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**THE POLYMYXIN-CLOTH ENZYME IMMUNOASSAY
FOR DETECTION OF SALMONELLAE AND ITS CONFIRMATION
BY POLYMERASE CHAIN REACTION**

by

Haiyan Wang, B.Sc., M.Sc.

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy**

Department of Biology

Carleton University

Ottawa, Ontario

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**THE POLYMYXIN-CLOTH ENZYME IMMUNOASSAY FOR DETECTION OF
SALMONELLAE AND ITS CONFIRMATION BY POLYMERASE CHAIN
REACTION**

submitted by

Haiyan Wang, B.Sc., M.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisor

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ABSTRACT

This thesis examines applications of a rapid, simple and economical method for the detection of the food pathogen Salmonella based on the polymyxin-cloth enzyme immunoassay (polymyxin-CEIA).

A monoclonal antibody to the common core of lipopolysaccharide (LPS) was examined in a comparison of several commercially available O-antigen polyclonal antisera as sources of detector antibodies for the Salmonella polymyxin-CEIA. The monoclonal antibody supplemented with a single factor O:35 rabbit antiserum was able to detect all of the 55 Salmonella serovars tested without causing any cross-reactions with non-Salmonella bacteria. Low levels of Salmonella cells spiked into various food samples were successfully detected after overnight enrichment.

An adaptation of the polymyxin-CEIA and a new enrichment culture method were developed to provide a rapid, simple and economical procedure for the detection of S. enteritidis in eggs. The egg samples were enriched in a minimal volume of a nutrient-rich broth overnight. The LPS antigens of S. enteritidis were then assayed by the polymyxin-CEIA using inexpensive anti-serogroup D (factor O:9) rabbit antiserum as detector antibody. This polymyxin-CEIA was highly specific for salmonellae bearing the factor O:9 antigen and was positive in the assay of 19 different S. enteritidis strains inoculated in eggs. The combined enrichment and polymyxin-CEIA required less than 24 h to complete and detected as few as 2 S. enteritidis cfu inoculated into a whole egg.

Presumptive positive results obtained in the polymyxin-CEIA were confirmed by subjecting pre-enriched samples directly to a PCR technique targeting Salmonella-

specific invA gene sequences developed for Salmonella detection in eggs. The entire procedure, from sample pre-enrichment, polymyxin-CEIA screening, to the final rapid confirmation by PCR, could be completed within 26 h.

A simple and inexpensive polymyxin-CEIA procedure was developed for serological identification of Salmonella O antigens by capture of the lipopolysaccharide antigens on polymyxin-coated polyester cloth followed by immunoenzymatic detection of the captured O antigens using rabbit Salmonella O antisera and an anti-rabbit IgG-peroxidase conjugate.

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ABBREVIATIONS

BACHS	biotinamidocaproate
	N-hydroxysuccinimide ester
BHI	brain heart infusion broth
BPW	buffered peptone water
°C	degrees celcius
CEIA	cloth-enzyme immunoassay
cfu	colony forming unit
EIA	enzyme immunoassay(s)
g	gram
h	hour(s)
HACCP	Hazard Analysis Critical Control Point
HRP	horseradish peroxidase
l	liter
LPS	lipopolysaccharide(s)
mg	milligram
ml	milliliter
μg	microgram (10 ⁻⁶ g)
μl	microliter (10 ⁻⁶ l)
min	minute(s)
Nal^r	nalidixic acid-resistant

NB	nutrient broth
O.D.	optical density
PBS	0.01 M phosphate buffer containing 0.85% NaCl (pH 7.2)
PBS⁺	0.01 M phosphate buffer containing 0.5 M NaCl (pH 7.3)
PBST	PBS containing 0.05% Tween 20
PBST-B	PBST containing 0.2% blocking reagent
PCR	polymerase chain reaction
PEG	polyethyleneglycol
polymyxin-CEIA	CEIA on polymyxin-coated cloth
RIA	radioimmunoassay
Strep-HRP	Streptavidine-peroxidase
TMB	3,3',5,5'-tetramethylbenzidine
TSA	trypticase soy agar
TSB	trypticase soy broth

GENERAL INTRODUCTION

Foods provide humans with nutrients and energy essential for survival. However, they can also serve as an important vehicle for the transmission of disease-causing agents, since humans are in close and frequent encounter with foods throughout their lives. There are many agents in foods which can cause disease in humans (Table 1). They may be intrinsic agents such as allergens naturally present in the food, or extrinsic agents such as chemical contaminants or pathogenic microorganisms.

Foodborne diseases are by no means a "new" problem. The first "Adulteration of Food and Drink Act" was passed in Britain in 1860 in response to the massive practice of food adulteration, which sometimes resulted in food poisoning (Varnam & Evans, 1991). The successful parliamentary passage of this Act was largely a result of a major food poisoning outbreak which affected hundreds of people and caused 21 deaths. The food involved was peppermint lozenges, which had been mistakenly adulterated with arsenic.

In the 20th century, microorganisms have become the major recognised agent of foodborne disease, although significant outbreaks of chemical food poisoning still occur (Varnam & Evans, 1991). Bacteria, such as Salmonella, Shigella, Campylobacter jejuni, C. coli and some strains of Escherichia coli are among the most important microorganisms associated with foodborne disease today. They may cause enteritis directly or indirectly by producing toxins in foods.

Statistics on the occurrence of foodborne disease outbreaks compiled by the Center for Disease Control (CDC) in Atlanta, Georgia, have shown that there is a great number of foodborne disease cases each year reported in the United States, among which

Table 1. Food poisoning agents.

Type	Agent	Example
Micro-organism	Bacteria	<u>Salmonella</u> Spp.
	Rickettsia	<u>Coxiella burnetii</u>
	Viruses	Hepatitis A
	Protozoa	<u>Giardia lamblia</u>
	Microfungi	Moulds
Chemical	Intrinsic	allergens
	Extrinsic	
	directly contaminated	Fungicide
	environmental	Agricultural chemicals
Parasite	Animal	Tapeworms
	Fish	Nematodes

the frequencies are highest for bacteria-caused diseases. For example, in 1988 there were almost 80,000 cases of reported foodborne disease in the U.S., which include 49,000 cases of salmonellosis and 30,000 cases of shigellosis (Zottola and Smith, 1990). Since the outbreaks of foodborne disease are actually estimated to be grossly underreported, the CDC estimates that more than 6 million cases of foodborne disease occur each year involving 9,100 fatalities. Todd (1989a,b) estimated the annual incidence of foodborne bacterial diseases at about 6.5 million cases in the U.S. and Canada, of which salmonellosis accounts for more than 50%. The annual cost in the U.S., including medical costs, loss of productivity, and product loss to the food industry through both voluntary and mandatory recalls, has been estimated by some researchers at approximately \$10 billion (Todd, 1984).

The genus Salmonella is a typical member of the family Enterobacteriaceae. They are Gram-negative, oxidase-negative, straight-sided, rod-shaped bacteria which are catalase-positive and show both respiratory and fermentative metabolism of carbohydrates. In addition, most salmonellae are motile by virtue of their peritrichous flagella (Varnam and Evans, 1991). There are more than 2,300 Salmonella serovars according to the Kauffmann-White antigenic scheme (Kauffmann, 1966; Varnam and Evans, 1991) for the identification of salmonellae, which is based on the somatic (O), flagellar (H) and capsular (Vi) antigenic profiles derived from agglutination reactions with homologous antisera. The O-antigens, which are most variable, are determined by the O-specific polysaccharide chain (O-chain) of lipopolysaccharides (LPS), the major components of the outer membrane of salmonellae and other members of

Fig.1. Cell wall of salmonellae and other gram negative bacteria showing the relative positions of outer membrane and other parts of the cell wall (Fomsgaard, 1990).

CM=cytoplasmatic membrane, PG=peptidoglycan layer, LP=lipoprotein, OM=outer membrane. Some bacteria have an outermost capsular layer of polysaccharides (KP).

Top: Section of the outer membrane (OM) showing LPS in the outer leaflet and the relative positions of lipid A, core and O-chain of LPS.

PL=phospholipids, P=proteins.

Enterobacteriaceae (Fig.1) (Fomsgaard, 1990).

Most members of the genus Salmonella cause the infectious disease salmonellosis in humans and other warm-blooded animals. There are two major types of human salmonellosis: typhoid (enteric) fever caused by S. typhi and nontyphoid infection (gastroenteritis) caused by other Salmonella serotypes. The symptoms of typhoid fever include malaise, anorexia, and headache followed by a gradual increase in temperature, which may last for several weeks and lead the patient to delirium (D'Aoust, 1989). Typhoid fever is relatively rare in the industrialized nations. The principal symptoms of nontyphoid infections are nonbloody diarrheal stools, abdominal pain, muscular pain, fever, nausea, vomiting, and prostration, which generally develop 8-72 h after the initial contact with the bacteria or bacteria-contaminated foods (D'Aoust, 1989).

Salmonella resides in the intestinal tract of humans and animals and can be spread in contaminated food, or from person to person (Gluic and Scrimgeour, 1994). The most frequently implicated foods include eggs, poultry, meat, meat products, and raw milk, etc.. Since salmonellae can multiply in a warm environment, the temperature abuse of Salmonella-contaminated foods often leads to the production of large numbers of this pathogen, which increase the risk of infection.

Although salmonellae have been recognised as causes of enteric disease for many years and control measures for their spread are well established, they are still the most important cause of foodborne infection worldwide. The dramatic increase in the number of salmonellosis cases in recent years has attracted increasing attention in industrialized countries, such as the United States, Canada and many European countries, due to the

high economic losses associated with this disease (Sockett, 1991). Infections of laying hens with one particular serotype, *S. enteritidis*, and the resultant contamination of eggs, particularly egg contents, is believed to be an important reason for this marked increase in human salmonellosis cases (Humphrey, 1994).

In order to control salmonellosis and other foodborne diseases more effectively, the Hazard Analysis and Critical Control Point (HACCP) system will be implemented in the food industries of the U.S., Canada and most European nations as a preventive food safety measure at all stages throughout the food chain from growing, harvesting, processing, manufacturing, distributing, and merchandising to preparing food for consumption (Stevenson, 1990; NACMF, 1992). In Canada, this plan is called the Food Safety Enhancement Program.

The HACCP system was first developed by Pillsbury Company in order to manage food processing and assure a safe food supply for the 1960s' U.S. space exploration program (Vail, 1994). Seven basic principles are included in the development of HACCP plans: 1) Hazard analysis; 2) Critical control point (CCP) identification; 3) Establishing critical limits for preventive measures associated with each identified CCP; 4) Establishing CCP monitoring requirements; 5) Establishing corrective actions when a deviation from an established critical limit occurs; 6) Effective record-keeping; 7) Verification procedure to ensure the HACCP system is working correctly. A critical control point is defined as a point, step or procedure during food manufacturing processes at which control can be applied and food safety hazard can be prevented, eliminated, or reduced to acceptable levels. The HACCP system applies not only to

microbial hazards (e.g. Salmonella) but also to chemical (e.g. pesticide residues) and physical (e.g. metal) hazards which also may cause a food to be unsafe for consumption.

In some food samples, particularly meats, salmonellae are usually present only in relatively small numbers and may be outnumbered by other bacteria. Some cells may be stressed by processing or environmental conditions. They need therefore to be first resuscitated and enriched to detectable levels in a nutrient-rich broth. The established cultural diagnosis procedure for Salmonella detection in foods requires the following steps (Andrews, 1985): pre-enrichment in nutrient-rich broth, selective enrichment in selective media, selective isolation on selective plates, biochemical characterization by proper tests, and finally serological confirmation using selected anti-Salmonella antisera. The whole procedure takes 4 days to obtain negative results and up to 7 days for positive ones (Andrews, 1985). Although these methods provide a theoretical level of sensitivity of one Salmonella cell per 25 g of food, the detection efficiency can be diminished by the presence of other microorganisms that can compete in growth with the salmonellae during cultural enrichment (Beckers *et al.*, 1987).

The cultural diagnosis procedure has been used widely for routinely monitoring food products for pathogens, however it is seldom an effective way for microbial hazard monitoring for HACCP plan since more frequent monitoring of microbial hazards in the food industry is required and the cultural diagnosis procedure is too time-consuming and too costly. There is a great need for rapid, sensitive, specific and economical methods for Salmonella detection which improve efficiency, reduce costs and can be applied in the HACCP system.

The commonly called "rapid methods" fall into two categories (Eyles, 1990). The first one includes those methods which aim primarily at reducing the amount of labour required. They automate conventional analyses (e.g. the Spiral Plater™ and automated colony counters) or use miniaturized kits and instruments for bacterial identification (e.g. API™ kits and Vitek™ instruments (Eyles, 1990). However, they do not substantially reduce the the time for final results. The second category may greatly reduce the time required for results. It includes measurements of metabolism, immunoassays, bacteriophage recognition, detection of bacterial DNA or RNA by gene probes or by the polymerase chain reaction (PCR) (Eyles, 1990; Blackburn, 1993).

One of the methods for Salmonella detection based on the measurement of metabolism is the use of inhibitory substances and elective substrates in growth media and the monitoring of the conductance by the commercially available conductance systems (e.g. the Bactometer® of bioMerieux UK Ltd) (Blackburn, 1993). Since salmonellae can reduce trimethylamine-N-oxide (TMAO) to trimethylamine (TMA), they can be detected by monitoring the conductance change in the pre-enrichment medium containing dulcitol and TMAO. The bacterial ability to produce ¹⁴CO₂ from ¹⁴C-labelled substrates was also used for Salmonella detection using a radiometer. A method was developed that used the specific inhibition of fermentation of ¹⁴C dulcitol in the presence of polyvalent H antisera. A large reduction in the production of ¹⁴CO₂ in the antiserum-medium indicates the presence of salmonellae. The conductance method was adopted by the Association of Analytical Chemists (AOAC) as a first action method (Andrews, 1993), however high false-positives were observed in radiometry method (Koning et al.,

1982).

Salmonella-specific bacteriophages such as Felix-O1 have been used for Salmonella detection. When Felix-O1 is introduced into the growth media during culturing of bacteria from foods, it replicates in most Salmonella cells and can be analysed by high-performance liquid chromatography (HPLC) (Hirsh and Martin, 1983). Since Felix-O1 can lyse its host cells, Salmonella can also be detected by introducing the bacteriophage into the selective solid media seeded with bacterial cells and observing the plaques formed on the plate (Blackburn, 1993). Most recently, genetically-modified bacteriophages have been constructed and applied to Salmonella detection. When Vibrio fischeri luciferase (*lux*) genes were introduced into the genome of Salmonella-specific bacteriophage, the infection of salmonellae by the phage led to the expression of the *lux* genes, resulting in bioluminescent bacteria (Schutzbank *et al.*, 1989; Turpin *et al.*, 1993). Another bacterial gene responsible for an ice-nucleating protein has also been incorporated into Salmonella-specific bacteriophage. The presence of Salmonella cells can be detected by the production of ice crystals in supercooled water (Worthy, 1990; Wolber and Green, 1990). However, the detection is determined by the effective infection of Salmonella cells by the bacteriophages and the infection is limited by the physiological state of the host bacteria, and physical and chemical conditions in the environment which is often difficult to control (Adams, 1959). The specificity of these assays has never been thoroughly evaluated.

Another approach involves the use of gene probes to detect Salmonella-specific nucleic acid sequences (e.g. virulence genes). A gene probe is a labelled DNA molecule

with a sequence complementary to DNA present in the pathogen to be detected, the target gene (Eyles, 1990). When the two strands of DNA containing the target gene are separated by heat or alkali, immobilized on solid phase (e.g. nylon membrane) and then allowed to recombine in the presence of the gene probe, the probe will bind to the complementary sequence of the target gene, and the label on the probe allows the target gene to be detected. The most commonly used labels are radioisotopes, biotin and digoxigenin. A mixture of several unique gene fragments isolated from S. typhimurium have been used as probes for specific detection of salmonellae in foods (Fitts *et al.*, 1983; Flowers, 1985). Many efforts have been exerted for the isolation of gene probes which can detect a wider range of Salmonella strains (Tsen *et al.*, 1991). Gene probes can also detect RNA sequences such as ribosomal RNA (rRNA) in bacterial cells (Eyles, 1990; Blackburn, 1993). The commercial Gene-Trak dipstick kit utilizes gene probes targeting 16s rRNA. Since there may be 1000-10,000 copies of rRNA per cell, while there is usually only one copy of a specific gene (DNA), testing for RNA instead of DNA improves the detectability (Sharpe, 1994). An unlabelled RNA probe has been used to detect Listeria monocytogenes (Blais and Phillippe, 1993a). In their procedure, the RNA-DNA hybrids were detected by an immunoenzymatic assay with an anti-RNA-DNA hybrid antibody.

