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**ENRICHMENT OF *SALMONELLA* IN POULTRY FOR DETECTION
BY POLYMYXIN-CLOTH ENZYME IMMUNOASSAY**

by

Satoko Hayashi, B. Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of Master of Science

Ottawa-Carleton Institute of Biology

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November 3, 1997

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acceptance of the thesis,

**Enrichment of *Salmonella* in Poultry for Detection
by Polymyxin-Cloth Enzyme Immunoassay**

submitted by

Satoko Hayashi, B. Sc.

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Chair, Department of Biology

Thesis Supervisor

November 3, 1997

Carleton University

Abstract

Selective enrichment of *Salmonella* in the presence of large excesses of *Escherichia coli* under agitation was developed using a combination of cholate and selenite for *Salmonella* detection by polymyxin-cloth enzyme immunoassay (pCEIA). Static enrichment for *Salmonella* detection in feces samples by the pCEIA was also developed. Various surfactants were examined for toxicity and capability of releasing *Salmonella* from poultry samples. The surfactants tested did not enhance the release. An anionic surfactant, 2-ethylhexyl sulfate, was found highly toxic to *Salmonella* and fecal microflora; thus it can be used as a disinfectant. The conditions for recovering both injured and uninjured *Salmonella* from poultry samples were investigated. The recovery of injured *Salmonella* requires prolonged (e.g., 20 hours) pre-enrichment in the presence of 0.5 % cholate, followed by 10-fold dilution and 20-hour static incubation with 0.3 % selenite. The advantages of the pCEIA in analyzing multiple samples have been demonstrated.

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Abbreviations

A	absorbance
AAFC	Agriculture and Agri-Food Canada
ADRI	Animal Diseases Research Institute
B58	Brij 58
BGSA	brilliant green sulfa agar
BPW	buffered peptone water
BY	brain heart infusion broth plus 0.5 % yeast extract
BYC	BY supplemented with 0.5 % sodium cholate
BYCS	BYC supplemented with 0.3 % sodium biselenite
cc	colony counts
cfu	colony forming units
dH ₂ O	distilled water
EIA	enzyme immunoassay
g	gram
h	hour(s)
HACCP	Hazards Analysis Critical Control Point
HPB	Health Protection Branch
LPS	lipopolysaccharide
M	molar
MCA	MacConkey agar
min	minute(s)

mg	milligram
ml	milliliter
μl	microliter
Nal	nalidixic acid
Nal ^r	nalidixic acid resistant
NP 4	Niaproof anionic surfactant 4
NP 8	Niaproof anionic surfactant 8
PBS	phosphate buffered saline
PBST	PBS containing 0.05 % Tween 20
pCEIA	polymyxin-cloth enzyme immunoassay
PCR	polymerase chain reaction
ppm	parts per million
rpm	revolution per minute
SC	sodium cholate
SDS	sodium dodecyl sulfate
sec	second(s)
TG7	Tergitol 7
TMB	3,3',5,5'-tetramethylbenzidine
TSA	trypticase soya agar
TX100	Triton X-100

Chapter 1

General Introduction

Food poisoning is one of the major concerns in our daily life as well as in the food industry. Food quality is becoming a non-tariff barrier to international trade, as the tariff barriers decline. Under the General Agreement on Tariffs and Trade (GATT), the World Trade Organization (WTO) is setting food safety requirements for over 150 countries (Anon, 1995; Caswell and Hooker, 1996). Hazard Analysis Critical Control Point (HACCP) system is a preventative approach recognized by WTO to control biological, chemical and physical hazards by determining risks at critical control points (ccp) in the course of food processing rather than by end-product inspection. The HACCP concept was pioneered in the 1960's by the Pillsbury Company to develop the production of safe foods for the space program in collaboration with the US Army and NASA. In 1985, the US National Academy of Science recommended that the HACCP approach be adopted in food processing establishments to ensure food safety and, in 1991, the Food Hygiene Committee of Codex Alimentarius of the World Health Organization, the world's reference organization for food safety, developed guidelines for HACCP application. Figure 1.1 shows seven principles of HACCP, which are in brief: (1) hazard analysis, (2) identification of critical control points (ccp), (3) establishment of critical limits, (4) monitoring procedures, (5) corrective actions, (6) record keeping, and (7) validation procedures (National Advisory Committee on Microbiological Criteria for Foods, 1992). The significance of HACCP is that all the measures are based on scientific verification replacing organoleptic, "touch and smell" type inspection that has been until now performed by food handlers. The benefits of HACCP could easily compensate for the costs of implementation, the restructuring of facilities and the training of personnel (Unnevehr and Jensen, 1996). With HACCP, food companies can more easily detect

Figure 1.1. Seven principles of Hazard Analysis Critical Control Points (HACCP)

potential problems and quickly react to them before the product is completely processed, packaged and distributed, and inspectors can focus more on verifying critical control points instead of checking each individual product or each package (Lammerding, 1995). Recently, US President Bill Clinton approved legislation requiring science-based food safety testing under HACCP in the meat and poultry industry to reduce the incidence of food poisoning (USDA, 1996). The regulation enforces, for instance, all slaughter plants and plants producing raw meat products to ensure that their *Salmonella* contamination rate is below the current national baseline prevalence (e.g., 20 % for chicken products). Plants which are not meeting that standard face penalties and could be ultimately closed (Lewis, 1996). In Canada, the Food Safety Enhancement Program (FSEP) has been functioning since 1991, as a guideline for HACCP-based production and compliance verification (Anon, 1995). Currently, agri-food processors in Canada are voluntarily implementing HACCP, although it has not yet been mandated. In order to monitor the microbial hazard level at each ccp, a large number of samples are required to be tested daily for hazards such as *Salmonella*.

Despite advances in modern technology, occurrence of foodborne outbreaks is rising and most are caused by bacteria (Bryan, 1981; USDA, 1988). The increasing incidence rate can be explained by increases in: (1) the consumption of meat and poultry, (2) the usage of contaminated feeds, particularly those of animal-origin, (3) cross-contamination rates due to mass-processing of carcasses, (4) mass catering and the consumption of restaurant food and pre-processed meals, (5) the number of aged and immuno-compromised consumers, (6) the resistance of pathogens to drugs, etc. (Maurice, 1994). Salmonellosis or *Salmonella* infection is the most common bacterial foodborne

illness; in Canada during 1973-1975, it accounted for 25 % of all reported outbreaks of foodborne diseases and 39 % of reported cases of foodborne diseases (Bryan, 1981). The economic impact of the diseases is also significant as Agriculture Canada (1988) has estimated that salmonellosis cost Canadians approximately 93 million dollars in 1987.

Salmonellae are short (1-2 μm), gram-negative, non-sporing rods which are motile, with the exception of the non-motile serotypes, *S. gallinarum* and *S. pullorum*. They are facultative anaerobes, biochemically characterized by their ability to ferment glucose with the production of acid and gas and their inability to attack lactose and sucrose (some serotypes such as *S. arizonae* are lactose and/or sucrose fermenting). Salmonellae grow optimally at 35 – 38 °C and fail to grow below 5 °C. They are relatively heat sensitive being killed at 60 °C in 15 – 20 minutes. Salmonellae thrive at pH levels from 6.5 to 7.5, but can survive and multiply at levels between pH 4.0 and 8.0. The bacteria are common in the intestinal tracts and feces of livestock, poultry, dogs, cats, rats, and other warm-blooded animals (Hayes, 1985; Agriculture Canada, 1988; USDA, 1988; FDA, 1992). The genus *Salmonella* is a prominent member of the family *Enterobacteriaceae* and contains more than 2,300 different serotypes, according to the Kauffmann-White antigenic scheme. This scheme is based on somatic (O), capsular (Vi) and flagellar (H) antigenic profiles derived from agglutination reactions with homologous antisera (Lindberg and Le Minor, 1984; Varnam and Evans, 1991). The most frequently reported *Salmonella* serotypes from humans in Canada in 1993 are listed in Table 1.1; all serotypes of salmonellae are potentially pathogenic to humans and/or animals. From the epidemiological point of view, salmonellae can be classified into three main groups: (1)

Table 1.1. The 10 most frequently reported *Salmonella* serotypes from humans in Canada (1993) (Khakhria *et al.*, 1994)

Rank	Serotypes	No.	%
1	typhimurium	1,341	17.0
2	enteritidis	1,298	16.4
3	hadar	933	11.8
4	heidelberg	607	7.7
5	thompson	274	3.5
6	agona	165	2.1
7	infantis	149	1.9
8	typhi	129	1.6
9	saint-paul	81	1.0
10	oranienburg	81	1.0
Total		5,058	64.0
Total	(all serotypes)	7,897	

S. typhi and *S. paratyphi* strains which infect only man and spread either directly or indirectly (via food and water) from person to person, (2) serotypes that are host-specific for particular species of vertebrates, e.g., *S. gallinarum* and *S. pullorum* in poultry, *S. dublin* in cattle, *S. abortus equi* in horses and *S. choleraesuis* in swine, some of which are also pathogenic to humans (especially *S. dublin* and *S. choleraesuis*) and (3) the majority of other *Salmonella* serotypes with no particular host preference that infect both humans and animals (WHO, 1988).

Salmonellae do not usually alter the odor, appearance, or taste of foods (Agriculture Canada, 1988), but can cause salmonellosis, the illness that possibly occurs when live *Salmonella* bacteria enter the human body, usually through foods. If the entered *Salmonella* cells survive the digestive acid in the stomach, penetration of the cells from the gut lumen into the epithelium of the small intestine will lead to inflammatory lesions in the lining of the intestinal tract; there is evidence that an enterotoxin may be produced, perhaps within the enterocyte. Salmonellosis is divided into two groups according to the clinical symptoms: (1) typhoid or paratyphoid fever, caused by *S. typhi* or *S. paratyphi* strains, which is characterized by septicemia, infecting virtually every organ via the blood stream and (2) gastroenteritis caused by other *Salmonella* serotypes, generally involving milder symptoms such as diarrhea, upset stomachs, chills, fever and headache. In typhoid fever, the symptoms gradually appear with an incubation period of at least one week (often 10 days to two weeks). The fever usually continues to rise stepwise, reaching a maximum in 7 to 10 days, and the spleen and liver become enlarged. Complications of typhoid infection include intestinal hemorrhage, necrosis of lymphoid tissues, hepatitis, infection of bones and joints, and inflammation of the lungs, gall

bladder and other organs (Guthrie, 1992). The latter type of salmonellosis causes stomach pain usually within 6 to 48 hours after the contaminated food is consumed and most people feel better within three to five days. Salmonellosis can be life-threatening; in the US about two deaths are reported for every 1,000 known cases, but experts believe that about 500 persons actually die each year from salmonellosis. The fatality rate of typhoid fever is 10 % compared to less than 1 % for the other forms of salmonellosis (USDA, 1988). An infective dose depends on the age and health of the host, the type of foods and strain differences. It is considered that about 10^6 viable *Salmonella* cells are necessary for a healthy adult to become clinically ill, though it has been reported that in some cases even a single cell could cause the illness. Silliker (1980) noted a low infective dose may be linked to the chemical composition of the food; the food may protect the organisms during passage through the stomach into the intestinal tract. D'Aoust (1977) also suggested that the high fat content in food (e.g., chocolate) could protect *Salmonella* from ingestive acids. Infants, the elderly and the immuno-compromised are susceptible to as few as 15 to 20 cells (Silliker, 1980; Hayes, 1985; FDA, 1992; Gerba *et al.*, 1996).

Salmonella can be found in a variety of foods such as raw meats, poultry, eggs, milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces and salad dressing, cake mixes, cream-filled desserts and toppings, dried gelatin, peanut butter, cocoa, and chocolate (FDA, 1992; Majaski, 1994). The trends of food consumption are changing; consumers are choosing more health-oriented foods (e.g., low-fat). Figure 1.2 shows the change in meat consumption by Canadians between 1979-1988. The consumption of poultry increased, as opposed to a slightly declining consumption of beef and pork. This is probably because poultry is relatively inexpensive, being produced on a large scale

Figure 1.2. Change in the consumption of meat and poultry in Canada during 1979-1988 ^a

^a Consumption in 1979 was taken as 0 % baseline. The data were adapted from Food Consumption Statistics, 1979-1988 (Organization for Economic Cooperation and Development, 1991).

^b Mutton, lamb and goat meat.

more easily than any other food animals, and is an ideal protein source which is low in fat and can provide tasty wholesome meals. As poultry consumption rises, it also becomes a more prevalent source of foodborne illnesses. Poultry has been historically associated with the presence of *Salmonella*. As shown in Figure 1.3 (a) and (b), during 1975-1984 poultry (turkey and chicken) was responsible for most *Salmonella* outbreaks (85.5 %) and cases (85.1 %) associated with meat. From 51 to 75 % of chicken carcasses are said to be contaminated with *Salmonella* (Agriculture Canada, 1988; Maurice, 1994; Todd and Harwig, 1996). Considering the increasing importance of HACCP implementation in poultry production and the high incidence of *Salmonella* in poultry, we have focused on developing an effective *Salmonella* detection procedure for poultry products.

The conventional culture method for the isolation of *Salmonella* from foods consists of: (1) sampling, (2) 18 – 24 h pre-enrichment with non-selective medium, (3) 18 – 24 h selective enrichment, (4) 24 – 48 h selective plating on differentiation plates, (5) 24 h purification on isolating plates, (6) 18 – 24 h biochemical confirmation, and (7) serological identification. Figure 1.4 shows the traditional *Salmonella* isolation procedure from processed meat by the Health Protection Branch, Health Canada (HPB, 1978). It takes four days (up to step four) to get results for negative samples, while samples which form suspected colonies at this stage are considered as merely ‘presumptive’ positive and subjected to confirmatory biochemical steps; this all adds up to six or seven days in total. Since the speed of the test results are important in order for food processors to reduce their storage time before shipping, a number of rapid detection methods have been developed with the intention of shortening the *Salmonella* isolation

Figure 1.3(a). Numbers of *Salmonella* outbreaks associated with various foods in Canada ^a

^a Adapted from Foodborne Disease in Canada, 10-year Summary 1975-1984, Health Protection Branch, Health Canada (1990).

Figure 1.3(b). Numbers of salmonellosis cases associated with various foods in Canada^a

^a Adapted from Foodborne Disease in Canada, 10-year Summary 1975-1984, Health Protection Branch, Health Canada (1990).

Figure 1.4. Methods for the isolation and identification of *Salmonella* from meat ^a

^a Constructed from HPB Method: *Salmonella* detection of Health Protection Branch, Health Canada (1978).

procedure. Biotechnology-based testing, including enzyme immunoassay (EIA), polymerase chain reaction (PCR), DNA probes and ATP bioluminescent technologies, have gained a foothold in this area. Some of these tests are available as kits which are less labor intensive and faster than the traditional methods; the results are usually available in 22 to 48 hours. For instance, Qualicon™ developed the BAX™ Pathogen Detection System for *Salmonella*, which is the first commercially available product to use PCR technology to detect bacteria in food. Gene- Trak™ has developed the *Salmonella* direct-labeled probe, a DNA hybridization test that uses *Salmonella* specific DNA probes directly labeled with horseradish peroxidase along with a colorimetric detection system (Cutt, 1996). In fact, Waterburton and Oggel (1995) of HPB suggested the use of 1-2 Test™, a rapid *Salmonella* detection test based on immunodiffusion of motile *Salmonella*, for *Salmonella* detection from feeds.

