conventional method, these assays can detect *S. enteritidis* within two days. They have the potential to be very sensitive and highly specific. This specificity however, relies on the quality of antibodies employed. Polyclonal antibodies (antisera) can be specific and are used in commercially available immunoassays. However, problems with the use of antisera are reproducibility and

cross-reactivity (Polin, 1984). Antiserum is a heterogenous mixture of antibodies and these can recognize a number of different antigens in a given bacteria or similar bacteria. The heterogeneity of antisera ultimately determines affinity, cross-reactivity and function of the antisera (Polin, 1984).

Hybridoma technology addresses the shortcomings of polyclonal antibodies. The major advantages of this technology, is the well defined chemical mixture that is obtained from an isolated antibody producing clone, a monoclonal antibody (Polin, 1984). When the MAb is targeted to a distinctive marker of a bacteria, specificity is assured. Monoclonal antibodies have been produced to a number of *Salmonella* antigens, LPS, fimbrial structures and outer membrane proteins (Tsang *et al*, 1991; Thorns *et al*, 1994; Jaradat and Zawistowski, 1995). Many of these MAbs have not been evaluated for the detection of *Salmonella* in foods and hence their performance is uncertain.

Furthermore, ELISAs are not free from drawbacks. The tests involve time-consuming enrichment incubations, exhibit varying degrees of cross-reaction, particularly between serogroups and both systems have been known to produce false positive reactions (Flowers *et al*, 1992; Lee *et al*, 1989; Wang *et al*, 1995).

The objective of the outlined study was to produce and characterize a MAb specific to *S. enteritidis*. In addition, the application of MAb to develop an immunoassay for the detection of *S. enteritidis* in eggs was investigated.

II. LITERATURE REVIEW

1 Introduction

There are over 2300 salmonellae serovars known and the number increases yearly (D'Aoust, 1995). The classification of these bacteria has been controversial, with many classification systems reported. In the past decade, there has been an increase in the number of human salmonellosis cases from *S. enteritidis* infections (Rodrigue *et al*, 1990). Poultry and poultry products have been implicated as a source of *S. enteritidis* (Humphrey *et al*, 1988; St. Louis *et al*, 1988). The conventional culture for the detection and isolation of *Salmonella* in foods is labour intensive and may require up to seven days for completion (Andrews, 1985). As a means to decrease the time involved for the detection of *Salmonella* in foods, a number of rapid detection methods have been developed (AOAC, 1995). The following review will detail information on the classification, sources and detection methods of *Salmonella*, with particular attention to *S. enteritidis*.

2. Classification of *Salmonella*

2.1 Historical naming of *Salmonella*

Due to their importance in pathology, the first salmonellae species were given names (in latinized form) that indicated the disease and animal from which they were isolated, e.g. *S. choleraesuis* (i.e. hog cholera). This form of nomenclature was

abandoned when it was discovered that some salmonellae species were not host specific (i.e. *S. typhimurium*, causative agent of mouse typhoid is also a human pathogen). Descriptive naming was replaced by naming newly identified serotypes from the places were they were first isolated, e.g. *S. london, S. havana*, etc. (Old, 1992).

2.2 The Kauffmann-White scheme

The Kauffmann-White scheme is intended as a diagnostic tool, by which salmonellae species are distinguishable from each other by their particular O (LPS or somatic), H (flagellar) and Vi (capsular) antigens present on the cell wall (Old, 1992). Thus, each salmonellae serotype is recognized by its distinct antigenic formula. *Salmonella* serovars sharing the same O-antigens are collected into serogroups (O groups) and arranged alphabetically by H antigens within these groups (Li Minor and Rohde, 1974). For example, *S. enteritidis* has the antigenic formula 1,9,12:g,m:- corresponding to O antigen: phase 1 H antigen: phase 2 antigen and is designated to serogroup D. All serovars in serogroup D have O-antigen 9 in common and share O-antigens 1 and 12 with serogroups A and B.

2.2.1 Lipopolysaccharides of *Salmonella*

Lipopolysaccharides are an integral part of the cell wall of gram negative bacteria (Rietschel and Brade, 1992). As a cell surface component, LPS can interact with its environment (i.e. a host during infection) and exhibit a number of biological activities, including pyrogenicity, lethal toxicity and complement activation (Kotina and Takada,

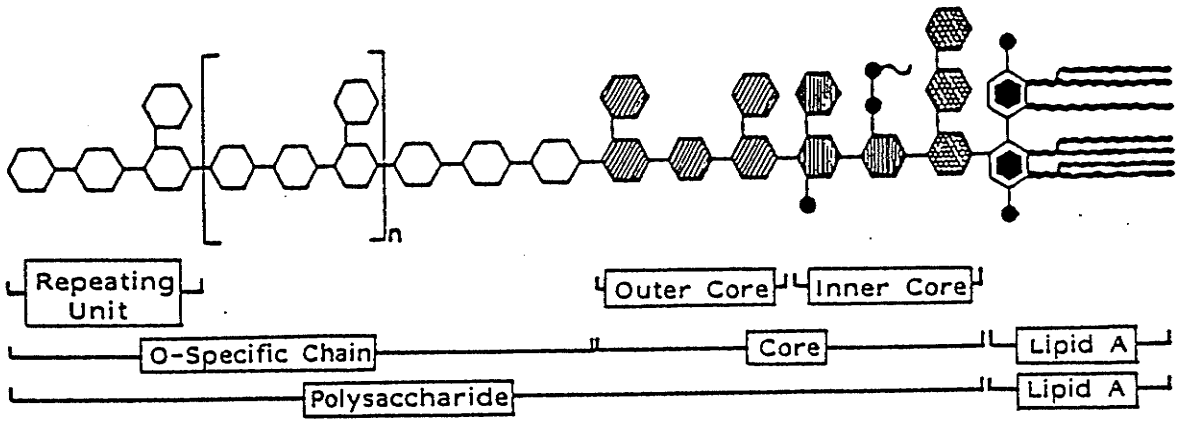
1990). Lipopolysaccharides are composed of a lipid moiety which is covalently bound to a polysaccharide portion (Figure 2.1). Lipopolysaccharide molecules are anchored to the outer membrane of the cell through their lipid moiety, lipid A. Virtually all biological activities of LPS reside in the lipid A portion. Lipid A is composed of a glucosamine disaccharide and four hydroxy fatty acids (Pearson, 1985). This backbone is ubiquitous and highly conserved among gram negative bacteria.

The polysaccharide part of LPS consists of two regions, the core and the O-specific chain. The core is subdivided into the lipid A-proximal inner core and the lipid A-distal outer core. The structural variability of the core within different bacterial species was thought to be limited. For example, *Escherichia coli* has six core types while *Salmonella* species consist of one. Recently, Tsang *et al* (1991) developed a MAb to an outer core structure of *Salmonella*, and used it to screen 176 *Salmonella* species. All Salmonella species belonging to serogroups A to E were recognized by the MAb, while 29% of Salmonella species from serogroups F to G_7 were undetected. This study points to the potential diversity of *Salmonella* core types.

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 Mc Groarty, 1985). The nature, ring form, type of linkage, and substitutions of the individual monosaccharide residues is characteristic and unique to a bacterial species. Thus, the O-chain is species-specific (Rietschel and Brade, 1992). In *S. enteritidis*, the oligosaccharide unit consists of a trisaccharide backbone composed of mannose, galactose and rhamnose and a side sugar tyvelose which is attached to the mannose residue. The

Figure 2.1 - Schematic structure of LPS

(Adapted from Kotina and Takada, 1990)


n
Repeating Unit
Outer Core
Inner Core
O-Specific Chain
Core
Lipid A
Polysaccharide
Lipid A

sugar tyvelose is the unique component of O-antigen factor 9 of Salmonella serogroup D. In contact with the immune system of vertebrate animal, LPS (particularly the O-specific chain) evokes the production of antibacterial antibodies which in turn can be used to identify these bacteria.

2.3 Subdividing *Salmonella* species

The Kauffmann-White scheme nomenclature results in each *Salmonella* serovar considered as a species (Le Minor and Popoff, 1987). The concept of "one serovar-one species" is untenable, as over 2300 *Salmonella* serovars have been identified (D'Aoust, 1995; Le Minor and Popoff, 1987). Several attempts to reduce the number of *Salmonella* species have been reported. In 1944, the concept of a three *Salmonella* species system was first described (*Borman et al*, 1944). In this scheme, there are three recognized species: *S. choleraesuis*, *S. typhosa* and *S. kauffmannii*. Kauffmann and Edwards (1952) proposed a similar scheme and identified *Salmonella* on the basis of *S. choleraesuis, S. typhosa* and *S. enterica*. In 1963, Ewing posed another three species concept based on *S. choleraesuis*, *S. typhi* and *S. enteritidis*. In the above schemes, all serovars except the first two are included in the last species named. For example, in the Ewing scheme both *S. choleraesuis* and *S. typhi* serotypes are considered *Salmonella* species, with all other *Salmonella* serotypes being grouped in the last category, *S. enteritidis*. Kauffmann (1966) divided the genus *Salmonella* based on biochemical characteristics into four subgenera, *S. kauffmannii* (subgenus I), *S. salamae* (subgenus

II), *S. arizonae* (subgenus III) and *S. houtenae* (IV). With the exception of a few species, all serovars in subgenera II to IV are designated by antigenic formula only, while all those in subgenus I are given names (Old, 1992). This four species system includes arizonae as *Salmonella* species. In the approved Lists of Bacterial Names, *Salmonella* is designated as five species, *S. arizonae, S. choleraesuis, S. enteritidis, S. typhi* and *S. typhimurium* (Skerman *et al*, 1980).

Recent DNA homology and numerical taxonomy studies have shown that all *Salmonella* serotypes belong to two species, *S. enterica* and *S. bongori*, with *S. enterica* subdivided into six subspecies (Table 2.1) (Le Minor and Popoff, 1987; D'Aoust, 1995). This two species concept is in use by the World Health Organization (WHO) collaborating centre for reference and research on *Salmonella* (D'Aoust, 1995). This concept uses the antigenic determinants of the Kauffmann-White scheme. Like the Kauffmann-White scheme, all serotypes belonging to subspecies I (enterica) are designated names, while the others are designated by antigenic formula only. In this concept serovars are named as follows, the old Kauffmann-White scheme designation of *S. enteritidis* is now *S. enterica* subspecies *enterica* serovar Enteritidis (D'Aoust, 1995). The two species concept is being challenged by microbiologist and acceptance of this system will be influenced by habit (D'Aoust, 1995; Old, 1992). In the following manuscripts, the Kauffmann-White designated names are used.

TABLE 2.1 - *Salmonella* species and subspecies[a]

Species	Subspecies no.	Name	Example	no. of serovars[b]
S. enterica	I	*enterica*	Enteritidis	1405
	II	*salamae*	4,12:b:1,5	471
	IIIa	*arizonae*	13,22:z_4,z_{23}:-	94
	IIIb	*diarizonae*	11:1,v:z	311
	IV	*houtenae*	6,14:z_4,z_{23}:-	65
	V	*indica*	11:b:1,7	10
S. bongori			44:r:-	19

[a] Adapted from Old, 1992

[b] Total number of serovars 2375 (D'Aoust,1995)

3. Sources of *Salmonella enteritidis* infections

3.1 Epidemiology of *S. enteritidis*

Human salmonellosis caused by *S. enteritidis* infections have increased dramatically worldwide in the past decade (Rodrigue *et al*, 1990). In many countries, *S. enteritidis* has surpassed *S. typhimurium* as the most commonly isolated serovar (Poppe *et al*, 1993). In the United Kingdom (UK), a 250% increase in human isolations of *S. enteritidis* has been reported for the years 1982 to 1992 (Roberts and Sockett, 1994). In the early 1970s, *S. enteritidis* human isolates in the United States (US) accounted only for 5% (Tauxe, 1991). This number however, increased to 20% by the year 1989, particularly in the northeastern states. In recent years, *S. enteritidis* outbreaks have occurred in most states (Madden, 1990). In Canada, the increase in the number *S. enteritidis* infections has not been as dramatic as in other countries (Poppe, 1994). However, *S. enteritidis* infections have increased to 16% ranking this pathogen second to *S. typhimurium* as the most commonly isolated serovar (Khakhria *et al*, 1994).

Phage typing has become an important diagnostic tool to subdivide species within a particular serotypes of salmonellae (Threlfall and Chart, 1993). Phage typing schemes have been developed for *S. enteritidis*, *S. typhimurium* and *S. typhi*. Unlike the UK, where the most common phage type (PT) isolates of *S. enteritidis* were PT 4, the most common phage type isolates in North America were *S. enteritidis* PT 8 (Poppe *et al*,

1992). Although some infections with *S. enteritidis* PT 4 occured in Canada, these isolates were more likely acquired abroad (Khakhria *et al*, 1994). Other common phage types isolated in Canada include PT 1, 13, 13a and 28 (Khakhria and Lior, 1991; Khakhria *et al*, 1994).

3.2 Poultry and poultry products as a source of *S. enteritidis*

Salmonellosis is a zoonotic disease of worldwide importance. *Salmonella* are natural inhabitants of the intestinal tract of animals such as domesticated fowl, cattle, swine and sheep. Most human infections are of foodborne origin, but contact with pets such as dogs, cats and turtles have been reported to cause human salmonellosis (Guthrie, 1992). In a study of domesticated farm animals, 3.5 times more *Salmonella* serotypes were identified from birds as compared to cattle, pigs and sheep (Haigh and Betts, 1991). Poultry, poultry products, including foods made with shell eggs are an important vehicle of *S. enteritidis* infections in humans (Humphrey *et al*, 1988; St Louis *et al*, 1988; Threlfall *et al*, 1993). Contaminated feed can introduce *Salmonella* serotypes to the farm and animals can become infected by either eating contaminated feeds or from the environment that has been contaminated (Bryan, 1981). Large scale farming confines large number of animals in crowded areas, they often share the same foods and water and *Salmonella* can spread by cross-infection. The presence of *S. enteritidis* in Canadian commercial layer flocks has been reported (Poppe et al, 1991). *S. enteritidis* was isolated from environmental samples (dust/fluff, eggbelt samples and faeces) in eight of 295 randomly selected flocks (2.7%). Naturally occurring infections of *S. enteritidis* in

layer flocks are usually mild or subclinical with little effect on egg production (Poppe *et al*, 1992). In the past, the main source of salmonellae in poultry was the caecal contents of infected birds (Rampling *et al*, 1989). However, this pathogen has been isolated from the ovaries, oviducts, liver, heart and spleen of condemned broilers and necropsied layer hens (Rampling *et al*, 1989; Poppe *et al*, 1992). *S. enteritidis* shares the same surface antigens with avian adapted *S. gallinarum* and *S. pullorum*. These similarities may account for the adaptation of certain *S. enteritidis* phage types to poultry (Rampling *et al*, 1989).

Shell eggs and foods containing eggs are a major vehicle of *S. enteritidis* infection in humans (Coyle *et al*, 1988; St. Louis *et al*, 1989). Implicated foods include ice cream, egg sandwiches, scrambled eggs, hollandaise sauce, eggnog, homemade pasta and bread coatings. Epidemiological studies of egg-associated *S. enteritidis* outbreaks show that improper food handling is the cause of these outbreaks (Morris, 1990). Pooling of eggs, exposing eggs to time and temperature abuse, eating raw or undercooked eggs, mixing egg shells with egg contents and poor blending sanitation have been identified as factors increasing the risk of infections (Morris, 1990). A recent outbreak in Canada of *S. enteritidis* occurred in an accredited care facility (Anon, 1992). This outbreak involved 95 confirmed cases of *S. enteritidis* PT 13. The most likely vehicle of infection was ready-to-eat foods contaminated by raw foods processed in the same vertical mixer. Once identified as the source of contamination, the mixer was examined and dried egg was found under the mixer blade. An environmental swab of the egg revealed *S.*

enteritidis PT 13.

3.3 How does *S. enteritidis* contaminate eggs?

Shell eggs are equipped with physical and chemical defence systems against bacteria (Parkinson, 1966). Physical barriers include the egg shell and the shell membranes. The pH of the albumen, which may reach 9 to 10, impedes microbial growth and is one the chemical defences of eggs. Antimicrobial agents such as lysozyme, which degrades the cell wall of gram-positive bacteria; conalbumin, which chelates iron; avidin, which binds biotin and apoprotein, which ties up riboflavin are also present in eggs (Frazier and Westhoff, 1988; Parkinson, 1966). Once the egg is broken, the physical defences are no longer a barrier and homogenization of the egg contents can neutralize chemical agents.