The polymerase chain reaction (PCR) is a more recently developed technology for the sensitive and specific detection of target DNA, which can be used alone or in combination with a gene probing technique for bacterial detection. The PCR is based on the ability of DNA polymerase to synthesize complementary strand of a DNA sequence

Fig.2. The steps involved in the first few rounds of a polymerase chain reaction. The original template is single-stranded DNA denatured from the target DNA and the leftward and the rightward oligonucleotide primers are shown as $\blacktriangleleft$ and $\blacktriangleright$, respectively. The products of the first few rounds of the amplification reaction are heterogeneous in size. However, the sequence of DNA lying between the two primers is preferentially amplified and quickly becomes the dominant product of the PCR (Sambrook *et al.*, 1989).

in the presence of a short priming region of DNA (primer) (Sambrook *et al.*, 1989). Two oligonucleotide primers are used which have different sequences and are complementary to the sequences of the opposite strands of the target DNA. The dominant product of the PCR is the DNA sequence lying between the two primers (Fig.2). The PCR product can be analysed by agarose-gel electrophoresis alone or by gene probing technique. Several PCR strategies targeting different gene sequences have been developed for salmonellae (Rahn *et al.*, 1992; Aabo *et al.*, 1993; Iida *et al.*, 1993). However, PCR reactions are often inhibited by substances in foods or in enrichment media, and therefore the separation of bacterial cells are usually necessary. Also, PCR is too technically complex and expensive to perform on a routine basis, especially for large numbers of samples.

Yet another approach for the rapid detection of food pathogens is the immunoassay technique. Immunoassays are based on the highly specific nature of immunological reactions between antigens and antibodies. The reactions are mediated by the sum of many relatively weak noncovalent forces, including hydrophobic and hydrogen bonds, van der Waals forces, and the ionic interactions. The conformational fit between the three-dimensional configurations of the antigens and antibodies is very important for the interactions since those weak forces become effective only when the antigen molecules are close enough to the recesses on the surface of the antibody (Alberts *et al.*, 1991).

There are several types of immunoassays for salmonellae, including fluorescent-antibody (FA) staining, radioimmunoassay (RIA), enzyme immunoassay (EIA) and immunosensing methods (Blackburn, 1993). The FA staining uses a fluorescein-labelled

anti-salmonella antibody to detect Salmonella cells present in enriched culture (Gibbs *et al.*, 1972). The bacterial cells must first be fixed on a slide, then incubated with FA, and finally viewed under a microscope equipped with fluorescence optics. The FA staining technique has been approved by AOAC as an official method for presumptive detection of salmonellae, however, it has several drawbacks, such as a high frequency of false positives, the need for expensive equipment and trained technicians, the tedium involved in examining many microscopic fields for each specimen and the problems associated with the subjective judgement of the fluorescence intensity from the bacterial cells. These factors have limited its use in industrial scale operations (Thomason, 1981; Swaminathan *et al.*, 1985).

The RIA technique was developed by Dr. Yalow and her colleagues for insulin detection in the study of diabetes (Berson and Yalow, 1960). When a known amount of radio-labelled antigen standard and unlabelled antigen in a sample are mixed with a fixed amount of antibody, the amount of radioactive antigen-antibody (Ag-Ab) complex will be inversely proportional to the amount of unlabelled antigen, since both labelled and unlabelled antigens will compete for the same binding sites on the antibody. Therefore, by determining the amount of radioactive Ag-Ab, it is possible to measure the amount of the unlabelled antigen (Yalow, 1978). The Ag-Ab complex has to be separated from free Ag before measuring the radioactivity of Ag-Ab. The separation can be accomplished by immunoprecipitation of Ag-Ab by the use of an anti-globulin reagent, the removal of free antigen by adsorption onto charcoal, or the use of a solid-phase RIA format where the antibody is first immobilized onto particles which can be separated by

centrifugation. Although RIA has been used for the detection of salmonellae in foods (Ibrahim *et al.*, 1985; 1986), its disadvantages such as the requirement for expensive instruments for radioactivity measurement, the short shelflife of the radioisotopes and the handling of hazardous radioisotopes limit its wide application in the food industry.

In EIAs, antigens or antibodies are labelled with an appropriate enzyme instead of a radioisotope. The reaction can be detected by using a chromogenic, fluorogenic or chemiluminescence-generating substrate for the labelled enzyme. They are based on the assumption that an antibody or antigen can be linked to an enzyme without significantly affecting the binding property between Ab and Ag and the activity of the enzyme (Swaminathan *et al.*, 1985). The enzyme-labelled immunoreagents are easy to prepare, relatively inexpensive and reasonably stable, and EIAs are potentially highly sensitive. These advantages of EIAs have led to their extensive applications (Tijssen, 1985; Swaminathan *et al.*, 1985).

EIAs can be classified into two classes: homogeneous and heterogeneous EIAs, which can be further divided into competitive and non-competitive categories. In homogeneous EIAs, antibody and antigen react in a homogeneous phase, while in heterogeneous EIAs, the reactions occur on a solid phase (Schuurs and van Weeman, 1977). As in RIA, the reacted and unreacted immunoreagents (eg. enzyme labelled antibody) have to be separated before addition of the substrate solution. In the latter class the separation can be easily accomplished by washing the solid phase with a suitable buffer solution.

Homogeneous EIAs are usually applied for the measurement of haptens (eg.

hormones and drugs), while heterogenous type is used for the measurement of substances with high molecular weight such as bacterial antigens (Schuurs and van Weeman, 1977; Swaminathan *et al.*, 1985). The most popular formats for heterogeneous EIAs used in the detection of salmonellae in foods are illustrated in Figure 3. In the competitive assay, antigen in the test solution competes with antigen bound to the solid phase for binding sites on enzyme-labelled antibody, such that the concentration of antigen in the test sample is inversely proportional to the concentration of enzyme product (Fig.3a). The other three formats are of the non-competitive type. The "sandwich" assay uses one immobilized antibody to capture the antigen which can then be detected with a enzyme-labelled antibody (Fig.3b). In the direct assay, the antigen reacts directly with enzyme-labelled antibody (Fig.3c). However, in the indirect assay, the antigen is first reacted with an unlabelled antibody followed by reaction with an enzyme-labelled antibody against the first antibody to allow its detection (Fig.3d).

The solid matrices often used in heterogenous EIAs include: 1) non-porous solid phases, such as plastic microtitration plates; 2) microporous membranes, such as nylon and nitrocellulose; 3) beads, such as magnetic beads. The immunoreaction rates in heterogeneous EIAs based on non-porous solid phases are limited by the small solid surface area and the slow diffusion of the immunoreactants to the immobilized antibody or antigen on the solid phase surface. Although the microtitration plates accomodate multiple samples, the need for repeated pipetting to individual wells for each immunoreaction and washing step makes the assay tedious and slow, and sophisticated automated equipment is often used to handle the washings and pipettings. Microporous

Fig. 3. The most popular formats of heterogeneous EIAs for salmonellae

membranes have larger surface areas and accommodate multiple samples, however the accessibility of immunoreagent is limited by the micropores. In addition, they are thin and brittle, which limit the sample volume and make them difficult to handle. Beads have large surface area, however they only allow the samples handled individually.

Recently, EIA based on macroporous hydrophobic polyester cloth (Fig.4) have been described (Blais and Yamazaki, 1989a; Blais, 1990). The macroporous polyester cloths have high surface area and short diffusional distances between the adsorbed immunoreactants and sample immunoreactants, therefore permitting easy sample application and fast immunoreactions. As compared to microporous membranes such as nitrocellulose, they are structurally stable and thus allow easier washing and handling. Furthermore, large sheets of cloth can accommodate multiple samples which can be processed simultaneously in subsequent washing and immunoreaction steps. Such cloths are readily available and economical.

Antibody-coated polyester cloths have been used in a sandwich cloth-enzyme immunoassay (CEIA) system for detecting Brucella antigen (Blais et al., 1989) and Salmonella antigen (Blais and Yamazaki, 1989b). More recently, cloth coated with the antibiotic polymyxin B was used as an alternative to antibody-coated cloth to capture S. typhimurium lipopolysaccharide (LPS) antigens for their subsequent immunoenzymatic assay (Blais and Yamazaki, 1990a,b,1991).

Salmonella LPS, like other gram-negative bacteria, especially other enterobacteriaceae, are composed of three structural regions: the O-specific polysaccharide, which consists of oligosaccharide repeating units and determines the O-

serological specificity of the molecule; the oligosaccharide core, which is believed to be common to most Salmonella LPS; and lipid A, the lipid moiety of the LPS and by which the LPS is anchored to the cell surface (Fig.1 & 6). LPS molecule contains a variety of ionic groups, with acidic phosphates and carboxyl moieties concentrated within the core and lipid A regions and therefore is associated with a variety of cations, including the metal ions Mg^{2+} and Ca^{2+} (Wilkinson, 1983). These divalent cations act as metal-ion bridges between phosphate groups of LPS and are important for the maintenance of outer-membrane structural integrity (Storm *et al.*, 1977).

Polycationic antibiotics such as polymyxin B can interact with LPS , and therefore destabilize the outer membrane structure by displacing the divalent cations from LPS. Structurally, polymyxin B produced by Bacillus polymyxa is a cyclic peptide containing a short peptide side chain acylated with a eight or nine carbon fatty acid at its N-terminus (Fig.5). Therefore, it can bind to polyester cloth hydrophobically through its fatty acid tail. The remaining portion of polymyxin can form stable molecular complexes with the lipid A portion of LPS. The polymyxin B-lipid interaction is determined by a fluorescent probe displacement technique as biphasic, with dissociation constants (K_d s) at $0.4 \mu\text{M}$ and $1.5 \mu\text{M}$ (David *et al.*, 1992).

Because polymyxin B is relatively inexpensive, stable and available in chemically pure and homogeneous form, the use of polymyxin as an LPS antigen capturing agent is more reproducible and economical than the use of an antibody. The captured Salmonella LPS on polymyxin- coated cloth can then be detected with LPS specific detector antibody (Blais, 1990; Blais and Yamazaki, 1990a).

Fig. 4. The structure of polyester.

Fig. 5. The structure of polymyxin B.

R = 6 - methyloctanoyl or 6 - methylheptanoyl

DAB = diaminobutyric acid

Fig.6. Molecular structure of Salmonella LPS (Fomsgaard, 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.

In lipid A region, the GlcN disaccharide carries 2 amide and 2 ester bound residues of C10-16 chain saturated fatty acids, which are themselves esterified with long chain fatty acids. Incomplete rough forms are produced from LPS mutants, which have defects in genes coding for glycosyl or phosphoryl transferases (or epimerases) (Hitchcock et al., 1986). They all lack O-antigens and are indicated as Ra, Rb, Rc and Re.

Ra is composed of lipid A and a complete core.

Re is composed of lipid A and 2-3 molecules of KDO.

Rb and Rc are composed of lipid A and incomplete cores with different lengths between Re and Ra.

Other cationic polypeptides and proteins, such as Poly (Lys) and lactoferrin, have also been reported to be membrane active agents (Bellamy *et al.*, 1992) and therefore may have potential use as LPS-capturing agents. In order to further improve the economy of the CEIA, several other polypeptides will be examined in Chapter 1. Salmonella-specific bacteriophage Felix-O1 binds Salmonella specifically and will be tested as a detecting reagent in polymyxin-cloth assay for salmonellae in Chapter 1 for the same purpose as above.

All members of the Salmonella genus are potential human or animal pathogens. In order to detect all the Salmonella serotypes by the polymyxin-CEIA, it is critical to select appropriate Salmonella LPS-specific detector antibodies. Both polyclonal and monoclonal anti-flagellar antibodies have been used in EIA for Salmonella detection, with polyclonal antibodies generally giving high rate of cross-reactivity (Flowers, 1985; Swaminathan *et al.*, 1985). During the early stage of the development of polymyxin-CEIA for salmonellae, a commercially prepared polyclonal antibody specific for an undefined common Salmonella antigen (CSA) has been used to demonstrate the effective capturing of Salmonella LPS by polymyxin coated cloth. However, later studies showed that the CSA antibody exhibited an unacceptable high degree of cross-reactivity with several non-Salmonella bacteria, which make them unsuitable as a detector antibody in polymyxin-CEIA for salmonellae. Therefore, further research was done to explore other sources of detector antibodies for specific and sensitive detection of salmonellae in the polymyxin-CEIA. Using a pool of commercially available polyclonal O antisera as detecting antibodies improved the performance for Salmonella detection (Blais *et al.*,

1993; Chapter 1), however, the use of polyclonal antibodies introduced potential batch to batch variations and difficulties in quality control, especially when many antisera have to be pooled in order to cover broad range of Salmonella serotypes.

As mentioned previously, most salmonellae share a common core LPS structure. Therefore another strategy for Salmonella detection at the genus level is the use of a anti-core LPS antibody. Chapter 2 will compare such a monoclonal anti-core Salmonella LPS antibody with other two sources of pooled polyclonal O-antisera for detection of salmonellae.

S. enteritidis is presently the most frequently isolated serotype in the U.S. and many European countries (Poppe *et al.*, 1993). This serotype has often been isolated from the contents of intact, clean shell eggs (Humphrey, 1990). To detect contamination of the eggs in egg-laying flocks, it is necessary to test large numbers of eggs for the presence of S. enteritidis. Therefore, simple rapid and economical methods for screening of this serotype are required. Chapter 3 will demonstrate the rapid and economical screening of S. enteritidis in eggs by the polymyxin-cloth enzyme immunoassay.

The positive results obtained by the polymyxin-CEIA are considered presumptive, and should be confirmed by a method based on a different principle. The use of traditional culturing methods might provide adequate confirmation, however such an approach adds considerable labour and time to the entire procedure. This will decrease the advantage of the rapid test strategy. As an alternative, a genus-specific PCR technique targeting the invA gene was used for the rapid confirmation of polymyxin-CEIA for S. enteritidis in eggs and the results will be shown in Chapter 4.

The wide transmission of Salmonella through food, water, poultry and human carriers makes elimination of this pathogen difficult. Serological identification of salmonellae helps to locate the source of the infection and thereby control or prevent its spread. The traditional slide agglutination method for serological analysis of salmonellae is cumbersome and time-consuming, especially when large numbers of isolates are to be analysed. The application of polymyxin-CEIA for Salmonella O serogroup identification will be presented in Chapter 5.

CHAPTER 1
PRELIMINARY INVESTIGATIONS

PREFACE

In order to further improve the economy of the polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) technology to optimize conditions for its application in antigen detection (eg., food pathogens), this thesis started with several preliminary investigations, including testing the possibility of using Salmonella specific bacteriophage Felix-O1 instead of the traditional antibodies as the lipopolysaccharide (LPS) detecting agent on polymyxin-coated polyester cloth, the use of sodium cholate to extract LPS antigens for the polymyxin-CEIA, and the examination of various polypeptides or proteins as LPS capturing agents.

Bacteriophage Felix-O1 infects and lyses Salmonella strains specifically. The initial stage of infection involves specific attachment of the phage to receptor molecules which are located in the core region of Salmonella LPS (Lindberg and Holme, 1969; Lindberg, 1973). The possibility of using Felix-O1 as Salmonella LPS detector reagent for the polymyxin-cloth was therefore examined.

As an LPS extracting agent, sodium deoxycholate (deoxycholate) is necessary for the production of strong signals in polymyxin-CEIA. However, deoxycholate has low solubility at room temperature and the stock solutions tend to form gels. Heating the stocking solution before use is usually needed to liquify it, which adds inconvenience to the assay. Therefore, a more soluble detergent sodium cholate (cholate) was examined as an LPS extracting agent in this chapter and its performance in a polymyxin-CEIA for detection of various serotypes of salmonellae was tested.

Polymyxin B is advantageous over antibody as an LPS capturing agent in several

aspects, especially in its chemical purity, absence of batch to batch variation in its preparation and economy. Since other polypeptides and proteins have also been reported as membrane active agents, they have potential capability to capture bacterial LPS, and these were compared to polymyxin B as Salmonella LPS capturing agents.

Preliminary Investigation I:

Use of bacteriophage Felix-O1 as a detection reagent for Salmonella LPS captured on polymyxin-coated cloth

When bacteriophage particles are introduced into an actively growing culture of susceptible bacteria, they will adsorb to the bacterial cells, multiply therein, and eventually cause the cell to lyse (Adams, 1959). Bacteriophage Felix-O1 was first named by Felix and Callow in 1943 (Felix and Callow, 1943). It lyses 96-99.5% of Salmonella strains studied and has been used in the identification of Salmonella (Cherry *et al.*, 1954; Lindberg, 1967; Welkos *et al.*, 1974). However strains of salmonellae belonging to the O-groups E1-E4 are less sensitive to this phage. They can be detected with another phage specific for these O-groups (Gudel and Fey, 1981; Pugh *et al.*, 1988).

Sensitivity of bacteria to bacteriophage is mainly determined by the presence of specific receptor sites on the cell surface (Lindberg and Holme, 1969). The Felix-O1 receptor sites are found in the common core of Salmonella LPS molecules (Lindberg, 1967; Jann & Westphal, 1975). The recognition between bacteriophage and the receptor LPS is a highly specific process and is part of the natural mechanism of host recognition (Lindberg, 1973).