Polymyxin-cloth enzyme immunoassay (pCEIA) has been developed to detect gram-negative bacteria such as *Salmonella* on polymyxin-coated macroporous hydrophobic cloth such as polyester cloth (Blais and Yamazaki, 1996). gram-negative bacteria possess an outer membrane (Figure 1.5) which serves as a molecular sieve protecting the sensitive cytoplasmic membrane from the environment (Woodcock, 1988). Lipopolysaccharides (LPS) are the major components of the outer membrane and have been hypothesized as the cause of the fever associated with salmonellosis (Bryan *et al.*, 1979). For the pCEIA, Blais and Yamazaki (1990) have developed a simple method for preparing solid-free LPS antigens by heating a sample in a solution of a detergent (e.g., deoxycholate) followed by centrifugation. Wang (1995) has demonstrated that cholate is better than deoxycholate as an LPS extracting reagent, not only because it remains in the

Figure 1.5. Schematic structure of cell wall and membrane from typical Gram-negative bacteria (Woodcock, 1988)

liquid state at concentration up to 20 % (w/v), which makes it more convenient to be used, but also because it gives higher sensitivity than deoxycholate. Therefore, cholate was used in our studies for the LPS extraction. Polymyxin adsorbed onto the cloth rapidly captures cholate-extracted LPS antigens of *Salmonella*. As an LPS capturing agent, polymyxin B (a cyclic peptide antibiotic with a hydrocarbon tail) is relatively inexpensive, homogeneous and stable compared to antibodies used in enzyme immunoassay (EIA). The captured LPS antigens are then detected using LPS-specific antibodies. Figure 1.6 shows the molecular structure of *Salmonella* LPS. The somatic (O) antigens, shown as O-specific chain, are the most variable part of the LPS among *Salmonella* serotypes, determining the O-serological specificity with repeated oligosaccharide units, while the core region is believed to be common to most *Salmonella* LPS (Tsang, *et al.*, 1991). The specificity of *Salmonella* detection by the pCEIA depends on the LPS-specific antibodies being used. In the pCEIA, multiple samples (e.g., 60 samples) applied onto a single sheet of polymyxin-cloth are simultaneously processed. The large surface of the cloth permits rapid EIA reactions, while the macroporosity of the cloth allows rapid and efficient washings. The pCEIA, simple and inexpensive compared to other available detection methods, is considered an ideal tool for routine *Salmonella* testing under HACCP.

Salmonella are normally found only in small numbers in food samples; thus an enrichment step is usually necessary to bring *Salmonella* to a detectable cell density. For example, *Salmonella* detection by the pCEIA requires more than 10^6 colony forming units (cfu) / ml for a positive reaction (Wang *et al.*, 1996). Unlike chemical residues or toxin

Figure 1.6. Molecular structure of *Salmonella* LPS (Fomsgaad, 1990) *

* Glu, D-glucose; Gal, D-galactose; AcGluN, N-acetyl-D-glucosamine; Hep, heptose; KDO, 2-keto-3-deoxy-manno-octonate; EtN, ethanolamine; AraN, 4-amino-4-deoxy-L-arabinose; P, phosphate; GlcN, D-glucosamine.

contamination, the analysis of microbial contamination of foods is complex because the population and physiological state of the target pathogens may be altered at any stage from farm to table due to physical and chemical environmental factors, as well as interaction with the other microflora existing in the food (Lammerding, 1995; Todd and Harwig, 1996). An enrichment procedure, therefore, should be able to recover cells injured by sublethal stresses during processing or storage and should inhibit the growth of the competing microflora present in the foods.

Poultry processing procedures are summarized as: (1) receiving, (2) scalding, (3) defeathering, (4) spray washing, (5) evisceration, (6) chilling, (7) decontamination (e.g., chlorine, organic acids), (8) packaging and (9) storage / shipping (Agriculture Canada, 1988). During processing, some microbial cells are susceptible to injury such as heat-injury at scalding which involves an immersion of the carcass in hot water (50 – 60 °C) for a few minutes to loosen muscles and facilitate defeathering. It is important to detect the injured pathogens, since they are still viable and could cause illness if allowed to resuscitate and multiply prior to consumption. Therefore, *Salmonella* present in poultry samples should be first resuscitated in a nutrient-rich medium to recover cells from the injured state.

Poultry carcasses, immediately after processing, carry numerous kinds of bacteria: *micrococci* (50 %), gram-positive rods (14 %), *flavobacteria* (14 %), *Enterobacteriaceae* (8 %), *Pseudomonas* (2%), *Acinetobacter* (7 %) and other unidentified microorganisms (5 %) (Russell *et al.*, 1996). During enrichment, the non-*Salmonella* microflora can compete for nutrients or inhibit *Salmonella* growth, especially when they are present in

greater numbers than *Salmonella* in the samples. Furthermore, LPS molecules from non-*Salmonella* gram-negative bacteria compete with *Salmonella* LPS for binding to the polymyxin-cloth. Therefore, selective enrichment procedure must suppress the growth of competing non-*Salmonella* microflora and bring the resuscitated *Salmonella* cells to a detectable level.

Chen *et al.* (1993) have shown that commonly used selective media for *Salmonella* detection, tetrathionate brilliant green and Rappaport-Vassiliadis are toxic to *Salmonella*. Growth of *Salmonella* in these media requires the generation of resistant variants; the minimum number of *Salmonella* cells required to generate such variants ranges from 10^3 to 10^5 cells. On the other hand, in selenite cystine broth (which contains 0.4 % selenite) *Salmonella* can outgrow from less than 5 cells. Selenite is said to react with a sulfhydryl group of certain cellular components and generate sulfur analogues (seleno-amino acids) (Weiss *et al.*, 1965; Rose *et al.*, 1971). Inactivation of cell proteins, as a result of seleno-amino acid incorporation, likely causes inhibition of cell growth. *Salmonella* are relatively resistant to selenite because they can reduce it to a much less toxic, insoluble, elemental selenium (Garbisu *et al.*, 1996). Although 0.4 % selenite is commonly used as a selective agent for the recovery of *Salmonella*, it may not be effective in the presence of a large number of background microflora; as Chen *et al.* (1994) have shown, some non-*Salmonella* bacteria (e.g., *Proteus vulgaris* and *Pseudomonas aeruginosa*) are resistant or other can generate variants resistant to 0.4 % selenite. The use of a higher concentration of selenite or a combination of selenite and other selective agents may be necessary for selective enrichment of *Salmonella*.

In our studies, cholate is added to pre-enrichment media to facilitate the selective enrichment with selenite. Cholate belongs to a group of bile acids, biological detergents synthesized from cholesterol (Nair and Kritchevsky, 1976). Bile acids are amphiphilic in nature and have distinct lipophilic and hydrophilic centers, though possessing a rigid cyclic ring structure common to all steroids (Figure 1.7). Some intestinal microflora hydrolyze bile salts and degrade the compounds, which presumably results in detoxification (Hylemon, 1985). Cholate is known to inhibit the growth of gram-positive bacteria, probably due to their inability to detoxify cholate. The addition of cholate to pre-enrichment medium provides advantages for *Salmonella* enrichment and detection by the pCEIA. While 0.5 % (w/v) cholate in brain heart infusion broth supplemented with yeast extract (BY) does not affect the growth of *S. typhimurium*, it inhibits the growth of gram-positive bacteria such as *Bacillus cereus* and *Staphylococcus aureus*, as well as some gram-negative bacteria including *Proteus vulgaris*, *E. coli*, *Citrobacter freundii* and *Pseudomonas aeruginosa* (Chen *et al.*, 1995a). A rich medium, BY was chosen as a basal medium since it provides a short doubling time of *Salmonella* (e.g., 22 minutes for *S. typhimurium*) (Chen *et al.*, 1995a). Moreover, upon heat-treatment of the culture samples, cholate facilitates the extraction of the LPS antigens from *Salmonella* cells in such a form that interacts efficiently with polymyxin-cloth, thus enhancing the sensitivity of the pCEIA (Blais and Yamazaki, 1990; Wang, 1995). Addition of cholate to the medium, from the beginning, eliminates the need for subsequent pipetting of cholate into multiple culture samples.

The major objective of my thesis research was to develop a procedure for the enrichment of *Salmonella* in poultry samples for the detection by the pCEIA, in which

Figure 1.7. Schematic diagram of the molecular structure of sodium cholate (Carey, 1985) ^a

^a The oxygens of the ionized carboxyl group of the side chain are depicted by open circles. The sodium ion is not displayed.

cholate and selenite are employed as selective agents. Chapter 2 describes the effects of higher selenite concentrations on *Salmonella* enrichment in the presence of high levels of *E. coli* under shaking incubation. Chapter 3 describes selective enrichment of *Salmonella* under static incubation for detection of small numbers of *Salmonella* in multiple chicken fecal samples for potential application in fecal screening of *Salmonella* in poultry and humans. Chapter 4 describes the evaluation of various surfactants in terms of their toxicity to *Salmonella* and their capability to liberate *Salmonella* cells from poultry skin. This information is useful in preparing samples for detection of *Salmonella* in poultry products. Chapter 4 also describes the strong bactericidal action of an anionic surfactant which can be used for disinfecting food processing plants. In Chapter 5, we report the marked inhibition on the recovery of injured *Salmonella* by poultry materials (presumably microflora) and present a solution to this problem.

Research described in Chapter 2 and the early part of Chapter 3 of this thesis was conducted under the close supervision of Ms. H. Chen who was a research associate of Dr. H. Yamazaki at that time. We were able to co-author two publications (Chen *et al.*, 1995a; 1995b). The latter part of Chapter 3 has also been published in a journal, Food Microbiology (Hayashi and Yamazaki, 1996).

The introductory section in the following chapters may contain repetitive statements. However, it is my intention that each chapter be self-explanatory without any reference to the General Introduction.

Chapter 2

**Effects of high levels of selenite and *Escherichia coli* on enrichment of *Salmonella*
and detection of *Salmonella* by polymyxin-cloth enzyme immunoassay**

ABSTRACT

The effect of various concentrations of selenite on the recovery of *Salmonella typhimurium* and *Escherichia coli* was examined after pre-enrichment with agitation in a rich medium containing 0.5 % cholate. For heat-injured *S. typhimurium* cells, longer pre-enrichment periods were required for recovery in higher concentrations of selenite. When 0.7 % selenite was added to six-hour pre-incubated cultures of heat-injured cells, *S. typhimurium* was fully recovered, while *E. coli*, whether heat-injured or uninjured, did not recover when present at numbers of less than 2×10^7 cells. The polymyxin-cloth enzyme immunoassay detected *S. typhimurium* grown from a small number (as few as one cell) of cells in the presence of a large excess of *E. coli*.

INTRODUCTION

Polymyxin, hydrophobically adsorbed onto polyester cloth, captures lipopolysaccharide (LPS) antigens which are detected by anti-*Salmonella* LPS antibodies. As a capturing agent, polymyxin is inexpensive, stable and homogeneous compared to antibodies. *Salmonella* detection by polymyxin-cloth enzyme immunoassay (pCEIA) requires enrichment of *Salmonella* to 10^6 colony forming units (cfu) / ml for a positive reaction.

In contaminated food samples, *Salmonella* are generally outnumbered by non-*Salmonella* bacteria. During enrichment, the presence of non-*Salmonella* bacteria (particularly coliforms) are known to interfere with the recovery of *Salmonella* (Speck, 1976). Although selenite (0.4 %) is commonly used as a selective agent for the recovery

of *Salmonella*, Chen *et al.* (1994) have found that non-*Salmonella* bacteria can generate variants resistant to 0.4 % selenite at various frequencies. For example, *Escherichia coli* can generate resistant variants at one per 7×10^3 cells under agitated culture condition. Since the variants can grow well in 0.4 % selenite, 0.4 % selenite may not be effective if the total number of *E. coli* exceeds the "minimum number of recovery" (7×10^3 cells). Therefore, use of a higher concentration of selenite could be useful for the recovery of *Salmonella* in the presence of a large excess of other microflora.

The present chapter describes the effects of high levels of selenite and *E. coli* on the recovery of *Salmonella* and on the performance of *Salmonella* detection by the pCEIA. A nalidixic-acid resistant strain of *Salmonella* was used to isolate *Salmonella* colonies from the cultures also containing *E. coli*.

MATERIALS AND METHODS

Media, chemicals and reagents. Brain heart infusion (no. 0037-01-6), yeast extract (no. 0127-01-7), and brilliant green sulfa agar (no.0717-01-3), a *Salmonella* selective plating medium, were purchased from Difco Laboratories (Detroit, MI). The non-selective plating medium trypticase soya agar (no. 11043) was purchased from BBL (Becton Dickinson Microbiology Systems, Cockeysville, MD). Sodium cholate (C-1254), sodium hydrogen selenite (S-5260) and nalidixic acid sodium salt (N-4382) were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of 25 % (w/v) sodium cholate (cholate) and 20 % (w/v) sodium hydrogen selenite (selenite) were separately prepared in distilled water and sterilized through 0.45 μ m filters. Brilliant green sulfa agar (BGSA) +

nalidixic acid (Nal) (BGSA + Nal) was prepared by adding 1.0 ml of 2.5 % (w/v) nalidixic acid stock solution to 1,000 ml of sterilized BGSA solution before pouring.

The detector antibody used was Bacto rabbit *Salmonella* O antisera poly A (serogroups A, B, D, E₁, E₂, E₃, E₄, and L) (no. 2534-47-6) purchased from Difco Laboratories, which has a high degree of specificity for *S. typhimurium* (serogroup B). Anti-rabbit IgG antibody peroxidase conjugate (A-6154) was supplied by Sigma Chemical Co. Ricoh Kyosan Inc. (Tokyo, Japan) donated the polymyxin-coated polyester cloth sheets (5.5 × 8 cm), CEIA sample applicator (60 apertures) and the TMB membrane peroxidase substrate system, a proprietary powder formulation based on the chromogen 3,3',5,5'- tetramethylbenzidine (TMB) and an unspecified enhancer.

Bacteria. *Salmonella typhimurium* 324, a nalidixic acid resistant strain, was kindly provided by Dr. R. Clarke, Health of Animals Laboratory, Guelph, Ontario, Canada. *E. coli*, isolated from food and animal origin, was obtained from the Animal Diseases Research Institute, Nepean, Ontario, Canada. All bacteria were stored on trypticase soya agar (TSA) slants at 4 °C. Overnight cultures were grown in brain heart infusion broth supplemented with 0.5 % (w/v) yeast extract (BY) by shaking (180 rpm) at 37 °C for 16 – 18 h.

Experiment 2.1 Effects of various concentrations of selenite on the recovery of heat-injured *S. typhimurium*. *S. typhimurium* cells were heat-injured by shaking at 56 °C for 30 min as previously described (Chen *et al.*, 1993). This heat-treatment generated the

injury rates of 85 to 95 %. The heat-injured cells were then washed and re-suspended in BY + 0.5 % (w/v) cholate (BYC) to a cell density of 10^5 cfu / ml and divided into 33 aliquots. Each aliquot (5 ml) was decimally diluted in BYC to produce average cell densities of 10^4 , 10^3 , 10^2 , 10^1 , 10^0 and 10^{-1} cfu / ml as previously described (Chen *et al.*, 1993). The selenite stock solution (20 %) was added to the first 8 aliquots without pre-enrichment (0 h) to produce final selenite concentrations of 0.3, 0.4, 0.5, 0.6, 0.7, 0.9 and 1.0 %. The dilutions from the second, third and fourth sets of 8 aliquots were first shaken at 37 °C for 1.5, 3.0 and 4.5 h, respectively, and then exposed to 0.3 to 1.0 % of selenite as above. The dilutions from the remaining aliquot were incubated at 37 °C for 20 h as an untreated control. After addition of selenite, all tubes were incubated at 37 °C for another 20 h. The presence (+) or absence (–) of growth in each dilution was determined by observing turbidity or by plating a 0.1 ml aliquot of culture on TSA plates and incubating the plates at 37 °C for 24 h.

Experiment 2.2 Effects of various concentrations of selenite on the recovery of *E. coli*. Four aliquots of the overnight culture of *E. coli* were serially diluted with BY to produce cell densities from 10^8 to 10^{-1} cfu / ml. The selenite stock solution (20 %) was added to four sets of the dilutions to produce final concentrations of 0.3, 0.5, 0.7 and 0.9 %, respectively. All the tubes were shaken at 37 °C for 24 h. The presence or absence of growth in each dilution was determined by plating a 0.1 ml aliquot of culture onto TSA plate, incubating the plate for at 37 °C for 24 h, and observing growth to determine the number of cells required for survival, as previously described (Chen *et al.*, 1994).