There are two possible routes for *Salmonella* to enter shell eggs, trans-shell infection (horizontal) and trans-ovarian infection (vertical). In trans-shell infections, *Salmonella* present on the shell can pass through the pores of the egg when water and temperature differences occur between the egg and the surrounding environment (Lock *et al*, 1992). A study by Haigh and Beets (1991) showed that immersing eggs into a broth containing *Salmonella* resulted in the contamination of the egg contents with the organisms. Egg pores are large enough (diameter of 30 μm) to allow the entry of bacteria (Haigh and Beets, 1991). Gast and Beard (1990) report a positive correlation between egg shell contamination and the presence of *S. enteritidis* PT 13 in faecal samples in hens artificially infected.

For *S. enteritidis*, there is evidence to suggest that the most probable route of entry to the egg is *via* trans-ovarian infection. *S. enteritidis* has been isolated from the contents of clean, intact shell eggs (Humphrey, 1994). Furthermore, *S. enteritidis* has been isolated from reproductive tissue of naturally and artificially infected hens in the absence of intestinal carriage (Gast and Beard, 1990; Humphrey , 1994; Lister, 1988; Poppe *et al*, 1992; Timoney *et al*, 1989). When *S. enteritidis* are isolated from naturally contaminated eggs, they are often present in pure culture (Humphrey, 1994). The most probable site of contamination within the egg appears to be either the albumen or the vitelline membrane (Humphrey et al, 1991). If *S. enteritidis* were infecting the yolk, larger number of bacteria are expected to be isolated from the egg contents (Humphrey *et al*, 1991). Humphrey *et al* (1991) found that 72% of naturally contaminated eggs have less than 20 cells of *S. enteritidis*. In a study of artificially infected hens, all yolk contents were salmonella-negative, even though *S. enteritidis* was isolated from the albumen (Gast and Beard, 1990).

4. Detection of *Salmonella* in foods

The detection of *Salmonella* in foods has been based on cultural methods designed to detect these bacteria in clinical samples (Andrews, 1985). However, determination of this pathogen in foods is more difficult for a number of reasons. The detection level of this pathogen is generally lower in foods than in clinical samples. The available method should allow for the detection as low as one bacterial cell per 25 g of food (Andrews, 1985). Furthermore, *Salmonella* can be subjected to stresses and/or injuries

during food processing and storage caused by heating, desiccation, preservatives, osmotic pressure and changes in pH. These bacteria may also be present along with a large number of competing microorganisms which represent the natural flora of foods (Jay and Davey, 1989). Due to these conditions, the methods for isolation of *Salmonella* must enhance survival and multiplication of these pathogens while suppressing competing organisms (Jay and Davey, 1989).

4.1 Conventional cultural method

The conventional cultural method for the detection, isolation and identification of *Salmonella* in foods consists of five steps (Andrews, 1985; Flowers *et al*, 1992):

- Pre-enrichment in a non-selective broth
- Selective enrichment
- Differential plating on selective agars
- Biochemical characterization
- Serological confirmation.

The sample size, number of samples, and technique used for testing must be defined by a sampling plan (Andrews, 1985). It has been generally understood that for adequate *Salmonella* recovery from a contaminated food material, a sample of at least 25 g should be tested (Jay and Davey, 1989). For high risk foods, such as dried milk powders, larger sample sizes of 50 to 100 g are recommended (Jay and Davey, 1989; AOAC, 1995).

Sample units are often measured by mass of the food material. However, for some foods it may be more appropriate to examine the external surface of the food

product. Samples can be obtained by swabbing, rinsing or excising a predefined area (Jay and Davey, 1989). Foods such as poultry or fish are likely to be examined by these techniques. The "whole-bird-rinse" technique developed by Surkiewicz *et al* (1969) has been recognized as a non-destructive and effective method for the routine testing of poultry (Jones *et al*, 1991; Notermans *et al*, 1975).

4.1.1. Pre-enrichment

Pre-enrichment is the first step of the conventional culture method in the detection of *Salmonella* in foods. Food processing such as heating or desiccation may sub-lethally damage bacteria (Andrews, 1985; Jay and Davey, 1989). Thus, pre-enrichment should provide nutrient capacity to favour the multiplication of *Salmonella*, repair cell damage, rehydrate bacterial cells, and dilute toxic inhibitory substance that may be present in food (Jay and Davey, 1989; Flowers *et al*, 1992).

One of the most widely used medium in the pre-enrichment step is lactose broth, developed by North (1961). Since *Salmonella* can not utilize lactose, North (1961) concluded that the fermentation of lactose by competing flora results in a change of pH, which in turn enables *Salmonella* to grow while inhibiting competing bacteria. Although lactose broth is widely used, it is not recommended for all food products and is one of many pre-enrichment broths available (Table 2.2).

Lactose broth has been adapted as the official pre-enrichment media for *Salmonella* detection in most foods by the Association of Official Analytical Chemists (AOAC) and the US Food and Drug Administration (FDA) (AOAC, 1990; Flowers *et al*, 1992). In Canada, the Health Protection Branch (HPB) recommends the use of

TABLE 2.2 - Media used for the detection of foodborne *Salmonella* recommended by different governing agencies[a]

Agency	Pre-enrichment	Selective enrichment	Selective plating
AOAC[b] and FDA[c]	Lactose Brilliant green water Nonfat dry milk with brilliant green Tryptic (trypticase) soy Nutrient broth	TBG SC	BSA XLD HE
HPB[d]	Nutrient broth Brilliant green water Trypticase soy	TBG SC	BSA BGS
ICMSF[e]	Lactose Buffered peptone Brilliant green water	TBG SC	BSA BGS third optional
USDA[f]	Lactose	TT SC	BGS XLD

a Adapted from Flowers et al, 1992
b Association of Official Analytical Chemists
c U.S. Food and Drug Administration
d Health Protection Branch, Canada
e International Commission on Microbiological Specification for Food
f U.S. Department of Agriculture
Abbreviations: TBG-tetrathionate broth with brilliant green; SC-selenite broth; TT-tetrathionate broth; BSA-bismuth sulfite agar; XLD-xylose lysine deoxycholate agar; BGS-brilliant green sulfa agar; HE-Hektoen enteric agar

nutrient broth, brilliant green water, T-soy broth and non-fat dried milk (NFDM) with brilliant green as pre-enrichment solutions (D'Aoust, 1991). For the detection of *Salmonella* in foods, the ratio of food to media of 1:10 is universally accepted, with an incubation protocol of 18-24 hours at 35-37°C (Jay and Davey, 1989; Flowers *et al*, 1992). For the isolation of *S. enteritidis* from eggs, the addition of ammonium-iron (III)-citrate, ferrous sulphate, ferrioxamine-type siderophores, novobiocin and cefsoludin to pre-enrichment broths have been reported (Van der Zee, 1994).

4.1.2 Selective enrichment

Selective enrichment media allow the proliferation of *Salmonella* while inhibiting the growth of competing non-*Salmonella* microorganisms, through the use of selective agents (Andrews, 1985). Selective enrichment broths are inoculated from the pre-enrichment media. However, raw or highly contaminated food samples may be directly enriched in selective media (Flowers *et al*, 1992). In unprocessed foods, *Salmonella* is not likely to be sublethally injured, pre-enrichment could lead to overgrowth by other microorganisms (Jay and Davey, 1989).

The most widely used selective media are those based on tetrathionate and selenite and there are many modifications of these (Table 2.2) (Jay and Davey, 1989). Selenite broths can be modified to contain cysteine or brilliant green (Flowers et al, 1992). Likewise, tetrathionate broth has been modified by the addition of thiosulphate, bile salts and brilliant green to favour the ratio of *Salmonella* to non-salmonellae bacteria (Flowers *et al*, 1992; Jay and Davey, 1989).

The incubation temperature during the selective enrichment step favourably influences the growth of *Salmonella*. Increased recovery of *Salmonella* has been shown by elevated temperature of 43°C in contrast to 35-37°C using the above media (Flowers *et al*, 1992; Jay and Davey, 1989). The AOAC (1995) method, however, does not recommend the use of elevated temperatures in their procedure. The incubation time has been generally implied to be 18-24 hours.

4.1.3 Selective Plating

A number of selective agar media have been developed for the further culturing of *Salmonella* from selective enrichment broths (Jay and Davey, 1989). These media support *Salmonella* growth in form of discrete colonies. They consist of nutritional agents as well as dyes, bile salts, antibiotics and other compounds (Flowers *et al*, 1992). To discriminate *Salmonella* against non-salmonellae, indicator systems such as H_2S production and/or fermentation of certain carbohydrates are used (Flowers *et al*, 1992; Jay and Davey, 1989). Samples of selective agars are brilliant green sulpha, bismuth sulphite, xylose lysine desoxycholate citrate and Heckton enteric. Table 2 lists a number of plating agars used by several governmental agencies. To optimize *Salmonella* isolation, two or more selective agars should be employed. Commonly, selective agars are inoculated from selective broths and incubated at 37°C for 24 hours. For biochemical and serological conformation, two to three typical *Salmonella* colonies should be selected. For multiple serotyping, more than 20 colonies should be selected for further testing (Jay and Davey, 1989).

4.1.4 Biochemical characterization

Characterization of biochemical traits of the test culture is performed on non-selective agars (biochemical media) by visual assessment of colour changes of the media (Flowers *et al*, 1992). Triple sugar iron (TSI) and lysine iron agar (LIA) are the most widely used differential agars. They are usually used together to provide preliminary biochemical screening of cultures. Use of TSI can provide information on the production of H_2S and the fermentation of glucose, lactose and sucrose, while the use of LIA measures H_2S production, the decarboxylation of lysine and the fermentation of sucrose (Flowers *et al*, 1992).

Presumptive identification of typical *Salmonella* cultures can be characterized by a number of biochemical tests. Bergey's manual of determinative bacteriology lists a comprehensive guide to biochemical and nutritional traits of *Salmonella*. Typical presumptive *Salmonella* identification can be based on biochemical traits such as growth in KCN, lysine decarboxylase and urease reactions and indole test (Flowers *et al*, 1992). A practical and economical method for the rapid biochemical identification of suspected *Salmonella* cultures can be obtained with the use of commercially available diagnostic kits. Several of these kits such as API 20E, Minitek, Enterotube and Micro ID have received approval by the AOAC (1995). To confirm suspect cultures from the results obtained by biochemical tests, serological identification must be performed (Flowers *et al*, 1992; Jay and Davey, 1989).

4.1.5 Serological confirmation

Serological characterization of *Salmonella* is based on the determination of specific antigens by immunological assays. Commercially available antisera to *Salmonella* antigens are commonly used. These antisera recognize somatic (O), flagellar (H) and capsular antigens (Flowers *et al*, 1992). When positive results are obtained from serological testing, the culture may be sent forth to a reference centre for complete identification which includes serotyping and phage typing. Phage typing of *Salmonella* cultures is done by using a standard set of typing bacteriophages (Flowers *et al*, 1992).

4.1.6 Advantages and disadvantages of the conventional culture method

The conventional culture method is the standard official and approved method used to detect *Salmonella* in foods (AOAC, 1995). This method is a laborious and time consuming procedure. To obtain a presumptive negative result four to five days are required, while complete identification of suspect colonies may require up to 7 days (Andrews, 1985). This time frame is impractical for the food industry, as food companies must place products on hold until negative results are obtained, which incurs additional costs. Thus, the development of a rapid *Salmonella* detection procedure is imperative for the food industry.

4.2 Rapid methods for *Salmonella* identification

The development of rapid methods to reduce the time for the detection of *Salmonella* in foods has brought about several advantages over the conventional method

(Swaminathan *et al*, 1985):

. Reduction of warehouse cost to the food industry
. Quick response to contamination problems
. Increased testing of food product for the presence of *Salmonella*
. The opportunity to buy raw meats, poultry and perishable foods that have been tested and found free of *Salmonella*.

The acceptance of rapid methods as alternatives to the conventional cultural method is attained after interlaboratory collaborative studies supervised by the AOAC. Several criteria for determining the acceptability of a rapid method for the detection of *Salmonella* must be met (Andrews, 1985). The sensitivity of a proposed method must be equal to, although not necessarily greater than the conventional method. A second criterion is applicability of the method, i.e. can the method be used for all foods or certain specific food groups. A third consideration is the rate of false positives or/and negative resulting from the method. When positive results are obtained by a rapid method, conventional cultural confirmation is required (AOAC, 1995). A common step in all rapid methods is enrichment of *Salmonella* in food, although the detection of *Salmonella* is usually completed one to two days earlier than by conventional methodology.

A number of rapid methods for the identification of *Salmonella* in foods have been devised and several have been approved by the AOAC (1995). These methods may be categorized as follows:

. Rapid cultural methods
. Immunoassays
. DNA Hybridization assays
. Conductance tests

. Other methods

The following section outlines the principles, advantages and disadvantages of these methods.

4.2.1 Rapid Cultural Methods

4.2.1.1 Shortened incubation period

The potential of a shorter enrichment incubation protocol has been reported by several authors (D'Aoust, 1981; D'Aoust *et al*, 1983). D'Aoust *et al*, (1983) have reported that a shorter selective enrichment incubation period of 6 h was as reliable as the conventional method. However, a shortened 6 h pre-enrichment incubation reduced the recovery of *Salmonella* and resulted in higher number of false negative results (D'Aoust, 1981).

4.2.1.2 Hydrophobic grid membrane filtration (HGMF)

Hydrophobic grid membrane filtration (HGMF) was developed by Sharpe and Michard (1974) to address the problems of limited counting ranges of conventional membrane filtration analysis of bacteria. This technique has been adapted for isolation of *Salmonella* in foods with a 16 h pre-enrichment, followed by a shortened 6 h selective enrichment (Entis *et al*, 1982). After enrichment, a one ml portion is filtered through a HGMF. The filter is then placed on a selective agar and further incubated. If *Salmonella* colonies are present, confirmation by the cultural method is required. A most

probable number value can be obtained by counting the number of occupied squares and using a formula specific for the method (Flowers *et al*, 1992).

A major advantage of HGMF is that a counting range in excess of 3 log cycles can be achieved on a single filter (Entis *et al*, 1982). A second advantage of this method is that HGMF can be subjected to computer scanning to facilitate colony counting (Jay and Davey, 1989). Presumptive positives are readily available for biochemical and serological testing (AOAC, 1995; Entis *et al*, 1982a).

An early limitation of this method was the clogging of the filters by food particles. This problem has been resolved by the use of non-bacterial enzymes and pre-filtering of food samples to remove large food particles (Entis *et al*, 1982b). The number of foods tested by this procedure was initially limited to six foods categories; chocolate, raw poultry meat, pepper, cheese powders, powdered eggs and nonfat dry milk (AOAC, 1990). However, use of this testing procedure has now been expanded to include all foods (AOAC, 1995). A disadvantage to this method is that the use of a single selective enrichment and plating agar may hinder the detection of some *Salmonella* strains which are more readily detected by other selective agents (Flowers *et al*, 1992).

To improve the HGMF procedure, immunological detection has been paired up with this method (Cerqueiro-Campos *et al*, 1986). Enzyme labelled antibodies are used to detect *Salmonella* colonies directly on the HGMF filters. The need for replicate plating on a number of selective agars is eliminated (Jay and Davey, 1989). This modified HGMF method uses antisera against *Salmonella*. The formed complex between bacterial cells and primary antibodies are detected with the aid of a protein A-horseradish

peroxidase conjugate with chloronaphtol as a substrate (Cerqueiro-Campos *et al*, 1986). With these modifications, results can be obtained within 48 hours. However, a major limitation of the use of antisera, is their high number of false positive resulting in cross-reactivity with other *Enterobacteriaceae*, mainly *Citrobacter freundii* (Jay and Davey, 1989). In 1991, the originally designed media, selective lysine agar was replaced by EF-18 agar (Entis and Boleszczuk, 1991). This media reduced the number of presumptive-positive results and thus increased specificity of the method.

4.2.1.3 Selective enrichment procedures

The use of M-broth as a selective enrichment step after a shortened pre- and selective enrichments has been investigated prior to serological agglutination of *Salmonella* with polyvalent flagella antiserum (Sperber and Diebel, 1969). This method was used to detect salmonellae in foods and feeds by Boothroyd and Baird-Parker (1973). It has been suggested that by incorporating M-broth enrichment a 95 % agreement with the conventional methods can be obtained (Boothroyd and Baird-Parker, 1973).

Anti-*Salmonella* antibodies have been coated on latex beads in order to increase sensitivity and agglutination visibility. Several of these systems including Spectrate™ Salmonella Coloured Latex Test (Rhone-Poulenc, Glasgow, Scotland), Microscreen™ Salmonella Latex Slide Agglutination Test (Mercia Diagnostics, Guildford, England) and Bactigen™ Salmonella-Shigella (Wampole Laboratories, Cranbury, NJ, USA) have been developed and commercialized (D'Aoust et al, 1991). When compared to the conventional methods, these commercial kits have shown 79.2%, 75% and 87.5%

detection agreement when used for analyzing food samples (D'Aoust *et al*, 1991).