Bacteriophage first attaches to the host bacterial surface, then injects its nucleic acid into the cell. The attachment rate is affected by the ionic environment, temperature, pH and organic cofactors (Lindberg, 1973). The T-even series of bacteriophages attach to their host cells by the tips of their tails and susceptible bacteria are clumped in the presence of purified T-even tail fibres (Wildy and Anderson, 1963). Although no study on Felix-O1 tail fibres has yet been done, the fact that Felix-O1 belongs to the same morphological group of Bradley's as the T-even bacteriophages (Bradley, 1967) suggests that the tail fibres of Felix-O1 may have the same function.

Based on the specific binding property between Felix O1 and Salmonella LPS, this section examined the possibility of using Felix-O1, instead of an antibody, for the detection of Salmonella LPS antigens captured on the polymyxin-cloth. Several experiments were performed and the summarized results presented here. Such an assay would have several advantages: 1) phage is easy to propagate in bacterial cultures; 2) no need for animal immunizations for antibody production; 3) no batch to batch variations in affinity to the LPS; and 4) Broad specificity for salmonellae.

Materials and Methods

Chemicals and immunoreagents

Polymyxin B sulfate (No. P-1004), deoxycholate (No. D-6750), biotinamidocaproate N-hydroxysuccinimide ester (BACHS) (No. B-2643), S. typhimurium LPS (No. L-6511), E. coli LPS (No. L-2880) and Streptavidine-peroxidase (Strep-HRP) (No. S-5512) were obtained from Sigma chemical Co.. Blocking reagent (No. 1093-657) was purchased

from Boehringer Mannheim. Nutrient broth was supplied by Difco Laboratories. The membrane peroxidase substrate system used in these studies is a proprietary formulation based on the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) and an unspecified membrane enhancer, and was a kind gift from Ricoh Kyosan, Inc. (Tokyo, Japan).

Propagation and titration of bacteriophage

S. typhimurium LT-2 strain SL1027 from Dr.K.E. Sanderson at University of Calgary was used as a host strain for propagating Felix-O1. Overnight culture (0.1 ml) in nutrient broth containing 0.15 M NaCl (NBS) of the host strain (about 10^8 cells/ml) was mixed with 0.1 ml of Felix-O1 phage stock diluted to about 10^6 plaque-forming units (pfu)/ml and 3.5 ml of molten soft nutrient agar (0.6%) containing 0.15 M NaCl were mixed gently and poured over the top of nutrient agar (1.5%) containing 0.15 M NaCl. The agar overlays were allowed to solidify, and the plates then were incubated at 37°C overnight. Semiconfluent or confluent lysis was observed. The soft-agar layers were scraped into 0.01M sodium phosphate buffer containing 0.5 M NaCl (pH 7.3) (PBS⁺) (2ml/plate) in centrifuge tubes by a sterile plastic loop and the tubes were placed on a Vortex mixer to disrupt pieces of agar. The lysates were centrifuged at 9,000 g for 30 min. The supernatant was collected and filtered through a 0.2 μ m milipore membrane and stored at 4°C.

The titre of the phage solution was determined by diluting the phage in sterile PBS⁺, and plating each dilution by agar-layer method as above. Plaques were counted 16 h later for each dilution, and the number of plaque-forming units per ml in the

original solution was calculated.

Purification of bacteriophage

The bacteriophage was first precipitated with polyethyleneglycol 8000 (PEG 8000). Varying amounts of PEG 8000 were added to the small aliquots of phage lysates in PBS⁺ and the mixtures were kept at 4°C for 60 min. They were then centrifuged at 8,000 g at 4°C for 10 min and the pellets were resuspended into PBS⁺ for titration. The optimum concentration of PEG 8000 was determined by measuring the highest percentage of phage recovery.

The bacteriophage was further purified by differential centrifugation in CsCl. Eleven ml of CsCl solutions with different densities: 1.7, 1.5 and 1.3 g/ml, were pipetted sequentially into 40 ml capacity tubes. Seven ml of PEG-purified phage was added onto the top layer. The tubes were then centrifuged at 45,000 rpm for 3 h. After centrifugation, the phage were visible as a blue band in the lower half of the tube and were collected by puncturing the tube right below the blue band. Dialysis to remove CsCl was done right afterwards against 0.01 M borate buffer (pH 8.0) (Thomas and Abelson, 1966).

Biotinylation of bacteriophage

Phage solution (0.3 ml) was incubated with a mixture of 30 μ l of 0.1 M borate buffer (pH 8.0) and 3 μ l of BACHS (10 mg/ml in dimethylformamide) for 30 min at room temperature. The reaction mixture was dialysed against 0.01 M phosphate buffer

containing 0.85% NaCl (pH 7.2) (PBS) for 24 h at 4°C with changes of buffer.

Preparation of bacterial LPS

LPS was either bought in purified form or extracted from bacterial culture. To extract LPS from bacterial culture, the cells were washed three times in PBS by centrifugation at 5,000g and diluted by 10 times in 10 mM EDTA-PBS and heated at 100°C for 10 min. The extract was cooled down before being used for coating.

Phage-enzyme assay on LPS coated polyester cloth

Cloth segments (4X5 cm) were coated overnight at room temperature either with 10 ug/ml EDTA (10 mM)-heat treated purified LPS or with LPS extracted from host bacteria or *E.coli* strain (ATCC 11775) by heating in the presence of deoxycholate. The segments were then washed with PBS containing 0.05% (w/w) Tween 20 (PBST). They were then blocked in PBST containing 0.2% blocking reagent (PBST-B) for one hour at room temperature. Serial dilutions of biotin-phage were spotted onto the LPS cloth and incubated for 20 min. The segments were then washed with PBST and saturated with streptavidine-peroxidase (Strept-HRP) (1:1000 diluted) for 15 min, then washed the same way as above. Membrane peroxidase substrate TMB solution was then added.

Phage-enzyme assay on polymyxin-coated cloth

Cloth segments were coated with 5 mg/ml polymyxin overnight at 37°C and then blocked with 0.2% blocking reagent. Five µl of heat-deoxycholate treated LPS of both

host bacteria and *E. coli* were spotted onto the polymyxin cloths and incubated for 30 min. After washing with PBST, the cloths were then saturated with phage-biotin dilutions (1:10, 1:100) for 15 min. After washing with PBST, the cloth was saturated with Strep-HRP solution (1:1000 diluted) and incubated for 15 min. The cloth was then washed with PBST and assayed with TMB substrate solution.

Results and Discussion

Page-enzyme assay on LPS-coated cloth

Felix-O1 purified by polyethylene glycol (PEG 8000) (Av. MW.8,000) was labelled with biotin and tested on LPS coated cloths. *S. typhimurium* LPS coated cloth shows stronger signals than *E. coli* LPS coated cloth, however, even the uncoated cloth shows equally strong signals as *S. typhimurium* LPS coated cloth (Table 1.1). This indicates that the biotin-labelled phage preparation can bind to polyester cloth non-specifically. Further purification of phage by CsCl differential centrifugation did not eliminate the problem (data not shown).

Phage was also labelled with horseradish peroxidase (HRP-phage) and tested as above. Unfortunately, this HRP-phage also bound non-specifically to the uncoated cloth. The non-specific binding to cloth most likely due to the phage itself, not the molecules used for labelling, since it binds to the cloth when labelled with biotin or HRP. Therefore, these results can not prove the specific binding between Felix-O1 and Salmonella LPS.

Phage-enzyme assay on polymyxin-coated cloth

Since the ultimate aim of this research is to use phage as detector agent for Salmonella LPS in the polymyxin-CEIA, the biotin-phage preparation was tested in the assay of LPS captured on polymyxin-coated cloth. The deoxycholate extracted LPS was spotted on polymyxin-cloth, then reacted sequentially with biotin-phage, Strept-HRP and TMB substrate. In this assay, white spots appeared where the LPS samples were spotted on the cloth, and intensive colour development occurred everywhere else on the cloth. This may again be due to the non-specific binding of biotin-phage to the polymyxin-cloth. The LPS did not bind to phage, or bound much less phage than polymyxin-cloth itself, most of the Strept-HRP would bind to the areas where no LPS were spotted. When the substrate was added, the colour reaction was thus developed mostly in those areas and produced a negative image of spots (white spots on blue cloth).

Although Felix-O1 was propagated successfully in this host S. typhimurium, labelled-Felix-O1 failed to produce any positive signals for LPS from S. typhimurium captured on polymyxin-cloth. This could be caused either by the lack of strong binding between phage and extracted LPS molecule, which may be due to that the propagated phage particles already bound some LPS fragments from host bacteria, or by too strong non-specific binding between labelled phage and the polymyxin-cloth, either of which make Felix-O1 unsuitable for use as detecting reagent for Salmonella LPS antigen captured on polymyxin-cloth.

Table 1.1. Phage-enzyme assay on LPS-coated cloth

Cloth coating	Origin of LPS	Phage dilution factor						
		4	8	16	32	64	128	256
<u>S. typhimurium</u>	culture	+	+	+	+/-	-	-	-
	pure	+	+	+	+	+/-	-	-
<u>E. coli</u>	culture	+/-	-	-	-	-	-	-
	pure	+	+	+	+/-	-	-	-
Uncoated		+	+	+	+	+/-	-	-

The LPS were either extracted from bacterial overnight culture or were bought in purified form.

Preliminary Investigation II:

Cholate as LPS extracting agent for detection in polymyxin-CEIA

In the earlier adaptations of the polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) methodology, the lipopolysaccharide (LPS) antigens were extracted by heating cell suspensions in the presence of 0.1% (w/v) sodium deoxycholate, which was shown to be necessary to produce strong positive reactions in the assay (Blais and Yamazaki, 1990b). However, deoxycholate solutions have a marked tendency to form a viscous gel at room temperature and neutral pH. On the other hand, sodium cholate (cholate) solutions remain liquid at concentrations of up to 20% (w/v) under the same conditions. The polymyxin-CEIA will ultimately be applied to the detection of salmonellae and other pathogens in various food samples, many of which may contain heavy amount of proteins or lipids. Therefore even higher concentration of detergent may be necessary for those lipid and protein-rich samples. Thus cholate was tested for use in the extraction of the LPS antigens for detection in polymyxin-CEIA.

Materials and Methods

Chemicals and immunoreagents

Cholate (No. C-1254) and anti-rabbit IgG-peroxidase conjugate (No. A-6154) were obtained from Sigma chemical Co.. Buffered peptone water (BPW) and Bacto Salmonella O rabbit antisera were supplied by Difco Laboratories. The antiserum was rehydrated according to the manufacturer's directions and stored at 4°C.

Polymyxin-CEIA

Polyester cloth (DuPont, Sontara 8100) was cut into 4 X 6 cm sheets, then saturated with ca. 3 ml of polymyxin B sulfate solution (10 mg/ml in PBS). After incubation for 16 h at 37°C, each sheet was washed with a total of about 6 X 10 ml of PBS containing 0.05% (v/v) Tween 20 (PBST) on a filter under suction. Non-specific binding sites remaining on the cloth were then blocked by saturating with ca. 3 ml of PBST containing 0.2% (w/v) Boehringer Mannheim blocking reagent (PBST-B) and incubating for 1 h at room temperature. The cloth was then washed with PBST, followed by a brief rinse with PBS, and stored moist in a sealed petri dish at 4°C until use.

To determine the optimum concentration of cholate, an overnight culture of S. typhimurium (ATCC 14028) was diluted in PBS into 10^{-1} , 10^{-2} and 10^{-3} . One 10th volume (0.1 volume) of 0, 1.25, 2.5, 5, 10, 20% of cholate were then added in each culture dilution and heated at 100°C for 10 min. All the following steps were conducted at room temperature. Five μ l of cholate-heat extracted LPS was spotted on polymyxin-coated polyester cloth and incubated for 20 min. The cloth was then washed with PBST as above, and then saturated with about 3 ml of 1:500 diluted anti-Salmonella group B antiserum in PBST-B and incubated for 10 min. The cloth was washed again as above and then incubated with 1:1000 diluted anti-rabbit-IgG-peroxidase conjugate in PBST-B for 10 min. The sheet was then washed with PBST, blotted, and incubated with 3 ml of TMB membrane peroxidase substrate system for 20 min at room temperature. Positive reactions produced a blue spot on the cloth at the point of sample application. Reactions

were graded qualitatively as follows: +, strong positive reaction (dark blue spot); +/-, weak positive reaction (light blue spot); -, no reaction (no spot).

For detection of salmonellae using pooled anti-O antigen antisera, the LPS antigens of bacterial broth cultures were extracted by the addition of one-tenth volume of 5.0% (w/v) cholate, followed by heating as before. Five μ l of the cholate-heat-treated samples were pipetted onto the polymyxin-cloth sheet. After 20 min incubation at room temperature, the sheet was washed with PBST as above, and then saturated with about 3 ml of PBST-B containing a mixture of Difco antisera poly A, B, C, D and group K, each diluted at 1:1000. After 10 min incubation at room temperature, the sheet was washed with PBST and then saturated with about 3 ml of PBST-B containing anti-rabbit IgG-peroxidase conjugate diluted at 1:1000, and incubated for 10 min at room temperature. The sheet was then washed with PBST and incubated with 3 ml of TMB membrane peroxidase substrate solution for 20 min at room temperature. Reactions were graded qualitatively as above.

Results and Discussion

Effect of cholate concentration on polymyxin-CEIA

Dilutions of an overnight culture of an *S. typhimurium* strain was heated in the presence of different concentrations of cholate. The heat-cholate treated samples (5 μ l of each) were spotted on polymyxin-cloth then detected with anti-group B antiserum as described above. As shown in Fig.1.1, samples without adding cholate (lane 7) only produced weak spots even in undiluted culture (27). Increasing the concentration of cholate enhanced the darkness and increased the sizes of the blue spots. It can be

speculated that more LPS was released by higher concentration of cholate, or cholate binds to LPS in such a way as to increase the affinity for polymyxin-cloth, therefore giving darker blue spots. However, the sensitivity of the assay reached to maximum at 0.5% cholate (lane 3), which produced a weak spot at 10^{-3} diluted culture (d3). These results suggest that the maximum amount of LPS can be extracted from the cell surface at 0.5% of cholate for sequential reactions in polymyxin-CEIA. Higher than 0.5% of cholate (lane 1 - lane 2) seems to lower the sensitivity of the assay. The extra amount of cholate may compete with extracted LPS for polymyxin-cloth binding sites. Therefore, the cholate concentration used for LPS extraction from broth culture of salmonellae should be 0.5% (w/v).

Detectability of polymyxin-CEIA

An overnight culture of *S. typhimurium* was serially diluted in PBS and the dilutions were extracted by heating in the presence of cholate (0.5% w/v) or deoxycholate (0.1% w/v). The extracted LPS were spotted onto polymyxin-cloth and then detected with an anti-core LPS antibody (see Chapter 2 for detailed explanations for this antibody).

Table 1.2 shows that cholate extraction resulted in 2 times higher sensitivity (higher dilution factor) than deoxycholate which may be speculated that the cholate extracted LPS binds to polymyxin-cloth stronger than deoxycholate extracted one.

Fig. 1.1. Effect of cholate concentration on polymyxin-CEIA.

Final concentration of cholate (w/v) in samples: 1, 2%; 2, 1%; 3, 0.5%; 4, 0.25%; 5, 0.125%; 6, 0.06%; 7, 0%.

Culture dilutions: a, undiluted; b, 10^1 ; c, 10^2 ; d, 10^3 .

(Overnight culture of S. typhimurium in BPW was diluted in PBS)

Table 1.2. Sensitivity of polymyxin-CEIA

LPS extracting agent	Culture dilution factor					
	20	40	80	160	320	640
Cholate	+	+	+	+/-	-	-
Deoxycholate	+	+/-	+/-	-	-	-
none	+/-	-	-	-	-	-

Specificity of polymyxin-CEIA

In a previous study, several Bacto O antisera including poly A, B, C, D and antigroup K were pooled and used in the polymyxin-CEIA to detect a broad range of Salmonella serotypes when they were extracted by deoxycholate (Blais *et al.*, 1993). This study examined several strains of Salmonella and non-Salmonella bacteria extracted by cholate in polymyxin-CEIA using pooled Bacto O antisera. Table 1.3 shows that all of the eight Salmonella serotypes belonging to different serogroups gave positive signals. Four of the five non-Salmonella tested were negative.

The cross-reactivity with Staphylococcus aureus was produced as in the previous study and was assumed to be due to the production of protein A in the cell walls of this strain (Blais *et al.*, 1993). The acidic protein A may have been present in cholate-heat-extracted culture of this organism and was probably adsorbed to the cationic polymyxin-cloth. This cross-reactivity problem was overcome by eliminating S. aureus during selective enrichment (Blais *et al.*, 1993).

These results demonstrate that cholate can replace deoxycholate as Salmonella LPS extracting agent for assay in polymyxin-CEIA. The optimum concentration for broth culture is 0.25-0.5%. It produced higher sensitivity than deoxycholate. Therefore, cholate was used to extract Salmonella LPS for polymyxin-CEIA throughout the following chapters. For food samples rich in proteins and lipids (e.g. eggs), higher concentration of cholate may be necessary. This aspect will be addressed in later chapters.