Experiment 2.3 Effect of cholate and selenite on the recovery of *E. coli* and *S. typhimurium*. *E. coli* and *S. typhimurium* cells were heat-injured as previously described (Chen *et al.*, 1993), washed and re-suspended in BY to a cell density of 10^7 cfu / ml. The cell suspension was treated in triplicate in three ways. Six 0.5 ml aliquots of the cell suspension were decimally diluted to extinction in 4.5 ml of BYC and were shaken at 37 °C. After 6 h shaking, the selenite stock solution (20 %) was added to the dilutions from the first 3 aliquots to a final concentration of 0.7 %. The same volume of sterile water (in place of selenite) was added to the dilutions from the second 3 aliquots. All the dilutions were shaken for another 20 h. As a control (no supplements), three 0.5 ml aliquots were similarly diluted in 4.5 ml of BY and shaken at 37 °C for 26 h. The presence of (+) or absence (–) of cell growth in each dilution was determined as above.

Experiment 2.4 Effect of *E. coli* on the growth and the pCEIA of *S. typhimurium* grown with or without cholate and selenite. Heat-injured *S. typhimurium* and *E. coli* cells were washed and diluted in BY or BYC. Four (for BY) or eight (for BYC) 5 ml mixtures containing 10^3 cfu / ml of *S. typhimurium* (S) cells and 10^4 , 10^5 , 10^6 , or 10^7 cfu / ml of *E. coli* (E) cells were prepared, generating E to S cells ratios of 10^1 , 10^2 , 10^3 , and 10^4 . All the mixtures were decimally diluted with the same dilution medium to a final *Salmonella* average cell density of 10^{-1} cfu / ml. All the dilutions were shaken at 37 °C for 6 h. The selenite stock solution was added to the dilutions from four of the BYC mixtures to a final concentration of 0.7 %. All the mixtures were shaken at 37 °C for another 20 h.

Uninjured *S. typhimurium* and *E. coli* cells were washed and diluted in BY, BYC or BYC + 0.7 % selenite. In each dilution medium, four 5 ml mixtures containing 10^3 cfu / ml of *Salmonella* cells and 10^4 , 10^5 , 10^6 , and 10^7 cfu / ml of *E. coli* were prepared. All the mixtures were decimally diluted with the same dilution medium to a final *Salmonella* average cell density of 10^{-1} cfu / ml. All the dilutions were shaken at 37 °C for 20 h.

Turbidity was observed as an indication of growth (+) in all the dilutions. The dilutions from the initial *Salmonella* average density of 1 cfu / ml (in a total volume of 4.5 ml) were plated on both TSA plates and BGSA + Nal plates. The colony count (cc) on TSA represents the total numbers of both S and E cells, while the cc on BSGA + Nal plates represents the numbers of S (nalidixic acid resistant) cells. Therefore, the number of E cells is equal to (cc on TSA – cc on BGSA + Nal).

All the dilutions were assayed for *S. typhimurium* using the pCEIA as follows: cholate (final concentration 0.5 %) was added to all the cultures that had previously received no cholate. All the mixtures were heated at 100 °C for 15 min to dissociate the LPS antigens from the cell surface; the heated cultures were allowed to cool to room temperature, and were then subjected to the pCEIA.

Polymyxin cloth enzyme immunoassay (pCEIA). The polymyxin cloth sheet (5.5 × 8 cm) was soaked in 10 ml of 0.01 M phosphate buffer (pH 7.3) - 0.85 % (w/v) NaCl containing 0.05 % (v/v) Tween 20 (PBST) and then blotted on Whatman 3MM paper. Five µl samples were pipetted through the apertures of the sample applicator onto the cloth sheet at about 0.7 cm intervals and incubated for 20 min after the final sample was

applied. The cloth sheet was washed with 4×25 ml of PBST on a macroporous filter under suction and blotted, then incubated with 4 ml of Bacto rabbit *Salmonella* O antisera poly A ($1:10^3$ diluted in PBST) for 15 min, and then washed with PBST as above. The cloth sheet was incubated with 4 ml of anti-rabbit IgG antibody peroxidase conjugate (diluted $1:10^3$ in PBST) for 15 min and then washed with PBST. The TMB substrate powder was dissolved in distilled water at a concentration of 35 mg / ml. The cloth was immersed in 4 ml of the TMB substrate solution. After a 5 min incubation, the cloth sheet was washed with PBST and blotted. The presence of the *Salmonella* LPS antigen in a sample was shown as a blue spot on a white background of the cloth sheet. The cloth sheet was then photocopied to obtain a permanent record of antigen spots. All incubations were performed at room temperature.

RESULTS

Effects of various concentrations of selenite on the recovery of heat-injured *S. typhimurium*. Chen *et al.* (1994) have found that *E. coli*, when present in excess of 7×10^3 cells (the minimum number of recovery), can recover in 0.4 % selenite because it generates resistant variants at one per 7×10^3 cells. Here, we hypothesized that a higher concentration of selenite should increase toxicity to *E. coli*, thereby increasing the minimum numbers required for recovery in selenite. The detection of *Salmonella* in foods normally requires pre-enrichment in non-selective media to resuscitate any injured *Salmonella* present. *Salmonella* cells recovering from heat-injury have shown a sensitivity to 0.4 % selenite, while uninjured cells did not (Chen *et al.*, 1993). Therefore,

the effects of higher concentrations of selenite on heat-injured *S. typhimurium* cells were first examined at various stages of resuscitation during pre-enrichment in BYC without selenite. Heat-injured *S. typhimurium* cells were serially diluted in BYC and then incubated at 37 °C for 0 to 4.5 h, after which 0.3 to 1.0 % of selenite was added. After an additional 20 h shaking, the growth was measured. As previously described (Chen *et al.*, 1993), the effects of selenite were determined by the maximal dilution (end point) which allowed the growth of *Salmonella*. Table 2.1 shows that longer pre-enrichment periods were necessary to overcome the toxic effect of higher concentrations of selenite. Full recovery of *Salmonella* was observed after 1.5 h pre-enrichment at 0.4 % selenite, after 3.0 h at 0.5 %, and after 4.5 h at 0.7 %. Higher selenite concentrations (> 0.7 %) did not allow full recovery even after 4.5 h pre-enrichment. A selenite concentration of 0.7 % was thus chosen for selective enrichment of *Salmonella*.

Effects of various concentrations of selenite on the recovery of *E. coli*. To compare the selectivity of these concentrations of selenite (in BYC) against *E. coli*, the minimum numbers of *E. coli* cells required for recovery in selenite (due to the selective-resistant variants) were determined as previously described (Chen *et al.*, 1994). The minimum numbers were 6×10^3 cells at 0.3 % selenite, 7×10^5 cells at 0.5 % selenite, 2×10^7 cells at 0.7 % selenite and 2×10^9 cells at 0.9 % selenite. This shows that increasing selenite concentration is accompanied by increasing selectivity against *E. coli*.

Table 2.1. Effects of various concentrations of selenite on recovery of heat-injured *S. typhimurium* ^a

Selenite (%)	Pre-incubation (h) prior to selenite addition			
	0	1.5	3.0	4.5
0	+++++ ^b			
0.3	+++---	+++++-	+++++-	+++++-
0.4	++-----	+++++-	+++++-	+++++-
0.5	++-----	+++++-	+++++-	+++++-
0.6	++-----	+++++-	+++++-	+++++-
0.7	++-----	+++++-	+++++-	+++++-
0.8	++-----	+++++-	+++++-	+++++-
0.9	+-----	+++--	+++--	+++--
1.0	+-----	+++--	+++--	+++--

^a *S. typhimurium* cells were heat-injured and decimally diluted to extinction in BY + 0.5 % cholate. After different periods (0 – 4.5 h) of pre-enrichment at 37 °C with shaking, various concentrations (0 – 1.0 %) of selenite were added, followed by shaking for another 20 h. The experimental procedure is detailed in the Methods (Experiment 2.1).

^b The presence (+) or absence (–) of growth in tubes containing (from left to right) 10⁴, 10³, 10², 10¹, 10⁰, 10⁻¹ cfu / ml *Salmonella* cells was determined by observing turbidity or by plating on TSA.

Effect of cholate and selenite in the recovery of heat-injured *E. coli* and *S. typhimurium* Full recovery of heat-injured *S. typhimurium* was observed in 0.7 % selenite if pre-incubated by shaking for 4.5 h (Table 2.1). To compare the effect of 0.7 % selenite on *E. coli* and *S. typhimurium*, both cells were identically heat-treated for injury and serially diluted to different cell densities in BY and BYC. After a 6 h pre-enrichment, one half of the dilutions in BYC were exposed to 0.7 % selenite. After an additional 20 h incubation, growth was measured. As before, the effect of 0.7 % selenite was determined by the maximal dilution which allowed growth. The recovery of heat-injured *E. coli* in 0.5 % cholate was not as complete as in medium without cholate (Table 2.2). While the addition of 0.7 % selenite to the cholate culture drastically reduced the recovery of *E. coli*, it had little effect on the recovery of *S. typhimurium*.

Effect of *E. coli* on the growth and pCEIA of *S. typhimurium* grown with or without cholate and selenite. A combination of 0.5 % cholate and 0.7 % selenite was shown to be selective against *E. coli* (Table 2.2). Therefore, we wanted to study the effect of *E. coli* on the growth of *Salmonella* in the absence or presence of these selective agents, and how the presence of excess *E. coli* and selective agents would affect the detection of *Salmonella* by the pCEIA.

E. coli and *S. typhimurium* were identically heated for injury and mixed in *E. coli* / *Salmonella* cells (E/S) ratios of 10 to 10⁴. All the mixtures were serially diluted to produce different *Salmonella* cell densities in BY and BYC. After 6 h incubation, one half of the dilutions in BYC were exposed to 0.7 % selenite. After a total of 26 h incubation, growth (turbidity) was recorded [Table 2.3(a) column (I)]. Since turbidity

Table 2.2. Effect of cholate and selenite on the recovery of heat-injured *E. coli* and *S. typhimurium* ^a

<u>Bacteria</u>								
Supplement(s)	Initial cell densities (cfu / ml)							
	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹
<u><i>E. coli</i></u>								
None	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
0.5 % cholate	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	—	—
	+	+	+	+	+	—	—	—
0.5 % cholate + 0.7% selenite ^b	+	+	—	—	—	—	—	—
	+	—	—	—	—	—	—	—
	+	—	—	—	—	—	—	—
<u><i>S. typhimurium</i></u>								
None	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
0.5 % cholate	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
0.5 % cholate + 0.7% selenite ^b	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	+	—
	+	+	+	+	+	+	—	—

^a Aliquots (4.5 ml) of cell suspension (in triplicate) at the initial cell densities indicated were shaken for a total of 26 h at 37 °C. The experimental procedure is detailed in the Methods (Experiment 2.3).

^b The samples consisting of cell suspensions in BY + 0.5 % cholate were first shaken for 6 h without 0.7 % selenite and then shaken for 20 h after the addition of 0.7 % selenite.

Table 2.3. Effect of *E. coli* on the growth and pCEIA of *S. typhimurium* grown with or without cholate and selenite.

Heat-injured (a) or uninjured (b) *S. typhimurium* (S) and *E. coli* (E) cells were mixed in the cell ratios indicated and decimally diluted in 5 ml of BY or BY + cholate. After 6 h preincubation, half of the dilutions in BY+ cholate were exposed to 0.7 % selenite and then incubated for an additional 20 h at 37 °C. The presence (+) or absence (–) of turbidity due to growth is recorded in column (I) Turbidity. The positive (+) or negative (–) reaction to *Salmonella* assay by pCEIA is recorded in column (II) pCEIA. The experimental procedure is detailed in the Methods (Experiment 2.4).

(a) Heat-injured	E/S cells	(I) Turbidity					(II) pCEIA				
		Initial <i>Salmonella</i> cfu / ml					Initial <i>Salmonella</i> cfu / ml				
		10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹	10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹
No Supplements	10	+	+	+	+	+	+	+	+	+	-
	10 ²	+	+	+	+	+	+	+	+	+	-
	10 ³	+	+	+	+	+	+	+	+	+	-
	10 ⁴	+	+	+	+	+	+	+	+	+	+
Cholate	10	+	+	+	+	+	+	+	+	+	+
	10 ²	+	+	+	+	+	+	+	+	+	-
	10 ³	+	+	+	+	+	+	+	+	+	-
	10 ⁴	+	+	+	+	+	+	+	+	+	-
Cholate + Selenite	10	+	+	+	+	-	+	+	+	+	-
	10 ²	+	+	+	+	-	+	+	+	+	-
	10 ³	+	+	+	+	+	+	+	+	+	+
	10 ⁴	+	+	+	+	-	+	+	+	+	-
<hr/>											
(b) Uninjured	E/S cells	10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹	10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹
No supplements	10	+	+	+	+	+	+	+	+	+	-
	10 ²	+	+	+	+	+	+	+	+	+	-
	10 ³	+	+	+	+	+	+	+	+	+	-
	10 ⁴	+	+	+	+	+	+	+	+	+	-
Cholate	10	+	+	+	+	-	+	+	+	+	-
	10 ²	+	+	+	+	+	+	+	+	+	-
	10 ³	+	+	+	+	+	+	+	+	+	+
	10 ⁴	+	+	+	+	+	+	+	+	+	-
Cholate + Selenite	10	+	+	+	+	-	+	+	+	+	-
	10 ²	+	+	+	+	-	+	+	+	+	-
	10 ³	+	+	+	+	+	+	+	+	+	-
	10 ⁴	+	+	+	+	-	+	+	+	+	-

represents growth of both *Salmonella* and *E. coli*, all the cultures derived from an initial *Salmonella* cell density of 10^0 cfu / ml (a total volume of 4.5 ml) were counted for viable *Salmonella* and *E. coli*. Figure 2.1(a)-(c) shows that a mixture of cholate and selenite showed strong selection against *E. coli*, whereas cholate alone provided some selection. In the presence of both cholate and selenite, *Salmonella* reached levels $> 10^8$ cfu / ml while levels of *E. coli* were below 10^6 cfu / ml.

In these latter experiments, both *E. coli* and *Salmonella* received the same heat-treatment and pre-incubation for 6 h prior to selenite addition. It can be argued that *E. coli* are more heat-sensitive than *Salmonella* and the higher degree of injury could have caused a greater sensitivity to the selective agents, even after a 6 h pre-enrichment. Therefore, the same set of experiments were carried out using uninjured *E. coli* and *Salmonella* [Table 2.3(b), Figure 2.2(a)-(c)]. Comparison of Figure 2.1(a)-(c) and Figure 2.2(a)-(c) shows that the selective action of cholate and selenite on *E. coli* was similar whether the cells were injured or uninjured.

The levels of *Salmonella* reached $> 10^6$ cfu / ml even when *E. coli* cells were allowed to grow without cholate or selenite. This cell density is higher than the limit of *Salmonella* detection by the pCEIA (Wang *et al.*, 1996). However, the LPS antigens from *E. coli* (grown with *Salmonella*) would compete for binding to polymyxin-cloth, which may interfere with *Salmonella* detection. Table 2.3 [column (II)] shows that the pCEIA successfully detected *Salmonella* even when large excesses of *E. coli* were allowed to grow without selective agents. Since the pCEIA did not give a positive reaction with *E. coli*, even when present at levels $> 10^9$ cfu / ml, any positive reaction was due to the presence of *Salmonella*. Furthermore, the addition of 0.7 % selenite did not

Figure 2.1(a). Effect of *E. coli* on the growth of *S. typhimurium* grown without cholate or selenite (heat-injured) ^a

^a Heat-injured *S. typhimurium* (1 cfu / ml) were mixed with 10, 10², 10³ or 10⁴ cfu / ml of identically heat-injured *E. coli* in 5 ml of BY. After 26 h incubation at 37 °C, all the cultures were plated on both TSA plates and BGSA + Nal plates for viable *Salmonella* and *E. coli* cells as described in the Methods (Experiment 2.4).