Another method which can be classified as a selective enrichment is based on immunodiffusion. The most popular technique is the 1-2 test™ (BioControl Systems, Inc., Bothell, W.A.) This method is used to screen for the presence of motile *Salmonella* in foods by employing polyvalent antibodies to flagellar antigens (H-antigens). *Salmonella* are detected in a self-contained disposable test unit, which has two interconnecting chambers: an inoculating chamber containing L-serine and tetrathionate broth with brilliant green dye, and a motility chamber also containing L-serine plus a peptone based selective motility medium (AOAC, 1995; Flowers *et al*, 1992). Food samples are pre-enriched for 24 hours and 0.1 ml of the broth is introduced into the inoculating chamber. The concentration of L-serine is higher in the inoculating chamber than in the motility chamber. Before depletion of the amino acid, *Salmonella* can proliferate into a high level. When the concentration of L-serine is used up, a concentration gradient is formed and *Salmonella* migrate to the motility chamber. Flagellar antisera present in the motility medium immunoreact with *Salmonella* and form white precipitable lines which are readily visible after 14 hours (AOAC, 1990; Flowers *et al*, 1992).

This method offers several advantages. It is easy to use, requires no specialized training or expensive equipment and can yield presumptive positive results in 2 days (Bailey *et al*, 1991). The major problem with this system is the inability to detect non-motile *Salmonella*, such as *S. gallinarum* and *S. pullorum*, which are important pathogens of poultry flocks. Furthermore, false positive and negative reactions can result with the

use of antisera. A 42% incidence of false negative results has been reported by D'Aoust and Sewell (1988). This method received first action approval by the AOAC in 1989.

Selective motility media have also been used for the detection of *Salmonella*. These pathogens can be detected as visible lines on modified semi-solid Rappaport-Vassiliadis (MSRV) medium (AOAC, 1995). The method is based on *Salmonella* migration by chemotaxis through a highly selective medium. After a 20 h pre-enrichment step, broth can be directly inoculated on the selective medium at 42°C for 24 h and *Salmonella* can be detected as visible grey lines extending from inoculated drops (direct motility enrichment). As an alternative, a selective enrichment broth can be inoculated from the pre-enrichment broth for 8 h and introduced on the selective medium (indirect motility enrichment). After 16 h, *Salmonella* can be detected as described above. Using this technique, presumptive positives can be attained within 48 h. The AOAC (1995) approved procedure, however, is limited to two food products, cocoa and chocolate and it only detects motile bacteria. Migrated cultures must be confirmed biochemically and serologically. Van der Zee (1994) reported on the use of MSRV to detect *S. enteritidis* from poultry and egg products. Poppe *et al* (1992) successfully used MSRV agar for the isolation of *S. enteritidis* from faeces and faeces-contaminated samples from commercial poultry flocks.

Direct plating of *Salmonella* spp. has also been reported. Gast (1993) evaluated the detection of *S. enteritidis* from pooled egg contents by direct plating on brilliant green agar supplemented with novobiocin after pre-enrichment and after selective enrichment. He found that pre-enrichment followed by direct plating yielded only a 47% recovery

rate, however, selective enrichment increased the recovery rate to 75% of samples. The use of two instead of one agar plating media may improve the method's capabilities (Gast, 1993).

4.2.2 Immunoassays

4.2.2.1 Fluorescent antibodies immunoassay

The fluorescent antibody (FA) method was the first officially recognized rapid method for the detection of *Salmonella* (AOAC, 1995; Jay and Davey; 1989). This method is based on an immunological reaction. *Salmonella* antigens are stained by fluorescent-labelled antibodies and detected by fluorescent microscopy. The AOAC approved method outlines a 52 hour enrichment protocol. Upon staining, the presence of fluorescent cells which are morphologically typical of *Salmonella* constitute a presumptive positive result. Such results must be confirmed by the cultural method (AOAC, 1995).

The advantage of this method is the ability to recognize negative results one day earlier than the cultural method. However, this method suffers from a high level of false positive results, as a result of the antibody cross-reactivity with a number of non-salmonellae bacteria (Thomason, 1981). The reading of stained slides is laborious, subjective and requires technical training and expensive instrumentation (Flowers *et al*, 1992; Jay and Davey, 1989). To improve the technique, an automated system has been devised (Thomason, 1981). The system consists of a slide processor for slide staining, reading and interpretation of results. This system, however, has failed to detect

Salmonella in certain foods and the number of false positive results still remained a problem (Flowers *et al*, 1992; Thomason, 1981). The above disadvantages of this procedure have contributed to the reluctance of the food industry to accept the method.

4.2.2.2 Enzyme Immunoassays

Enzyme immunoassays (EIA) were developed as a replacement for radioimmmunoassays (RIA) to minimize health hazards (Beumer et al, 1991). EIA use enzyme as labels instead of radioisotopes which are used in RIA. Antibodies used in EIA may be either polyclonal or monoclonal and the enzyme activity of these labelled antibodies can be determined chromatically, fluoremetrically or by other means (Maggio, 1980).

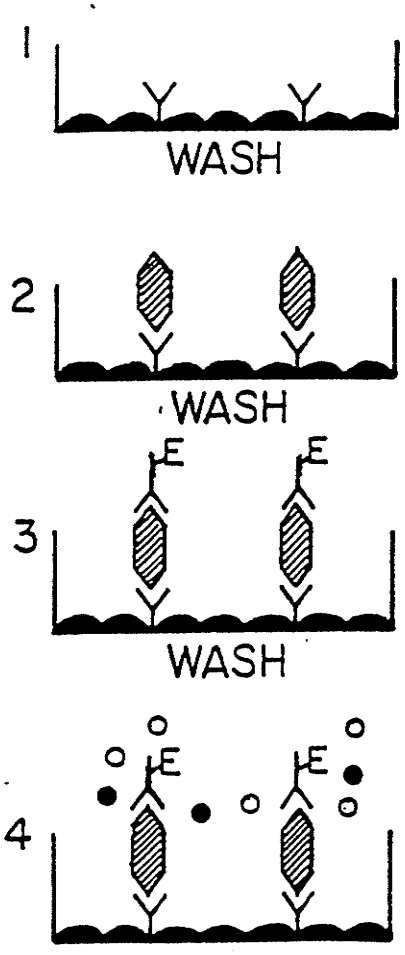
The detection of *Salmonella* by a direct sandwich ELISA (Figure 2.2) has been approved by the AOAC. The procedure is simple and composed of four major steps:

. The capture Ab is adsorbed onto a solid phase (ie. microplates, etc)
. An Ag containing solution is added and allowed to attach to the capture Ab
. An enzyme labelled Ab is then allowed to attach to the Ag;
. The enzyme substrate is then added to form either a chromogenic or fluorescent product which can be spectrophotometrically measured.

After each step, except the last, excess reagents are washed off (Maggio, 1980). The measured product is compared to an end point determinant value to assess the amount of Ag present. The following discussion will mainly pertain to the AOAC approved EIA, although a number of other EIA are commercially available (Feng, 1992).

Figure 2.2 - Direct Sandwich Elisa

(Adapted from Zawistowski, 1995)


1
WASH
2
WASH
E
E
3
WASH
E
E
4

4.2.2.2.1 Polyclonal antibody based enzyme immunoassays

There are two AOAC approved EIA commercially available for the detection of *Salmonella* employing polyclonal antibodies (PAbs). The Tecra™ system (Bioenterprises Pty Ltd, Australia) and the Assurance *Salmonella* EIA test kit (BioControl Systems, Inc., Bothwell, WA).

The Tecra™ system uses highly purified PAbs to detect a flagellar determinant of *Salmonella*. This test employs a pre-enrichment, selective enrichment and post-enrichment procedure and depending on the food sample it requires 44 to 62 hours to be completed. The assay itself takes approximately 2 hours to perform (AOAC, 1995). This assay may be performed on a microtiter plate or strip wells. The results can be read visually (using reference cards) or spectrophotometrically (AOAC, 1995). The false positive rate for this system when compared against the conventional culture method was found to be 4.1% with a false negative rate of 1.6%. No differences were found between visual and instrumental interpretation of results (Flowers *et al*, 1988).

There are several advantages to this system. Reduction of detection time by one day and no requirement for sophisticated instruments as wash steps can be performed manually and results can be read visually. However, this method employs PAbs which may cross-react with other *Enterobacteriaceae* leading to a high number of false positives (AOAC, 1995). Furthermore, non-motile *Salmonella* are not recognized by this method. It's been found that some foods and the selective broths used may affect the assay (Lambiri *et al*, 1990).

The second polyclonal test kit approved by the AOAC (1995) is the Assurance

Salmonella EIA kit. This test utilizes highly specific PAbs to both somatic and flagellar antigens of *Salmonella*. For processed foods, an enrichment protocol of a minimum of 44 h is recommended while for raw foods, an enrichment of 48 h should be used. The assay itself can be performed in 2 h (AOAC, 1995). This kit has one advantage over the Tecra™ system, the ability to detect non-motile *Salmonella* with the use of somatic-specific-antibodies. However, the use of polyclonal Abs may cross-react with other *Enterobacteriaceae* present in the food and the results must be read spectrophotometrically (AOAC, 1995).

4.2.2.2.2 Monoclonal antibody based enzyme immunoassays

The AOAC has approved the use of two monoclonal antibody (MAb)-based EIAs, Salmonella-Tek (Organon Teknika Corp, Durham, NC) and Q-Trol™ (Dynatech Laboratories, Chantily, VA) (AOAC, 1995).

Salmonella-Tek utilizes two MAbs for the detection of *Salmonella* in foods. This EIA replaces the former BioEnzabead™ kit method (Flowers et al, 1992) and utilizes the same MAb that recognizes a flagellar determinant of *Salmonella*. The manufacturers suggest different enrichment protocols for use with raw and processed foods, however, both enrichment steps total 56 h. The EIA itself takes less than 2 h to complete. The results must be read spectrophotometrically (AOAC, 1995).

Salmonella-Tek test kit has been assessed by several authors. Van Poucke (1990) evaluated different enrichment methods and their effect on Salmonella Tek. Tetrathionate broth, selenite cysteine broth, Rapport broth and salmosyst broth were tested. Of all

enrichment media, salmosyst broth gave the quickest detection time of 31 h. St. Claire and Klenk (1990) evaluated Salmonella Tek and found an incidence of 42.3% false positives. Bailey *et al* (1991) using processed broiler carcasses found a 6% false positive rate. The AOAC (1995) reported a false positive rate of 4%. This decrease may be attributed to recommendation made by Eckner *et al* (1992) to improve the test. They recommended that selective and post selective enrichment steps be held at 42°C to reduce the number of competing bacteria. Another recommendation is the addition of novobiocin to the post selective medium, to discourage the growth of *Proteus* species. Furthermore, the elimination of agitation of microtiter plates during incubation and centrifugation of samples before EIA is advised. All these recommendation have been incorporated in the latest, AOAC approved version of Salmonella Tek (AOAC, 1995).

The advantage of using this kit is that presumptive positives are attained one day earlier than by using the conventional method. Problems with this EIA include cross-reactivity with other enteric bacteria, mainly *Citrobacter freundii* (D'Aoust and Sewell, 1988). Furthermore, the MAbs used have greater affinity for some strains and not others. For instance, *S. typhimurium* and *S. typhi* are better recognized than *S. paratyphi*. The sensitivity for *S. typhimurium* is 10^5 CFU while for *S. paratyphi* is 10^7 CFU (Organon Teknika manual, 1990). Non-motile *Salmonella* are also not detected by this test (Paterson and Tiffin, 1988).

The Qtrol™ Salmonella EIA detection kit utilizes a fluorescent substrate EIA (AOAC, 1995). This kit uses a fluorescent substrate to measure alkaline phosphatase activity conjugated to MAbs (Curiela *et al*, 1990). The enrichment procedure can take

up to two days and the assay itself requires 2 h to complete (AOAC, 1995). To read fluorescence, an expensive reader is required which most food laboratories would find of limited use. Therefore, there are two versions of colorimetric immunoassay using components of the fluorescent substrate assay : the Qtrol Microelisa kit and the Qtrol visible kit. These modified kits follow the same enrichment steps as the fluorescent assay. However, the assay portion time is reduced to 1 h. The total detection time for *Salmonella* by using any of the version available is reduced by one day. Even though this method is quicker than the conventional method, the level of organisms required for detection by the MAbs should be near or above 10^8 cells ml^{-1}. Levels of 10^7 cells ml^{-1} may give false positive or negative results. This assay therefore relies heavily on the enrichment procedure. A further disadvantage is that non-flagellated *Salmonella* are not detected by the MAbs used in this assay (Curiela et al, 1990).

Recently, a number of monoclonal antibodies of various antigenic specificity against *Salmonella* have been developed in several laboratories. These antibodies are potentially useful for EIA, although their effectiveness was not yet fully evaluated via interlaboratory trials.

Patterson and Tiffin (1988) have developed MAbs against an outer membrane protein closely associated with LPS extracts. Thorns *et al* (1990) have produced a MAb specific to a fimbrial structure of *S. enteritidis* and *S. dublin*. A number of MAbs have been developed towards LPS components. Tsang *et al* (1987) produced a MAb to an outer core oligosaccharide from *Salmonella* serogroups A, B, C, D and E. Various MAbs have also been produced to specific the O-Ag factors of LPS such as O-Ag factor

8 (Duffey et al, 1992), O-Ag factor 4 (Tsang et al, 1991) and O-Ag factor 7 (Tsang et al, 1992). Most of the above MAbs were tested for the detection of *Salmonella* in clinical samples. Two MAbs specific for the detection of *Salmonella* in foods have also been described. A rapid ELISA has been developed for the detection of *S. enteritidis* in eggs (Lee et al, 1989). This one can detect a minimum of 10 *S. enteritidis* cell per egg in less than 24 h. A problem with this assay is that the MAb cross-reacted with a number of B-serogroup *Salmonella*. The same workers have developed a one day EIA for the detection of *S. typhimurium* in food (Lee et al, 1990). The detection limit is 10 cells per 25 g of food and the assay can be completed in 19 h. By using high affinity MAbs, both selective and post-enrichment steps used by most commercially available ELISA can be eliminated, thus, a reduction of total time involved for the detection of Salmonella in food (Lee et al, 1989).

4.2.2.2.3 Alternative solid phase surfaces for enzyme immunoassays

The solid phase of most EIAs are microtiter plates, but other alternatives have been reported including the use of polyester cloth for the capturing of *Salmonella* antigen (Blais and Yamazaki, 1989; Wang *et al*, 1995). The cloth is saturated with either polymyxin B or PAbs to capture salmonellae antigens. An assay for the detection of *S. enteritidis* from eggs has been reported. Salmonella LPS are released from the egg by heating in the presence of sodium cholate and captured by a polymyxin-saturated- cloth (Wang *et al*, 1995). The assay is performed as a dot blot in less than 24 h and has a 10^6 cfu ml^{-1} of egg detection limit. However, the anti-LPS PAbs used in this assay exhibit

cross reactivity with other enteric bacteria.

4.2.3 Nucleic acid hybridization assays

Deoxyribonucleic acid (DNA) probes potentially detects any microorganism in foods, as these probes possess genes that encode toxins, virulence factors or plasmid DNA (Fitts, 1985). Unlike *E. coli* and *Yersinia enterocolitica, Salmonella* has no virulence factors, toxin genes or widely distributed plasmid DNA that can be used to detect all *Salmonella* species (Fitts, 1985; Wolcott, 1991). However, a *Salmonella* genome for a chromosomal sequence specific to all *Salmonella* species was found and has been successfully used in commercially available DNA-Hybridization assays (Gene-Trak™; Gene Trak Systems, Framingham, USA) (AOAC, 1995; Fitts, 1985).

The DNA-Hybridization method (DNAH) for the detection of *Salmonella* in foods is very similar to the EIA. Following the enrichment procedure, the bacteria are collected on filters, where they are lysed and DNA is denatured. Single-stranded DNA is fixed to a solid phase and incubated in hybridization solutions containing a detector probe (AOAC, 1995). If *Salmonella* target DNA is present in the test sample, the labelled probe will hybridize to the target sequence and the label can be detected.

There are two DNAH screening methods approved by the AOAC (1995). The first DNAH method was introduced in 1987, the GENE-TRAK™ test kit which uses an isotopic-labelled detector probe. This kit outlines a 42 and 48 h pre-enrichment incubation step for processed and raw or highly contaminated foods, respectively. The DNAH assay takes approximately 3 h to complete. This kit has been evaluated by a

number of researchers. St. Claire and Klenk (1990) reported false positive and negative rates of 4% and 9.2%, respectively, when compared to the AOAC cultural method. False positive and negative rates found by Bailey *et al* (1991) were 1.4% and 2.5%, respectively.