Table 1.3. Reactivity of Salmonella and non-Salmonella in polymyxin-CEIA

Bacteria	Polymyxin-CEIA
<u>Salmonella</u> (serogroup)	
<u>S. paratyphi</u> A (A)	+
<u>S. abony</u> (B)	+
<u>S. braenderup</u> (C1)	+
<u>S. blockley</u> (C2)	+
<u>S. enteritidis</u> (D1)	+
<u>S. amsterdam</u> (E1)	+
<u>S. rubislaw</u> (F)	+
<u>S. blukwa</u> (K)	+
<u>Non-Salmonella</u>	
<u>Escherichia coli</u>	-
<u>Shigella sonnei</u>	-
<u>Enterobacter aerogenes</u>	-
<u>Yersinia enterocolitica</u>	-
<u>Staphylococcus aureus</u>	+

Preliminary Investigation III:

Examination of various cationic polypeptides as alternative LPS-capturing agents in cloth enzyme immunoassay (CEIA)

As stated previously, Lipopolysaccharides (LPS) are the major components of the outer membrane of gram-negative bacteria. LPS of gram-negative bacteria contains a variety of ionic groups, with acidic phosphates and carboxyl moieties concentrated within the core and lipid A regions. Therefore, LPS is associated with a variety of cations, including the metal ions Mg^{2+} and Ca^{2+} (Wilkinson, 1983). Polycationic polymyxin B can bind to the LPS by displacing the divalent cations from LPS. Other polycationic polypeptides such as Poly (Lys) and the glycoprotein lactoferrin have also been reported to be membrane active agents (Bellamy *et al.*, 1992), but have not been tested in CEIA applications.

A capturing agent used in CEIAs must meet two conditions: 1) Binds to polyester cloth, 2) Captures LPS when bound to cloth. Binding to the cloth should be in such a way which accommodates or at least does not affect the later binding to the LPS. However, it is not the purpose of this research to examine these two steps separately. Instead, the cloth was coated with various polypeptides or glycoprotein lactoferin the same way as for polymyxin B and the detectability for *S. typhimurium* LPS was compared with different polypeptides.

Materials and Methods

Chemicals and immunoreagents

Polymyxin B sulfate (No. P-1004), Poly (Orn, Leu) (No. P-7414), Poly-L-lysine (No. P-2636), Poly (Lys, Phe) (No. P-3150), Poly-L-histidine (No. P-2534), Poly-L-ornithine (No. P-3655), Melittin (No. M-7391), and Lactoferrin (L-4765) were obtained from Sigma chemical Co..

Cloth enzyme immunoassay using various agents as capturing agents

Polyester cloth (DuPont, Sontara 8100) was cut into 3 X 6 cm sheets, then saturated with 2.5 ml of coating reagent solutions (2 mg/ml in PBS). After incubation for 16 h at 37°C, each sheet was washed with a total of about 6 X 10 ml of PBST on a filter under suction.

An overnight broth culture of *S. typhimurium* was diluted in BPW into 1/10, 1/100, 1/200, 1/400, 1/800, 1/1000, 1/2000, 1/4000 and 1/8000. The 1/10 volume of 5% cholate was added into each dilution and heated at 100°C for 10 min. After the samples were cooled down, the 1/10 volume of 10% blocking reagent was mixed with each sample. Five μ l of each sample was spotted on cloth sheets and incubated for 5 min. The cloth sheets were then washed as above. The cloth sheets were then saturated with Bacto anti-group B antiserum diluted at 1/200 and incubated for 10 min. The cloth sheets were washed and incubated with anti-rabbit IgG-peroxidase conjugate diluted at 1/1000 for 10 min. After the final wash, the sheets were incubated with TMB substrate solution for 20 min.

Results and Discussion

Detectability of *S. typhimurim* by CEIA

Table 1.4 shows that higher sensitivity was produced on the cloth sheets coated with most of the capturing agents tested than on uncoated cloth, of which polymyxin B produced highest sensitivity and darkest spots. Uncoated polyester cloth binds to the extracted LPS non-specifically through hydrophobic forces. Coating with ploy (lys, phe) or lactoferrin did not enhance the sensitivity, giving the same level of detectability as uncoated cloth. Poly-L-histidine coated cloth had blue background with clear spots on the sites where the extracted LPS were pipettd on. Binding between polymyxin B and LPS molecules involves both electrostatic and hydrophobic forces, therefore is much stronger than the non-specific binding between uncoated-cloth and LPS.

The lower sensitivity given by those agents than polymyxin B may be due to either one of the two factors, or combination of both: 1) their lower affinity for polyester cloth, which leads to less amount of agents bound on the cloth; 2) their weaker affinity to LPS molecules. The blue background produced on poly-L-histidine coated cloth may be caused by the strong ionic interaction between peroxidase conjugate and poly-L-histidine.

In addition, polymyxin B is much cheaper than the other agents tested, it is therefore the best polyester cloth coating agent among those tested so far for the CEIA of Salmonella LPS.

Table 1.4. Detection limit on polyester cloth coated with various agents

Coating agent (2 mg/ml)	Culture dilution factor							
	100	200	400	800	1000	2000	4000	8000
Uncoated	+	+/-	+/-	-	-	-	-	-
Polymyxin	+	+	+	+/-	+/-	+/-	+/-	-
PO	+	+/-	+/-	+/-	+/-	-	-	-
Melittin	+	+	+/-	+/-	+/-	-	-	-
PL	.	+	+/-	+/-	+/-	+/-	-	-
POL	+	+	+/-	+/-	+/-	+/-	-	-
PLP	+	+/-	+/-	-	-	-	-	-
LF	+	+/-	+/-	-	-	-	-	-

* POL, Poly (Orn, Leu); PL, Poly-L-lysine, PLP, Poly (Lys, Phe); PO, Poly-L-ornithine; LF, Lactoferrin.

^b Overnight culture of *S. typhimurium* ATCC 14028 was diluted in BPW, treated and assayed in polymyxin-CEIA as described in method section.

CHAPTER 2

Salmonella DETECTION BY THE POLYMYXIN-CLOTH ENZYME IMMUNOASSAY USING A MONOCLONAL ANTI-CORE LPS ANTIBODY AS DETECTOR ANTIBODY

ABSTRACT

A monoclonal antibody to the core region of lipopolysaccharide (LPS) was examined in comparison with several commercially available O-antigen polyclonal antisera as sources of detector antibodies in a polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) for Salmonella. In this assay, polymyxin-coated polyester cloth captured the LPS antigens from Salmonella broth cultures, followed by immunoenzymatic detection of the captured LPS using specific antibodies. Pools of polyvalent antisera reacted with all of the Salmonella strains tested, but also gave cross-reactions with some non-Salmonella bacteria. On the other hand, the monoclonal antibody gave positive reactions with all of the Salmonella tested except serogroup O strains, but did not react with any of the non-Salmonella bacteria. The monoclonal antibody supplemented with a single factor O:35 rabbit antiserum was able to detect the serogroup O strains without causing any cross-reactions with the non-Salmonella bacteria. As an example of the applicability of this assay system, low levels of Salmonella cells spiked into various food samples were successfully detected after an overnight enrichment in broth.

INTRODUCTION

Members of the genus Salmonella cause human enteritis worldwide through the consumption of a variety of contaminated foods, including meats, dairy products, eggs, and frozen seafoods. The effective control of Salmonella in foods may be achieved by the rigorous application of the Hazard Analysis and Critical Control Point (HACCP) system, which seeks to control microbial hazards at all levels of the transmission chain.

Current approaches of endproduct testing using traditional culture diagnostic techniques are time consuming and labour-intensive. The availability of rapid, simple and inexpensive methods for the detection of Salmonella will be essential to monitoring the efficacy of the HACCP program in achieving a safe and wholesome food supply.

A polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) system has been developed for Salmonella antigens which utilizes polyester cloth coated with polymyxin B (polymyxin-cloth) as an inexpensive high surface area adsorbent for the capture and subsequent immunoenzymatic assay of lipopolysaccharide (LPS) antigens (Blais, 1990; Blais and Yamazaki, 1990a,b; 1991). In this system, the macroporous polyester cloth support provides rapid immunoreactions, and the polymyxin B used as the LPS capturing agent provides a stable, inexpensive and reliable alternative to the traditional approach utilizing capture antibodies.

Recently, Nielsen *et al.* (1994) reported on an adaptation of this LPS capture assay based on the use of polymyxin immobilized in microtiter plates. Although successful in the sensitive assay of Salmonella LPS antigens, the limited surface area of the microtiter plate wells resulted in severe inhibition of target LPS capture when non-target LPS was present. On the other hand, previous studies have shown that the use of polymyxin-coated polyester cloth provides a sufficiently large surface for the capture of the target LPS even in the presence of large excess of non-target LPS (Blais and Yamazaki, 1990b). Furthermore, the use of large sheets of polymyxin-cloth in a dot blot format greatly facilitates the simultaneous assay of multiple samples (e.g., in enrichment broth cultures of food samples).

In order to detect various serotypes of salmonellae, a pooled mixture of commercially available rabbit Salmonella O antisera containing antibodies which react with each of the many different types of O-antigens has been used in polymyxin-CEIA (Blais *et al.*, 1993). This approach provided satisfactory detection of a variety of Salmonella serotypes on the basis of the reaction of the polyclonal antibodies with the O-polysaccharide of the LPS antigen. While useful for preliminary screening of samples, the use of polyclonal antibodies is a potential source of variability in the assay, since it may be difficult to standardize different batches of antisera, especially when a large number of antisera must be pooled to ensure detection of a broad variety of serotypes. In addition, polyclonal antibodies may introduce cross-reactivity with non-salmonellae since the antibodies to the common antigenic factors shared by other Enterobacteriaceae can not always be eliminated.

Since the salmonellae are believed to share a common core structure in their LPS (core LPS, see Fig.6), core oligosaccharide of LPS might serve as an ideal target as an alternative approach to general detection of salmonellae. Therefore this chapter examined the applicability of a monoclonal antibody which is specific for a core oligosaccharide of Salmonella LPS for general detection of salmonellae in polymyxin-CEIA. The performance of this monoclonal antibody in the polymyxin-CEIA was compared to that of pooled mixtures of antisera available from different commercial sources and a combination of the monoclonal antibody with a single factor antiserum.

MATERIALS AND METHODS

Chemicals and immunoreagents

The following were purchased from Difco Laboratories: Bacto Salmonella O rabbit antisera poly A (No. 2534-47), poly B (No. 2535-47), poly C (No. 2536-47), poly D (No. 2537-47), anti-group K (factor 18, No. 2518-47), anti-group O (factor 35, No. 2522-47), poly A-I and Vi (No. 2264-47) and bacterial culture media buffered peptone water (BPW), yeast extract, nutrient broth (NB), and brain heart infusion broth (BHI). Rabbit Salmonella antisera Polyvalent O (310 308) and Polyvalent O1 (310 309) were obtained from Denka Seiken Co., Ltd. (Tokyo, Japan), and rabbit antisera poly A-S (No. PL6002) was obtained from Pro-Lab Diagnostics. Polymyxin B sulfate (No. P-1004), sodium cholate (No. C-1254), anti-rabbit IgG-peroxidase conjugate (No. A-6154), anti-mouse IgG-peroxidase conjugate (No. A-4416), and Salmonella Ra-LPS (No. L-6016), Rc-LPS (No. L-5891) were obtained from Sigma Chemical Co.. Blocking reagent (No. 1093-657) was obtained from Boehringer Mannheim. The membrane peroxidase substrate system used in polymyxin-CEIA is a proprietary formulation based on the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) and an unspecified membrane enhancer, and was a kind gift from Ricoh Kyosan Inc. (Tokyo, Japan).

Production of monoclonal antibody M181

Monoclonal antibody M181 specific for the Salmonella core oligosaccharide was produced at the Animal Diseases Research Institute (Nepean, Ont.) according to the procedure of Kennett *et al.* (1978) by fusing Sp2/0 myeloma cells with spleen cells from

BALB/c mice immunized with Salmonella antigens. The Salmonella antigens used to immunize mice were a mixture of purified LPS from S. typhimurium, S. typhosa, S. minnesota, S. abortusequi and S. enteritidis (Sigma, St. Louis, Missouri); water-extracted antigens from S. typhimurium, S. hadar, S. infantis, S. saintpaul, S. anatum; and Bacto Salmonella O antigen groups A, C, D and E (Difco Laboratories, Detroit, Michigan). Hybridomas were grown in ascitic fluid according to the procedure of Henning and Nielsen (1987), and the antibodies were used without further purification. The isotype was determined as IgG2a using an enzyme-linked immunosorbent assay kit (GIBCO BRL, Burlington, Ontario) (Hoszowski *et al.*, 1995).

Bacterial strains and foods

The Salmonella strains tested (see Table 2.1) represent 99.9% of all the serotypes recovered from over 2460 Salmonella-positive foods, feeds and environmental samples submitted for routine analysis at Laboratory Services Division (LSD), Agriculture and Agri-Food Canada, during a 15-year period (Blais *et al.*, 1993). In addition, Salmonella serotypes from O-serogroups A, H, I, J, L, P, T, U and Y, from which no Salmonella were isolated at LSD, were also tested (Table 2.1). A variety of non-Salmonella gram-negative and gram-positive bacteria were also examined (Table 2.3).

Growth conditions for both Salmonella and non-Salmonella strains were as previously described (Blais *et al.*, 1993). All the Salmonella and most of the non-Salmonella bacteria were routinely grown in BPW by shaking at 37°C for 16-20 h. Enterococcus faecalis and Listeria spp. were grown in BHI by shaking for 16-20 h at 37

Table 2.1. *Salmonella* isolates examined in this study.

Serovar	Serogroup	Serovar	Serogroup
<i>S. paratyphi</i> A	A	<i>S. binza</i>	E2
<i>S. abony</i>	B	<i>S. newington</i>	
<i>S. agona</i>		<i>S. thomasville</i>	E3
<i>S. banana</i>		<i>S. senftenberg</i>	E4
<i>S. bredeney</i>		<i>S. rubislaw</i>	F
<i>S. heidelberg</i>		<i>S. poona</i>	G1
<i>S. java</i>		<i>S. worthington</i>	G2
<i>S. schwarzengrund</i>		<i>S. schalkwijk</i>	H
<i>S. typhimurium</i>		<i>S. gaminara</i>	I
<i>S. vom</i>		<i>S. zwickau</i>	
<i>S. braenderup</i>	C1	<i>S. saphra</i>	
<i>S. infantis</i>		<i>S. bleedon</i>	J
<i>S. livingstone</i>		<i>S. blukwa</i>	K
<i>S. mbandaka</i>		<i>S. cerro</i>	
<i>S. montevideo</i>		<i>S. minnesota</i>	L
<i>S. ohio</i>		<i>S. pomona</i>	M
<i>S. oranienburg</i>		<i>S. urbana</i>	N
<i>S. blockley</i>	C2	<i>S. alachua</i> (5)*	O
<i>S. hadar</i>		<i>S. ealing</i>	
<i>S. newport</i>		<i>S. inverness</i>	P
<i>S. haardt</i>	C3	<i>S. johannesburg</i>	R
<i>S. kentucky</i>		<i>S. kahla</i>	T
<i>S. eastbourne</i>	D1	<i>S. bongor</i>	Y
<i>S. enteritidis</i> (18)*		<i>S. subsp.</i> (2)*	Non-typable
<i>S. fresno</i>	D2		
<i>S. amsterdam</i>	E1		
<i>S. anatum</i>			
<i>S. london</i>			
<i>S. orion</i>			
<i>S. westhampton</i>			

*Total number of strains tested.

and 30°C, respectively; Bacillus spp. and Serratia marcescens were grown in NB by shaking for 16-20 h at 30°C and 26°C, respectively. Whenever necessary, viable counts were obtained by plating serial dilutions of the broth cultures on nutrient agar and incubating the plates at 37°C for 20 h.

The foods used in spiking studies were butter, skim milk powder, egg powder and semi-soft cheese samples submitted by Agriculture and Agri-Food Canada inspection staff for analysis at LSD. These samples were determined to be Salmonella-free by standard cultural analysis (A. Zebchuk, LSD, personal communication). For spiking, 15 g of food sample was transferred aseptically to 135 ml of nutrient broth in a stomacher bag and stomached for 60 sec in a Lab Blender 400 stomacher (Seward Medical, Inc.). The food-broth mixtures were dispensed in 10 ml portions into test tubes and then spiked with 0 or ca. 5 cfu of S. typhimurium as verified by viable count. Three replicate spiked samples were prepared for each food. These samples were then incubated at 37°C for 18 h, and then subjected to the polymyxin-CEIA procedure.