Figure 2.1(b). Effect of *E. coli* on the growth of *S. typhimurium* grown with cholate (heat-injured) ^a

^a Heat-injured *S. typhimurium* (1 cfu / ml) were mixed with 10, 10², 10³ or 10⁴ cfu / ml of identically heat-injured *E. coli* in 5 ml BY + 0.5 % cholate. After 26 h incubation at 37 °C, all the cultures were plated on both TSA plates and BGSA + Nal plates for viable *Salmonella* and *E. coli* cells as described in the Methods (Experiment 2.4).

Figure 2.1(c). Effect of *E. coli* on the growth of *S. typhimurium* grown with cholate and selenite (heat-injured) ^a

^a Heat-injured *S. typhimurium* (1 cfu / ml) were mixed with 10, 10^2 , 10^3 or 10^4 cfu / ml of identically heat-injured *E. coli* in 5 ml of BY + 0.5 % cholate. After 6 h pre-enrichment, the cultures were exposed to 0.7 % selenite and then incubated for an additional 20 h at 37 °C. All the cultures were plated on both TSA plates and BGSA + Nal plates for viable *Salmonella* and *E. coli* cells as described in the Methods (Experiment 2.4).

^b The levels of *E. coli* were below 10^6 cfu / ml.

Figure 2.2(a). Effect of *E. coli* on the growth of *S. typhimurium* grown without cholate or selenite (uninjured) ^a

^a Uninjured *S. typhimurium* (1 cfu / ml) were mixed with 10 , 10^2 , 10^3 or 10^4 cfu / ml of uninjured *E. coli* in 5 ml of BY. After 20 h incubation at 37 °C, all the cultures were plated on both TSA plates and BGSA + Nal plates for viable *Salmonella* and *E. coli* cells as described in the Methods (Experiment 2.4).

Figure 2.2(b). Effect of *E. coli* on the growth of *S. typhimurium* grown with cholate (uninjured)^a

^a Uninjured *S. typhimurium* (1 cfu / ml) were mixed with 10, 10², 10³ or 10⁴ cfu / ml of uninjured *E. coli* in 5 ml BY + 0.5 % cholate. After 20 h incubation at 37 °C, all the cultures were plated on both TSA plates and BGSA + Nal plates for viable *Salmonella* and *E. coli* cells as described in the Methods (Experiment 2.4).

Figure 2.2(c). Effect of *E. coli* on the growth of *S. typhimurium* grown with cholate and selenite (uninjured)^a

^a Uninjured *S. typhimurium* (1 cfu / ml) were mixed with 10, 10², 10³ or 10⁴ cfu / ml of uninjured *E. coli* in 5 ml BY + 0.5 % cholate + 0.7 % selenite. After 20 h incubation at 37 °C, all the cultures were plated on both TSA plates and BGSA + Nal plates for viable *Salmonella* and *E. coli* cells as described in the Methods (Experiment 2.4).

^b The levels of *E. coli* were below 10⁷ cfu / ml.

affect *Salmonella* detection by the pCEIA, which is useful to know whenever 0.7 % selenite is used as a selective agent.

DISCUSSION

We have demonstrated that under agitation the use of higher concentrations (e.g., 0.7 %) of selenite can provide greater selectivity against an *E. coli* strain without decreasing the recovery of a *S. typhimurium* strain. Although a nalidixic acid-resistant *S. typhimurium* strain was used in the present studies, a wild type nalidixic acid-sensitive *S. typhimurium* strain is equally resistant to 0.7 % selenite (Chen *et al.*, 1995a). However, this differential effect may need to be confirmed on other *E. coli* and *Salmonella* strains. The inclusion of 0.5 % cholate inhibits not only gram-positive but also some non-*Salmonella* gram-negative bacteria. Six hours of pre-enrichment was found to be sufficient to resuscitate heat-injured *S. typhimurium* prior to addition of 0.7 % selenite. By contrast, the conventional pre-enrichment generally requires overnight or one day pre-enrichment in a non-selective medium. The direct addition of selenite to pre-enrichment medium after a 6 h incubation further simplifies the enrichment procedure. We have demonstrated that the pCEIA can detect *Salmonella* grown from a small number (as few as 1 cell) of initial cells in the presence of 10^4 excess of *E. coli* cells, even without the use of selective agents. Since the presence of *E. coli* did not lower the sensitivity of *Salmonella* detection, it can be interpreted that the amount of polymyxin adsorbed onto a large surface of the cloth is so large that competition for LPS antigens does not occur. We have also shown that a high concentration of selenite did not affect the pCEIA. Since one cannot predict when large numbers of *E. coli* will appear in samples, under agitation

(e.g., shaking) we recommend the inclusion of high concentrations (e.g., 0.7 %) of selenite as well as cholate, during enrichment for the detection of *Salmonella*. The application of the enrichment with a combination of cholate and selenite will be evaluated in the following chapters for *Salmonella* detection in feces and uncooked animal foods (e.g., poultry meats).

Chapter 3

**Selective enrichment of *Salmonella* with cholate and selenite under static incubation
for detection in chicken feces by polymyxin-cloth enzyme immunoassay**

ABSTRACT

Enrichment of *Salmonella* using a combination of 0.3 % selenite and 0.5 % cholate under static incubation was examined for five common serotypes in the presence of *Salmonella*-free chicken feces. This selective enrichment method was effective in suppressing large excesses of non-*Salmonella* chicken fecal microflora, while permitting the growth of small numbers of *Salmonella*, which produced strong signals in the pCEIA. When the medium contained no selective agents, the ability of the pCEIA to detect *Salmonella* was greatly reduced in the presence of large excesses of chicken fecal microflora. The total procedure to detect *Salmonella* in chicken feces took about 22 h. This procedure permitted the detection of endogenous *Salmonella* in feces of chicken orally inoculated with *Salmonella* and the measurement of the fecal *Salmonella* level. This simple, rapid and sensitive procedure for *Salmonella* detection in feces should be useful not only for routine screening of *Salmonella* fecal shedding in poultry, but also testing stool from human patients and food handlers for *Salmonella*.

INTRODUCTION

The primary source of *Salmonella* contamination of processed poultry is *Salmonella* infected poultry, which often shed *Salmonella* (as many as 10^6 cfu / g of feces) without showing apparent infectious symptoms (Budnick, 1989; Grau 1989). The cross-contamination between infected flocks and uninfected flocks occurs in the breeding house, during transportation and also during poultry processing in the plant (Agriculture Canada, 1988). *Salmonella* screening of poultry feces enables the separation of infected flocks from uninfected flocks, which would reduce the level of cross-contamination in

processed poultry. Detecting *Salmonella* and measuring its levels in poultry feces by the pCEIA would allow such an intervention.

In Chapter 2, we have shown that in an agitated culture, a combination of 0.7 % selenite and 0.5 % cholate as selective agents enriches *Salmonella* while inhibiting *E. coli*. Selenite does not interfere with the pCEIA, while cholate also helps to extract LPS and facilitates its interaction with polymyxin. This selective enrichment permits the pCEIA detection of a small number (as few as 1 cell) of *Salmonella* cells grown in the presence of a large excess of *Escherichia coli* cells. For the microbial assay of a large number of samples, static incubation (for culturing) is more convenient since a static incubator can accommodate a larger volume of samples and is less expensive than a shaking incubator. Furthermore, culturing by shaking can generate aerosols containing the pathogens. Considering these points, we set out to investigate the effectiveness of a static enrichment procedure using cholate and selenite.

The present chapter describes static selective enrichment of *Salmonella* in a selenite-cholate medium for *Salmonella* detection by the pCEIA in the presence of chicken fecal microflora. The application of this procedure is also described for measuring the level of *Salmonella* shed in feces.

MATERIALS AND METHODS

Media and chemicals. Buffered peptone water (no. 1810-17-9), brain heart infusion (no. 0037-01-6), yeast extract (no. 0127-01-7) and brilliant green sulfa agar (no. 0717-17-5) were purchased from Difco Laboratories (Detroit, MI). Trypticase soya agar (no. 11043) was supplied by BBL (Becton Dickinson Microbiology systems, Cockeysville, MD).

Sodium cholate (C-1254) was obtained from Sigma Chemical Co. (St. Louis, MO) and sodium hydrogen selenite (no.2630) from BDH Ltd. (Poole, UK). Stock solutions of 25 % (w/v) sodium cholate (cholate) and 15 % (w/v) sodium hydrogen selenite (selenite) were separately prepared in distilled water and sterilized through 0.45 μ m filters.

Immunoreagents for the pCEIA. The procedure of the pCEIA is described in the Methods in Chapter 2. Bacto *Salmonella* O antisera poly A (serogroups A, B, D, E₁, E₂, E₃, E₄, and L) (no. 2534-47-6) and poly B (serogroups C₁, C₂, F, G and H) (no. 2535-47), and rabbit anti-*Salmonella* group O: factor 35 antiserum (no. 2522-47) were purchased from Difco Laboratories. Mouse monoclonal antibody, M-181 (ascites) was obtained from Dr. B. W. Brooks of Animal Diseases Research Institute (ADRI), Agriculture and Agri-Food Canada (AAFC) (Nepean, ON). Anti-rabbit IgG antibody peroxidase conjugate (A-6154) and anti-mouse IgG antibody-peroxidase conjugate (A-4416) were supplied by Sigma Chemical Co. *Salmonella* LPS antigens captured by polymyxin-cloth was detected by an equal mixture of Bacto *Salmonella* O antisera poly A and poly B, followed by the reaction with anti-rabbit IgG antibody-peroxidase conjugate. The antibody mixture (diluted 10³ times) and conjugate (diluted 10³ times) were used in phosphate-buffered saline (pH 7.3) containing 0.05 % Tween 20 (PBST). Alternatively for the detection of endogenous *Salmonella* in feces, an antibody mixture that detects all the *Salmonella* serogroups was used as previously described (Wang *et al.*, 1996). The detector antibody was an equal mixture of the mouse monoclonal anti-*Salmonella* LPS antibody and the rabbit anti-*Salmonella* group O: factor 35 antiserum. Before use, the

detector antibody mixture was diluted 400 times with PBST. The detector antibodies, bound to the captured LPS antigens, were detected by immuno-reaction with an equal mixture of the anti-mouse IgG antibody-peroxidase conjugate (diluted 10^3 times) and the anti-rabbit IgG antibody-peroxidase conjugate (diluted 10^3 times).

Bacteria. Dr. B. W. Blais of Laboratory Services Division, AAFC (Ottawa, ON) provided the following *Salmonella* strains: *S. typhimurium* ATCC 14028, *S. enteritidis* ATCC 13076, *S. hadar*, *S. heidelberg* and *S. infantis*. These serotypes represent those most commonly found in poultry products, as well as in human patients (Pope *et al.*, 1991a,b; Lior and Khakhria, 1993).

All *Salmonella* stock cultures were kept on trypticase soya agar (TSA) slants at 4 °C and transferred every two months. Overnight cultures of *Salmonella* were prepared by inoculating in 30 ml of buffered peptone water (BPW) and incubating at 37 °C, without shaking, for 16 – 18 h. Viable colony counts were performed by the spread-plate method on TSA plates.

Fecal samples. Chicken feces were collected from Single Comb White Leghorns at ADRI. The feces taken from *Salmonella*-free flocks were homogenized, stored frozen in 5 g aliquots at -70 °C, and thawed at room temperature for 30 min prior to the subsequent experiments. The absence of *Salmonella* in the feces was determined by both the cultural method and the pCEIA.

Feces were collected from two groups of flocks, orally inoculated three days earlier with either *S. typhimurium* (Group 1) or *S. enteritidis* (Group 2), at ADRI (E. Riche, personal communication). These feces were analyzed without freezing for the presence of *Salmonella* and its levels by the pCEIA. The aerobic count of the fecal microflora was estimated upon incubation of serially diluted fecal suspension on TSA plates at 37 °C for 48 h. The absence or presence of *Salmonella* in feces was confirmed by inoculating serially diluted fecal suspensions on brilliant green sulfa agar (BGSA) plates at 37 °C for 48 h.

Experiment 3.1 Effects of various concentrations of selenite on the growth of *S. typhimurium* under static culture. Overnight culture of *S. typhimurium* was diluted in saline (0.85 % NaCl in H₂O) to a cell density of approximately 10⁶ cfu / ml. One ml of the cell suspension was mixed with 40 ml of brain heart infusion supplemented with 0.5 % (w/v) yeast extract and 0.5 % (w/v) cholate (BYC), BYC + 0.3 % selenite, BYC + 0.5 % selenite, or BYC + 0.7 % selenite. The cultures were incubated at 37 °C without shaking. At various intervals, 0.1 ml aliquots of the cultures were withdrawn and appropriately diluted with saline and plated onto TSA plates. Colonies on TSA plates were counted after incubating the plates at 37 °C for 24 h, and cell densities were plotted on a logarithmic scale against time.

Experiment 3.2 Effect of 0.6 % selenite on recovery of *Salmonella* under static culture. Overnight cultures of *S. typhimurium*, *S. enteritidis*, *S. hadar*, *S. heidelberg* and

S. infantis were diluted in saline to a cell density of approximately 10^3 cfu / ml. Triplicate 1 ml aliquots of the *Salmonella* cell suspension were decimally diluted to extinction in 9 ml of either BYC or BYC + 0.6 % selenite. All the dilutions were incubated at 37 °C without shaking for 22 – 24 h. The presence (+) or absence (–) of growth in each dilution was determined by observing turbidity or by plating a 0.1 ml aliquot of culture on TSA plates and incubating the plates at 37 °C for 24 h. The dilutions that showed weak turbidity were recorded as $\pm$.

Experiment 3.3 Effects of cholate and selenite on static enrichment of *Salmonella* with various amounts of chicken fecal microflora assayed by the pCEIA. One g of the thawed chicken feces were homogenized with 9 ml of saline. After a brief sedimentation, the upper portion of the chicken feces suspension was assayed for aerobic count on TSA after incubation for 24 h at 37 °C. The upper portion containing aerobic counts of 5×10^7 cfu / ml was used as a source of non-*Salmonella* bacteria. Triplicate 1 ml aliquots of feces suspension were decimally diluted to a final average cell number of 5 cells in 9 ml of either BY, BYC, BY + 0.3 % selenite, or BYC + 0.3 % selenite. Overnight cultures *S. typhimurium*, *S. enteritidis*, *S. hadar*, *S. heidelberg* and *S. infantis* were diluted in saline to produce an average cell density of 40 – 80 cfu / ml. One-tenth ml of the *Salmonella* suspension was mixed with each of the above feces dilutions and incubated at 37 °C, without shaking, for 22 – 24 h. Duplicate 0.1 ml of the same *Salmonella* suspensions were also plated on TSA for viable colony counts.

All the dilutions were tested for *Salmonella* using the pCEIA. Cholate was added to a final concentration of 0.5 % to the dilutions that had previously received no cholate. To extract the LPS antigens and kill *Salmonella*, all dilutions were autoclaved at 121 °C for 5 min. The dilutions were allowed to cool to room temperature before storing at 4 °C. The stored dilutions were subjected to the pCEIA, at a later time.

Experiment 3.4 Detection of *Salmonella* in chicken feces. A cotton swab (0.5 cm diameter head) was moistened with 0.1 ml sterile saline and inserted into chicken feces. The swab was placed into a culture tube containing 5 ml BYC + 0.3 % selenite (BYCS). The culture tube containing the swab was vortexed for 10 sec and incubated at 37 °C for 20 h without shaking. The tube was then heated at 100 °C for 10 min or autoclaved at 121°C for 5 min in order to extract LPS antigens. The sample was cooled to room temperature before analysis with the pCEIA. All the experiments were repeated three or more times.