The major advantage of this method is that microorganisms possess their unique DNA regardless of environmental influences (Jay and Davey, 1989). Therefore, even mutated *Salmonella* strains could be detected. In some instances, DNA probe methods have yielded superior results to cultural methods (Wolcott, 1991). The use of radioactive DNA probes and the expensive equipment required for the assay are serious disadvantages. To overcome these drawbacks, a ribosomal RNA (rRNA) hybridization kit was developed (Wilson *et al*, 1989).

The second hybridization screening method (cDNAH) approved by AOAC (1995) for the detection of *Salmonella* is a combination of DNA probe and EIA in a dipstick format. The kit uses two probes, one labelled with fluorescein and another with deoxyadenylic acid. The detecting probe is different from the one on the isotopic DNAH method and it targets ribosomal RNA (Wilson *et al*, 1989). The enrichment procedure is identical to the isotopic DNAH method, the assay, however, is completed in 2 h (AOAC, 1995). The two labelled probes are allowed to hybridize with salmonellae rRNA to form a complex. A dipstick coated with deoxythymidinylic acid is then used to capture the complex. Consequently, the complex is detected by the addition of anti-fluorescein antibodies conjugated with horseradish peroxidase.

A significant advantage of a probe against rRNA, is the sensitivity of the assay.

This is attained because of the high number of ribosomes present in the cell (5000 to 20000 copies per bacterial cell) when compared to genomic DNA. In addition, this method eliminates the use of isotopes (Wolcott, 1991).

4.2.4 Conductance method

During growth of microorganisms, small molecular weight, highly charged molecules such as amino acids, fatty acids and organic acids are secreted into a culture medium. These cause changes in electrical conductance of a culture medium which can be measured by an electronic detection device (Jay and Davey, 1989)

The AOAC (1995) has approved an automated conductance method for the detection of *Salmonella* in foods. The food sample must be pre-enriched in a lactose-based broth (16-24 h) followed by a two tube conductance assay in selenite-based media containing trimethylamine-N-oxide, dulcitol (Salmonella Medium 1) and lysine (Salmonella Medium 2). Typical *Salmonella* species will give large conductance changes in these medium when compared to non-salmonellae (AOAC, 1995).

The advantages of this method is that presumptive results can be obtained within 48 h, and consequently, *Salmonella* isolated can be subcultured directly onto selective agars for confirmation. The drawbacks of this system is that false positive results can occur due to the presence of *C. freundii.* and *E. coli* (Jay and Davey, 1989). Furthermore, the high cost of the equipment required for this assay may restrict its use.

4.2.5 Other methods

4.2.5.1. Bacteriophage assays

A method involving the bacteriophage Felix-O1 in conjunction with high performance liquid chromatography (HPLC) for the detection of *Salmonella* has been described (Hirsh and Martin, 1983). In this method, the proliferation of bacteriophage is assayed by HPLC and correlated with the number of *Salmonella* originally present in the sample.

Another bacteriophage assay, the Bacterial Ice Nucleation Diagnostic (BIND) test by DNA Plant Technology Corporation (Oakland, CA) has recently been developed. This test involves the use of bacteriophage (P22) encoded with an ice-nucleating protein (Blackburn, 1993; Feng, 1992). This protein naturally present in certain bacteria (*Pseudomonas, Erwinia* and *Xanthomonas*) binds water to stimulate the formation of ice crystals. The food sample is incubated with the bacteriophage and chilled to -5°C, *Salmonella*-positive samples will form ice crystals which are readily detected by an indicator dye medium (Feng, 1992).

4.2.5.2 Separation techniques

Methods to separate *Salmonella* from competitive bacteria have been described. The use of ion-exchange resins, immunosorbent columns, antibody-coated petri plates, magnetic particles coated with *Salmonella*-specific antibodies, and lectins have been studied (Blackburn, 1993; Payne *et al*, 1992: Wyatt *et al*, 1992).

There is a large number of reported rapid methods for the detection of *Salmonella*

in foods each having certain drawbacks, the most common being the time involved for the detection of the bacteria. This thesis describes the production and characterization of a monoclonal antibody which can be utilized as a probe for the detection of *S. enteritidis* in eggs.

III. MANUSCRIPT 1

DETECTION OF LIVE AND HEAT-TREATED *SALMONELLA ENTERITIDIS* BY A D_1-SEROSPECIFIC ANTI-LPS O-9 MONOCLONAL ANTIBODY

3.1 ABSTRACT

A murine monoclonal antibody 2F11 (IgG_{2a}) against *Salmonella enteritidis* was produced by a fusion of P3X63-Ag8.653 myeloma cells with spleenocytes of a mouse immunized with heat-attenuated (80°C, 20 min) *S. enteritidis* cells. The specificity of this antibody was tested in an indirect ELISA and SDS-PAGE followed by immunoblotting. The MAb was specific to D_1-serogroup *Salmonella* and exhibited the highest reactivity with all of the tested phage types of *S. enteritidis* (1, 4, 8, 13, 13a). The MAb was reactive with heat-attenuated as well as live *S. enteritidis* cells. In addition, this antibody exhibited high and equal avidity to LPSs isolated from *S. enteritidis* regardless of phage types. The MAb 2F11 proved to be specific to LPS O-9 present in D_1-serogroup *Salmonella.* Immunoblotting and ELISA results demonstrated that the epitope recognized by this antibody was partially composed of tyvelose and mannose and also determined by the nature of glycosidic bonds between monosaccharides in the polysaccharide backbone region. Employing poly-L-lysine pre-coated microplates, this antibody had a detection limit of 10^4 *S. enteritidis* cells/ml of buffer as assessed by ELISA.

3.2 INTRODUCTION

In the past decade incidence of *Salmonella enteritidis* as a cause of human salmonellosis has increased dramatically worldwide (Rodrigue *et al*, 1990). In Great Britain reported infections involving mainly *S. enteritidis* phage type (PT) 4, have risen by 250% from 12,000 cases/year in 1982 to 31,000 cases/year in 1992 (Roberts and Sockett, 1994). The number of *S. enteritidis* outbreaks in the US increased from 26 in

1985 to 77 in 1989, involving 44 deaths (Mason, 1994). In Canada, although the increase in prevalence of this pathogen in people has not been as dramatic as in Europe and the US, infections due to *S. enteritidis* increased to 16% in 1993 ranking it the second most commonly isolated serovar after *S. typhimurium* (Khakhria *et al.*, 1994, Poppe, 1994).

Epidemiological investigations have identified that fresh shell eggs and egg products are important vehicles of infection by *Salmonella enteritidis*. Recently, an increase in the isolation of *S. enteritidis* from poultry has been reported worldwide (Kim *et al.*, 1991). Infection by *Salmonella enteritidis* threatens the safety of human consumers and the economic soundness of the egg and poultry industry. Its control and elimination requires early detection in raw and processed food.

Traditional methods for detection of *S. enteritidis* are scarce and require up to one week in order to culture and identify bacterial isolates. These methods are also labour-intensive, as they involve the isolation of the organism using pre-enrichment as well as selective enrichment procedures and serological confirmation tests (Andrews, 1985, Van der Zee, 1994). More rapid methodology available for serological detection of *S. enteritidis* is represented by two basic enzyme linked immunoassay (ELISA) procedures, the sandwich and indirect ELISA. In contrast to conventional methods these tests can detect *Salmonella* in two days or even less than 24 h (Lee *et al.*, 1989). However, they are not free of drawbacks. Most of them involve time-consuming pre-enrichment, selective enrichment and often post-enrichment incubation steps. Some of them also exhibit varying degrees of cross-reactions, particularly with serogroup B salmonellae. These methods employ antisera as well as monoclonal antibodies produced against

flagellin (Van Zijderveld *et al.*, 1992), lipopolysaccharide (LPS) (Lim and Folk, 1987, Lu *et al.*, 1991, Brigmon *et al.*, 1992, Torensma *et al.*, 1992) and fimbrial (Thorns *et al.* 1990) antigens. Even the most specific antibody for *S. enteritidis* (Thorns *et al.* 1990) cross-reacted with other D_1 serovars. In addition, the majority of them required a further attenuation of bacteria in order to expose the epitopes.

This paper reports the production of a D_1-serospecific anti-LPS O-9 monoclonal antibody which can be used as a probe for the detection of heat-treated as well as live *S. enteritidis*.

3.3 MATERIAL AND METHODS

3.3.1 Materials

Nutrient broth (NB; Difco Laboratories, Detroit, MI, USA), standard plate count agar (SPC; Difco), tetrathionate broth (Difco), selenite cysteine broth (Difco) and M-broth (Difco) were obtained from BDH Inc. (Toronto, ON, Canada).

Brain heart infusion broth (BHI) and RPMI-1640 medium were from Gibco (Grand Island, NY, USA). Lipopolysaccharides from *Escherichia coli, S. typhimurium* and *S. enteritidis*, bovine pancreatic ribonuclease and deoxyribonuclease, hypoxanthine, aminopterin and thymidine, poly-L-lysine (PLL), *p*-nitrophenyl phosphate (*p*npp), polymyxin B sulphate and, concanavalin A were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Phenol, formalin and pristane (2, 6, 10, 14-tetramethylpentadecane) were from Aldrich Chemical Co. (Milwaukee, WI, USA). Myeloma cells P3X63-Ag8.653 (ATCC

CRL 1580) were obtained from American Type Culture Collection (Rockville, MD, USA) while polyethylene glycol 4000 (cat. no. 9727) was from Merck, (Germany). Fetal calf serum was a product of Bocknek Ltd, (Toronto, ON, Canada).

Nitrocellulose membrane (0.45 μm), diethanolamine buffer, alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, mouse-type subisotyping kit, 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitroblue tetrazolium chloride (NBT) were purchased from BioRad, (Hercules, CA, USA). Microtest III polyvinyl flexible tissue culture plates (Falcon 3912) were obtained from Canlab, (Mississauga, ON, Canada).

3.3.2 Bacteria and Culture Conditions

Table 3.1 lists bacteria used in this study and their sources. All *Salmonella* strains were prepared by the following procedure. A loopful of stock bacterial culture which was maintained in SPC slants was inoculated into 5 ml of NB and incubated at 37°C for 16 h. One ml of turbid NB was placed in 10 ml of tetrathionate broth or selenite cysteine broth and incubated 24 h at 42°C. One ml of each of the selective broths were added to 200 ml of M broth and incubated with agitation at 37°C for 16 h. The cells were collected by centrifugation, washed twice with saline (0.85% NaCl) and resuspended in saline. Non-salmonellae bacteria were grown in BHI for 16 h at 37°C, harvested by centrifugation, washed and resuspended in saline. The cell suspensions were diluted to the required concentration, heated for 20 min at 80°C and then used for ELISA as described below. Bacteria for some experiments were attenuated by addition of either 5% phenol or 3% formalin in saline and incubated overnight at room temperature. The

live bacteria were obtained by washing slant cultures with saline.

3.3.3 Lipopolysaccharide preparation

Bacterial lipopolysaccharide (LPS) extracts were prepared using the modified method of Johnson and Perry (1976). One gram of freeze dried *Salmonella* cells was resuspended in 20 ml of buffer L and sonicated three times for 30 sec at 300 W at 4°C using the Braun-Sonic 1510 (B. Braun, Melsungen AG). The suspension was diluted to 100 ml with 20 mM $MgCl_2$ containing 0.1 μg/ml of bovine pancreatic ribonuclease and deoxyribonuclease and then incubated for 10 minutes at 37°C, an additional 10 minutes at 60°C. Then, the suspension was mixed with an equal volume of preheated 90% phenol and incubated for 15 minutes at 70°C. The final suspension was centrifuged at 1500 x g for one hour. After that, the aqueous phase was collected and dialysed for one week at 4°C against distilled water which was changed daily. The LPS extracts were then lyophilized and stored at -20°C until use.

3.3.4 Production of monoclonal antibodies

3.3.4.1 Immunization.

Female BALB/c mice (6-8 weeks old) were immunized five times intraperitoneally with 5 x 10^7 heat attenuated (80°C for 20 minutes) cells of *S. enteritidis* PT 4 or with LPS (10μg/ml) at two week intervals. Tailbleedings were performed before the first immunization, a week after each subsequent injection and shortly before the mice were

sacrificed. Diluted sera from the tailbleedings were tested for the presence of antibodies against *S. enteritidis* by an indirect ELISA. The mouse with the highest titre was given a final booster injection, sacrificed 5 days later, the spleen was removed and used for a fusion.

3.3.4.2 Hybridoma production.

The fusion was performed according to Goding (1983). Spleenocytes were fused with P3X63-Ag8.653 myeloma cells at a ratio of 4:1 in RPMI-1640 in the presence of 40% polyethylene glycol 4000. The fused cells were then resuspended in HAT medium (RPMI-1640 with 10 % FCS supplemented with 10^{-4} M hypoxanthine, 4 x 10^{-7} M aminopterin and 1.6 x 10^{-5} M thymidine) to a concentration of 10^6 cells ml^{-1}, and plated out onto six 96-well tissue culture plates containing 10^4 peritoneal macrophage feeder cells per well. After two weeks, hybridomas were screened for antibody production against *S. enteritidis*. Positive hybridomas were cloned at least twice by limiting dilution into 96-well tissue culture microplates containing standard tissue culture medium supplemented with 5 x 10^4 feeder cells per well. Growing hybridomas were screened against *S. enteritidis*, *S. typhimurium* and *E. coli* whole cells and LPS using an indirect ELISA.

3.3.4.3 MAb propagation and isotyping.

Large quantities of antibodies from stable hybridoma lines were expanded *in vitro* in tissue culture flasks. Spent media were collected, aliquoted and stored either at 4^0C

for immediate use or -20^{0}C for future use. The established MAb 2F11 was propagated *in vivo* as ascites fluid according to the procedure of Harlow and Lane (1988). Adult male BALB/c mice were primed with 500 μl of pristane. After a week, the mice were injected intraperitoneally with hybridoma cells (10^6). Ascites fluid was collected, clarified by centrifugation, and purified by precipitation with 50% saturated ammonium sulphate. Purified ascites fluid was stored at -20°C until use.

Monoclonal antibodies were isotyped using a mouse isotyping kit following the manufacturers instructions. Immunoglobulin classes, subclasses and light chain specificity were performed on MAbs from hybridoma spent media and ascites fluid.

3.3.4.4 ELISA protocol

Screening antisera, spent media and ascites for the presence of antibodies towards *S. enteritidis* was performed by an indirect non-competitive ELISA using polyvinyl chloride Microtest III plates which were coated with either bacterial cell suspensions or LPS extracts.

Bacteria whole cells (100 μl, 10^7 cells/well) in carbonate buffer (pH 9.6) were added to the plates and incubated overnight at 4°C. After the plates were washed five times with PBST (PBS with 0.5% Tween 20), they were blocked by incubating for 2 h at room temperature with 200 μl/well of PBST containing 5 % (w/v) skim milk. After washing four times with PBST, 100 μl of monoclonal antibody (hybridoma spent media, ascites fluid or appropriate dilution of antiserum) in PBST containing 0.1 % (w/v) skim milk was added to plates followed by 1 h incubation at 37^{0}C. Hybridoma spent medium

was diluted 1:1, while ascitic fluid was diluted 1:1,000 prior to use. The plates were washed four times with PBST, and 100 μl/well of goat anti-mouse IgG alkaline phosphatase conjugate (diluted 1:3000 in PBST containing 0.1% skim milk) was added, followed by incubation for 1 h at 37°C. The plates were again washed four times with PBST and then developed by adding 100 μl/well (1 mg/ml) of ρ-nitrophenol phosphate in 0.1 M diethanolamine buffer, pH 9.8, and incubated overnight at room temperature. The plates were read at λ 405 nm using a Titertek Multiskan ELISA plate reader (Flow Laboratories, McLean, VA). Pre-immune sera were used as a negative control.

When LPS was used as an antigen, microplates were pre-coated with poly-L-lysine. Briefly, 100 μl/well of poly-L-lysine (20 μg/ml) in PBS (pH 7.2) was used to coat 96-well plates for 30 min at 37°C. After washing three times with distilled water, plates were coated with 100 μl/well of LPS (10 μg/ml) in distilled water overnight at 4°C. The plates were developed as described above.

To study the effect of pre-coating of microplates on the reactivity of MAb 2F11 with *Salmonella* whole cells, plates were coated with 100 μl/well of either poly-L-lysine (20 μg/ml), polymyxin B (50 μg/ml) or concanavalin A (50 μg/ml) in distilled water for 30 min at 37°C and then were used for ELISA as described above.

3.3.5 Electrophoresis and immunoblotting

3.3.5.1 SDS-PAGE. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) in a Protean II Cell vertical electrophoresis apparatus (BioRad, Hercules, CA, USA).