Preparation of samples for the polymyxin-CEIA

LPS antigens in broth cultures and in enrichment cultures of butter and milk powder were extracted by the addition of 0.1 volume of 5% (w/v) sodium cholate in 0.01 M sodium phosphate buffer (pH 7.4)/0.85% (w/v) NaCl (PBS), followed by heating at 100°C for 10 min. These preparations were then used directly in the polymyxin-CEIA. For extraction of the antigens from enrichment cultures of cheese and egg powder, 0.1 volume of 15% (w/v) sodium cholate in PBS was added followed by heating as above.

The cholate-heat-treated mixtures were then centrifuged at 3000 g for 5 min, and the resulting supernatant was used in polymyxin-CEIA.

Polymyxin-CEIA

Polyester cloth (DuPont, Sontara 8100) was cut into 6 X 4 cm sheets and then saturated with 3 ml of polymyxin B sulfate solution at 5 mg/ml in PBS. After incubation at 37°C for 16h, each sheet was washed 6 times with about 10 ml of PBS containing 0.05% (v/v) Tween 20 (PBST) each time on a filter under suction, and then blocked by incubating in PBST containing 0.2% blocking reagent (PBST-B) for 1 h at room temperature. The following reactions were all performed at room temperature. Five μ l of extracted LPS sample was pipetted onto the polymyxin-cloth sheet at 0.5 cm intervals, so that each cloth sheet accommodated a total of 40 samples. After 20 min incubation, the cloth was washed with PBST as above, and then saturated with 3 ml of detector antibodies diluted in PBST-B. Four detector antibodies were examined individually: (1) monoclonal antibody M181 diluted 1:400; (2) an equal mixture of Difco antisera poly A, poly B, poly C, poly D and anti-group K, each diluted 1:500; (3) an equal mixture of Denka Seiken antisera polyvalent O and polyvalent O1, each diluted 1:500; and, (4) M181 (1:400 diluted) supplemented with single factor antiserum O:35 (1:500 diluted). After 10 min incubation with the detector antibodies, the cloth was washed with PBST and then saturated with either (1) anti-mouse IgG-peroxidase conjugate diluted 1:1000 in PBST-B for detection of bound M-181 detector antibodies, or (2) anti-rabbit IgG-peroxidase conjugate diluted 1:1000 PBST-B for detection of bound polyclonal

antibodies. An equal mixture of anti-mouse IgG-peroxidase and anti-rabbit IgG-peroxidase conjugates (each diluted 1:1000 in PBST-B) was used for the assay of bound M181 and rabbit anti-group O antibodies. After 10 min incubation with the conjugate, the cloth was washed with PBST and then incubated with 3 ml of tetramethylbenzidine (TMB) membrane peroxidase substrate for 20 min. Reactions were graded qualitatively as follows: positive reaction (blue spot), +; negative reaction (no spot), -.

RESULTS AND DISCUSSION

Specificity of polymyxin-CEIA using polyclonal antisera

Although Salmonella O antigen-specific antisera from Difco were previously used in the polymyxin-CEIA for Salmonella detection (Blais et al., 1993), the need to pool several antisera (poly A, poly B, poly C, poly D and anti-group K) to permit detection of the Salmonella strains listed in Table 2.1 introduces a potential quality control problem in their use as immunoreagents, since quite a few antisera are included and different batches of antisera might be difficult to standardize for this assay system. Therefore, we also examined the applicability of other commercially available Salmonella O antisera.

In preliminary experiments, cultures of various Salmonella strains belonging to serogroups A to E and several non-Salmonella organisms were tested in the polymyxin-CEIA using broadly reactive O antisera from different commercial suppliers as the detector antibodies: (1) antisera poly A-I and Vi (Difco); (2) antisera poly A-S (Pro-Lab

Diagnostics); and (3) antisera poly O (Denka Seiken). Antisera poly A-I and Vi (Difco) and poly A-S (Pro-Lab) gave positive reactions with all of the Salmonella strains tested within serogroups A-E but cross-reacted extensively with various Gram positive and Gram negative non-Salmonella bacteria, whereas antisera poly O (Denka Seiken) gave positive reactions with the Salmonella strains and no cross-reactivity with the non-Salmonella bacteria.

However, in further studies, antisera poly O (Denka Seiken) failed to detect Salmonella strains outside of serogroups A-E, and these antibodies were therefore supplemented with antisera poly O1 (Denka Seiken) to enable detection of the other serogroups. The performance of this mixture of antisera poly O and poly O1 (Denka Seiken) as the detector antibodies in the polymyxin-CEIA is summarized in Table 2.2, along with the results obtained using a pool of Difco antisera. Table 2.2 shows that the combined antisera permitted the detection of all of the Salmonella strains tested. However, they cross-reacted with non-Salmonella bacteria (Table 2.3). Thus, while these polyclonal antisera effectively detected Salmonella by the polymyxin-CEIA, perhaps the best strategy for their use would involve their application to the screening of selectively enriched cultures, followed by more definitive identification testing of presumptive positives (e.g., cultural and biochemical testing).

Specificity of the polymyxin-CEIA using a monoclonal antibody

LPS is the major cell surface antigenic component of Salmonella, consisting of a polysaccharide chain formed from repeating oligosaccharide units (O-chain), a core

Table 2.2. *Reactivity of Salmonella serogroups in polymyxin-CEIA using different detector antibodies.

Serogroup	no. positives/total ^b		
	Denka Seiken Pool	Difco Pool	M181
A	1/1	1/1	1/1
B	9/9	9/9	9/9
C1	7/7	7/7	7/7
C2	3/3	3/3	3/3
C3	2/2	2/2	2/2
D1	19/19	19/19	19/19
D2	1/1	1/1	1/1
E1	5/5	5/5	5/5
E2	2/2	2/2	2/2
E3	1/1	1/1	1/1
E4	1/1	1/1	1/1
F	1/1	1/1	1/1
G1	1/1	1/1	1/1
G2	1/1	1/1	1/1
H	1/1	1/1	1/1
I	3/3	3/3	3/3
J	1/1	1/1	1/1
K	2/2	2/2	2/2
L	1/1	1/1	1/1
M	1/1 ^c	1/1	1/1
N	1/1 ^c	1/1	1/1
O	6/6	6/6	0/6
P	1/1 ^c	1/1	1/1
R	1/1	1/1	1/1
T	1/1 ^c	1/1	1/1
Y	1/1 ^c	1/1 ^c	1/1
Non-typable	2/2	2/2	2/2

* Salmonella cultures were grown in BPW to a density of ca. 10^8 cfu/ml, and then subjected to the polymyxin-CEIA using the various detector antibodies as described in Methods. Denka Seiken Pool contains Poly O and Poly O1 antisera; Difco Pool contains Poly A-D and group K antisera.

^b No. of strains giving positive reactions in polymyxin-CEIA per total number tested.

^c Weak reaction was produced.

oligosaccharide complex (core region), and lipid A (Rietschel *et al.*, 1982; Fomsgaard, 1990). In the polymyxin-CEIA, cholate-heat extracted LPS is assumed to bind to polymyxin on polyester cloth through the lipid A portion, while the O-chain and core region are exposed for subsequent immunoreaction with specific antibodies. Since most salmonellae are believed to share a common core oligosaccharide structure (Tsang *et al.*, 1991) it should be possible to utilize an antibody targeting a common portion of the core region for *Salmonella* detection at genus level. Therefore the performance of several murine monoclonal antibodies raised against the *Salmonella* LPS core region was examined in the polymyxin-CEIA. The product of one hybridoma in particular, monoclonal antibody M181, reacted strongly with purified *Salmonella* Ra-LPS but not with Rc-LPS in the polymyxin-CEIA (Fig.2.1), suggesting that M181 is specific for the outer core region of LPS, where the common *Salmonella* antigen is believed to reside (Tsang *et al.*, 1991). Therefore, the reactivity of M181 in the polymyxin-CEIA was tested with the *Salmonella* and non-*Salmonella* bacteria and listed in Tables 2.2 & 2.3. With the exception of the 6 serogroup O salmonellae (5 *S. alachua* and 1 *S. ealing* strains), all of the *Salmonella* strains tested produced positive results (Table 2.2). None of the non-*Salmonella* bacteria tested gave positive reactions (Table 2.3), suggesting that M181 is a highly *Salmonella*-specific detector antibody. The lack of reactivity of this antibody with the serogroup O *Salmonella* strains suggests that these organisms bear a different outer core structure. All of the *S. alachua* and *S. ealing* strains were detected when M181 was supplemented with the single factor antiserum O:35 (serogroup O-specific) from Difco diluted 1:500. No cross-reactivity with the non-*Salmonella* bacteria

Fig.2.1. Reactivity of M181 with various *S. typhimurium* (St) LPS.

a, LPS were treated with cholate at 100°C for 10 min;

b, LPS were heated without adding any cholate.

1, St wild type LPS; 2, St Ra-LPS; 3, St Rc-LPS; 4, St Re-LPS.

Refer to Fig. 6 for the structure of St LPS.

Table 2.3. Reactivity of non-Salmonella bacteria in polymyxin-CEIA using different detector antibodies^a.

Bacteria	no. positives/total ^b		
	Denka Seiken Pool	Difco Pool	Mab M181
<u>Escherichia coli</u>	3/3 ^c	1/3	0/3
<u>Shigella sonnei</u>	1/1 ^c	0/1	0/1
<u>Enterobacter aerogenes</u>	1/1 ^c	0/1	0/1
<u>Enterobacter cloacae</u>	0/1	0/1	0/1
<u>Klebsiella pneumoniae</u>	1/1 ^c	0/1	0/1
<u>Proteus vulgaris</u>	0/1	0/1	0/1
<u>Serratia marcescens</u>	1/1 ^c	0/1	0/1
<u>Yersinia enterocolitica</u>	2/5 ^c	2/5 ^c	0/5
<u>Yersinia intermedia</u>	0/1	0/1	0/1
<u>Pseudomonas aeruginosa</u>	0/1	1/1 ^c	0/1
<u>Citrobacter freundii</u>	1/1 ^c	0/1	0/1
<u>Staphylococcus aureus</u>	1/1 ^c	1/1	0/1
<u>Bacillus cereus</u>	0/1	0/1	0/1
<u>Bacillus subtilis</u>	0/1	0/1	0/1
<u>Listeria welshimeri</u>	0/1	0/1	0/1
<u>Listeria innocua</u>	0/1	0/1	0/1
<u>Listeria ivanovii</u>	0/1	0/1	0/1
<u>Enterococcus faecalis</u>	0/1	0/1	0/1

^a Broth cultures of different Gram positive and Gram negative non-Salmonella bacteria (ca. 10⁸ cfu/ml) were subjected to the polymyxin-CEIA using the various detector antibodies as described in Methods. Contents of Denka Seiken and Difco pools are as indicated in Table 2.2.

^bNo. of strains giving positive reactions in polymyxin-CEIA per total number tested.

^cWeak reaction was produced.cfu/ml for the Difco antisera.

was observed with this mixture of detector antibodies (data not shown).

Limit of detection for Salmonella

The detection limit of the polymyxin-CEIA for four Salmonella serogroups was determined using the pooled Difco and pooled Denka Seiken antisera and M181 as the detector antibodies. Table 2.4 shows that the detection limit of polymyxin-CEIA varied with pooled Difco, pooled Denka Seiken or M181 detector antibodies for four different serogroups ranging from $0.4-18 \times 10^6$ cfu/ml for the Denka Seiken and $0.7-8.8 \times 10^6$ cfu/ml for the Difco antisera. The detection limit for monoclonal antibody M181 was in the range of $1.4-8.8 \times 10^6$ cfu/ml. The detectability is slightly lower for M181 probably because of lower avidity typical for monoclonal antibodies compared to polyclonals. The variability in the detection limit for the antisera may be due to the heterogeneity of the single factor-specific antibodies and the variability of the O-antigens present on the surface of the different serotypes. These detection limits would allow detection of Salmonella in enrichment cultures of foods and other samples, where initially low numbers of cells are grown to a detectable level.

Detection of Salmonella in enrichment cultures of foods

The preceding results show that monoclonal antibody M181 supplemented with single factor antiserum O:35 (Difco) detected pure cultures of all the Salmonella strains tested in the polymyxin-CEIA without cross-reacting with the non-Salmonella bacteria tested. To demonstrate the applicability of this system in the detection of Salmonella

Table 2.4. Detection limit of the polymyxin-CEIA using different antibodies*

Strain	Serogroup	Detection limit (X10 ⁶ cfu/ml)		
		Denka Seiken Pool	Difco Pool	Mab M181
<u>S. typhimurium</u>	B	1.1	1.1	4.4
<u>S. braenderup</u>	C1	18.0	8.8	8.8
<u>S. enteritidis</u>	D1	0.4	5.5	1.4
<u>S. amsterdam</u>	E1	0.7	0.7	1.4

* Salmonella cells suspended in buffered peptone water (BPW) at different densities were subjected to the polymyxin-CEIA using the various detector antibodies as described in Methods. The detection limit was determined on the basis of the lowest cell density giving a positive reaction (blue spot) on the cloth. Contents of Denka Seiken and Difco pools are as indicated in Table 2.1.

in actual food samples, enrichment broth cultures of various foods spiked with 5 cfu of S. typhimurium were subjected to the polymyxin-CEIA employing M181 supplemented with anti-factor O:35. Table 2.5 shows that all of the spiked food samples and none of the unspiked samples gave positive results in the polymyxin-CEIA after enrichment. This confirms the specificity of the assay for Salmonella, and demonstrates its suitability for the detection of low numbers of this pathogen in a variety of food types.

This chapter has demonstrated the detection of Salmonella using various O-chain-specific polyclonal antibodies and a core oligosaccharide-specific monoclonal antibody as the detector antibodies for the polymyxin-CEIA. The use of the polyclonal antibodies alone provides an inexpensive source of detector antibodies for the polymyxin-CEIA. However, these antibodies exhibited some cross-reactivity with non-Salmonella bacteria, and may best be suited for preliminary screening of enrichment cultures of foods. On the other hand, monoclonal antibody M181 supplemented with single factor antiserum O:35 (Difco) reacted with all of the Salmonella strains and none of the non-Salmonella bacteria tested, suggesting that the polymyxin-CEIA utilizing this combination of detector antibodies will provide a highly specific tool for the detection of Salmonella in foods. The fact that M181 alone failed to react with serogroup O salmonellae is in agreement with the previous report that some degree of variability exists in the Salmonella outer core oligosaccharide structure (Tsang *et al.*, 1991).

Table 2.5. Detection of *S. typhimurium* in spiked foods using M181^a

<i>Salmonella</i> inoculated (cfu)	No. positive/total no. of replicates ^b			
	Butter	Milk Powder	Egg Powder	Cheese
5	3/3	3/3	3/3	3/3
0	0/3	0/3	0/3	0/3

^a Various food samples were spiked with 0 or 5 *S. typhimurium* cells and then enriched in nutrient broth. The O/N enrichment cultures were then subjected to the polymyxin-CEIA using M181 supplemented with single factor antiserum O:35 as the detector antibodies as described in Methods.

^b No. of replicates giving positive results in polymyxin-CEIA per total number tested.

The work done in this chapter has been presented in the following paper which was accepted for publication:

Wang, Haiyan, Burton W. Blais, Brian W. Brooks and Hiroshi Yamazaki (1995) Salmonella detection by the polymyxin-cloth enzyme immunoassay using polyclonal and monoclonal detector antibodies. International Journal of Food Microbiology (In press).

CHAPTER 3

RAPID AND ECONOMICAL DETECTION OF Salmonella enteritidis IN EGGS

BY THE POLYMYXIN-CLOTH ENZYME IMMUNOASSAY

ABSTRACT

A rapid, simple and economical procedure for the detection of *S. enteritidis* in eggs was developed. The contents of whole eggs inoculated with low numbers of *S. enteritidis* were mixed with a minimal volume of a nutrient-rich broth (1:2 ratio of egg to broth) and incubated overnight. The lipopolysaccharide (LPS) antigens of *S. enteritidis* were extracted by heating in the presence of cholate. The antigens were captured on polymyxin-coated polyester cloth, and the captured antigens were detected by sequential reactions with factor O:9 rabbit antiserum, anti-rabbit antibody-peroxidase conjugate and peroxidase substrate. This polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) was highly specific for salmonellae bearing the factor O:9 antigen, reacting in the assay of 19 *S. enteritidis* strains tested, including two rough isolates, but not with salmonellae lacking the factor O:9 antigen or non-*Salmonella* bacteria. This combined enrichment culture and polymyxin-CEIA required less than 24 h to complete and detected as few as 2 *S. enteritidis* cfu inoculated into a whole egg. This procedure should facilitate the routine monitoring of *S. enteritidis* in large numbers of egg samples.