Experiment 3.5 Measurement of the fecal *Salmonella* level. The feces collected from orally inoculated flocks were homogenized. One gram was taken from the feces in duplicate, suspended in 9 ml BYSC, and decimally diluted in BYSC to a final dilution of 10^{-10} . All the dilutions were then incubated at 37 °C for 20 h without shaking, heated as above, and analyzed by the pCEIA. Presumptive *Salmonella* colonies were counted after incubating appropriate dilutions of the fecal suspension on BGSA plates at 37 °C for 48h.

RESULTS

Effects of various concentrations of selenite on the growth of *S. typhimurium* under static culture. Chapter 2 has demonstrated that, under agitation (shaking), a nutrient rich medium containing 0.5 % cholate (BYC) and 0.7 % selenite enriches low numbers (as few as 1 cell) of *S. typhimurium* to sufficient cell densities for detection by the pCEIA. Static culturing is well suited to culturing a large number of samples for HACCP, but it may influence the recovery of *Salmonella* using a high concentration of selenite such as 0.7 %. Therefore, we examined the growth of *S. typhimurium* under static culturing in the presence of various concentrations of selenite in BYC. Figure 3.1 shows that, under static culturing, 0.7 % selenite significantly reduced net growth, while 0.3 % selenite showed little effect on net growth during early and mid-exponential growth phases. In the presence of 0.3 – 0.5 % selenite, the culture reached a maximum viable cell density that was lower than the culture without selenite, with the viable cell density declining beyond the maximum, suggesting cell death. We speculated, however, that because LPS antigens are stable, the pCEIA should be able to detect LPS antigens from dead cells.

Choice of a selenite concentration for quantitative recovery of *Salmonella*. In Chapter 2, we have found that 0.7 % selenite showed little effect on the quantitative recovery of *S. typhimurium* under agitation. We postulated that static culturing may influence the recovery of *Salmonella* in the presence of selenite at a high concentration (e.g., 0.6 %). Because different serotypes of *Salmonella* may show different sensitivities to selenite, five serotypes commonly encountered in poultry samples in Canada were

Figure 3.1. Effects of various concentrations of selenite on the growth of *S. typhimurium* under static culturing ^a

^a One ml of *S. typhimurium* cell suspension (ca. 10^9 cfu / ml) was mixed with 40 ml of BYC containing various concentrations (0 – 0.7 %) of selenite and incubated at 37 °C without shaking. The cell densities were measured by plating on TSA at various intervals and plotted on a logarithm scale against time as described in the Methods (Experiment 3.1).

examined for their sensitivity to 0.6 % selenite. These *Salmonella* cells were diluted to extinction with BYC medium with or without 0.6 % selenite. After 24 h incubation, growth was determined by observing turbidity. Table 3.1 shows that *S. enteritidis* was the most sensitive serotype to 0.6 % selenite, with *S. hadar* and *S. heidelberg* being the least sensitive. The highest concentration of selenite that allowed for the recovery of *S. enteritidis* was then determined by comparing the effect of various selenite concentrations ranging from 0.3 – 0.6 %. While the presence of 0.3 % selenite had no effect on the recovery of *S. enteritidis*, selenite concentrations greater than 0.3 % resulted in lower recovery (results not shown). Therefore, for static culturing, 0.3 % selenite was used in subsequent experiments.

Effect of selenite and cholate on pCEIA of *Salmonella* statically cultured in the presence of microflora from chicken feces. We have previously demonstrated that under agitation a combination of 0.7 % selenite and 0.5 % cholate was effective in selecting against *E. coli* (Chapter 2). There are many other non-*Salmonella* microflora in poultry intestines which are regarded as sources of microbial contaminants in poultry products (Poppe *et al.*, 1991a,b). During *Salmonella* enrichment in fecal samples, these non-*Salmonella* microflora may interfere with the amplification and subsequent detection of *Salmonella*. The selective action of 0.3 % selenite and 0.5 % cholate, therefore, was investigated in *Salmonella*-free chicken feces as a source of non-*Salmonella* fecal microflora. For reproducibility, pooled fresh feces, frozen in small aliquots, were used throughout the present study. The fecal suspensions were decimally diluted with BY ±

Table 3.1. Effects of 0.6 % selenite on the recovery of *Salmonella* under static culturing ^a

<i>Salmonella</i> serotype	Supplement	Initial cell number (cfu / 9 ml)			
		10 ²	10 ¹	10 ⁰	10 ⁻¹
<i>S. typhimurium</i>	None	+	+	+	—
		+	+	+	—
		+	+	+	—
	0.6 % selenite	+	+	±	—
		+	±	±	—
		+	±	±	—
<i>S. enteritidis</i>	None	+	+	+	+
		+	+	+	—
		+	+	—	—
	0.6 % selenite	+	±	±	—
		+	±	—	—
		+	±	—	—
<i>S. hadar</i>	None	+	+	+	—
		+	+	+	—
		+	+	—	—
	0.6 % selenite	+	+	+	—
		+	+	+	—
		+	+	+	—
<i>S. heidelberg</i>	None	+	+	+	+
		+	+	+	—
		+	+	+	—
	0.6 % selenite	+	+	+	—
		+	+	+	—
		+	+	+	—
<i>S. infantis</i>	None	+	+	+	—
		+	+	+	—
		+	+	+	—
	0.6 % selenite	+	±	±	—
		+	±	±	—
		+	±	—	—

^a Overnight cultures of *Salmonella* were serially diluted in triplicates in BYC with or without 0.6 % selenite. Growth was determined after 24 h static culture as described in the Methods (Experiment 3.2). (+), strong turbidity; (—), no turbidity; (±), weak turbidity.

0.5 % cholate and ± 0.3 % selenite. An average number of 4 – 8 cells of five *Salmonella* serotypes was added to these dilutions, and resulting mixtures were incubated for 22 – 24 h. The recovery of *Salmonella* in each dilution was assayed by the pCEIA. Figure 3.2 shows the results of the pCEIA in the dot-blot format, in which the intensity of the spot reflects the degree of *Salmonella* enrichment in each sample. The pCEIA of feces alone, without *Salmonella*, incubated in the presence of both selenite and cholate, gave no spot. The feces alone, in the absence of selenite and cholate, gave very faint spots whose intensity was much weaker than the weakest spots shown in Figure 3.2. As expected, the presence of large amounts of feces resulted in a greater reduction in the intensity of the LPS antigen spots. However, with a combination of selenite and cholate, small numbers (4 – 8 cells) of *Salmonella* cells multiplied to give clear LPS spots (part **d**, Figure 3.2), even in the presence of the highest aerobic counts (10^7 cfu / ml) of fecal microflora. With cholate alone (part **b**), the degree of *Salmonella* enrichment was lower. The enrichment of *S. typhimurium* and *S. heidelberg*, however, benefited from the presence of cholate. When selenite alone was present (part **c**), *S. hadar* and *S. infantis* gave similar spots, as when both selenite and cholate were used. Since we cannot predict the serotypes of *Salmonella* present in samples, the use of both selenite and cholate is recommended. The missing spots from the first triplicate dilutions on cloth **4b** and cloth **4d** are likely due to the low *Salmonella* inocula introduced into the fecal dilutions, statistically distributing no *Salmonella* in the mixtures. The difference in intensity of the spots produced by different *Salmonella* serotypes can be explained in terms of the difference in the amounts and avidity of the detection antibodies to different serotypes in the commercial preparation

Figure 3.2. Effects of cholate and selenite on polymyxin-cloth enzyme immunoassay (pCEIA) of *Salmonella* statically cultured in the presence of microflora from chicken feces.

A suspension of chicken feces (aerobic count of 5×10^7 cfu / ml) was decimally diluted in triplicate with (a) BY; (b) BYC; (c) BY + 0.3 % selenite; and (d) BYC + 0.3 % selenite to produce tubes containing average total aerobic counts of 5×10^7 , 5×10^6 , 5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 , 50 and 5 (from left to right on each cloth sheet). To these dilutions, small numbers (4 – 8 cells) of five serotypes of *Salmonella* were added to all the feces dilutions in each column (1–5): 8 cells of *S. typhimurium* (column 1); 5 cells of *S. enteritidis* (column 2); 5 cells of *S. hadar* (column 3); 4 cells of *S. heidelberg* (column 4); and 7 cells of *S. infantis* (column 5). The feces-*Salmonella* mixtures were cultured at 37 °C for 22 – 24 h without shaking and assayed for *Salmonella* by the pCEIA as described in the Methods (Experiment 3.3).

Each cloth sheet (e.g., 1d) contains six rows of eight spots (representing *Salmonella* lipopolysaccharide antigens captured on polymyxin-cloth). The first and second rows represent duplicate samples of the first triplicate dilutions; the third and fourth rows, duplicate samples of the second triplicate dilutions; the fifth and sixth rows, duplicate samples of the third triplicate dilutions.

used, as well as the difference in the ability of different *Salmonella* serotypes to compete with non-*Salmonella* microflora.

Detection of *Salmonella* and measurement of *Salmonella* level in chicken feces.

Figure 3.2 shows that the intensity of the pCEIA signals with *Salmonella* O-antisera is not equal for all serotypes. Furthermore, pooled *Salmonella* O-antisera in the pCEIA has been shown to cross-react with *Pseudomonas aeruginosa* grown in medium containing 0.1 % deoxycholate (Blais *et al.*, 1993). Thus, we considered the use of monoclonal antibodies to an epitope common to all *Salmonella* serotypes. Wang *et al.* (1996) have shown that the use of M-181, a mouse monoclonal antibody to the outer-core region of *Salmonella* LPS (Figure 1.6, Chapter 1), permits the detection of all *Salmonella* serogroups, except the O serogroup, which can be detected by supplementing M-181 with a single factor rabbit antiserum to *Salmonella* O serogroup. According Wang *et al.* (1996), all of the 76 strains of salmonellae tested, which comprise all the serogroups, were detected in pure cultures by M-181 supplemented with this rabbit antiserum, whereas none of the 24 non-salmonellae was detected. In the use of this mixed antibody as a detector antibody, the detection should be highly specific for all serogroups of *Salmonella*. *Salmonella* detection in chicken feces by the pCEIA was thus studied using this mixed antibody. A portion (50 mg) of salmonellae-free chicken feces was mixed with 5 ml of BYC + 0.3 % selenite (BYCS) containing 5 – 15 cells of three salmonellae serotypes, incubated at 37 °C for 20 h, without shaking, and then assayed for *Salmonella* by the pCEIA. The maximum amount of feces collectable on the tested cotton swab was

about 50 mg. A fixed amount (50 mg rather than a swab-full) of the feces was used in this study to provide a reproducible number of fecal non-*Salmonella* microflora (an aerobic count of about 10^7 cfu / ml) in all the samples, although the use of a cotton swab is recommended for routine collection of fecal samples. Performing the procedure described in the Methods (Experiment 3.4), the presence of a small number (1 cfu / ml) of *S. typhimurium*, *S. enteritidis*, or *S. hadar* in 5 ml of chicken fecal suspension in BYCS was detected by the pCEIA after the selective enrichment. Therefore, we could apply this procedure to detect a small number of *Salmonella* in feces shed by infected birds. This detector antibody mixture was thus used in subsequent experiments.

To study the detection of endogenous *Salmonella* present in chicken feces, fecal droppings were collected from two chicken flocks orally inoculated 3 days earlier with either *S. typhimurium* (Group 1) or *S. enteritidis* (Group 2). Fifty mg of an individual dropping was homogenized by vortexing it with 5 ml of BYSC, incubated at 37 °C for 20 h without shaking and prepared for *Salmonella* detection by the pCEIA as described in the Methods (Experiment 3.4). The pCEIA of the selectively enriched cultures indicated the presence of *Salmonella* in four out of five fecal droppings from Group 1 and all five droppings from Group 2.

The overall level of *Salmonella* infection in the flocks is important in the sanitation of poultry processing plants, as the total number of *Salmonella* entering a plant influences the fecal *Salmonella* in a flock. About 100 g of feces from Group 1 or Group 2 was combined and homogenized. A one g portion was taken in duplicate from each fecal sample pool, suspended in 9 ml BYSC, and decimally diluted in BYSC to a final

dilution of 10^{-10} . After a 20 h static incubation at 37 °C, the presence or absence of *Salmonella* in all the dilutions was determined by the pCEIA. Table 3.2 shows that *Salmonella* was not detected after a 10^{-4} dilution for the Group 1 sample and after 10^{-2} dilution for the Group 2 sample. Thus, it could be estimated that the Group 1 feces contained $10^4 - 10^5$ *Salmonella* cells / g while the Group 2 feces contained $10^2 - 10^3$ *Salmonella* cells / g. These results agreed with presumptive plate counts for fecal samples, 2.8×10^4 cfu / g for Group 1, and 10^3 cfu / g for Group 2.

DISCUSSION

We have found that under static culturing a combination of 0.3 % selenite and 0.5 % cholate was effective in selectively enriching small numbers of *Salmonella* in the presence of large excesses of chicken fecal microflora, so that strong signals in the pCEIA were achieved. Assessment of *Salmonella* levels based on a dilution end-point principle needs the analysis of all the dilutions generated (e.g., 10 dilutions per sample). Static incubation is not only convenient in accommodating such a large number of samples, but is also safer to perform compared to shaking cultures, which could generate aerosols that are capable of spreading the pathogen.

Freezing the feces may have caused some degree of injury to fecal microflora which in turn may have reduced the ability of the fecal microflora to interfere with enrichment and the pCEIA for *Salmonella* detection. However, the detection capacity of the pCEIA was greatly reduced in the presence of large excesses of pre-frozen fecal microflora when the enrichment medium contained no selenite and cholate. Thus, we

Table 3.2. The level of endogenous *Salmonella* in feces from infected chicken flocks ^a

	Dilution of fecal samples									
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰
Group 1	+	+	+	+	–	–	–	–	–	–
	+	+	+	+	–	–	–	–	–	–
Group 2	+	+	–	–	–	–	–	–	–	–
	+	+	–	–	–	–	–	–	–	–

^aOne g of feces was collected in duplicate from two flocks orally infected with either *S. typhimurium* (Group 1) or *S. enteritidis* (Group 2), mixed with 9 ml of selective medium (BYSC), and decimally diluted with BYSC. After a 20 h static incubation, the samples were assayed for the presence (+) or absence (–) of *Salmonella* by polymyxin-cloth enzyme immunoassay. The experimental procedure is detailed in the Methods (Experiment 3.5).

could conclude that this selective condition suppresses the growth of non-*Salmonella* gram-negative bacteria (whose LPS would compete with *Salmonella* LPS for binding to polymyxin), as well as fecal microflora which would compete for nutrients or inactivate *Salmonella*. The presence of such microflora in chicken feces has been documented (Pivnick and Nurmi, 1982). The total procedure of this *Salmonella* detection in feces took less than 24 h. The use of the monoclonal antibody supplemented with the rabbit antiserum to *Salmonella* O serogroup should permit a highly specific *Salmonella* detection (Wang, *et al.*, 1996). This simple procedure can be applied to routine screening of *Salmonella* shedding in poultry as well as to *Salmonella* testing of stool from human patients and the detection of *Salmonella* carriers among food handlers.

The present procedure could be utilized to determine the presence and levels of *Salmonella* in poultry carcass meat, considering that the competing microflora in poultry are derived from feces. Uninjured cells were used here as we assumed that *Salmonella* cells present in feces are most unlikely injured. However, when employing this procedure for *Salmonella* detection in poultry carcasses, the presence of injured *Salmonella* should be taken into account. In the following chapters, we attempt to implement this procedure for *Salmonella* detection in poultry products.