Separating and stacking gels contained 15 %, and 4 % acrylamide, respectively. LPS extracts were dissolved in sample buffer containing β-mercaptoethanol, heated for 5 minutes at 100°C and loaded on the gel at a concentration of 10 μg (20 μl) per well. The gels were run at a constant current (30 mA per slab gel) and after electrophoresis was completed, the gels were either developed or submitted to electroblotting. The development of gels were performed using a silver staining procedure according to Kittelberg and Hilbink (1993).

3.3.5.2 Immunoblotting.

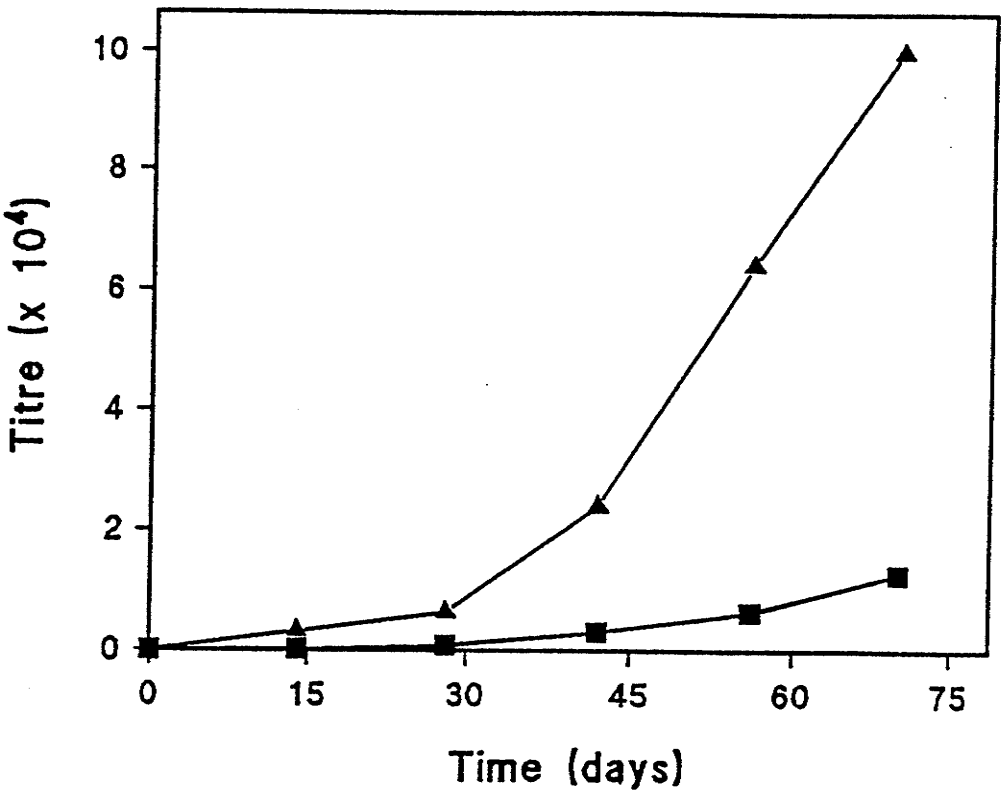
Immediately after electrophoresis, separated LPSs were electrophoretically transferred from the SDS-PAGE gel onto 0.45 μm nitrocellulose membrane using a Trans-Blot cell (BioRad, Richmond, CA, USA). The electroblotting was carried out for 16 hours at a constant current (180 mA) at 4°C (Weintraub *et al.*, 1992). The NC membrane was blocked by incubating in 5% skim milk in TBS (20 mM Tris, 500 mM NaCL, pH 7.5) for 2 h with shaking at room temperature. Then, the membrane was washed four times with TBST (TBS with 0.05% Tween 20) and incubated with MAb diluted 1:25 in Blotto (TBST containing 1% skim milk) overnight at 4°C. Next, the NC membrane was washed four times with TBST and incubated with goat anti-mouse IgG alkaline phosphatase conjugate (diluted 1:3000 in Blotto) for 1 h at room temperature. The membrane was washed four times with TBST and then developed by incubating in BCIP/NBT substrate solution for 30 minutes at room temperature. Colour development was stopped by rinsing the membrane with warm distilled water.

3.4 RESULTS AND DISCUSSION

In order to produce monoclonal antibodies (MAbs) specific to *Salmonella enteritidis*, two approaches were attempted; one group of mice was immunized with heat-attenuated *S. enteritidis* PT 4 cells while another group with purified *S. enteritidis* lipopolysacharides (LPS). Repeated immunization with LPS resulted only in a weak antibody response which was not sufficient to proceed with a fusion. In contrast, mice immunized with whole cells responded well yielding after five injections a dramatic increase in the titre up to 1/100 000 (Figure 3.1). Consequently, mice from this group were sacrificed and three fusions were performed yielding over 500 hybridomas. Of this number, 20 hybridoma lines were found to secrete antibodies which reacted with either cells and/or LPS extracts of *S. enteritidis*. Positive hybridomas were cloned at least two times by limiting dilution and after each cloning, they were screened against a panel of *Salmonella* and non-salmonellae spp. Most of the tested hybridomas secreted antibodies which were relatively non-specific, reacting with salmonellae and non-salmonellae spp. In contrast, the clone 2F11 secreted antibodies that were highly reactive with *S. enteritidis* and showed no reactivity towards other *Salmonella* serogroups. This clone was propagated as ascitic fluid and the resulting antibodies were purified and used for further studies. The MAb 2F11 was of the IgG_{2a} type immunoglobulin with a lambda light chain as analyzed by a mouse-type subisotyping assay.

Table 3.1 shows the specificity of MAb 2F11 as determined for the panel of heat-attenuated (80°C, 20 min) bacteria using an ELISA procedure. Antibodies prominently reacted with all tested phage types of *S. enteritidis* (PT 1, 4, 8, 13, 13a) exhibiting

FIG. 3.1 Typical titration curves of mouse sera following repeated intraperitoneal injections of whole cells (▲), and LPS extract (■) obtained from *Salmonella enteritidis* PT 4. About 100μl of blood was collected from the lateral tail vein.


Titre (x 10⁴)
10
8
6
4
2
0
0
15
30
45
60
75
Time (days)

the highest avidity towards PT 1. The MAb 2F11 reacted also with other D_1-serovars, displaying different degrees of binding. It appeared also that monoclonal antibody showed no reactivity towards antigenically similar *S. maarseen* of D_2-serogroup as well as towards eight other tested *Salmonella* serogroups: B, C_1, C_2, D_2, E_1, E_3, E_4, F, G_2 and a *S. arizonae* strain. In addition, a number of *Enterobacteriaceae* (*E. coli, Y. enterocolitica, C. freundii, E. cloacae, S. flexneri*) and other bacteria (*M. fortuitum, Ps. fluorescens, A. hydrophila*) did not bind to MAb 2F11.

Moreover, MAb 2F11 reacted with live *S. enteritidis* giving similar absorbance values for all tested phage types as assessed by ELISA. Somewhat lower absorbance values were observed with other D_1-serovars such as *S. gallinarum, berta* and *pullorum* and negative results were obtained for other bacteria (Table 3.2). The ability of anti-LPS monoclonal antibodies to detect live bacteria was rarely reported (Torensma *et al.*, 1992) and it appears that LPS O-9 antigenic sites are easy accessible by MAb 2F11 without need for further heat or chemical treatment in order to expose the epitope. In addition, these properties make the MAb 2F11 suitable for use for the isolation of live *S. enteritidis* from food, clinical, and environmental samples in a single immunocapture step in ELISA. An immunocapture step that employs antibodies immunobilized on latex or magnetic beads, plastic tubes or dipsticks could significantly reduce the time needed for the detection of bacteria by elimination of a selective enrichment procedure (Wyatt, 1992).

Antibody specificity was evaluated by ELISA using polyvinyl chloride microplates which have a limited antigen binding capacity. To enhance the concentration of cells on

TABLE 3.1 Specificity of MAb 2F11 to *Salmonella enteritidis*

Serogroup	Bacteria	Antigenic Formula	Absorbance (405 nm)
D_1	*S. enteritidis* PT 1[a]	9,12:g,m:-	1.925
	S. enteritidis PT 4[a]	9,12:g,m:-	1.199
	S. enteritidis PT 8[a]	9,12:g,m:-	1.048
	S. enteritidis PT 13[a]	9,12:g,m:-	1.222
	S. enteritidis PT 13a[a]	9,12:g,m:-	1.449
	S. berta[b]	9,12:f,g,t:-	1.105
	S. gallinarum[c]	9,12:-:-	0.949
	S. pullorum[b]	9,12:-:-	0.268
D_2	*S. maarseen*[b]	9,46:z_4z_{24};$z_{39}z_{42}$	0.017
B	*S. typhimurium*[b]	4,5,12:i:1,2	0.052
	S. stanley[c]	4,5,12:d:1,2	0.021
	S. brandenburg[d]	4,12:1,v:e,n,z_{15}	0.017
	S. agona[d]	4,12:f,g,s:-	0.020
	S. schottmuelleri[c]	4,5,12:b:1,2	0.020
	S. albert[d]	4,12:z_{10}:e,n,x	0.031
	S. kingston[d]	4,12,27:g,s,t:-	0.031
C_1	*S. thompson*[c]	6,7:k:1,5	0.016
	S. cholerasuis[d]	6,7:k:1,5	0.030
	S. mbandaka[d]	6,7:z_{10}:e,n,z_{15}	0.058
C_2	*S. hadar*[d]	6,8:z_{10}:e,n,x	0.033
	S. muenchen[d]	6,8:d:1,2	0.018
E_1	*S. anatum*[d]	3,10:e,h:1,6	0.036
E_3	*S. thomasville*[d]	15,34:y:1,5	0.043
E_4	*S. senftenberg*[e]	3,9:g,s,t:-	0.045
F	*S. rubislaw*[d]	11:r,:e,n,x	0.029
G_2	*S. havana*[d]	13,23:f,g(s):-	0.035
	S. arizonae[c]	ND	0.034
	E. coli[b]	ND	0.054
	E. coli[b]	ND	0.044
	Yersinia enterocolitica[d]	ND	0.027
	Citrobacter freundii[b]	ND	0.045
	Enterobacter cloacae[d]	ND	0.046
	Shigella flexneri[d]	ND	0.031
	Mycobacterium fortuitum[d]	ND	0.059
	Pseudomonas fluorescens[d]	ND	0.052
	Aeromonas hydrophila[d]	ND	0.048

[a]Laboratory Centre for Disease Control, Ottawa, Canada.
[b]American Type Culture Collection, Rockville, MD, USA.
[c]University of Manitoba Microbiology Department, Winnipeg, Canada.
[d]Economic Innovation Technical Centre, Winnipeg, Canada.
ND- not determined.

TABLE 3.2. Binding of MAb 2F11 to live *Salmonella* as assessed by ELISA

Bacteria	Absorbance
S. enteritidis PT 1	0.916
S. enteritidis PT 4	0.980
S. enteritidis PT 8	1.092
S. enteritidis PT 13	0.919
S. enteritidis PT 13a	0.989
S. gallinarum	0.831
S. berta	0.809
S. pullorum	0.313
S. maarseen	0.069
S. typhimurium	0.054
E. coli	0.058

the solid phase and subsequently increase the detection limit of the assay, various coating agents: poly-L-lysine, polymyxin B and concanavalin A were investigated. The use of poly-L-lysine was aimed at the utilization of binding between the anionic bacterial cell wall and the cationic poly-L-lysine surface (Verschoor *et al.*, 1990), while the use of polymyxin B, and concanavalin A was targeted at the utilization of affinity between these compounds and bacterial LPS (Lugtenberg and van Alphen, 1983), and polysaccharides *via* the mannose residues (Sharon and Lis, 1989), respectively. Pre-coating of microplates with either polymyxin B or concanavalin A had an adverse effect on detection of *Salmonella* (Table 3.3). Since polymyxin B has been shown to increase binding of bacterial LPS to various solid phases (Takahashi *et at.*, 1992, Blais *et al.*, 1993), it is possible that pre-coating resulted in the inhibition of MAbs binding because some of the epitopes may be unpredictably masked. This phenomenon has been reported in respect to other antibodies (Kenneth *et al*, 1985). In contrast, pre-coating of microplates with poly-L-lysine increased absorbance values in ELISA by 54% and 34% for detection of *S.* enteritidis PT 4, and PT 1, respectively, while S. *typhimurium* (negative control) remained undetectable. The use of poly-L-lysine as a pre-coating agent in MAb 2F11-based ELISA improved the detection limit of the assay setting it at 10^4 cells/ml (Figure 3.2). This significant improvement of the ELISA was observed for detection of heat-treated cells but no differences were found when live bacteria were used (data not shown). Thus, it is most likely that the improvement of ELISA sensitivity was due to increasing of microplates binding capacity for free lipopolysaccharides released from bacterial cells upon heat treatment. It has been reported that pre-coating with

TABLE 3.3. Effect of pre-coating of microtiter plates on the reactivity of MAb 2F11 with whole cells of *Salmonella* as assessed by ELISA

	Absorbance (405 nm)		
Pre-coating	*S. enteritidis* PT 4	*S. enteritidis* PT 1	*S. typhimurium*
Untreated	1.199	1.449	0.052
Poly-L-lysine	1.844	1.939	0.070
Polymyxin B	0.354	0.544	0.027
Concanavalin A	0.333	0.426	0.042

poly-L-lysine significantly enhances detectability of free lipopolysaccharide as well as lipid A isolated from various gram-negative bacteria (Takahashi *et al.*, 1992).

The antigenic specificity of the MAb 2F11 was analyzed by ELISA using poly-L-lysine pre-coated microplates and LPSs as coating antigens extracted from: a) various *S. enteritidis* phage types as well as other D_1 serovars (O:1, 9, 12); b) *S. typhimurium* of serogroup B (O:1, 4, 5, 12), which share the same trisaccharide backbone c) *S. maarseen* of serogroup D_2 (O:9, 46), which share the same tetrasaccharide repeating unit. The antibody was highly reactive to all tested *S. enteritidis* strains exhibiting equal avidity regardless of phage type (Table 3.4). This property makes MAb 2F11 a suitable probe for the detection of various phage types of *S. enteritidis* with equal sensitivity. Furthermore, the antibody was neither reactive with LPS of B-serogroup nor LPS isolated from serovars D_2 (Table 3.4). This indicated that MAb 2F11 was highly specific for factor 9 (LPS-O9) present in D_1 salmonellae. Similar specificity was reported for a monoclonal antibody produced against *S. typhi* (Lu *et al*, 1991). Factor 9 is part of the D-*Salmonella* O-antigen and is composed of two monosaccharides; tyvelose and mannose linked through α1,3. The tyvelose residue is a side sugar attached to the trisaccharide repeating unit backbone comprised of mannose, rhamnose and galactose (Figure 3.3). The chemical structure of O-antigen is responsible for serogroup specificity of salmonellae (Lindberg and Le Minor, 1984). Thus substitution of tyvelose by abequose yields factor 4 which is unique for B salmonellae (Figure 3.3). Although the trisaccharide backbone remains the same, LPS O4 was not recognized by MAb 2F11. This suggested that tyvelose is an essential part of the epitope recognized by this antibody. It appeared also that the nature of the glycosidic bonds between monosaccharides in the trisaccharide backbone play a significant role in the specificity of the

Figure 3.2 ELISA standard curve of *S. enteritidis* in PBS buffer. Each point represents the mean of three replicates. Poly-L-lysine pre-coated microplates were used for ELISA.