INTRODUCTION

The incidence of salmonellosis outbreaks in humans caused by *Salmonella enteritidis* has increased markedly in recent years, and this serotype is presently among the most frequently isolated in the United States and many European countries (Poppe et al., 1993). The consumption of contaminated eggs and egg products is the major cause of infection by *S. enteritidis* (Kim et al., 1989). The contamination of eggs with *S.*

enteritidis appears to originate in the ovaries and oviducts of egg-laying hens, and this serotype is frequently isolated from the contents of intact, clean shell eggs, where it can survive certain forms of cooking (Humphrey, 1990). The number of bacteria within contaminated eggs is generally very low, usually within the range of one to a few cells per egg (Gast, 1993). To detect contamination of the eggs in egg-laying flocks, it is necessary to test large numbers of eggs for the presence of *S. enteritidis*. Therefore, simple and economical methods for the isolation and detection of low numbers of this pathogen in eggs are required. The traditional method for detecting *Salmonella* in eggs involves homogenization of eggs in a large volume of enrichment broth followed by cultural diagnoses and biochemical tests, a labour-intensive procedure which can take up to 7 days to complete (Andrews *et al.*, 1984). Recently, Gast (1993) used direct plating of incubated egg contents on selective agar to detect *S. enteritidis*. Although this method was simpler and more economical than the traditional culture technique, it was less sensitive and still required 4-5 days to obtain results.

As a rapid and economical approach for screening *Salmonella* in foods and other samples, we have previously described a polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) for capturing the lipopolysaccharide (LPS) antigens in test samples followed by immunoenzymatic detection (Chapter 2; Blais and Yamazaki, 1990a,b; 1991). When a pooled mixture of commercially available O antisera was used as a source of detector antibodies, the polymyxin-CEIA was able to specifically detect a variety of different *Salmonella* serotypes in enrichment broth (Blais *et al.*, 1993; Chapter 2). A monoclonal anti-core LPS antibody was also used in polymyxin-CEIA and showed better

performance than polyclonal antisera for general detection of different Salmonella serovars (Chapter 2). Development of a S. enteritidis specific assay is important to control the problem raised by this special pathogen. An assay based on polymyxin-CEIA should be possible for specific detection of S. enteritidis by using an anti-group D antiserum. In this chapter the development of a rapid, simple and inexpensive method for the detection of S. enteritidis in eggs will be described, which involves rapid pre-enrichment of egg samples in a small volume of enrichment broth and their subsequent analysis by the polymyxin-CEIA in a dot blot format employing a commercially available serogroup D-specific antiserum as the detector antibody.

MATERIALS AND METHODS

Chemicals and Immunoreagents

The following were obtained from Sigma Chemical Co.: polymyxin B sulfate (No. P-1004), sodium cholate (No. C-1254), nalidixic acid (sodium salt)(No. N-4382) and anti-rabbit IgG-peroxidase conjugate (No. A-6154). Anti-rabbit IgM-peroxidase conjugate (No. A120-110P-3) was obtained from Bethyl Laboratories, Inc.. Blocking reagent (No. 1093-657) was obtained from Boehringer Mannheim. Bacto Salmonella factor O:9 rabbit antiserum (No. 2818-47), buffered peptone water (BPW), yeast extract (YE), nutrient broth (NB), brain heart infusion broth (BHI) and brilliant green sulfa agar (BGS) were obtained from Difco Laboratories, and trypticase soy broth (TSB) was from BDH. All conjugates and antisera were rehydrated and stored according to the manufacturers' instructions. The membrane peroxidase substrate system used in this

study is a proprietary formulation based on the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) and an unspecified membrane enhancer and was a kind gift from Ricoh Kyosan Inc. (Tokyo, Japan).

Bacterial strains and Eggs

A total of 18 *S. enteritidis* strains isolated from egg, poultry and environmental samples at several Agriculture and Agri-Food Canada laboratories, and one ATCC strain (ATCC 13076), were examined in this study. Included among the 18 *S. enteritidis* strains were two rough isolates, one biotype C (untypable by phage typing), and the other biotype A (phage type 23)(Poppe *et al.*, 1993). Other *Salmonella* serotypes examined (Table 3.1) were isolated from food, feed and environmental samples analyzed at Laboratory Services Division (LSD), Ottawa, Agriculture and Agri-Food Canada, and represent over 99.9% of all serogroups recovered at LSD during a 15 year period (Blais *et al.*, 1993). Several non-*Salmonella* gram-positive and gram-negative bacteria were also examined (Table 3.1).

Salmonella strains were routinely grown in BPW by shaking at 37°C for 16-20 h. Growth conditions for the other bacteria were as previously described (Chapter 2). Whenever necessary, viable counts were obtained by plating serial dilutions of the broth cultures on nutrient agar and incubating the plates at 37°C for 20 h.

The eggs used in this study were from Canadian producers and were purchased from local retailers. In some experiments, the contents of several eggs were aseptically transferred to a stomacher bag and blended by stomaching for 60 sec in a Lab Blender

Table 3.1. Salmonella and non-Salmonella organisms examined in this study^{a,b}**Salmonella:**

S. abony (B)
S. agona (B)
S. banana (B)
S. bredeney (B)
S. heidelberg (B)
S. javiana (B)
S. schwarzengrund (B)
S. typhimurium (B)
S. vom (B)
S. braenderup (C1)
S. infantis (C1)
S. livingstone (C1)
S. mbandaka (C1)
S. montevideo (C1)
S. ohio (C1)
S. oranienburg (C1)
S. blockley (C2)
S. hadar (C2)
S. newport (C2)
S. haardt (C3)
S. kentucky (C3)
S. eastbourne (D1)
S. enteritidis (D1)
S. fresno (D2)
S. amsterdam (E1)
S. anatum (E1)
S. london (E1)
S. orion (E1)
S. westhampton (E1)
S. orion var. 15⁺ (E1)
S. anatum var. 15⁺ (E1)
S. orion var. 15⁺, 34⁺ (E1)
S. senftenberg (E4)

S. rubislaw (F)
S. poona (G)
S. worthington (G)
S. subsp. II 17:g,t:[e,n,x,z₁] (J)
S. blukwa (K)
S. cerro (K)
S. minnesota (L)
S. alachua (O)
S. johannesburg (R)
S. subsp. (non-typable)

Non-Salmonella:

Bacillus cereus
Bacillus subtilis
Citrobacter freundii
Escherichia coli (3 strains)
Enterobacter aerogenes
Enterobacter cloacae
Enterococcus faecalis
Klebsiella pneumoniae
Listeria innocua
Listeria ivanovii
Listeria welshimeri
Proteus vulgaris
Pseudomonas aeruginosa
Shigella sonnei
Staphylococcus aureus
Yersinia enterocolitica (5 strains)
Yersinia intermedia

^a Nomenclature as provided by the WHO Collaborating Centre for Reference and Research on Salmonella. Institut Pasteur, Paris, France.

^b Salmonella serogroups are indicated in brackets.

400 stomacher (Seward Medical, Inc.). The blended eggs were then divided into 60 g portions and spiked with 20 μ l of *S. enteritidis* cell suspensions. The spiked samples were mixed with enrichment broth (BHI broth supplemented with 0.5% (w/v) yeast extract (BY) and further stomached as before. These samples were then incubated at 37°C for 16 h. In other experiments, the contents of an individual whole egg were weighed, aseptically transferred to a stomacher bag, and spiked with 20 μ l suspensions of a nalidixic acid-resistant *S. enteritidis* strain. Enrichment broth (BY or BY containing 25 μ g/ml nalidixic acid) was added to the bag in the proportion of 2 ml of broth per gram of egg, and the mixture was blended by stomaching as before, followed by incubation at 37°C for 20 h.

Nalidixic acid-resistant *S. enteritidis* strain

A nalidixic acid-resistant (Nal^r) *S. enteritidis* strain was isolated according to a modification of the method described by Miller (1972). *S. enteritidis* (ATCC 13076) was grown for 16 h at 37°C in NB. Five ml of the broth culture was centrifuged at 8000 X g for 5 min. The bacterial pellet was resuspended in 0.1 ml of NB and spread onto a nutrient agar plate containing 25 μ g/ml of nalidixic acid (NAL plate). After incubation of the plate at 37°C for 16 h, a large colony was picked and further purified by incubation on a NAL plate as before. To confirm the identity of the Nal^r strain, biochemical testing was performed using an automated diagnostic bacteriology system (Vitek Systems) (see Appendix II) and the Enterotube II system (Roche Diagnostic System, Inc.) (see Appendix I). Serological confirmation was obtained by slide

agglutination using Difco antisera factor O:9 and factors O:1,9,12. This NaI' *S. enteritidis* strain was maintained on NAL plates.

Preparation of heat-injured Salmonella cells

S. enteritidis (ATCC 13076) cells from 20 ml of a broth culture (ca. 10^9 cfu/ml) were harvested by centrifugation at 9000 X g for 5 min, and resuspended in 20 ml of 0.01 M phosphate-buffered (pH 7.4)/0.85% NaCl (PBS). Ten ml of cell suspension was then placed in a pre-heated 125 ml flask and incubated at 55°C, for 20 min, with constant gentle shaking (90 rpm). Viable counts were determined by plating serial dilutions of the cell suspension on TSA and BGS plates before and after the heat treatment, and the injury rate was calculated according to the method of Chen *et al.* (1993).

Salmonella enteritidis polymyxin-Cloth EIA (SE polymyxin-CEIA)

Polyester cloth (DuPont, Sontara 8100) was cut into 6 X 4 cm sheets, then saturated with 3 ml of polymyxin B sulfate solution (5 mg/ml in PBS). After incubation at 37°C for 16 h, each sheet was washed with a total of about 6 X 10 ml of PBS containing 0.05% (v/v) Tween 20™ (PBST) on a filter under suction. Lipopolysaccharide (LPS) antigens in broth cultures without eggs were extracted by the addition of 0.1 volume of 5% (w/v) cholate in PBS, followed by heating for 10 min at 100°C. For enrichment cultures of egg samples, the LPS antigens were extracted by the addition of 0.1 volume of 15% (w/v) cholate, followed by heating as before. The

cholate-heat-treated mixture was then centrifuged at 3000 X g for 5 min to sediment the coagulated egg material, and an aliquot of the resulting supernatant was used in the assay of SE by the polymyxin-CEIA. Five μ l of extracted LPS sample was pipetted onto the polymyxin-cloth sheet at 0.5 cm intervals, so that each cloth sheet accommodated a total of 40 samples. After 20 min at room temperature, the cloth was washed with PBST as above, and then saturated with 3 ml of rabbit factor O:9 antiserum diluted 1:200 in PBST containing 0.2% (w/v) blocking reagent (PBST-B). After 10 min at room temperature, the cloth was washed with PBST and saturated with 3 ml of a mixture of anti-rabbit IgG-peroxidase and anti-rabbit IgM-peroxidase conjugates, each diluted 1:1000 in PBST-B, and incubated for 10 min at room temperature. The cloth was then washed with PBST and incubated with 3 ml of TMB membrane peroxidase substrate for 20 min at room temperature. Reactions were graded qualitatively as follows: +, strong positive reaction (dark blue spot); +/-, weak positive reaction (light blue spot); -, no reaction (no spot).

RESULTS AND DISCUSSION

Salmonella enteritidis polymyxin-CEIA

A cholate concentration of 0.5% (w/v) was necessary to produce strong positive reactions in the assay of Salmonella broth suspensions by the polymyxin-CEIA (Chapter 1). Therefore, LPS antigens were routinely extracted by heating in 0.5% (w/v) cholate for broth cultures of Salmonella. When the samples contain a high content of fatty materials, such as eggs, the cholate concentration had to be increased to 1.5% (w/v) to achieve the same effect, possibly to overcome the adsorption of the detergent by the egg

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PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
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Table 3.2. *Effect of cholate concentration on polymyxin-CEIA of egg samples

Concentration of Cholate (%) (w/v)	Polymyxin-CEIA at enrichment time		
	16 h	24 h	40 h
0	-	+/-	+/-
0.5	+/-	+ ^b	+
1.0	+ ^b	+	+
1.5	+	+	+
2.0	+	+	+

* Egg-BY mixtures (1:10 ratio) were inoculated with ca. 10 *S. enteritidis* cells and enriched for 16, 24 and 40 h. They were treated with different concentrations of cholate (see method section) and assayed in polymyxin-CEIA. The experiment was done in triplicate with egg-broth mixtures without inoculation as negative controls. All the uninoculated eggs gave negative reactivity at all incubation times with all different cholate treatment.

^b Diameters of dark blue spots are less than 3 mm, while the diameters for other dark blue spots are ca. 6 mm.

material (Table 3.2).

Pooled preparations of commercially available O antigen-specific antisera have previously been used as the detector antibodies in a polymyxin-CEIA system for the Salmonella serotypes most commonly isolated from foods, feeds and environmental samples (Chapter 2; Blais *et al.*, 1993). Since this approach was successful, It was surmised that an antiserum recognizing the serogroup D-specific factor O:9 antigen might be suitable as the source of detector antibodies in a polymyxin-CEIA for the detection of S. enteritidis in eggs. To test the specificity of the factor O:9 antiserum as the detector antibody, 19 different S. enteritidis strains and several other Salmonella and non-Salmonella strains (Table 3.1) were screened by the polymyxin-CEIA employing this antiserum. Of the different Salmonella serotypes tested, only those bearing the factor O:9 antigen (serogroups D1 and D2), which included all of the S. enteritidis isolates, gave positive reactions in the assay (Table 3.3). One of the rough S. enteritidis strains, which was not typable by phage typing (Poppe *et al.*, 1993), gave only a weak reaction. The total or partial loss of O side chain in the LPS of this strain may account for the weak reactivity with the detector antibody. Interestingly, the other rough strain (phage type 23) (Poppe *et al.*, 1993) gave a strong positive reaction in the assay. None of the non-Salmonella organisms tested (Table 3.1) was reactive in the SE polymyxin-CEIA.

The sensitivity of the SE polymyxin-CEIA was then determined for 7 S. enteritidis strains by the assay of BPW suspensions containing different cell densities. Table 3.4 shows that the detectability of the assay ranged from 0.5×10^6 to 2.1×10^6 cfu/ml, depending on the strain. The level of sensitivity achieved and the specificity of

Table 3.3. Specificity of the SE polymyxin-CEIA in the assay of various Salmonella serogroups.

Serogroup	Positive/Total ^a	Serogroup	Positive/Total ^a
B	0/8	F	0/1
C1	0/7	G1	0/1
C2	0/3	G2	0/1
C3	0/2	J	0/1
D1	20/20 ^b	K	0/2
D2	1/1	L	0/1
E1	0/8	O	0/1
E4	0/1	R	0/1
		Non-typable	0/1

^a No. of strains giving positive reactions in the SE polymyxin-CEIA per total number tested.

^b Includes 19 S. enteritidis strains.

Table 3.4. Sensitivity of the SE polymyxin-CEIA.

Strain/source	Detectability* (cfu/ml X 10 ⁶)
ATCC 13076	0.5
Poultry	1.1
Poultry	1.1
Egg	1.3
Egg	1.0
Environmental	0.6
Environmental	2.1

* Minimum cell density (determined by viable counts) giving a positive reaction in the SE polymyxin-CEIA.

the S. enteritidis polymyxin-CEIA were deemed suitable for its application in the detection of S. enteritidis in primary enrichment cultures from eggs.

Detection of SE in enrichment cultures from eggs

Traditionally, pre-enrichment of S. enteritidis in eggs is carried out using an egg sample to enrichment broth ratio of 1:9 (Andrews *et al.*, 1984), which requires the use of 540 ml of enrichment broth for a typical 60 g egg. Such a practice is uneconomical and cumbersome for the routine screening of large numbers of eggs. Therefore, I studied the possibility of reducing the quantity of enrichment broth needed to enrich low numbers of cells inoculated into eggs for detection by the SE polymyxin-CEIA. BHI broth containing 0.5% (w/v) yeast extract (BY) was chosen as a nutrient-rich broth for the rapid enrichment of S. enteritidis in eggs. A blended pool of the contents of several eggs was divided into several 60 g portions which were inoculated with different numbers of fresh cells. The portions were then mixed with either 120, 240 or 480 ml of BY, giving egg to broth ratios of 1:2, 1:4 and 1:8, respectively, and incubated at 37°C for 16 h. The enrichment cultures were then assayed by the SE polymyxin-CEIA. Each experiment was carried out in triplicate.

Fewer than 5 S. enteritidis cfu could be detected by the SE polymyxin-CEIA after enrichment in samples with an egg to broth ratio of 1:2 (Table 3.5). The failure to detect cells in some of the replicates with the lower inocula reflects the low statistical probability of a sample receiving even a single cell at these levels of inoculation. The positive reactions obtained in the assay of BY broth (devoid of egg sample) inoculated with different numbers of cfu is consistent with the patterns observed for the egg-broth

Table 3.5. Assay of *S. enteritidis* in eggs using different ratios of egg:broth

cfu inoculated (egg:BY)	Positive/total			
	5	0.5	0.05	0
1:2	3/3	2/3	0/3	0/3
1:4	3/3	0/3	0/3	0/3
1:8	3/3	0/3	0/3	0/3
BY alone ^a	3/3	1/3	0/3	0/3

^a No. of replicates per triplicate samples giving a positive reaction in the SE polymyxin-CEIA.

^b 100 ml portions of BY devoid of egg sample.

mixtures, suggesting that the presence of egg material in the enrichment cultures did not interfere with cell growth. None of the uninoculated egg-broth mixtures gave a positive reaction in the assay, indicating that this egg pool likely did not contain endogenous S. enteritidis.