Chapter 4

Evaluation of surfactants for toxicity to *Salmonella* and the release of *Salmonella* from chicken skin

ABSTRACT

A surfactant in rinse sampling fluid may facilitate the dislodging of *Salmonella* cells in the fatty layer of chicken carcass skin, but should be non-toxic to *Salmonella*. Sodium cholate and Brij 58 at a concentration of 0.5 % did not show toxicity to either *Salmonella typhimurium* ATCC 14028 (wild type) or *Salmonella typhimurium* 324 (nalidixic acid resistant), while sodium dodecyl sulfate and Niaproof 4 showed moderate toxicity. Niaproof 8 and Tergitol 7 showed strong bactericidal action on *S. typhimurium* 324. The presence of 0.5 % cholate or Brij 58 in the rinsing fluid did not show any advantages in releasing cells from chicken skin inoculated with *Salmonella*. Niaproof 8 (2-ethylhexyl sulfate) in a neutralized form showed strong bactericidal action on other *Salmonella* serotypes and caused a significant reduction in aerobic counts of chicken fecal microflora. Thus, this surfactant could become an effective component of a neutral or alkaline detergent system as a disinfecting agent for food processing plants.

INTRODUCTION

The sensitivity of *Salmonella* detection in poultry is clearly affected by the sampling method used. Before applying the enrichment procedure using 0.5 % cholate and 0.3 % selenite described in Chapter 3 to *Salmonella* detection in poultry, an effective sampling method must be sought. Bacterial attachment to carcasses involves multiple mechanisms due to the complexity of both bacterial cells and carcass surfaces. After the scalding and plucking of the processing, the poultry carcass retains a film of processing water, which contains soluble and insoluble organic matters and a large number of bacteria. Bacterial attachment occurs in the scald and chill tanks, where water absorption

probably plays a role in the attachment by causing chicken skin to swell. The bacteria rapidly become attached to the skin; salmonellae have been shown to attach to poultry skin within 15 seconds of contact (Agriculture Canada, 1988). Although *Salmonella* existing in the interior of the poultry carcasses are more likely killed during cooking before consumption, those present on the skin can be readily transferred to surfaces, utensils and hands, which cause cross-contamination (Notermans and Kampelmacher, 1975). Therefore, the detection of the cells attached to chicken skin is important for the food safety of the consumer.

Todd and Harwig (1996) noted the superior sensitivity of whole carcass rinses for microbial detection over the blending of a portion of the carcass or surface swab sampling. By rinse sampling, however, only a portion of the bacterial flora present on the carcass is counted, due to incomplete dislodging of the bacteria from the skin surfaces (Fromm, 1959; Avens and Miller, 1970). Dickson *et al.* (1989) explained that the rupture of fat cells and the subsequent lipid coating of surfaces which commonly occurs at all stages of poultry processing, would account for a protective barrier for bacterial cells from sampling fluid. Thus, the addition of surfactants to sampling fluid may benefit the dislodging of *Salmonella*, since the surfactants can emulsify the fat-layer surface and dissociate the hydrophobic link which accounts for this protective barrier. The addition of 0.6 % Tergitol 7, sodium heptadecyl sulfate has been recommended in sampling fluid for *Salmonella* isolation from food samples, which is also to be used as pre-enrichment medium when the food sample contains large amounts of fat (Galton *et al.*, 1968; AOAC, 1984; FDA, 1992). The use of Tergitol 7 was probably first introduced by Galton *et al.* (1954) for the isolation of salmonellae naturally present in pork sausages, in order to

emulsify the top fatty layer of enrichment broth and facilitate free dispersion of the sample contents. They also claimed that Tergitol 7 enhanced the growth of salmonellae in culture. However, a number of scientists have found no significance in the addition of 0.6 % Tergitol 7 to sampling fluids for isolation of *Salmonella* from foods (Morris and Dunn., 1970; Cox and Blankenship, 1975). D'Aoust *et al.* (1982) even observed that the growth of *Salmonella typhimurium* was inhibited by this surfactant. Therefore, samples obtained from rinsing with 0.6 % Tergitol 7 may not represent a true occurrence of *Salmonella*, because some cells may be inactivated during sampling.

The purpose of the present study was to examine the bactericidal effects of the surfactants and the effects of non-toxic surfactants on the release of *Salmonella* cells from chicken skins. We hypothesized that anionic surfactants bound to the skin would be able to repulse the negatively charged *Salmonella* cells, thus, providing effective means for liberating the cells from the skin.

MATERIALS AND METHODS

Media and chemicals. Buffered peptone water (BPW) (no. 1810-17-9) and MacConkey agar base (no. 0818-01) were purchased from Difco Laboratories (Detroit, MI). Trypticase soy agar (TSA) (no. 11043) was obtained from Becton Dickinson Microbiology Systems (Cockeysville, MD). Nalidixic acid, sodium salt (N-4382), sodium cholate (C-1254), sodium dodecyl sulfate (SDS) (L-5750), Triton X-100 (T-6878), Niaproof 4 (N-1404 type 4) and Niaproof 8 (N-1404 type 8) were supplied by Sigma Chemical Co. (St. Louis, MO). Brij 58 (no. 4233B) was from Atlas Chemical Industrial Inc. and Tergitol 7 (no. 2-3549) from J. T. Baker Chemical Co. (Phillipsburg,

NJ).

Bacteria and growth condition. *Salmonella typhimurium* 324, nalidixic acid resistant (Nal^r) was obtained from the Health of Animal Laboratory (Guelph, ON). *S. typhimurium* ATCC 14028 (wild type), *S. enteritidis* ATCC 13076, *S. hadar*, *S. heidelberg* and *S. infantis* were supplied by Dr. B. W. Blais, Laboratory Services Division, Agriculture and Agri-Food Canada (AAFC) (Ottawa, ON). The bacteria were grown in BPW at 37 °C for 16 – 18h, without shaking. The cell densities are determined by colony counts on TSA plates, except for *S. typhimurium* 324 on TSA containing 25 µg/ml nalidixic acid (TSA + Nal) plates incubated at 37 °C for 24 h. MacConkey agar containing 25 µg/ml nalidixic acid (MCA+Nal) plates were used to enumerate the released *S. typhimurium* 324 cells from chicken skin.

Chicken feces were collected from Single Comb White Leghorns at the Animal Diseases Research Institute, AAFC (Nepean, ON). The feces were homogenized and stored in 2 g aliquots at –70 °C. Prior to the experiments, the feces were thawed at room temperature for 30 min. The aerobic colony counts were obtained by plating on TSA at 37 °C for 24 h.

Experiment 4.1 Toxicity of surfactants to *Salmonella*. Overnight cultures of *S. typhimurium* (wild type) and *S. typhimurium* (Nal^r) were diluted 10² times to a cell density of ca. 10⁷ cfu / ml in 0 or 0.5 % (w/v) solutions of: sodium cholate, SDS, Brij 58, Triton X-100, Tergitol 7, Niaproof 4 or Niaproof 8 in 0.9 % NaCl. After 5 min

incubation at room temperature without shaking, the surviving cells were determined by plating a 100 µl portion of appropriately diluted solution on TSA plate and incubating at 37 °C for 24 h.

Experiment 4.2 Release of *Salmonella* cells from chicken skin. Carcass skin was removed from chicken legs (purchased from a local retail store) and autoclaved with 200 ml of distilled water (dH₂O) at 121°C for 15 min. The fatty portion was removed from the upper oily layer and autoclaved at 121 °C for another 15 min. This chicken fat was used as a diluent of *Salmonella* overnight culture for inoculation. Fresh chicken thighs were purchased from the local retail store. The skin was removed from the thighs and cut into about 5 × 5 cm pieces (weighed 5-6 g), washed thoroughly with dH₂O and blotted with paper towels. One ml of an overnight culture of *S. typhimurium* (Nal^r) was mixed with 3 ml of the above liquefied chicken fat, and one ml (containing ca. 5×10^7 cfu / ml) of the inoculated chicken fat was placed on a piece of chicken thigh skin in a sterile beaker. After 2 h incubation at room temperature, the inoculated skin was shaken at 150 rpm, at 25 °C, for 5 min with 100 ml of 0.5 % (w/v) sodium cholate, 0.5 % (w/v) Brij 58, 0.9 % NaCl or dH₂O. Each rinse solution was diluted 10³ times and 100 µl portions were spread and incubated on MCA+Nal plates at 37 °C for 24 h for colony counts. The presence of surfactants (5 ppm) in the diluted mixtures did not affect the plating efficiency of *S. typhimurium* (Nal^r) on MCA + Nal.

Experiment 4.3 Effect of NP 8 on growth of *Salmonella enteritidis*. An overnight culture (ca. 5×10^8 cfu / ml) of *S. enteritidis* was diluted 100 times with BPW and shaken at 150 rpm at 37 °C for 2 h. The culture was divided into 6 portions, and 10 % (w/v) NP 8 stock solution was added to various concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1.0 %). The growth was followed by absorbance at 500 nm (A_{500}) at 30 min intervals.

Experiment 4.4 Effect of NP 8 on viability of *Salmonella* strains. Overnight cultures (ca. 5×10^8 cfu / ml) of five different *Salmonella* serotypes were mixed individually with 100 volumes of 1 % (w/v) NP 8 in 0.9 % NaCl in triplicate. After 5 min incubation at room temperature (21 – 22 °C), the mixtures were immediately diluted 100 times with BPW, and 100 µl of the diluted solution was plated on TSA. The presence of NP 8 (0.01 %) in the diluted mixtures did not affect the plating efficiency of *Salmonella* on TSA.

Experiment 4.5 Determination of killing efficiency of NP 8. *S. enteritidis* cells were pelleted by centrifugation and suspended to a final cell density of ca. 10^{10} cfu / ml in 0.9 % (w/v) NaCl (saline) or 1 % NP 8 in saline. After 5 min incubation at room temperature, the cell suspension was decimally diluted and 100 µl of the dilutions were spread on TSA, to determine viable colony counts. The killing efficiency was expressed as the log of the number of cells exposed to the surfactant / the number of cells survived.

Experiment 4.6 Effect of NP 8 concentrations and treatment temperature on chicken fecal microflora. The chicken feces were vortexed in 9 volumes of BPW and left to

stand for a few min to let the solids settle. The upper portion of the settled suspension was removed, diluted 10 times with various concentrations (0 – 3 %) of NP 8 in saline, and incubated for various periods of time (0.5 – 5 min) at various temperatures (room temperature of 21 – 22 °C to 85 °C). The treated feces suspensions were decimally diluted and 100 µl of the dilutions were spread on TSA to determine aerobic colony counts. The NP 8 present in the dilutions that were used for colony counts (cc) was at concentrations of not more than 0.01 %. The presence of NP 8 (0.01 %) in the diluted mixtures did not affect the plating efficiency of *Salmonella* on TSA. The survival rate was calculated as $100 \times (\text{cc after treatment} / \text{cc before treatment}) \%$.

RESULTS AND DISCUSSION

Toxicity of surfactants to *Salmonella*. The surfactant to be used in a rinsing solution should be non-toxic to *Salmonella*, otherwise the cells may be killed during rinsing. To test the toxicity of various surfactants, overnight cultures of *S. typhimurium* (wild type) and *S. typhimurium* (Nal^r) were diluted to a cell density of ca. 10^7 cfu / ml in 0.5 % surfactant solutions listed on Table 4.1. Niaproof 4 (sodium 7-ethyl-2-methyl-undecyl sulfate) and Niaproof 8 (sodium 2-ethylhexyl sulfate) were tested here as possible substitutes for Tergitol 7 (sodium monoheptadecyl sulfate), since Tergitol 7 is no longer produced due to the unavailability of its primary precursor in the world market. After a 5 min incubation at room temperature, the surviving cells were counted and compared with the initial cell numbers (before exposure to the surfactant). Table 4.2 shows survival rates of *Salmonella* after 5 min exposure to the surfactants. Sodium cholate and Brij 58

Table 4.1. Chemical nomenclature of surfactants

Customary / trade name	Systematic name
Cholate	3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24oic acid
SDS	sodium dodecyl sulfate
Brij 58	polyoxyethylene(20)octyl ether
Triton X-100	octyl phenoxy polyethoxy ethanol
Tergitol 7	sodium monoheptadecyl sulfate
Niaproof 4	sodium 7-ethyl-2-methyl-4-undecyl sulfate
Niaproof 8	sodium 2-ethylhexyl sulfate

Table 4.2. Survival rates of *S. typhimurium* ATCC 14028 and *S. typhimurium* 324 upon 5 min exposure to various surfactants^a

	SC	SDS	B58	TX100	TG7	NP4	NP8	saline
<i>S. typhimurium</i> ATCC 14028	100	91	100	18	n/d	95	0	97
<i>S. typhimurium</i> 324	96	94	100	25	0	80	0	96

^a Overnight cultures of *S. typhimurium* ATCC 14028 and *S. typhimurium* 324 (Nal^r) were diluted 10² times to a cell density of ca. 10⁷ cfu/ml in 0.5 % (w/v) of sodium cholate (SC), sodium dodecyl sulfate (SDS), Brij 58 (B58), Triton X-100 (TX100), Tergitol 7 (TG7), Niaproof 4 (NP4), Niaproof 8 (NP8) or 0.9 % NaCl (saline). After a 5 min incubation at room temperature, the surviving cells were determined by plating in triplicate on TSA plate at 37 °C for 24 h. The survival rates (%) are shown as 100 × colony counts after exposure / colony counts before exposure; n/d, not determined.

did not show toxicity to either strain, while SDS and Niaproof 4 showed moderate toxicity. No colonies were found after 10^3 times dilution of the cell suspension exposed to 0.5 % Tergitol 7 and Niaproof 8. That is, these surfactants have bactericidal effects on the *Salmonella* strains. Since bactericidal surfactants can be used to reduce *Salmonella* incidence in poultry meats, the bactericidal effect of Niaproof 8 was further investigated and will be discussed later in this chapter. Because the surfactant solutions of sodium cholate and Brij 58 were found to be non-toxic, they were chosen as additives to the rinse solution to test releasing efficiency.

Release of *Salmonella* cells from chicken carcass skin. Since the cells on the chicken carcasses are considered protected by the fat-layer of the chicken skin (Dickson and Koohmaraie, 1989), liquefied chicken fat was used as diluent of *Salmonella* overnight culture to inoculate chicken skin (5×5 cm). A nalidixic acid resistant strain (Nal^r) of *S. typhimurium* was used to distinguish inoculated *Salmonella* from the endogenous *Salmonella* on the skin. The skin inoculated with ca. 5×10^7 cells of *S. typhimurium* 324 (Nal^r) was incubated at room temperature for 2 h, because it probably takes 2 h to process a whole chicken carcass. Table 4.3 shows the release rates of the cells from chicken skin, after rinsing with 100 ml of 0.5 % sodium cholate, 0.5 % Brij 58, 0.9 % NaCl (saline) or dH₂O. Rinsing with saline or dH₂O showed release rates of above 100 %, though with large deviations. The reason for this unexpectedly high recovery is unclear. The cells might have multiplied during the 2 h incubation, in which case the recovery of the cells in cholate or Brij 58 was less effective. Therefore, the notion that the presence of a

Table 4.3. Release of *S. typhimurium* 324 cells from chicken carcass skin ^a

	Cholate	Brij 58	saline	dH ₂ O
Release rates (%)	99 ± 1	40 ± 11	123 ± 57	126 ± 57

^a Skin removed from chicken thighs (5 × 5 cm pieces) was washed thoroughly with distilled water (dH₂O) and blotted with paper towels. One ml of chicken fat contained ca. 5×10^7 cfu / ml of *S. typhimurium* 324 (Nal^r) was placed on a piece of chicken carcass skin in a sterile beaker. After 2 h incubation at room temperature, the inoculated skin was shaken at 150 rpm at 25 °C for 5 min with 100 ml of 0.5 % sodium cholate, 0.5 % Brij 58, saline or dH₂O. Each rinse solution was diluted 10³ times and 100 µl portions were plated on MCA+Nal plates at 37 °C for 24 h to determine the number of cells released from the skin. The release rates (%) were obtained by $100 \times \text{colony counts in the initial inoculum} / \text{colony counts in the solution after rinsing}$ with standard deviations (n=3).

surfactant in rinsing fluid can facilitate the release of the *Salmonella* cells from chicken skin cannot be supported. D'Aoust *et al.* (1982b) have similarly concluded that no benefits or even erroneous results were found when various surfactants (including Tergitol-7 and Triton X-100) were tested as additives to the rinsing fluid. Therefore, in subsequent studies, no surfactants were used in the preparation of samples for the detection of *Salmonella* from poultry products.