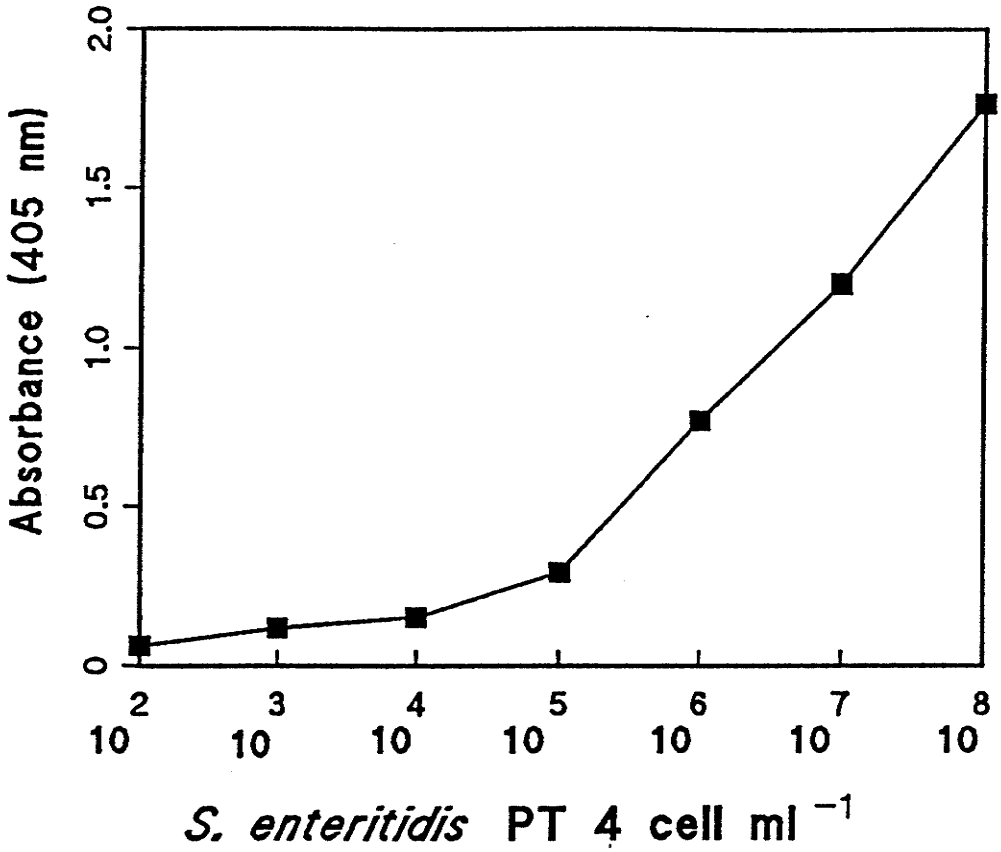

Absorbance (405 nm)
0
0.5
1.0
1.5
2.0
2
10
3
10
4
10
5
10
6
10
7
10
8
10
S. enteritidis PT 4 cell ml −1

TABLE 3.4 Binding of MAb 2F11 to lipopolysaccharides isolated from different bacteria as assessed by ELISA[a]

LPS Extract	Absorbance (405 nm)
S. enteritidis PT 1	1.786
S. enteritidis PT 4	1.992
S. enteritidis PT 8	1.840
S. enteritidis PT 13	1.891
S. enteritidis PT 13a	1.966
S. enteritidis[b]	1.803
S. gallinarum	1.423
S. berta	0.844
S. pullorum	0.235
S. maarseen	0.133
S. typhimurium[b]	0.086
E. coli[b]	0.056

[a] Using microplates pre-coated with poly-L-lysine.
[b] Commercial LPS from Sigma Co.

antibody. In D_2 salmonellae which contain factor 9, mannose is bound to rhamnose *via* β1,4 instead of α1,4 (D_1) while the trisaccharide backbone is linked through α1,6 instead of α1,2 (D_1) (Figure 3.3). Because of this difference, MAb 2F11 did not react with LPS O9 from D_2 salmonellae (Table 3.4).

To further investigate the epitope specificity of the MAb 2F11, the LPS from various phage types of *Salmonella enteritidis* and other D_1 serovars as well as from serogroups B, D_2 and *E. coli* were studied by electrophoresis and immunoblotting. Silver staining after SDS-PAGE electrophoresis revealed the heterogeneity of the tested LPS (Figure 3.4). All LPS samples yielded ladder-like migration patterns typical for LPS from gram-negative smooth bacteria. A similar banding pattern has been reported by others (Palva and Makela, 1980) and represents LPS molecules containing increasing numbers of O-antigen repeating units. The highest mobility molecules (closer to the bottom of the electrophoregram) is believed to correspond to LPS containing a complete core oligosaccharide but lacking O-antigen units, while slow-migrating molecules (at the higher molecular weight region) represent LPS molecules containing long chain O-antigen (Parent et al, 1992).

The specificity of MAb 2F11 for LPS O-antigen was confirmed by immunoblotting (Figure 3.5). The antibody reacted selectively with LPS isolated from all tested phage types of *S. enteritidis* (Figure 3.5, lanes 3-8) and other D_1-serovars (Figure 3.5, lanes 9-11) but it did not bind to LPS from serovars B (Figure 3.5, lane 2), D_2 (Figure 3.5, lane 12) and E.coli (Figure 3.5, lane 1). The MAb preferentially reacted with a high-molecular weight population of LPS indicating that the sites recognized were

Figure 3.3 Partial structures of B, D_1 and D_2-serogroup *Salmonella* O-antigens (adapted from Griffiths and Davies, 1991 & Linderg and Le Minor, 1984)

D_1 *Salmonella*

→2)-α-D-Mannose-(1→4)-α-L-Rhamnose-(1→3)-α-D-Galactose-(1→
3
↑
1
Tyvelose

B *Salmonella*

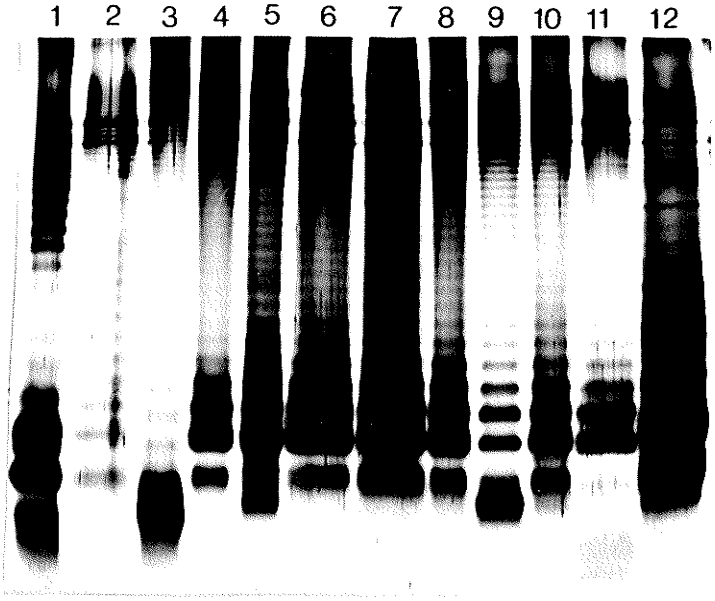
→2)-α-D-Mannose-(1→4)-α-L-Rhamnose-(1→3)-α-D-Galactose-(1→
3
↑
1
Abequose

D_2 *Salmonella*

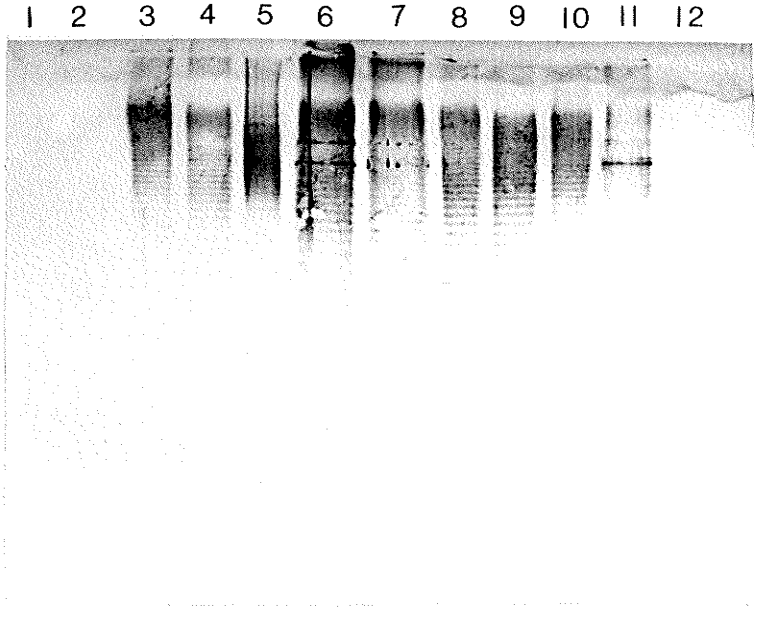
→6)-β-D-Mannose-(1→4)-α-L-Rhamnose-(1→3)-α-D-Galactose-(1→
3
↑
1
Tyvelose

Figure 3.4 SDS-PAGE of LPSs extracted from different bacteria and developed by silver staining. Lanes: (1) *E. coli* (Sigma), (2) *S. typhimurium* (Sigma), (3) *S. enteritidis* (Sigma), (4) *S. enteritidis* PT 1, (5) *S. enteritidis* PT 4, (6) *S. enteritidis* PT 8, (7) *S. enteritidis* PT 13, (8) *S. enteritidis* PT 13a, (9) *S. gallinarum*, (10) *S. pullorum*, (11) *S. berta*, (12) *S. maarseen.*

Figure 3.5 Immunoblot of SDS-PAGE of LPSs extracted from different bacteria corresponding to gel of Fig. 3.4 and probed with MAb 2F11. Lanes: (1) E. coli (Sigma), (2) *S. typhimurium* (Sigma), (3) *S. enteritidis* (Sigma), (4) *S. enteritidis* PT 1, (5) *S. enteritidis* PT 4, (6) *S. enteritidis* PT 8, (7) *S. enteritidis PT* 13, (8) *S. enteritidis* PT 13a, (9) *S. gallinarum*, (10) *S. pullorum*, (11) *S. berta*, (12) *S. maarseen.*



A



B

in the long chain O-antigen region bearing a specific epitope.

Immunoblotting in concert with ELISA results demonstrated that this epitope, at least in part, was composed of tyvelose and the α-mannose residue in the backbone region, since it was shared by all *S. enteritidis* strains but not *S. typhimurium*, and *S. maarseen*. The exact size of epitope remains to be determined, although it could be similar to the size of epitope recognized by another serospecific monoclonal antibody which was produced against LPS O-4. This antibody has been shown to react with an antigenic structure not smaller than a trisaccharide but no larger than a pentasaccharide (Lind and Linderberg, 1992).

Since LPS antigen O forms the external leaflet of the bacterial outer membrane (Lugtenberg and van Alphen, 1983), its antigenicity and the accessibility by antibodies would be dependent on the method used for the attenuation of bacteria. In this respect, the effect of various attenuations of *S. enteritidis* PT 4, *S. typhimurium* and *S. maarseen* on reactivity of MAb 2F11 was investigated. Table 3.5 shows that heat treatment is the best way to enhance binding of MAb 2F11 to *S. enteritidis* while treatment with phenol and formalin resulted in a reduction of absorbance values as assessed by ELISA using microplates without pre-coating of 86%, and 72%, respectively. Although temperature treatment releases free LPS from the surface of bacterial cells, the increase of absorbance value for heat-treated *Salmonella* was most likely not due to the MAb binding to free LPS because of their poor adsorption to plastic (Takahashi *et al.*, 1992). It was rather due to enhanced accessibility of MAb to the membrane bound LPS yielding a higher molecular ratio between MAb molecules bound per *Salmonella* cell as compared with

results obtained for live bacteria (Table 3.5). It is worthwhile to note that none of these treatments enhanced binding of MAb to B or D_2 *Salmonella* confirming the high specificity of MAb 2F11 to D_1-serogroup *Salmonella*.

3.5 CONCLUSION

Monoclonal antibody 2F11 was reactive against various PT of *S. enteritidis* and as well as to other D_1-serovars. Both heat-treated and live *S. enteritidis* were bound by the MAb 2F11 with approximately equal avidity. The antibody was specific to LPS 0-9 factor of D_1-serovars. Antigenically similar B- and D_2-serogroup *Salmonella* were not recognized by MAb 2F11.

TABLE 3.5. Effect of attenuation treatments of *Salmonella* on reactivity of MAb 2F11 as assessed by ELISA

	Absorbance (405 nm)		
Treatment	*S. enteritidis* PT 4	*S. typhimurium*	*S. maarsee*
Live	0.916	0.054	0.069
Heated[a]	1.199	0.052	0.017
Phenol	0.172	0.045	0.037
Formalin	0.340	0.045	0.041

[a] Bacterial cells were heated at 80°C for 20 min.

IV. MANUSCRIPT 2

DEVELOPMENT OF A DOT-BLOT ASSAY FOR THE DETECTION OF *SALMONELLA ENTERITIDIS* IN EGGS

4.1 ABSTRACT

This study describes the development of a simple method for the detection of *Salmonella enteritidis* in eggs. The method utilizes a two step procedure comprising an enrichment step and a monoclonal antibody-based dot-blot assay. A small concentration of *S. enteritidis* cells (one cell/25 g of egg) was enriched to 10^6 cells/ml directly in the homogenized whole egg by incubation for 20 h at 37° C. The enrichment step was carried out without the need to isolate the organism or the use of an enrichment broth. The obtained bacteria concentration level was readily detected by the dot-blot assay. Egg mixture containing enriched *Salmonella* (10^6 cells/ml) was heated in the presence of sodium cholate to release the lipopolysaccharide antigen from the bacterial cells. The antigen was uniformally dispersed and trapped in the gelled egg network formed upon heating. This permitted easy blotting of the antigen onto a solid support, such as nitrocellulose membrane and the subsequent detection by MAb 2F11 specific to the lipopolysaccharide O-9 antigen of *S. enteritidis*. A total of 90 egg samples were assessed for the presence of *S. enteritidis* by the developed method. Artificially inoculated egg samples in the range of one to 500 bacterial cells/25 g were positive by both the dot-blot assay and the culture method. The developed procedure was equally effective as the culture method, yet it can be performed in less time.

4.2 INTRODUCTION

The association of *Salmonella enteritidis* outbreaks with the consumption of eggs

or egg containing foods are a concern to egg producers, regulatory agencies and the public (St. Louis *et al*, 1988). Recently, *S. enteritidis* has been isolated from intact shell eggs (Humphrey, 1994). Several surveys have revealed that eggs laid by hens infected with *S. enteritidis* contained low level of this bacterium (Humphrey *et al*, 1991; Poppe *et al*, 1991). Data from epidemiological studies of *S. enteritidis* outbreaks show that improper food handling techniques may be responsible for most of the outbreaks (Morris, 1990). An increase in infections can results from prolonged exposure of eggs to time and temperature abuse, eating raw or undercooked eggs, mixing eggs with shells and poor equipment sanitation (Morris, 1990; Anonymous, 1992). In Canada, an outbreak of *S. enteritidis* PT 13 was traced back to an improperly cleaned vertical mixer (Anonymous, 1992).

Two possible routes for the infection of eggs with *S. enteritidis*, trans-shell (horizontal) and trans-ovarian transmission, have been described. Horizontal infections refer to egg shell contamination with *Salmonella* as a result of faecal contamination and subsequent translocation of the bacteria into the egg through the egg pores (Lock *et al*, 1992). It appears that for *S. enteritidis* infections, vertical transmission is of more importance. It occurs as a result of infections of the reproductive tissue and contamination of the egg prior to the development of the egg shell (Humphrey, 1994). Evidence for this type of infection is the isolation of *S. enteritidis* from the reproductive tissue of naturally and artificially contaminated hens in the absence of intestinal carriage (Lister, 1988; Timoney et al, 1989). Furthermore, *S. enteritidis* has been isolated from the contents of clean, intact eggs (Humphrey *et al.* 1989). Regardless of how *Salmonella*

contaminate eggs, problems arise when the contaminated eggs are stored or cooked improperly (Morris, 1990).

Cultural methods for detection of *S. enteritidis* in eggs are scarce, time consuming, labour intensive and expensive if a large number of egg samples are to be tested. Furthermore, some selective broths may be inhibitory towards *S. enteritidis* (Van der Zee, 1994). As an alternative to the cultural method, direct plating was studied. Gast (1993) plated egg contaminated with *S. enteritidis* on selective agar. The procedure is both simple and economical, but less sensitive and still requires 4 days for a presumptive positive result. More rapid methods available for detection of this pathogen are represented by immunoassays employing both monoclonal (MAb) and polyclonal antibodies (PAb) (Lee *et al*, 1989; Wang *et al*, 1995). The assay described by Lee and coworkers (1989) uses MAb in a sandwich ELISA format. The assay can detect about 10 cells of *S. enteritidis*/g of egg in less than 24 h. More recently, Wang *et al* (1995) described the use of a polymyxin-coated polyester cloth enzyme immunoassay utilizing rabbit PAbs against factor O:9 *S. enteritidis*. The cloth captures lipopolysaccharides liberated from *S. enteritidis* by extraction with heat in the presence of sodium cholate. The assay has a sensitivity of detecting 10^6 cfu/ml of egg in less than 24 h.

Immunoassay procedures have the potential of being highly specific and sensitive. Their specificity, however, is dependent upon the quality of antibodies (Abs) employed. The MAb produced by Lee *et al* (1989) recognized not only *S. enteritidis* but other B-serogroup *Salmonella*. Likewise, polyclonal antibodies suffer from non-specific reaction with other enteric bacteria. To reach the sensitivity level of the assays, the above

procedures use pre-enrichment in non-selective broths.

This paper presents a MAb-based immunoassay for the detection of *S. enteritidis* in eggs. The assay utilizes an one-step pre-enrichment procedure directly in eggs.

4.3 MATERIAL AND METHODS

4.3.1 Monoclonal antibody - MAb 2F11 (ATCC HB-11891) described previously by Masi and Zawistowski (1995) was used in the assay. Ammonium sulphate purified tissue culture supernatant in a 1/25 dilution was used.