Since the 1:2 egg to broth ratio used in the present enrichment procedure could conceivably prove detrimental to the recovery of injured Salmonella cells in naturally contaminated eggs, which may contain anti-bacterial components, I studied the recovery and assay of heat-injured cells inoculated into egg sample. A heat-treated suspension of S. enteritidis containing 84% injured cells (determined on the basis of the fraction of heat-treated cells exhibiting growth on TSA but not on BGS plates, according to Chen et al., 1993) was diluted in sterile PBS to give different cell densities, which were then inoculated into 10 ml portions of a mixture containing a 1:2 ratio of egg to broth. After a 20 h incubation at 37°C, the enrichment cultures were assayed by the SE polymyxin-CEIA. Each experiment was carried out in triplicate. Table 3.6 shows that on average even one heat-injured cfu inoculated into either the egg-broth mixture or BY broth alone could be recovered and detected by this combined enrichment and polymyxin-CEIA procedure. This suggests that the high egg to broth ratio is not detrimental to the recovery of injured cells.

Characterization of Nal^r S. enteritidis

Some studies to estimate recovery of low numbers S. enteritidis inoculated into eggs require use of eggs from specific pathogen-free flocks to ensure that the endogenous

Table 3.6. Assay of heat-injured *S. enteritidis* in egg-broth mixture

Sample	Positives/Triplicate*			
	cfu inoculated			
	100	10	1	0
Egg-broth	3/3	3/3	3/3	0/3
BY alone	3/3	3/3	2/3	0/3

* No. of replicates per triplicate samples giving a positive reaction in the SE polymyxin-CEIA.

S. enteritidis does not present (Gast, 1993). Such flocks are difficult to obtain and maintain. As an alternative, some researchers use Nal^r mutant to study the growth of S. enteritidis in experimentally inoculated eggs (Kim et al., 1989). Creation of Nal^r mutant is very easy and inexpensive. Nalidixic acid (NAL) in growth media suppress the growth of any possibly present endogenous S. enteritidis, while allow the artificially inoculated Nal^r strain to amplify.

Before applying the assay system to the detection of very low numbers of S. enteritidis in individual eggs, a Nal^r S. enteritidis strain was produced (see methods section) from a S. enteritidis strain ATCC 13076. Its biochemical and serological properties confirmed that it was still a serogroup D Salmonella. Its growth was compared with the parent strain and Fig.3.1 shows that Nal^r strain grows slower than parent strain.

Assay of individual eggs inoculated with a Nal^r S. enteritidis strain

In the previous experiments, a number of eggs were pooled and blended to provide egg samples for the development of the assay procedure. However, in practice it will be necessary to assay eggs individually for the presence of S. enteritidis. In order to demonstrate the applicability of the present procedure in the detection of S. enteritidis inoculated into individual eggs, it was necessary to preclude the possibility that the interpretation of results obtained with the inoculation of very low numbers of cells would not be affected by the possible presence of endogenous Salmonella in the eggs. Thus, the recovery and assay of a nalidixic acid-resistant (Nal^r) S. enteritidis mutant inoculated

Fig.3.1. Growth curves of *S. enteritidis* comparing Nal^r strain and the parent strain.

The bacteria were first grown in BHI supplemented with yeast extract (BY) at 37 °C overnight, then 0.5 ml of overnight culture was transferred into 50 ml prewarmed BY. Samples were taken every 30 min afterwards, diluted in 1:2 and the O.D. readings at 550 nm were recorded.

into individual eggs and enriched in the presence of nalidixic acid were studied. The contents of individual eggs were first inoculated with different numbers of Nal^r cells, blended in a stomacher, and mixed with 2 parts of BY broth containing 25 µg/ml nalidixic acid (BY-Nal). This condition inhibited the growth of any endogenous S. enteritidis but allowed the growth of the inoculated Nal^r S. enteritidis. Five eggs were used for each level of inoculation. The egg-broth mixtures were then incubated at 37°C for 20 h and assayed by the SE polymyxin-CEIA. Table 3.7 shows that when the eggs were inoculated with an average of 20 cfu all of the replicates gave a positive reaction in the assay. Four out of five replicates produced positive reactions with an average inoculum of 2 cfu, and none of the replicates were positive with an average of 0.2 cfu per egg. A similar trend was obtained when BY-Nal devoid of egg sample was inoculated with the different cell numbers. After 20 h incubation, the eggs receiving an inoculum of 2 cfu gave an average cell density of ca. 10⁶ cfu/ml in the cultures (as determined by viable counts on NAL plates), which was more than sufficient to meet the detectability of the polymyxin-CEIA. Therefore, these results suggest that as few as 2 cfu could be detected in an individual egg by this procedure.

This chapter has demonstrated the applicability of the polymyxin-CEIA as a screening tool for the detection of S. enteritidis in enrichment cultures from eggs. Although the polymyxin-CEIA employing the serogroup D detector antibodies was reactive in the assay of other Salmonella spp. bearing the Factor O:9 antigen (e.g., S. eastbourne and S. fresno), any salmonellae occurring in the contents of intact eggs are

Table 3.7. Assay of Nal^a *S. enteritidis* strain in individual eggs

Sample	Positives/Total replicates ^a		
	cfu inoculated		
	20	2	0.2
Egg	5/5	4/5	0/5
BY-Nal alone^b	5/5	5/5	0/5

^a No. of replicates per total no. of samples inoculated giving a positive reaction in the SE polymyxin-CEIA.

^b 100 ml portions of BY-Nal devoid of egg sample.

potential health hazards, and their detection would therefore be of interest.

In the interest of shortening the time required to complete the analysis, I chose to apply the polymyxin-CEIA in the assay of primary enrichment cultures of the eggs. Thus, this inexpensive assay should permit the identification of negative cultures in less than 2 days. However, the polymyxin-CEIA performed equally well in the assay of S. enteritidis in eggs using the traditional enrichment protocol (Andrews et al., 1984) involving 24 h of pre-enrichment in nutrient broth using a ca. 1:9 egg to broth ratio, followed by 24 h selective enrichment in selenite cystine broth. The lower volume of broth used per egg in the present enrichment method, which uses a volume reduction factor of ca. 4.5 (as compared to the traditional protocol), further simplifies and economizes the procedure, making it particularly attractive for large scale users such as the food industry and government testing facilities.

The results presented in this chapter has been published in the following paper:

Wang, Haiyan, Burton W. Blais and Hiroshi Yamazaki (1995) Rapid and economical detection of Salmonella enteritidis in eggs by the polymyxin-cloth enzyme immunoassay. International Journal of Food Microbiology 24:397-406.

CHAPTER 4

RAPID CONFIRMATION OF POLYMYXIN-CLOTH ENZYME

IMMUNOASSAY FOR Salmonella enteritidis IN EGGS

BY POLYMERASE CHAIN REACTION

ABSTRACT

A rapid, simple and inexpensive combined screening test and confirmation procedure was developed for Salmonella enteritidis in eggs. S. enteritidis inoculated into egg samples was pre-enriched in a minimal volume of enrichment broth, which was then assayed by the polymyxin-cloth enzyme immunoassay (polymyxin-CEIA): cholate-extracted lipopolysaccharide antigens were captured on polymyxin-coated polyester cloth and detected by sequential reactions with factor O:9 rabbit antiserum, anti-rabbit antibody-peroxidase conjugate and peroxidase substrate. Presumptive positive results obtained in the polymyxin-CEIA were confirmed by subjecting pre-enriched samples directly to a polymerase chain reaction (PCR) technique targeting Salmonella-specific invA gene sequences. The polymyxin-CEIA gave positive results in the assay of 18 different S. enteritidis strains inoculated into eggs, all of which were confirmed by the invA PCR. This entire procedure, combining the steps of sample pre-enrichment in a minimal volume of broth, polymyxin-CEIA screening and rapid confirmation by PCR, gave a short turn around time from sample collection to completion of the analysis of within 26 h.

INTRODUCTION

The occurrence of Salmonella enteritidis in the contents of whole-shell chicken eggs has emerged as an important food safety issue in the United States and several European countries (Poppe *et al.*, 1993; Madden, 1994). The traditional culture method for detection of this pathogen in eggs is cumbersome, time-consuming (up to 7 days) and

expensive. In order to facilitate the routine monitoring of large numbers of samples rapid, simple and inexpensive polymyxin-cloth enzyme immunoassays (polymyxin-CEIA) have been developed for Salmonella in general (Chapters 2; Blais *et al.*, 1993), and S. enteritidis in particular (Chapter 3; Wang *et al.*, 1995), for screening pre-enrichment cultures. The economy of the detection procedure for S. enteritidis was further improved by minimizing the volume of pre-enrichment broth needed to enrich the samples (1:2 ratio of egg to broth) (Chapter 3; Wang *et al.*, 1995). However, positive results obtained in any such rapid methods are considered presumptive because immunoassays can still give false positive results especially when using polyclonal detector antibodies (Chapter 2; Blais *et al.*, 1993), and should be confirmed by a method based on a different principle. Although further testing of presumptive positive samples by the traditional battery of biochemical and immunological tests might provide adequate confirmation, such an approach adds considerable labour and time (i.e., several days) to the entire procedure, somewhat diminishing the advantages of the rapid test strategy (eg. polymyxin-CEIA). The highly sensitive and specific polymerase chain reaction (PCR) techniques amplifying unique DNA sequences may provide an excellent alternative method for the rapid confirmation of results of the polymyxin-CEIA. A group of genes, invABC and invD, allow S. typhimurium to penetrate cultured epithelial cells. Regions of high sequence similarity to invABC genes have been found in all the Salmonella isolates, but not in those non-salmonellae examined in one study (Galan and Curtiss III, 1991). A Salmonella genus-specific PCR technique targeting invA gene sequences was therefore developed and its specificity has been thoroughly evaluated (Rahn *et al.*, 1992).

However, the PCR is usually strongly inhibited when applied directly to samples derived from foods because of inhibitory substances in foods and enrichment media (Rossen *et al.*, 1992; Blais and Phillippe, 1993b). Extraction of DNA or removal of inhibitory substances adds labour, time and uncertainty because of possible DNA loss.

This chapter demonstrates that the *invA* PCR technique can be directly applied to samples of high egg content containing *S. enteritidis*, and that the polymyxin-CEIA followed by this PCR provides a rapid screening and subsequent confirmation procedure for this pathogen.

MATERIALS AND METHODS

Bacterial strains and Eggs

A total of 18 *S. enteritidis* strains isolated from eggs, poultry and environmental samples at several Agriculture and Agri-Food Canada laboratories were examined in this study. Other *Salmonella* and non-*Salmonella* bacteria examined are listed in Table 4.2. *Salmonella* strains were routinely grown by shaking in buffered peptone water (BPW; Difco) at 37°C for 16-20 h. Media and growth conditions for other bacteria were as previously described (Chapter 2; Blais *et al.*, 1993). Whenever necessary, viable counts were determined by plating serial dilutions of broth cultures on nutrient agar and incubating the plates at 37°C for 20 h.

The eggs used in this study were from Canadian producers and were purchased from local retailers. The contents of eggs were aseptically transferred to a stomacher bag and mixed with enrichment broth (Brain Heart Infusion broth (Difco) supplemented with

0.5% (w/v) yeast extract (Difco), BY) at an egg to broth ratio of 1:2 (egg-broth mixture) by stomaching as previously described (Wang *et al.*, 1995). Ten ml of the egg-broth mixture was then inoculated with *S. enteritidis* and incubated at 37°C for 20 h.

Preparation of samples for Polymyxin-CEIA and Polymerase chain reaction

Lipopolysaccharide (LPS) antigens in broth cultures without eggs were extracted by the addition of 0.1 volume of 5 % (w/v) sodium cholate in 0.01 M phosphate-buffered (pH 7.2)/0.85 % NaCl (PBS), followed by heating at 100°C for 10 min, and then used directly in the polymyxin-CEIA as described below. LPS in pre-enriched egg-broth samples were extracted by the addition of 0.1 vol. of 15 % (w/v) sodium cholate, followed by heating as before. The cholate-heat-treated egg-broth mixture was then centrifuged at 3000 X g for 5 min to sediment coagulated egg material, and an aliquot of the resulting supernatant was used in the polymyxin-CEIA. For the polymerase chain reaction, aliquots of broth cultures without eggs were added directly to the reaction mixture, and pre-enriched egg-broth samples were used directly before or after the cholate-heat treatment and centrifugation as above.

Polymyxin-CEIA

Polyester cloth (DuPont, Sontara 8100) was cut into 6 X 4 cm sheets, then saturated with 3 ml of polymyxin B sulfate (Sigma, No. P-1004) solution (5 mg/ml in PBS). After incubation at 37°C for 16 h, each sheet was washed with a total of about 6 X 10 ml of PBS containing 0.05 % (v/v) Tween 20™ (PBST) on a filter under suction.

Once coated, the polymyxin-cloth sheets could be stored moistened with PBS at 4°C until use. Five μ l of extracted LPS sample was pipetted onto the polymyxin-cloth sheet at 0.5 cm intervals, so that each cloth sheet accommodated a total of 40 samples. After 20 min at room temperature, the cloth was washed with PBST as above, and then saturated with 3 ml of rabbit factor O:9 antiserum (Difco, No. 2818-47) diluted 1:200 in PBST containing 0.2 % (w/v) blocking reagent (Boehringer Mannheim, No. 1093-657)(PBST-B). After 10 min at room temperature, the cloth was washed with PBST and saturated with 3 ml of a mixture of anti-rabbit IgG-peroxidase (Sigma, No. A-6154) and anti-rabbit IgM-peroxidase (Bethyl Laboratories, Inc., No. A120-110P-3) conjugates, each diluted 1:1000 in PBST-B, and incubated for 10 min at room temperature. The cloth was then washed with PBST and incubated with 3 ml of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate system (RicoH Kyosan Inc., Tokyo, Japan) for 20 min at room temperature. Reactions were graded qualitatively as follows: strong positive reaction (dark blue spot), weak positive reaction (light blue spot), no reaction (no spot).

Oligonucleotide primers and Polymerase chain reaction (PCR)

Oligonucleotide primers for the amplification of a 284-bp *invA* DNA fragment were as described by Rahn *et al.* (1992) (Table 4.1), and were synthesized on a DNA synthesizer (model 391, PCR-Mate-EP; Applied Biosystems) using phosphoramidite chemistry (Applied Biosystems) as specified by the manufacturer. For the PCR, 10 μ l of sample was added to 89.5 μ l of PCR mixture containing 0.22 mM of each deoxynucleoside triphosphate (dNTP), 1.1 μ M of each primer, 2.2 mM MgCl₂, 55 mM

KCl, 11 mM Tris-HCl (pH 8.3), and 0.11% (w/v) Triton X-100. The mixture was then overlaid with mineral oil, placed in a thermal cycler (model TC480; Perkin-Elmer Cetus) and held at 100°C for 10 min for cell lysis. The temperature was then adjusted to 80°C for 10 min before adding 0.5 μ l (2 units) of Taq DNA polymerase (Boehringer Mannheim, No. 1596594), then raised to 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 2 min. An additional 2 min at 72°C was allowed for the completion of primer extension after the last cycle. Amplicons were analyzed by electrophoresing 10 μ l of PCR product on a 1.2% (w/v) agarose gel at 80 V for 1.5 h, followed by staining for 20 min in ethidium bromide solution (10 μ g/ml). DNA on the gels was visualized by fluorescence under UV light and photographed onto Polaroid 667 film. The size of the amplicon was determined by including a 123-bp ladder DNA molecular size marker (GIBCO-BRL, No. 5613SA) in each gel.

Table 4.1. Base sequences and locations of oligonucleotide primers

Primer	Sequence (5'-3')	Location within <i>invA</i> gene
U	gtgaaattatcgccacgttcgggcaa	287-312
V	tcatcgaccgtcaaaggaacc	571-550

Preparation of invA gene probe and hybridization

The 284-bp *invA* PCR fragment was labelled with digoxigenin and used as a probe to test the specificity of the PCR method. The 284-bp DNA fragment was purified by electrophoresis of the PCR product on a low-melting point agarose gel and excision of the appropriate band according to the method of Sambrook *et al.* (1989). Purified DNA was labelled with digoxigenin-dUTP using a random primed DNA labelling and detection kit (Boehringer Mannheim, No. 1093657) as specified by the manufacturer.

For southern hybridization experiments, PCR products from *S. enteritidis* strains and non-*Salmonella* were first electrophoresed on a 1.2 % (w/v) agarose gel and then transferred onto a nylon membrane (Boehringer Mannheim, No. 1209-299) using a nucleic acid transfer apparatus (model 785 Vacuum Blotter; BIO-RAD) following the manufacturer's instructions. The membrane was allowed to air dry and the DNA was fixed by a 1 min exposure to UV (254 nm) at 120 mJ/cm² using a UV box (model CL-1000 Ultraviolet Crosslinker; Ultraviolet Products LTD.). The membrane was then hybridized with the digoxigenin-labelled DNA probe and detected according to the instructions provided in the random primed DNA Labelling and Detection Kit.