Effect of NP 8 on growth of *Salmonella enteritidis*. At low pH levels (pH 2 – 3), some anionic surfactants show bactericidal action which generally diminishes at neutral or higher pH levels (Dychdala, 1977). Table 4.2 shows that NP 8 (sodium 2-ethylhexyl sulfate) in a neutralized form (pH 8.2 at 1 %) shows a strong bactericidal action on *S. typhimurium*. Since this surfactant could be added to a neutral detergent system as a disinfecting agent for sanitizing poultry processing equipment, the bactericidal action of NP 8 was further investigated using another common *Salmonella* serotype, *S. enteritidis*. An overnight culture of *S. enteritidis* was diluted 100 times with BPW and shaken at 150 rpm at 37 °C for 2 h. NP 8 was then added at various concentrations (0, 0.2, 0.4, 0.6, 0.8, or 1.0 %). The growth was followed by absorbance at 500 nm (A_{500}) at 30 min intervals. Figure 4.1 shows that the addition of NP 8 at an initial stage of the exponential phase caused an immediate cessation in the growth of *S. enteritidis*, when the NP 8 concentration was greater than 0.4 %.

Effect of NP 8 on viability of *Salmonella* strains. In order to see if the effect (shown in

Figure 4.1. Effect of NP 8 on *Salmonella* growth ^a

^a At time 0, the overnight culture of *S. enteritidis* was diluted 100 times with BPW. After 2 h shaking, the culture was exposed to various concentrations of NP 8 (0, 0.2, 0.4, 0.6, 0.8, and 1.0 %) and the growth was followed by A_{500} as described in the Methods (Experiment 4.4). The effects of concentrations higher than 0.4 % (0.6 – 1.0 %) were not shown here since they were practically identical to that of 0.4 %.

Figure 4.1) resulted from the bactericidal action of NP 8, cell suspensions (ca. 5×10^6 cfu / ml) of *S. enteritidis*, *S. typhimurium*, *S. hadar*, *S. heidelberg* and *S. infantis* were exposed to 1 % NP 8 for 5 min at room temperature, then diluted 100 times, and 100 μ l of the diluted mixtures were plated on TSA. None of the treated *Salmonella* samples produced colonies, while untreated samples produced the expected numbers of colonies. Another Niaproof anionic surfactant, NP 4 (sodium tetradecyl sulfate) as well as sodium dodecyl sulfate did not show a significant bactericidal effect on *Salmonella*, under the same test conditions. It is speculated that NP 8's bactericidal effect is due to its relatively small hydrocarbon portion which permits its permeation through the protective layers (e.g., outer membrane of *Salmonella*) to cause killing.

Determination of killing efficiency of NP 8. To determine the killing efficiency of NP 8, it was necessary to have some cells survive the treatment. Therefore, a highly concentrated cell suspension (ca. 10^{10} cfu / ml) of *S. enteritidis* was exposed to 1 % NP 8 for 5 min at room temperature. The killing efficiency expressed as the log of the number of treated cells / the number of survived cells, was 5.94. That is, 1 % NP 8 caused reduction by a factor of ca. 10^6 within 5 min at 21 – 22 °C.

Effect of NP 8 concentrations and treatment temperature on chicken fecal microflora. Since microbial contamination of poultry products often originates from fecal materials, it is important to study the bactericidal effect of NP 8 on fecal microflora for its application in poultry processing. The effect of 1 % NP 8 on chicken fecal

microflora was studied at various temperatures because, in practice, a disinfectant is often used at elevated temperatures. In order to see the effects of temperature, the NP 8 treatment time was reduced to 1 min rather than 5 min. Figure 4.2 shows that the aerobic counts of the fecal microflora was reduced to 20 % after treatment with 1 % NP 8 at 20 °C, and the survival rate decreased as the treatment temperature was elevated. For example, when the temperature was elevated to 41 – 42 °C, the survival rate was reduced to 5.9 %, while little effect was observed at the same temperature in the absence of NP 8. At this elevated temperature (41 – 42 °C), the survival rate was mildly decreased to 4.2 % when the treatment time was increased to 2 min or longer, indicating the presence of some microflora which were resistant to 1 % NP 8. Instead of elevating temperature, higher concentrations of NP 8 were examined for bactericidal effects at room temperature (21 – 22 °C) with a 1 min treatment. The survival rate of the fecal microflora markedly decreased when treated with higher concentrations of NP 8: 20 % survived at 1 % NP 8; 9 % at 2 % NP 8; and 0.8 % at 3 % NP 8. These results suggest the possibility of nearly complete disinfection of fecal contaminants by using a combination of higher concentrations of NP 8 and elevated temperatures. All the experiments were done using cell suspensions. Since planktonic cells exhibit lower thermotolerance and higher susceptibility to disinfectants than surface-attached cells (Dhir and Dodd, 1995), the bactericidal action on surface-attached cells (e.g., biofilms) should be studied for the use of NP 8 for cleaning poultry processing equipment.

NP 8 is used to assist the lye peeling of fruits and vegetables as a well-established commercial operation. Lye solutions increase the surface tension of water; therefore,

Figure 4.2. Effect of 1% NP 8 on chicken fecal microflora at various temperatures ^a

^a Chicken fecal microflora were treated with 1 % NP 8 or H₂O (control) for 1 min at various temperatures and the survival rates were determined by measuring aerobic counts before and after treatment, as described in the Methods (Experiment 4.6).

they wet most surfaces poorly and unevenly without penetrating the surface soil. NP 8 enhances the penetration and cleaning action of lye solutions by lowering the surface tension of water, thus permitting rapid wetting of the soil. In addition, NP 8 helps in solubilizing oily soil, dispersing solid soil and preventing their re-deposition. NP 8 is available as a 40 % liquid which is transparent and has no offensive odor. Its use, at a concentration less than 0.2 % in the lye solutions, has been approved by the US Food and Drug Administration (Anon, 1992); therefore, its use at a higher concentration as a disinfectant in the food industry can be approved if the NP 8 treatment is followed by sufficient rinsing with potable water.

Chapter 5

Enrichment of injured *salmonella* in poultry products for detection by polymyxin-cloth enzyme immunoassay

ABSTRACT

As injured *Salmonella* cells have potential to cause salmonellosis, their detection in foods is important to food safety. In pure cultures, a small number of heat-injured *Salmonella enteritidis* cells were detected by the polymyxin-cloth enzyme immunoassay after a two-hour static pre-enrichment in a brain heart infusion medium supplemented with 0.5 % yeast extract and 0.5 % cholate (BYC), followed by overnight static enrichment in the presence of 0.3 % selenite. However, in chicken carcass rinse, heat-injured *S. enteritidis* cells (400 cfu / ml) could not be detected even after a six-hour pre-enrichment in BYC, followed by overnight static enrichment in the presence of 0.3 % selenite; uninjured cells were detectable. Prolonged (e.g., 20 hours) pre-enrichment in BYC, followed by 20-hour incubation of a 10-fold dilution with BYC supplemented with 0.3 % selenite, permitted the detection of heat-injured *S. enteritidis* (less than 7 cfu / ml) in the chicken carcass rinse.

INTRODUCTION

While commonly used selective media such as tetrathionate brilliant green and Rappaport-Vassiliadis are toxic to *Salmonella* (Chen *et al.*, 1993), we have shown that a combination of 0.5 % cholate and 0.3 % selenite permits selective enrichment of small numbers of uninjured *Salmonella* for detection by the pCEIA, in large excesses of chicken fecal microflora with static incubation (Chapter 3). Feces are regarded as the sources of microbial contamination in poultry products (Poppe *et al.*, 1991a,b). Since this selective enrichment suppresses the growth of non-*Salmonella* fecal microflora, the

application of this selective enrichment to *Salmonella* detection in poultry carcasses is conceivable.

In processed foods, a substantial portion of the microbial population is injured due to sublethal physical or chemical treatments during the processing (Andrews, 1986). Since injured *Salmonella* have the potential to cause salmonellosis, the detection of injured *Salmonella* is important. Poultry products are common sources of *Salmonella* which may be in the injured state, as poultry processing includes treatments that cause cell damage, such as heat at the scalding stage (Agriculture Canada, 1988), and chemical treatments with sanitizers (e.g., trisodium phosphate and organic acid) (Bautista *et al.*, 1997). Therefore, an enrichment procedure is required to first resuscitate the damaged cells and then selectively enrich *Salmonella* to a detectable cell density in the presence of other poultry microflora.

The main objective of this thesis is to enrich injured *Salmonella* cells in the presence of poultry microflora for detection by the P-CEIA. Here, we describe the enrichment of heat-injured *Salmonella enteritidis* using a combination of 0.5 % cholate and 0.3 % selenite with static incubation.

MATERIALS AND METHODS

Media and chemicals. Trypticase soy agar (TSA) (no. 11043) was obtained from BBL (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). Buffered peptone water (BPW) (no. 1810-17-9), brain heart infusion (no. 0037-17-8), yeast extract (no. 0127-01-7), and brilliant green sulfa agar (BGSA) (no. 0717-01-3) were purchased from Difco Laboratories (Detroit, MI, USA). Sodium cholate (C-1254) was supplied by Sigma

Chemical Co. (St. Louis, MO, USA) and sodium hydrogen selenite (no. 2630) was purchased from BDH Inc. (Toronto, ON, Canada). Stock solutions of 25 % (w/v) sodium cholate (cholate) and 15 % (w/v) sodium hydrogen selenite (selenite) were separately prepared in distilled water and sterilized through 0.45 µm filters.

Immunoreagents for the pCEIA. The procedure of the pCEIA is described in the Methods in Chapter 2. We used the mouse monoclonal anti-*Salmonella* LPS antibody, M-181 (obtained from Dr. B. W. Brooks of ADRI, AAFC) at a 400-fold dilution, as it can practically detect all *Salmonella* occurring in poultry. Its use in *Salmonella* detection by the pCEIA has been previously described (Wang *et al.*, 1996). The detector antibody bound to the captured LPS antigens was detected by its reaction with anti-mouse IgG antibody-peroxidase conjugate (A-4416, Sigma Chemical Co.) diluted 10^3 times.

Bacteria, growth conditions and heat-injury. *S. enteritidis* (ATCC 13076) was grown in BPW for 16 – 18 h at 37 °C, without shaking. The cells were harvested by centrifugation at 10,000 g for 10 min and then suspended in 0.01 M phosphate buffer (pH 6.0) - 0.85 % (w/v) NaCl to a cell density of about 10^8 cfu / ml. For heat-injury, 10 ml of the cell suspension was shaken at 54 °C for 10 min at 140 rpm in a 250 ml flask and immediately chilled in an ice bath. The heat-injury rate was determined by plating appropriately diluted cell suspensions on non-selective TSA plates and selective BGSA plates, as previously described (Chen *et al.*, 1993). The heat-injury rate (%) was expressed as a proportion of injured cells in total population; $100 \times (\text{cc on TSA plate} - \text{cc$

on BGSA plate) / (cc on TSA plate). This heat-treatment generated injury rates of 80 to 95 %.

Experiment 5.1 Recovery of heat-injured *Salmonella* in media. The suspension of heat-injured cells was serially diluted in brain heart infusion medium containing 0.5 % (w/v) yeast extract (BY) or BY supplemented with 0.5 % (w/v) cholate (BYC). One triplicate set of the diluted sample culture was incubated for 20 h at 37 °C, without shaking, and autoclaved at 121 °C for 10 min to kill the cells and extract the LPS antigens for the pCEIA facilitated by the presence of 0.5 % cholate. The other four triplicate sets of the diluted cultures in BYC were incubated for 0, 2, 4, or 8 h at 37 °C, without shaking, and then a 1/50th volume of 15 % (w/v) selenite solution was added to each dilution to obtain a final concentration of 0.3 % selenite. After an additional 20 h incubation at 37 °C, all the cultures were heat-treated, as above, for *Salmonella* detection by the pCEIA.

Experiment 5.2 Recovery of *Salmonella* in inoculated poultry samples. Ground chicken and turkey meats were purchased from local retail stores. The meat samples were inoculated with various levels of uninjured or injured *S. enteritidis*, homogenized in nine parts of BYC and the insoluble particles were allowed to sediment. A whole chicken carcass (weighed 1 – 1.2 kg) was manually massaged with one liter of BPW in a sealed sterile plastic bag for 2 min and the rinse solution was collected in a sterile container. The carcass rinse was then inoculated with various levels of uninjured or injured *S.*

enteritidis. The upper homogenate portion of inoculated meat samples and inoculated carcass rinse samples were decimally diluted in triplicate with BYC to extinction. All the dilutions were incubated for 6 h at 37 °C, without shaking, and then 15 % selenite stock solution was added to a final concentration of 0.3 % (w/v). After an additional 20 h static incubation, all the cultures were autoclaved at 121 °C for 10 min and assayed by the pCEIA for the presence or absence of *Salmonella* LPS antigens. The uninoculated samples showed negative results by this procedure.

Experiment 5.3 Effect of 20 h pre-enrichment in cholate on the recovery of injured

***S. enteritidis*.** A whole chicken carcass was rinsed with one liter of BY as described above. The carcass rinse (150 ml) was inoculated with approximately 10^3 cells of injured or uninjured *S. enteritidis*. The inoculated bacteria in the rinse were pre-enriched with the addition of 0.5 % cholate (BYC), or without cholate (BY), for 20 h at 37 °C, in a stationary incubator. The pre-enrichment cultures were then decimally diluted to extinction in triplicate with BYC + 0.3 % selenite (BYCS), and all the dilutions were incubated for 20 h at 37 °C, without shaking. All the cultures were autoclaved at 121 °C for 10 min and assayed for the presence or absence of *Salmonella* LPS antigens by the pCEIA. The uninoculated carcass rinse showed negative results by this procedure.

RESULTS AND DISCUSSION

Effect of cholate and selenite on the recovery of heat-injured *S. enteritidis* in media.

We have previously demonstrated that with agitation (shaking) the presence of 0.5 %

cholate in a rich medium (BY) does not affect the recovery of heat-injured *Salmonella typhimurium* (Chapter 2), but does inhibit the growth of gram-positive bacteria. Since static culturing (in a stationary incubator) allows the safe processing of a large number of samples, the effect of cholate on the recovery of injured *Salmonella* under static culturing was examined. Heat-injury was used as a model for cell damage. *S. enteritidis* was chosen because it is the most sensitive *Salmonella* serotype to selenite (Chapter 3). *S. enteritidis* cells were severely heat-injured to an injury rate of 95 % and decimally diluted in the brain heart infusion-yeast extract broth (BY), or BY containing 0.5 % sodium cholate (BYC). After 20 h static culturing, the recovery of *S. enteritidis* was assayed by the pCEIA. Table 5.1 shows that 0.5 % cholate did not affect the recovery of highly injured *S. enteritidis* under static culturing.

Injured cells develop sensitivity to many chemical compounds such as selective agents and fail to multiply in selective media (Andrews, 1986). Therefore, the effect of selenite on the recovery of heat-injured *S. enteritidis* during resuscitation under static culturing was examined. Table 5.2 shows that 0.3 % selenite only mildly affected the recovery of low numbers (2 – 8 cells / 4.5 ml) of heat-injured *S. enteritidis*. However, the addition of selenite after 2 h culturing in BYC did not show any significant effect on the recovery when compared with the control (no selenite). Thus, when selenite is added after 2 h resuscitation in BYC (containing no other microflora), injured *S. enteritidis* can be recovered for detection by the pCEIA.