4.3.2 Inoculum preparations

Salmonella enteritidis phage type (PT) 4 was obtained from the Laboratory Centre for Disease Control, Ottawa, Canada. The culture was maintained on standard plate count (SPC; Difco, Detroit, MI) slants. *Salmonella enteritidis* PT 4 were grown in M-Broth (Difco) and attenuated at 80°C for 20 minutes as described earlier by Masi and Zawistowski (1995). Live *S. enteritidis* PT 4 cells were grown overnight at 37°C in 5 ml of nutrient broth (Difco). A loopful of broth was then streaked on SPC slants and incubated for 16 h at 37°C. Next, the slant was washed with 1 ml of 0.85% saline to obtain a 10^9 CFU/ml *Salmonella* suspension. Viable counts were determined by plating on SPC agar at 37°C for 48 hours. The *Salmonella* suspension was serially diluted to 1, 5, 50, 500 cell/ml. The suspension was used within 1 h of preparation.

4.3.3 Preparation of nitrocellulose membrane strips

Nitrocellulose (NC) membrane (BioRad, Richmond, CA) was cut into 8.5 x 2.5 cm strips and saturated in either (10 ml) phosphate buffered saline (PBS; pH 7.2), poly-L-Lysine (PLL; Sigma, St Louis, MO) in PBS (PLL; 1μl/ml) or polymyxin B (Sigma) in PBS (5μg/μl) at 4°C for 16 h. The strips were then washed twice in PBS and used within 30 minutes of washing.

4.3.4 Preparation of Egg samples

Large grade A eggs (~ 50 g) were purchased from a local supermarket. All eggs purchased had at least four weeks to expiry date. To detect attenuated *S. enteritidis*, four eggs were scrubbed with 70% ethanol, opened aseptically and the contents placed in stomacher bags (Tekmar CO). The eggs were mixed for 20 sec in a stomacher and 25 g portions were aliquoted into 50 ml plastic centrifuge tubes (Falcon, Oxnard CA). To each tube, one ml of attenuated *S. enteritidis* was added to a final concentration of 10^6, 10^7, and 10^8 cell/ ml of egg. To the negative control, one ml of saline was added. The tubes were vortexed and 2.5 ml (0.1% volume) of a 15% sodium cholate (Sigma) solution in PBS was added to the eggs and mixed. The tubes were then heated for 10 minutes at 100°C to allow eggs to solidify. The samples were then cooled for 30 minutes at 4°C. With the aid of a sterile core borer (10 mm in diameter), 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, egg (50 g) were artificially inoculated with *S. enteritidis*. Three eggs (150 g) were aseptically opened, mixed as above and a 3 ml inoculum of either a 1, 5, 50, 500 cell/ml of live *S. enteritidis* PT 4

was added. To the negative control, 3 ml of saline was added. The egg mixtures were incubated at 37°C for 20 h. Following incubation for each inoculum, 25 g of egg mixture was distributed into 50 ml centrifuge tubes. Samples in each tube were then mixed with sodium cholate and prepared as described above.

4.3.5 Culture Method

Following incubation, one ml of egg mixture was sampled from each tube to determine viable counts. Egg samples were diluted, plated on SPC and incubated at 37°C for 48 h. In addition, one ml of the egg mixture from each egg sample (inoculated and control) was added to 9 ml of selenite cysteine broth (SC; Difco) and incubated at 37°C overnight. Recovery of *Salmonella* was confirmed by streaking SC broth onto Brilliant Green Agar (Difco) and MacConkey Agar (Difco) followed by incubation at 37°C for an additional 48 h. Isolated *Salmonella*-suspect colonies were then transferred to triple sugar iron (TSI; Difco) and lysine iron agar and grown for 24 h at 37°C. Serological confirmation was performed using Salmonella O-antigen antiserum (Difco).

4.3.6 Dot-Blot Assay

Egg discs were placed on a pre-wet 0.45 μm NC membrane strips and incubated for five minutes at room temperature. All further incubations were performed at room temperature, unless otherwise stated. The discs were removed and the strip was washed twice with PBS (pH 7.2) for 2 minutes and blocked by incubating for two hours with 5% skim milk in Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCL, pH 7.5). After

washing twice for 5 minutes with TTBS (TBS with 0.05% Tween 20), the strips were incubated with MAb 2F11 overnight at 4°C. Next, the strips were washed with TTBS as above and incubated for one hour with either goat-anti-mouse IgG alkaline phosphatase (GAM-AP; 1/3000 diluted in 0.1% skim milk in TTBS; Biorad) or biotinylated goat-anti-mouse IgG (B-GAM; 1/3000 diluted in 0.1% skim milk in TTBS; Sigma) for 1 h. After washing twice with TTBS, the GAM-AP strips were developed with NBT (nitroblue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolylphosphate) substrate solutions (BioRad) for 40 minutes and the B-GAM strips were further incubated for 1 h with either avidin-alkaline phosphatase (A-AP; 1/5000 diluted in 0.1% skim milk in TTBS; BioRad) or streptavidin-alkaline phosphatase (S-AP; 1/30000 in 0.1% skim milk in TTBS; Cedarlane Laboratories Ltd., Hornby, Ont.). The A-AP and S-AP strips were washed and developed with NBT/BCIP substrate solution for 40 minutes. To stop colour formation, the strips were washed twice in warm distilled water.

4.4 RESULTS AND DISCUSSION

Immunoassays for the detection of *Salmonella* in food offer a significant advantage over culture methods. However, these methods still involve a complex enrichment procedure and suffer from low sensitivity.

The purpose of this study was to develop a simple and sensitive method for the detection of *S. enteritidis* in eggs. The method utilizes a two-step procedure comprising an enrichment step and a dot-blot immunoassay. The dot-blot employs monoclonal antibody (MAb) 2F11 specific to various phage types (PT) of *S. enteritidis* and

characterized earlier (Masi and Zawistowski, 1995).

Salmonella cells were enriched to a detectable level directly in the homogenized whole eggs without the need to isolate the organism and use of large volumes of enrichment broth, thus overcoming a disadvantage of culture methods. Table 4.1 shows the recovery of *S. enteritidis* artificially inoculated eggs by both the culture method and the dot-blot assay. Inoculum levels of five, 50 and 500 cells per 25 g of homogenized eggs resulted in 10^7, 10^8, and 10^9 *S. enteritidis*, respectively, after a 20 h incubation. At these concentrations, cells were readily detected by the dot-blot assay within 10 minutes of substrate addition (Figure 1). The one cell inoculum, yielded 0.8 to 5.2 x 10^6 cell of *S. enteritidis* and was detected by the assay within 20 minutes of the addition of the substrate.

The outlined procedure does not require the use of a pre-enrichment broth to increase the number of *Salmonella* needed for the detection (Table 4.1). Eggs are a good substrate for microbial growth and *Salmonella* species have been shown to grow easily in yolk/white mixtures (Baker et al, 1983; Galyean and Cotterill, 1972). The antimicrobial agents present in eggs (ie. albumin or conalbumin) are neutralized when the yolk and egg white are homogenized, thereby allowing bacteria to grow (Galyean and Cotterill, 1972). In contrast to other rapid methods for the detection of *S. enteritidis* in eggs, the absence of a pre-enrichment broth for this procedure does not hinder bacterial growth, as egg mixtures appear to be sufficient. It is worthwhile to note that *S. enteritidis* in eggs originating from vertical infections is often present in pure culture, although organisms were isolated in small numbers (about 1 cell/ml) from

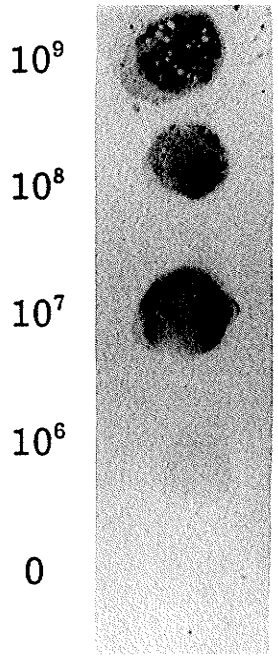
TABLE 4.1 - Recovery of *S. enteritidis* from artificially inoculated eggs by dot-blot and culture methods

Initial Inoculum (cells/25 g of egg)	Counts after incubation for 20 h at 37°C[a]	Positive by dot-blot[b]	Positive by culture method
500 cells	2.1×10^9 (1.0 - 3.4)	+++	Yes
50 cells	3.3×10^8 (1.7 - 4.8)	+++	Yes
5 cells	$1.9 \times 10^{7\ (1.1 - 3.7)}$	++	Yes
1 cell	2.8×10^6 (0.8 - 5.2)	+	Yes
0 cell	$< 10^1$	-	No

a - Mean and range ( ) of 18 samples.
b - Blot assay was determined for the mean of the viable count at each inoculum level. Egg-B Reaction rating was determined by how quickly the colour development was evident af substrate addition. A rating of +++ colour development was noted within 5 minutes; + indicates a colour development within 10 minutes;
+ indicates colour development within 20 minutes; - No colour development within 40 minu of substrate addition.

FIG 4.1 Typical results of the MAb 2F11-dot-blot assay using PBS pre-coated strips, from eggs inoculated with 0, 1, 5, 50 and 500 live *S. enteritidis* PT 4 cells yielding <1, 10^6, 10^7, 10^8 and 10^9 cells/ml of egg respectively, after a 20 h incubation at 37°C. Egg samples were heated in the presence of sodium cholate and detected by biotinylated antibodies, streptavidin-alkaline phosphatase and NBT/BCIP substrate solution.

S. enteritidis PT 4
(CFU/ml of egg)



naturally contaminated eggs (Humphrey *et al*, 1989). In eggs stored above 30°C, coliforms attain dominance over *Pseudomonas* when inoculated together in the air cell of hens' eggs and *S. enteritidis* PT 4 outcompeted other ram negative bacteria including *Escherichia coli* at incubation temperatures of 37°C (Dolman and Board, 1992;). The outlined procedure considers these two facts and exploits their potential. Humphrey and Whitehead (1992) demonstrated the importance of techniques for the isolation of *S. enteritidis* in eggs, they recommend the homogenization of contents and incubation before subculturing into selective agars. Unlike their techniques, which employ a 48 h incubation of egg contents, our method can detect *S. enteritidis* with a 20 h incubation period.

The second feature of the developed procedure is based on the discovery that the LPS antigen of *S. enteritidis*, once released from the bacterial cells, can be uniformally distributed in the egg gel network formed by heating. This permits easy blotting of the antigen onto a solid support, such as porous membranes. The solidified egg mixture was cut into discs of a convenient size and the discs were placed on a pre-wet 0.45 μm nitrocellulose membrane strip, and left in contact with this strip for 5 minutes at room temperature. This produced a blot transfer of the LPS antigen of *S. enteritidis* from the solidified egg to the membrane via diffusional forces. Positive results appeared as purple dots on the membrane (Figure 4.1). Other detergents such as Triton X, EDTA and sodium deoxycholate can be used for the extraction of LPS antigen (Blais and Yamazaki, 1990; Wang *et al*, 1995). Like the assay described by Wang and coworkers, our procedure employs a 15% sodium cholate solution. Sodium cholate is necessary for the

detection of LPS antigen in eggs, as illustrated in Figure 4.2. Egg samples inoculated with attenuated 10^6 to 10^8 cells/ml of egg and heated in the absence of cholate produced no reaction on PBS, PLL or polymyxin B pre-coated strips (Figure 4.2). However, the same inoculum introduced in egg samples heated in the presence of cholate produced strong reaction at the 10^8 cell/ml density (Figure 4.3).

Poly-L-lysine and polymyxin B were examined as NC precoating agents. The sensitivity of PLL-coated NC strips was lower than those seen on PBS-coated strips. PLL has been successfully used as a coating agent for LPS antigens on microtiter plates (Masi and Zawistowski, 1995; Takahashi et al, 1992). However, when used as a pre-coating agent on NC, high backgrounds were observed. It appeared that PLL bound non-specifically to the detector Abs. This tendency increased when biotinylated Abs were used, particularly in conjunction with avidin. In general, the streptavidin detecting system gave better results than avidin (Figure 4.5). It is likely, that the neutral character of streptavidin accounted for the lower background as opposed to the higher background produced by negatively charged avidin (Stott, 1989).

Unlike PLL-coated strips, polymyxin B-coated strips produced low background levels, although, sensitivity was varied. On strips detected without biotinylated Abs, the sensitivity was similar to PBS-coated strips (Figure 4.3). When avidin was used for detection, sensitivity was enhanced but the negative control was detected (Figure 4.4c). Incubation with streptavidin eliminated this problem but increased the detection limit to 10^8 cells/ml sample (Figure 4.5c). The optimal system for the assay was PBS-coated NC strips in conjunction with streptavidin-alkaline phosphatase. With this arrangement the

FIG. 4.2 Typical results of the MAb 2F11-dot-blot assay from eggs inoculated with heat attenuated *S. enteritidis* PT 4 cells heated in the absence of sodium cholate. The blot was developed by goat-anti-mouse-IgG-alkaline phosphatase and NBT/BCIP substrate solution. Strips were pre-coated in a: PBS, b: PLL and c: polymyxin B.

S. enteritidis PT 4
(CFU/ml of egg)

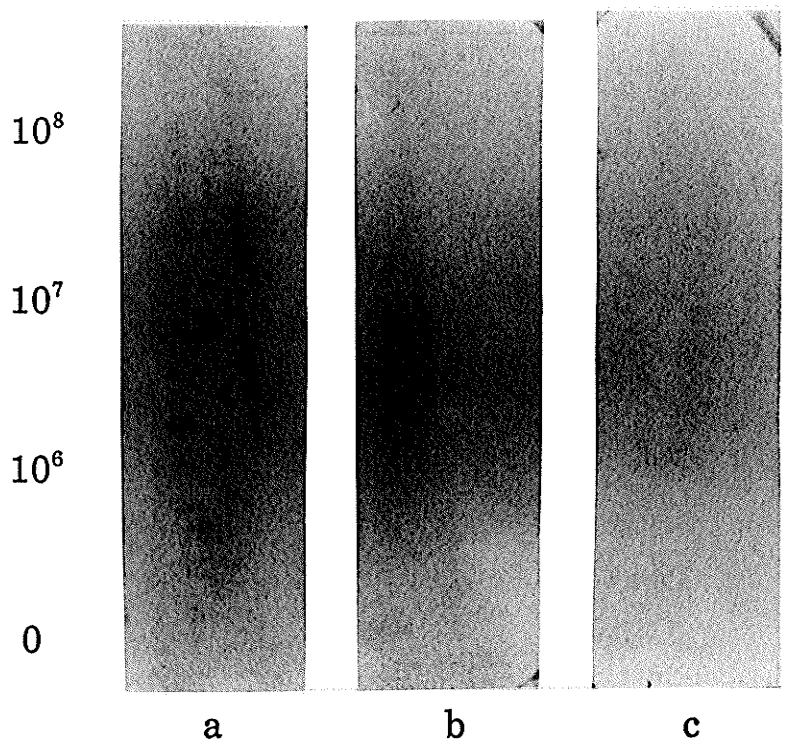
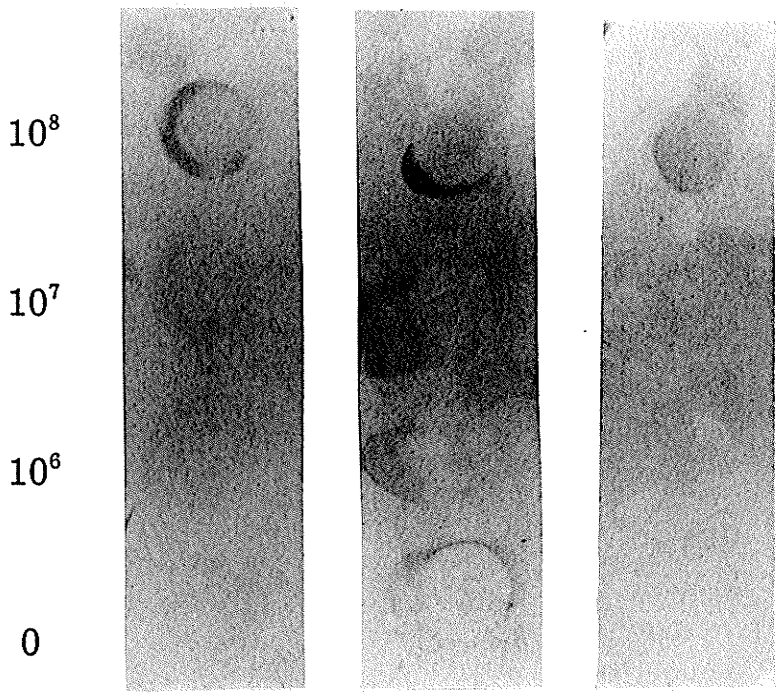


FIG. 4.3 Typical results of the MAb 2F11-dot-blot assay from eggs inoculated with heat attenuated *S. enteritidis* PT 4 cells heated in the presence of sodium cholate. The blot was detected by goat-anti-mouse-IgG- alkaline phosphatase and NBT/BCIP substrate solution. Strips were pre-coated in a:PBS, b: PLL and c: polymyxin B.

S. enteritidis PT 4
(CFU/ml of egg)



detection limit of the assay was 10^6 S. enteritidis cells/ml of egg (Figure 4.1). A total of 90 egg samples were assessed for the presence of *S. enteritidis*. Artificially inoculated egg samples (72) tested positive by both dot-blot assay and culture method (Table 4.1). Uninoculated eggs (18) yielded negative results. The obtained results showed that the dot-blot assay was equally effective as the culture method, yet it can be performed faster and with less labour.

4.5 CONCLUSION

The MAb 2F11 was highly specific to *S. enteritidis*. The described MAb 2F11-dot-blot assay has been shown to detect 10^6 *S. enteritidis* PT 4 cells/ml of egg, resulting from an inoculum of one cell/25 g of egg. The optimization of this assay is currently being investigated. When optimized, the assay will be useful for screening large number eggs, which should be of particular interest to governmental testing agencies.

FIG. 4.4 Typical results of the MAb 2F11-dot-blot assay from eggs inoculated with heat attenuated *S. enteritidis* PT 4 cells heated in the presence of sodium cholate. The blot was detected by biotinylated antibodies and avidin-alkaline phosphatase and NBT/BCIP substrate solution. Strips were pre-coated in a: PBS, b: PLL and c: polymyxin B.

S. enteritidis PT 4
(CFU/ml of egg)

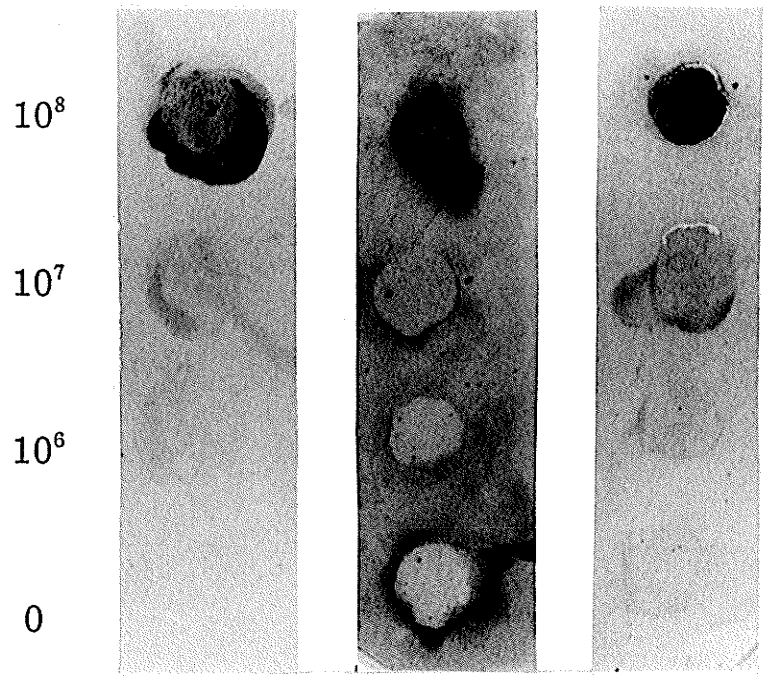
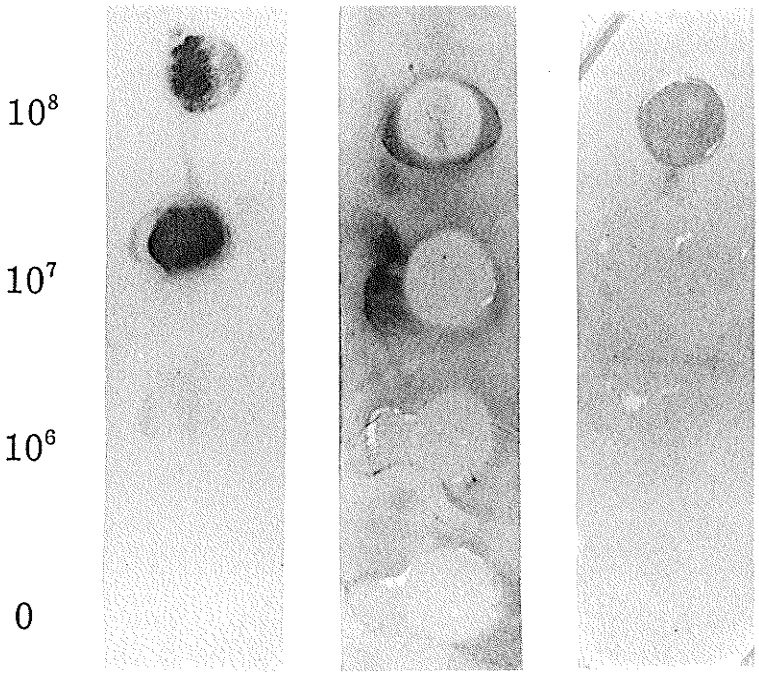


FIG 4.5 Typical results of the MAb 2F11-dot-blot assay from eggs inoculated with heat attenuated *S. enteritidis* PT 4 cells heated in the presence sodium cholate. The blot was detected by biotinylated antibodies and streptavidin-alkaline phosphatase and NBT/BCIP substrate solution. Strips were pre-coated in a:PBS, b:PLL, c: polymyxin B.

S. enteritidis PT 4
(CFU/ml of egg)



V. GENERAL DISCUSSION

To produce monoclonal antibodies specific to *Salmonella enteritidis*, two immunization approaches were attempted. Immunizing mice with purified *S. enteritidis* lipopolysaccharides (LPS) resulted in a weak titre response which was not sufficient to proceed with a fusion. In contrast, when the mice were immunized with attenuated *S. enteritidis* phage type (PT) 4 cells, a dramatic increase in the titre was observed. Three fusions were performed with over 500 hybrids produced, but only 20 hybridoma lines were found to secrete antibodies reactive to either cells and/or LPS of *S. enteritidis*. After screening the lines against a panel of *Salmonella* and non-*Salmonella* species, line 2F11 was found to be reactive with *S. enteritidis* and showed no reactivity towards other *Salmonella* serogroups.

The specificity of monoclonal antibody (MAb) 2F11 was determined by an ELISA using attenuated bacteria. The MAb 2F11 was reactive towards all phage types (PT) of *S. enteritidis* tested (PT 1, 4, 8, 13, 13a), exhibiting the highest reaction with PT 1. The antibody also reacted with other D_1-serovars such as *S. berta, S. gallinarum* and *S. pullorum* with different degrees of binding. The MAb did not recognize the antigenically similar D_2-serogroup, *S. maarseen*. In addition, 2F11 did not react with eight other serogroups: B, C_1, C_2, E_1, E_3, E_4, F, G_2 and a *S. arizonae* serotype as well as with all tested non-*Salmonella* bacteria: *Escherichia coli, Yersinia enterocolitica, Citrobacter freundii, Enterobacter cloacae, Shigella flexneri, Mycobacterium fortuitum, Pseudomonas*

fluorescens and *Aeromonas hydrophila*. The MAb 2F11 line was shown to be D_1-serogroup LPS specific.

The effect of different attenuation methods on the specificity of a MAb 2F11-based ELISA was investigated using *S. enteritidis PT 4, S. typhimurium* and *S. maarseen*. The bacteria were attenuated by heating at 80°C for 20 minutes, treated with 5% phenol, and 3% formalin. Heat treatment was the best way to enhance binding of MAb 2F11 to *S. enteritidis*, although live bacteria (control) were also detected. Treatment with phenol and formalin resulted in a reduction of absorbance values. When a selected panel of live bacteria was assayed, MAb 2F11 gave similar absorbance values for all phage types of *S. enteritidis* tested and lower absorbance values for the other D_1-serovars. Live *S. typhimurium*, *S. maarseen* and *E. coli* were not recognized by 2F11.

The specificity of MAb 2F11 was determined by an indirect non-competitive ELISA using polyvinyl chloride microplates of low bacterial cell binding capacity. To enhance the attachment of cells to plastic, a number of different coating agents were investigated: poly-L-lysine (PLL), polymyxin B and concanavalin A. The use of polymyxin B and concanavalin A had an adverse effect on the detection of *Salmonella*. Pre-coating plates with PLL increased the detection of *S. enteritidis* PT 4 and PT 1 by 54% and 34%, respectively, while *S. typhimurium* (negative control) remained undetected. The detection limit of a MAb 2F11-based ELISA employing PLL pre-coated plates was 10^4 *S. enteritidis* PT 4 cells/ml.

The O-antigens of salmonellae are a distinctive marker of each serogroup. In *Salmonella* serogroup D, the O-antigen is made up of a trisaccharide backbone (mannose,

rhamnose and galactose) and tyvelose which is attached to the mannose residue (Lindberg and Le Minor, 1984). Mannose and tyvelose form LPS O-antigen factor 9 and it is the distinctive feature of all D-serogroup *Salmonella*. The MAb 2F11 recognized all D_1-serovars tested and was highly specific to factor 9. *Salmonella* B-serogroup are antigenically similar to D-serogroup, as they share the same trisaccharide backbone. However, tyvelose is substituted by abequose yielding O-antigen factor 4, which was not recognized by MAb 2F11. This finding suggests that tyvelose is an important component of the epitope recognized by this antibody. The nature of the glycosidic bond between the monosaccharides in the trisaccharide backbone also plays an important role in the specificity of the antibody. In D_2 *Salmonella*, mannose is bound to rhamnose via β1,4 instead of α1,4 (D_1) and the trisaccharide is linked through α1,6 instead of α1,2 (D_1). Even though factor 9 is present in D_2 *Salmonella*, the linkage difference, does not allow MAb 2F11 to react with D_2 *Salmonella*.

The specificity of MAb 2F11 was further studied by electrophoresis followed by immunoblotting. The LPS extracts of various phage types of *S. enteritidis*, other D_1-serovars and serogroups B, D_2 as well as *E. coli* were silver stained after SDS-PAGE electrophoresis. The LPS electrophoretical profile showed the ladder-like pattern typical of LPS from smooth Gram-negative bacteria. Immunoblotting and subsequent incubation with MAb 2F11 revealed that the antibody selectively reacted with the LPS from all the D_1-serovars tested, including the various phage types of *S. enteritidis*. The MAb 2F11 however, did not recognize the LPS extracts of serovars B and D_2 as well as *E. coli*.

The conventional culture method for detection of *S. enteritidis* in foods is time

consuming, labour intensive and it requires up to one week for full identification of this pathogen. Furthermore, some selective broths were shown to be inhibitory towards *S. enteritidis* (Van der Zee, 1994). Therefore, rapid methods for the detection of *S. enteritidis* are highly desired by the food industry. Two rapid immunoassays for the detection of *S. enteritidis* in eggs have been described (Lee *et al*, 1989; Wang *et al*, 1995). The problem with the assay developed by Lee and coworkers (1989) is the monoclonal antibody employed, which not only recognizes *S. enteritidis* but a number of B-serogroup *Salmonella*. The assay developed by Wang et al (1995) uses rabbit antisera for the detection of *S. enteritidis* and can suffer from non-specific reactions with other enteric bacteria.

The results of the second manuscript of this study describe the development of a simple procedure for the detection of *S. enteritidis* in eggs. The methods utilizes a two-step procedure comprising an enrichment step and a MAb 2F11-based dot-blot assay. Eggs are a good substrate for microbial growth and *Salmonella* species have been shown to grow easily in yolk/white egg mixtures (Baker *et al*, 1983; Galyean and Cotterill, 1972). Thus, a small concentration of cells of *S. enteritidis* can be enriched to a detectable level directly in the homogenized whole egg without the need to isolate the organism and use of large volumes of enrichment broth, thus overcoming a disadvantage of the culture method. Egg samples inoculated with one *S. enteritidis* cell/25 g yielded over 10^6 cells/ml after a 20 h incubation at 37° C. This level of *S. enteritidis* was detectable by the developed dot-blot assay. Prior to the assay, the egg mixture

containing enriched *Salmonella* was heated in the presence of sodium cholate. This released the LPS antigen from the bacterial cells and caused an uniform distribution of LPS within a solidified egg gel network formed by heating. A solid disc of cooked egg was cut and applied to the nitrocellulose membrane employed by the dot-blot. The antigen was transfered to the membrane *via* diffusional forces. The membrane was blocked with skim milk, probed with MAb 2F11 and incubated with either goat-anti-mouse IgG alkaline phosphatase or biotinylated goat-anti-mouse IgG. When biotinylated antibodies were used, the membrane was further incubated with avidin or streptavidin-alkaline phosphatase and developed with the addition of BCIP/NBT substrate solutions. Positive results were observed as purple dots on the NC. The sensitivity of the outlined assay was comparable to the conventional culture method, which dictates the detection of one cell of *Salmonella* per 25 g of food. The detection limit of the assay was 10^6 *S. enteritidis* cells/ml of egg.

The use of different NC pre-coating agents to increase the sensitivity of the assay were also investigated. The application of phosphate buffered saline (PBS), poly-L-lysine and polymyxin B were studied. The sensitivity of PLL and polymyxin B-coated strips was lower than for those strips that were coated with PBS alone. Poly-L-lysine has been successfully used as a coating agent for LPS antigens on microplates (Masi and Zawistowski, 1995; Takahashi *et al*, 1992). However, when used as a pre-coating agent on NC, high backgrounds were observed, particularly when biotinylated antibodies were used. Unlike PLL-coated strips, polymyxin B-coated strips exhibited a low background, but sensitivity was compromised. The optimal system for the assay was PBS-coated NC

strips in conjunction with streptavidin-alkaline phosphatase as a detector. In this arrangement, the detection limit of the assay was 10^6 *S. enteritidis* cells per ml of egg.

VI. CONCLUSION AND RECOMMENDATIONS

Monoclonal antibody 2F11 (IgG_{2a}) reactive with various phage types (PT) of *S. enteritidis* and other D_1-serovars was produced. The antibody was specific to LPS O-9 factor of D_1-serovars. Antigenically similar B- and D_2-serogroup salmonellae were not recognized by MAb 2F11. Results of ELISA and SDS-PAGE followed by immunoblotting, show that the presence of tyvelose and the glycosidic bonds between the sugar and the trisaccharide backbone are important parts of the epitope recognized by the antibody. Both, heat-treated and live *S. enteritidis* were bound by MAb 2F11 with approximately equal avidity. To further confirm the ability of MAb 2F11 to bind live bacteria immunoelectron microscopy should be performed.

Further characterization of MAb 2F11 should include all the various phage types of *S. enteritidis* available, particularly those that do not express long-chain LPS (PT 7, 7a and 23, 30) (Therfall *et al*, 1993). Long-chain LPS (of smooth phenotypes) appears to be a virulence factor of *S. enteritidis* and short-chained phenotypes (rough strains) confer avirulence in mice. Immunoblotting results indicated that MAb 2F11 recognized sites in the long-chain O-antigen region of the LPS of *S. enteritidis*. MAb 2F11, thus may be useful in distingushing between long and short-chain phenotypes, without performing electrophoresis and immunoblotting.

During the course of this study, a dot-blot assay utilizing MAb 2F11 was developed. The assay with a pre-enrichment step allowed for the detection of one cell

of *S. enteritidis*/25 g of egg. The assay exhibited a detection limit of 10^6 *S. enteritidis* However, further optimization of the assay is required. The enrichment step may be enhanced by the addition of ammonium-iron (III) citrate, ferrous sulphate, ferroxamine-type siderophores, novobiocin and cefsoludin, as these agents have been shown to increase the isolation of *S. enteritidis* from eggs (Van der Zee, 1194). Increasing the incubation time of homogenized egg will allow more salmonellae to proliferate and thus give stronger reactions on the NC upon staining.

The use of different solid matrices and coating agents should be investigated. The dot-blot assay can employ various solid supports; nylon (positively and neutrally charged) and polyvinyl diflouride. The time and temperature of incubation employed by the procedure as well as pre-coating agent used should be optimized.

Optimization of the assay should also focus on reducing the detection time of the assay. It is possible to shorten certain steps of the assay. The blocking time may be reduced by the use of a more efficient agents, such as bovine serum albumin or gelatin. The use of either biotinylated or enzyme conjugated antibodies will also reduce assay time. In this respect, conjugation of MAb 2F11 to enzymes such as horseradish peroxidase or alkaline phosphatase, should be considered for further study.

MAb 2F11 is highly specific to both heat-treated and live *S. enteritidis*. This characteristic makes it a good candidate for use in separating *S.enteritidis* from competitive bacteria by the use of capture techniques. This MAb could be immobilized on magnetic particle beads, dipsticks or immunosorbent columns and used as a capture probe.

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