Recovery of *S. enteritidis* in inoculated poultry samples. Numerous kinds of bacteria

Table 5.1. Effect of cholate on the recovery of heat-injured *S. enteritidis* ^a

	Approximate no. of cells / tube				
	10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹
BY	+	+	+	—	—
	+	+	+	—	—
	+	+	+	—	—
BYC	+	+	+	+	—
	+	+	+	—	—
	+	+	+	—	—

^a A *S. enteritidis* cell suspension was heated to an injury rate of 95 % and diluted in triplicate with BY or BY containing 0.5 % cholate (BYC) to colony forming units / tube (4.5 ml) indicated above. After 20 h static incubation at 37 °C, BYC cultures and BY cultures after supplementing with 0.5 % cholate were autoclaved at 121 °C for 10 min and assayed for the presence (+) and absence (—) of *Salmonella* by the pCEIA.

Table 5.2. Effect of selenite on the recovery of heat-injured *S. enteritidis*^a

Time of selenite addition (hours after suspension in BY)	Approximate no. of cells / tube				
	200	40	8	2	0.4
No addition	+	+	+	+	–
	+	+	+	+	–
	+	+	+	–	–
0 h	+	+	+	+	–
	+	+	+	–	–
	+	+	–	–	–
2 h	+	+	+	+	–
	+	+	+	+	–
	+	+	+	–	–
4 h	+	+	+	+	–
	+	+	+	–	–
	+	+	+	–	–
8 h	+	+	+	+	–
	+	+	+	–	–
	+	+	+	–	–

^a Heat-injured *S. enteritidis* cells (injury rate of 81 %) were diluted in triplicate with BYC to various cfu / 4.5 ml. During static incubation at 37 °C, selenite was added at various times to its final concentration of 0.3 %, and incubated for additional 20 h. Triplicate samples were assayed for the presence (+) or absence (–) of *Salmonella* by the pCEIA, as described in the Methods (Experiment 5.1).

including spoilage bacteria cling to poultry carcass skin. Such microflora or other materials present in poultry meats could interfere with the recovery of *S. enteritidis*, particularly in the injured state. To examine this possibility, ground turkey and chicken meats were inoculated with various levels of uninjured or injured *S. enteritidis* and homogenized in nine parts of BYC. Similarly, the carcass rinse of a whole chicken carcass was inoculated. No *Salmonella* was detected from the uninoculated samples. The inoculated samples were then decimally diluted to extinction with BYC. All the dilutions were incubated without shaking for 6 h, as recommended by Chen *et al.* (1993), and then exposed to 0.3 % selenite. After an additional 20 h static incubation, the dilutions were assayed by the pCEIA for the presence or absence of *Salmonella* LPS antigens. Table 5.3 shows that while uninjured *S. enteritidis* cells recovered as efficiently as in the poultry-free media, the recovery of injured cells was markedly reduced in the poultry samples. Ground chicken meat caused more marked inhibition than ground turkey meat, whereas chicken carcass rinse caused total inhibition. This shows that the environment of the turkey meat was more favorable for *Salmonella* recovery, and it may explain the higher *Salmonella* incidence associated with turkey in Canada (Figure 1.3(a) and (b), Chapter 1). It is now clear that a 6 h pre-enrichment in BYC followed by a 20 h selective enrichment in selenite was not adequate for the recovery of the injured *Salmonella* in poultry samples. It is important to detect the injured cells in poultry for complete *Salmonella* surveillance; the enrichment must allow sufficient growth of injured *Salmonella* to the detection range by the detection method being used (e.g., $>10^6$ cfu / ml for the pCEIA).

Table 5.3. Recovery of *S. enteritidis* in inoculated poultry samples^a

Samples	Approx. cfu / 4.5 ml before dilution (10^0)	Uninjured					Injured				
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Ground turkey	30	+	+	-	-	-	+	-	-	-	-
		+	+	-	-	-	±	+	-	-	-
		+	+	-	-	-	±	±	-	-	-
	300	+	+	+	-	-	+	-	-	-	-
		+	+	+	-	-	+	-	+	-	-
		+	+	+	-	-	-	+	+	-	-
	3000	+	+	+	+	-	+	+	-	-	-
		+	+	+	+	-	+	±	+	-	-
		+	+	+	+	-	+	+	+	-	-
Ground chicken	10	+	-	-	-	-	-	-	-	-	-
		+	+	-	-	-	-	-	-	-	-
		+	+	-	-	-	-	-	-	-	-
	100	+	+	-	-	-	-	-	-	-	-
		+	+	-	-	-	-	-	-	-	-
		+	+	+	-	-	-	-	-	-	-
	1000	+	+	+	-	-	+	+	-	-	-
		+	+	+	+	-	±	±	±	-	-
		+	+	+	+	-	±	-	+	-	-
Chicken Carcass Rinse	20	+	+	-	-	-	-	-	-	-	-
		+	+	-	-	-	-	-	-	-	-
		+	+	-	-	-	-	-	-	-	-
	200	+	+	+	-	-	-	-	-	-	-
		+	+	+	-	-	-	-	-	-	-
		+	+	+	-	-	-	-	-	-	-
	2000	+	+	+	+	-	-	-	-	-	-
		+	+	+	+	-	-	-	-	-	-
		+	+	+	+	-	-	-	-	-	-

^a The heat-injury rates of *S. enteritidis* inoculated in ground turkey, ground chicken, and chicken carcass rinse were 84, 80, and 87 %, respectively. The experimental details were described in the Methods (Experiment 5.2). (±); weak pCEIA signals.

Effect of 20 h pre-enrichment in cholate on the recovery of injured *S. enteritidis*.

We postulated that the failure to recover the injured *Salmonella* in the carcass rinse (Table 5.3) resulted from the activities of non-*Salmonella* microflora in the rinse, as the carcass surface is a major site of microbial contamination (Mountney, 1976). Non-*Salmonella* microflora could either compete for nutrients or produce substances which inhibit the resuscitation or growth of injured *Salmonella*. The conventional *Salmonella* enrichment consists of 18-24 h pre-enrichment in non-selective media, followed by 18-24 h culturing of the 10-fold diluted pre-enriched culture with selective media (HPB, 1978; FDA, 1992). Unlike commonly used pre-enrichment media, such as nutrient broth and buffered peptone water, cholate in the present pre-enrichment medium (BYC) should suppress the growth of cholate-sensitive non-*Salmonella* microflora, which may inhibit the recovery of injured *S. enteritidis*. To test this, 150 ml of *Salmonella*-free chicken carcass rinse in BY was inoculated with approximately 10^3 cells of heat-injured or uninjured *S. enteritidis*, and incubated for 20 h with 0.5 % cholate (BYC) or without cholate (BY). The pre-enrichment cultures were then decimally diluted to extinction with BYCS (which contained 0.3 % selenite). All the dilutions were further incubated for 20 h and then assayed for the presence or absence of *Salmonella* LPS antigens by the pCEIA. The detection of *Salmonella* in the first decimal dilutions would be equivalent to the detection of *Salmonella* with the conventional *Salmonella* enrichment procedure (which uses 10 fold dilution of pre-enriched cultures). Minimum dilutions (in the above decimal dilution series) that show no detectable *Salmonella* after selective enrichment would indicate the extent of *Salmonella* growth in the pre-enriched cultures. Table 5.4

Table 5.4. Effect of 20 h pre-enrichment in cholate on the recovery of injured *S. enteritidis*^a

Pre-enrichment medium	<i>S. enteritidis</i> inoculum	Dilution factor of pre-enrichment cultures									
		10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹	10 ¹⁰
BY	Injured	-	+	+	+	+	-	±	-	-	-
		-	+	+	+	+	+	-	-	-	-
		-	+	+	+	+	+	-	-	-	-
BYC	Injured	+	+	+	+	+	+	-	-	-	-
		+	+	+	+	+	-	-	-	-	-
		+	+	+	+	+	-	-	-	-	-
BY	Uninjured	+	+	+	+	+	+	+	-	-	-
		+	+	+	+	+	+	+	-	-	-
		+	+	+	+	+	+	-	-	-	-
BYC	Uninjured	+	+	+	+	+	+	+	+	-	-
		+	+	+	+	+	+	+	+	-	-
		+	+	+	+	+	+	+	-	-	-

^a Chicken carcass rinse (in BY) was inoculated with injured (87 %) or uninjured *S. enteritidis* at ca 10³ cfu / 150 ml, and incubated for 20 h with 0.5 % cholate (BYC) or without cholate (BY). The pre-enriched cultures were decimally diluted with BYCS which contained 0.3 % selenite. After 20 h incubation, the presence (+) or absence (-) of *Salmonella* was assayed by the pCEIA. The experimental details were described in the Methods (Experiment 5.3).

shows that when injured *S. enteritidis* in the carcass rinse were pre-enriched without cholate for 20 h and incubated for 20 h after 10 fold dilution with BYCS, no *Salmonella* was detected by the pCEIA, whereas cholate in the pre-enrichment allowed for *Salmonella* detection. However, cholate did not show much effect on the extent of growth of injured *Salmonella* in the pre-enrichment. When uninjured *S. enteritidis* was enriched after 10 fold dilution of the pre-enrichment culture, the pCEIA detected *Salmonella*, regardless of the presence or absence of cholate in the pre-enrichment (though the extent of growth was somewhat greater in the presence of cholate). These results suggest that the absence of cholate in the pre-enrichment likely produces false-negative results in the detection of injured *Salmonella*, when the conventional enrichment procedure was employed for the carcass rinse.

The majority of microflora on chicken carcasses have their origin in feces and some chicken fecal microflora are known inhibit the growth of *Salmonella* in poultry intestine (Pivnick and Nurmi 1982). One interpretation of the present observations is that some microflora in the carcass rinse accumulate substances that are particularly inhibitory to the growth of injured *Salmonella* during the pre-enrichment, while the presence of cholate reduces the production of such inhibitory substances. This study has shown that overnight pre-enrichment with cholate followed by selective enrichment with selenite allows detection of heat-injured *Salmonella* in chicken carcass rinse. We are currently applying this enrichment procedure for the detection of endogenous *Salmonella* in chicken meats.

Chapter 6

General Conclusion

The application of a new food safety enhancement program, Hazard Analysis Critical Control Point (HACCP), will benefit from rapid, simple and economical methods of screening, and enumeration of foodborne pathogens such as *Salmonella*. For this purpose, polymyxin-cloth enzyme immunoassay (pCEIA) has been developed for the assay of gram-negative bacteria (Blais and Yamazaki, 1996). When a dot-blot format is used, the pCEIA permits the rapid and simultaneous analysis of *Salmonella* in multiple samples.

The major objective of my thesis research was to develop a procedure for enrichment of *Salmonella* in poultry samples for detection by the pCEIA, in which cholate and selenite are employed as selective agents. *Salmonella* normally represent a small portion of the total microbial population in foods and usually receive sublethal stress during processing and storage; therefore, it is necessary to first resuscitate the injured cells with pre-enrichment, and then selectively enrich *Salmonella* to a detectable cell density. A rich medium, brain heart infusion broth supplemented with 0.5 % yeast extract has been used, with the addition of 0.5 % cholate (BYC), as pre-enrichment medium. The addition of cholate to the pre-enrichment medium is beneficial not only for the suppression of some non-*Salmonella* microflora growth, but also for the facilitation of LPS extraction from the cell membrane for the pCEIA. Selenite is non-toxic to *Salmonella*, while some commonly used selective agents show toxicity even to uninjured *Salmonella* (Chen *et al.*, 1993). In order to effectively enrich injured *Salmonella* in foods, selenite has been chosen as a selective agent to assure cell recovery.

In this thesis, we have shown that longer pre-enrichment periods are required for the recovery of heat-injured *Salmonella* in high concentrations of selenite. With an

increasing concentration of selenite, the selectivity has increased against *E. coli* in the medium. The best combination of selenite concentration and the pre-enrichment period must be sought, in order to maximize the efficacy of selenite for the selective enrichment of heat-injured *Salmonella* in the presence of non-*Salmonella* microflora.

The implementation of HACCP will likely involve the microbial assay of a large number of samples. Culturing in a stationary incubator is suitable for the microbial analysis of multiple food samples, since it can accommodate a large volume of samples and reduce chances of spreading the pathogens via aerosols. In this thesis, we have shown that *Salmonella* cells become more susceptible to selenite when cultured in a static incubator than cultured under agitation. Therefore, static culturing in the selenite cystine selective medium, a commercial preparation (containing 0.4 % selenite), may not recover *Salmonella* (especially *S. enteritidis*). Similarly, the toxicity of selective media should be first investigated, before they are employed in the static condition.

We have also shown that static culturing, with a combination of 0.5 % cholate and 0.3 % selenite, enriches small numbers of *Salmonella* to sufficient cell densities for detection by the pCEIA, even in the presence of a large excess of chicken fecal microflora. This combination should be effective for static selective enrichment of *Salmonella* in highly contaminated samples. Based on the above findings, we have developed a rapid and simple detection procedure for *Salmonella* in feces. After sampling feces with a swab, *Salmonella* can be detected in about 22 h. Early detection of infected broiler flocks by this procedure will help prevent the cross-contamination among birds. This procedure should be also useful for *Salmonella* testing of stool from human patients and food handlers.

We have shown that poultry materials interfere with the recovery of injured *Salmonella*, while uninjured cells were not affected. Prolonged pre-enrichment was necessary for the detection of a small number of heat-injured *S. enteritidis* in the chicken carcass rinse. This implies a complex ecosystem of microflora in processed poultry carcasses. Thus, injured cells, in the presence of microflora other than the target pathogen, should be used when any selective enrichment media are evaluated for the detection of pathogens in foods.

The presence of cholate in the pre-enrichment medium has facilitated the recovery of injured *Salmonella* in the chicken carcass rinse, presumably suppressing the growth of poultry microflora, which compete with or inactivate *Salmonella* during the pre-enrichment. The results also suggest that the absence of cholate in the pre-enrichment, as seen in conventional enrichment procedure, will likely produces false-negative results in the detection of injured *Salmonella*. The duration and type of pre-enrichment is often discussed because the conventional pre-enrichment also promotes non-*Salmonella* microflora to outnumber *Salmonella*, and consequently *Salmonella* fail to multiply to a detectable level. The addition of cholate in the pre-enrichment medium would be a solution to this problem, as it acts as a mild selective agent.

The presence of non-toxic surfactants in the rinsing fluid has not shown advantages in releasing *Salmonella* cells from chicken skin. In the course of the surfactant evaluation, we have shown the strong bactericidal action of Niaproof 8 (2-ethylhexyl sulfate) in a neutralized form. Considering that FDA has approved its use in the lye peeling of fruits and vegetables, this surfactant could be used as a disinfectant in the food industry.

The usefulness of the pCEIA has been also demonstrated in this thesis. The presence of LPS molecules derived from large numbers (e.g., 10^9 cells) of *E. coli* did not interfere with the detection of *Salmonella* LPS by the pCEIA; therefore, it demonstrates the large capacity of polymyxin-cloth to capture LPS molecules. The enrichment of *Salmonella* for detection by the pCEIA thus does not need to be completely selective because the pCEIA allows *Salmonella* detection even in the presence of large numbers of other gram-negative bacteria.

As Blais and Yamazaki (1991) have demonstrated with food samples spiked with uninjured *Salmonella*, using a dilution end-point principle, the present procedure would be applicable to semi-quantitative analysis of *Salmonella* contamination of poultry products. Being able to simultaneously handle multiple samples, the pCEIA enables this type of semi-quantitative analysis of a large number of samples. In addition, static culturing accommodates large volumes of samples and is safer to perform compared to shaking cultures which could generate aerosols. These points are particularly important for measuring the contamination levels of poultry within the processing plants.

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