For dot-blot hybridization, bacteria were lysed as following: an overnight bacterial culture (ca. 10⁹ cells/ml) was centrifuged at 8,000g for 15 min and the cell pellet was resuspended in 1/2 volume of 0.4N NaOH, followed by incubation at 80°C for 20 min. Five µl of lysate was spotted onto a nylon membrane and air-dried. The membrane was rinsed briefly with 2 X SSC (1 X SSC is 0.15 M NaCl and 0.015 M sodium citrate) and then hybridized with the digoxigenin-labelled DNA probe and detected as above.

RESULTS AND DISCUSSION

Specificity of the polymyxin-CEIA and invA PCR methods

The specificity of the polymyxin-CEIA and invA PCR methods were examined in the analysis of several S. enteritidis, other Salmonella and a variety of non-Salmonella strains. Pure cultures of the 18 Salmonella enteritidis strains were all reactive in the invA PCR, giving the expected 284-bp amplicon (as shown in Fig.4.1), as well as in the polymyxin-CEIA. All other Salmonella serovars tested, and none of the non-Salmonella bacteria, were positive for the production of the 284-bp amplicon (Table 4.2). The S. eastbourne and S. fresno strains gave positive results in the polymyxin-CEIA (Table 4.2), since these are serogroup D Salmonella and would therefore be reactive with the detector antibodies (factor O:9) used in the assay. All of the other Salmonella (non-serogroup D) and non-Salmonella bacteria tested were negative in the polymyxin-CEIA. Several of the non-Salmonella bacteria (Enterobacter cloacae, Shigella sonnei and one Escherichia coli strain) gave non-specific amplification products in the PCR (none of these products corresponded to the size of the 284-bp amplicon by agarose gel electrophoresis analysis).

Fig.4.1. Application of the invA PCR directly to enrichment cultures from eggs. Egg-broth mixtures were inoculated with 10 S. enteritidis (ATCC 13076) cfu and incubated at 37°C for 20 h to give a final density of ca. 10⁸ cfu/ml. Samples were then subjected to the invA PCR before (lane 1) or after (lane 2) cholera-heat treatment and centrifugation as described in Methods. For comparison, a pure culture of S. enteritidis in BPW (ca. 10⁸ cfu/ml) was also subjected to PCR (lane 3). Lane m is 123-bp DNA molecular size marker. Arrowhead denotes the position of 284 bp PCR amplicon.

Table 4.2. Reactivity of non-*S. enteritidis* in the polymyxin-CEIA and *invA* PCR tests

Strain	Source	Results by:	
		Polymyxin-CEIA ^a	PCR ^b
<u>Salmonella</u>(serogroup)			
<u>S. abony</u> (B)	Feed	-	+
<u>S. agona</u> (B)	Feed	-	+
<u>S. amsterdam</u> (E1)	Feed	-	+
<u>S. binza</u> (E2)	Feed	-	+
<u>S. blockley</u> (C2)	Environmental	-	+
<u>S. braenderup</u> (C1)	Environmental	-	+
<u>S. eastbourne</u> (D1)	Egg	+	+
<u>S. fresno</u> (D2)	Feed	+	+
<u>S. haardt</u> (C3)	Environmental	-	+
<u>S. newington</u> (E2)	ATCC 29628	-	+
<u>S. rubislaw</u> (F)	ATCC 10717	-	+
<u>S. typhimurium</u> (B)	ATCC 14028	-	+
<u>Non-Salmonella</u>			
<u>Bacillus cereus</u>	ATCC 14579	-	-
<u>B. subtilis</u>	ATCC 6051	-	-
<u>Citrobacter freundii</u>	ATCC 8090	-	-
<u>Enterobacter aerogenes</u>	ATCC 13048	-	-
<u>E. cloacae</u>	ATCC 13047	-	NS
<u>Enterococcus faecalis</u>	ATCC 19433	-	-
<u>Escherichia coli</u>	ATCC 11775	-	NS
	Feed	-	-
	Dairy	-	-
<u>Klebsiella pneumoniae</u>	ATCC 13883	-	-
<u>Listeria innocua</u>	ATCC 33090	-	-
<u>L. ivanovii</u>	ATCC 19119	-	-
<u>L. welshimeri</u>	Dairy	-	-
<u>Proteus vulgaris</u>	ATCC 13315	-	-
<u>Shigella sonnei</u>	ATCC 29938	-	NS
<u>Staphylococcus aureus</u>	ATCC 12600	-	-
<u>Yersinia enterocolitica</u>	ATCC 9610	-	-

^a +, production of a blue spot; -, no spot.

^b +, production of a 284-bp amplicon; NS, non-specific amplicon; -, no amplicon.

Southern hybridization experiments in which digoxigenin-labelled invA PCR DNA from S. typhimurium (ATCC 14028) was used as a probe for PCR products from 4 S. enteritidis and the 3 non-Salmonella strains showed that the amplification products from non-salmonellae did not hybridize with the Salmonella-derived PCR probe, whereas the probe did hybridize with the 284-bp amplicon from the S. enteritidis strains (Fig.4.2). The PCR products from these non-Salmonella strains are therefore non-specific. Conversely, dot-blot hybridization experiments in which digoxigenin-labelled invA PCR DNA from S. enteritidis (ATCC 13076) was used to probe total DNA from lysates of several Salmonella and non-Salmonella strains showed that only the Salmonella strains hybridized with the probe (Fig.4.3). These experiments confirm the specificity of the invA PCR for Salmonella, and demonstrate that the 284-bp amplicons generated from S. typhimurium and S. enteritidis are homologous. Previously, Rahn et al. (1992) tested the specificity of the invA PCR by demonstrating hybridization of the PCR product with a 1.5 Kb Sal I-Eco RI fragment of the cloned invA gene. Taken together, all of these results strongly support the use of the invA PCR method as a reliable tool for the identification of Salmonella isolates.

Fig. 4.2. Southern hybridization to check the specificity of the products invA PCR.

PCR products from 4 strains of S. enteritidis and 3 strains of non-salmonellae were hybridized with a DNA probe from S. typhimurium invA PCR product.

A. Agarose gel electrophoresis of PCR products.

B. Southern hybridization on nylon membrane.

Lane 1-4, S. enteritidis strains; Lane 5, E. coli ATCC 11775; Lane 6, Shigella sonnei; Lane 7, Enterobacter cloacae.

Fig. 4.3. Dot blot hybridization to check the specificity of invA PCR.

Cell lysates from several Salmonella and non-Salmonella strains were spotted on nylon membrane and hybridized with a DNA probe from S. enteritidis invA PCR product.

a1, S. abony; a2, S. agona; a3, S. braenderup; a4, S. blockley; a5, S. haardt;
 a6, S. eastbourne; b1, S. enteritidis; b2, S. fresno; b3, S. amsterdam; b4, S. binza;
 b5, S. newington; b6, S. rubislaw; c1, E. coli; c2, Shigella sonnei; c3, Enterobacter aerogenes;
 c4, Staphylococcus aureus; c5, Bacillus cereus; c6, B. subtilis.

	1	2	3	4	5	6
a	●	●	●	●	●	●
b	●	●	●	●	●	●
c						

Performance of the *invA* PCR using enrichment cultures from eggs

In performing PCR analyses on a routine basis, it would be most convenient to sample enrichment cultures directly to the reaction mixture without any prior manipulation of the target cells. However, certain substances present in foods and enrichment media can inhibit the PCR when introduced into the reaction mixture (Rossen *et al.*, 1992; Blais and Phillippe, 1993). Therefore, it was necessary to examine the performance of the *invA* PCR using *S. enteritidis* cells enriched in egg-broth mixture. Fig. 4.1 shows that the *invA* PCR was able to amplify the expected 284-bp fragment from *S. enteritidis* cells directly in an egg-broth culture (lane 1). The PCR performed equally well when the egg-broth culture was first subjected to cholate-heat treatment and centrifugation (lane 2), which is required in the preparation of samples for the polymyxin-CEIA. The amplicons from the egg-broth cultures did not differ qualitatively from that of a pure culture in BPW (lane 3).

The effect of the media and the presence of egg material in the cell suspension on the detection limit of the PCR was examined by suspending *S. enteritidis* cells at various densities in different combinations of enrichment broth, Tris-buffered saline (TBS) and egg sample. Fig. 4.4 shows that the PCR gave maximum detectability and quality of the 284-bp amplicon when the cells were suspended in TBS (i.e., in the absence of enrichment media or egg material) (lanes 1-3). Suspending the cells in enrichment broth resulted in extensive smearing of the amplicon band (lanes 4-6), an effect probably due to the presence of interfering substances in BHI (Blais and Phillippe, 1993). The presence of egg material in the enrichment broth (lanes 7-9) and

Fig.4.4. Effect of different sample compositions on the invA PCR. *S. enteritidis* (ATCC 13076) cells were suspended at various densities in either 0.01 M Tris-buffered (pH 8.0)/0.85 % NaCl (TBS), lanes 1-3; enrichment broth (BY), lanes 4-6; egg-broth mixture, lanes 7-9; egg-TBS (1:2) mixture, lanes 10-12. Samples of each were directly subjected to the invA PCR and the products analyzed by agarose gel electrophoresis as described in Methods. Cell densities were as follows: lanes 1, 4, 7 and 10, 8×10^7 cfu/ml; lanes 2, 5, 8 and 11, 8×10^6 cfu/ml; lanes 3, 6, 9 and 12, 8×10^5 cfu/ml.

m 1 2 3 4 5 6 7 8 9 10 11 12

— 284

TBS (lanes 10-12) suspensions produced some interference with the PCR, diminishing the detectability of the method. The sensitivity of this PCR for *S. enteritidis* in TBS is ca. 10^5 cfu/ml (data not shown), there is ca. 80-fold reduction in sensitivity by the presence of egg in TBS. Interestingly, the presence of egg material in the enrichment broth eliminated the smearing noted in lanes 4-6. The reason for this is not known.

To be useful in the confirmation of presumptive positive enrichment cultures initially screened by the polymyxin-CEIA, the *invA* PCR should achieve or surpass the detection limit (minimum number of cells detectable) of the initial screening assay. The detectability of the polymyxin-CEIA for *S. enteritidis* was previously determined to be in the range of $0.5-2 \times 10^6$ cfu/ml (Chapter 3; Wang et al., 1994). Table 4.3 shows that the detectability of the *invA* PCR for different *S. enteritidis* strains suspended in egg-broth mixture ranged from $0.8-3.0 \times 10^5$ cfu/ml, which is at least 10-fold greater than that of the polymyxin-CEIA. Thus, despite the effect of egg-broth mixture on the detectability of the *invA* PCR, the minimum cell density detectable by this technique was sufficiently low to exceed the detection limit of the polymyxin-CEIA by a comfortable margin.

Table 4.3. Detection limit of *invA* PCR for various *S. enteritidis* strains in egg-broth mixture*

Source	Detection limit (cfu/ml X 10 ⁶)
ATCC 13076	2.3
Environmental	0.8
Egg	1.1
Poultry	3.0

* *S. enteritidis* isolates from various sources were suspended in egg-broth mixture at various cell densities, and the suspensions were directly subjected to the *invA* PCR. The detection limit is reported as the minimum cell density giving a detectable 284-bp amplicon upon agarose gel electrophoresis analysis.

These experiments have demonstrated the suitability of the invA PCR method for the confirmation of S. enteritidis in pre-enrichment cultures of egg samples initially screened by the polymyxin-CEIA. This strategy enabled the confirmation of polymyxin-CEIA results within several hours, as opposed to several days necessary using the traditional cultural and biochemical identification technique. The ability of the method to amplify target sequences directly in egg-broth mixtures, without the need for prior separation of the target cells to remove interfering substances, provides convenience for routine monitoring applications. On the basis of these results the following procedure is recommended for the detection of S. enteritidis in eggs: (1) overnight pre-enrichment in 1:2 ratio of egg to broth, (2) screen pre-enrichment cultures by the polymyxin-CEIA to rapidly identify presumptive positive samples, and (3) confirm positives by the invA PCR method. This entire procedure, from sample collection to completion of the analyses, can be finished within 26 h.

The work done in this chapter has been presented in the following paper which was accepted for publication:

Wang, Haiyan, Burton W. Blais and Hiroshi Yamazaki (1995) Rapid confirmation of polymyxin-cloth enzyme immunoassay for Salmonella enteritidis in eggs by polymerase chain reaction. Food Control (In press).

CHAPTER 5

A POLYMYXIN-CLOTH ENZYME IMMUNOASSAY FOR Salmonella

O SEROGROUP IDENTIFICATION

ABSTRACT

The preceding chapters (Chapter 2-4) have demonstrated the applicability of the polymyxin-CEIA as a tool for the detection of Salmonella in foods. In this chapter, the applications of the polymyxin-CEIA technology are expanded to include a simple method for the serological identification of Salmonella O antigens by capture of the lipopolysaccharide antigens on polymyxin-coated polyester cloth followed by immunoenzymatic detection of the captured O antigens using rabbit Salmonella O antisera and an anti-rabbit IgG-peroxidase conjugate. By using a strip of polymyxin-coated cloth, it was possible to characterize a given isolate with multiple antisera dot blotted on the strip. Conversely, the same format enabled the simultaneous characterization of multiple isolates with a given antiserum on a single strip. This method was much less cumbersome and time-consuming than the traditional agglutination technique, and utilized very little of the antisera.

INTRODUCTION

Members of the genus Salmonella are an important public health concern worldwide, and infections in humans are most commonly acquired through the ingestion of contaminated foods, milk and water. More than 2300 Salmonella serotypes have been identified, based on the O (lipopolysaccharide), H (flagellar) and Vi (capsular) antigenic properties of individual isolates. In diagnosing salmonellosis or detecting Salmonella contamination in foods and other samples, the organisms are usually first isolated using cultural methods, followed by identification of the isolates using a battery of biochemical

and serological tests (D'Aoust *et al.*, 1992). The serological identification of Salmonella isolates helps to trace the source or origin of the contamination.

The most widely used technique for serological characterization involves the determination of O and H antigens by agglutination of the bacterial cells with highly specific antisera (Ewing, 1986). Since the O antigens are highly variable, with the classification of salmonellae into 46 O-serogroups, establishing the O antigenic serogroup of an isolate is an important step in its identification. Most diagnostic laboratories routinely perform partial or complete O antigenic analyses of isolates.

Bacto Salmonella O antisera are employed in the identification of the somatic (O) antigens of members of genus Salmonella by the slide agglutination technique. Briefly, O antigens have been assigned Arabic numbers, in consecutive order, from 1-64. Some somatic antigens were deleted from the schema for one reason or another, therefore there is not a complete continuity of the numbers. For convenience, the salmonellae are placed into "serogroups" according to their somatic content. There is at least one somatic antigen which is shared in common by all members of a given serogroup. Bacto Salmonella O Group Antisera contain all factors present in the named group, Bacto Salmonella O Factor Antisera contain only the factors indicative of the individual groups, while Bacto Salmonella O Antisera Poly A through G are polyvalent antisera containing a number of different groups to help to narrow the number of individual antisera which must be tested (Table 5.1).

The traditional approach to serological analysis involving slide agglutination of bacterial cells with O antisera is cumbersome and time-consuming, especially when large

Table 5.1. The contents of the Salmonella O Antisera Poly A through G

Salmonella Poly Groups	Somatic Groups Present
Poly A	A, B, D, E, L
Poly B	C, F, G, H
Poly C	I, J, K, M, N, O
Poly D	P, Q, R, S, T, U
Poly E	V, W, X, Y, Z
Poly F	51-55
Poly G	56-61

numbers of isolates need to be analyzed, and consumes large amount of the precious typing antisera. Therefore, there is a need for simpler and less expensive methods for the identification of Salmonella O antigens in the routine analysis of isolates.

In previous chapters (Chapter 2-4), I have described a polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) for Salmonella detection in foods which utilizes polymyxin-coated polyester cloth as an adsorbent for the capture of LPS antigens and their subsequent immunoenzymatic assay using O antigen or core LPS specific antibodies and an antibody-enzyme conjugate. As an LPS capturing agent, polymyxin has the advantages of being more stable and much less expensive than antibodies. Furthermore, the large surface area of the cloth gives superior solid phase immunoreaction kinetics, making this test more rapid than enzyme immunoassays carried out in microtiter plates. The macroporosity of the cloth permits rapid and efficient washings between each immunoreaction step. In this Chapter I will demonstrate the applicability of the polymyxin-CEIA in the O antigenic analysis and identification of Salmonella isolates using a simple dot blot format where large strips or sheets of polymyxin-coated cloth permit the simultaneous processing of multiple samples.

MATERIALS AND METHODS

Chemicals and Immunoreagents

The following reagents were purchased from Sigma Chemical Co.: polymyxin B sulfate (No. P-1004); sodium cholate (No. C-1254) and anti-rabbit IgG-peroxidase conjugate (No. A-6154). Blocking reagent (No. 1093-657) was purchased from

Boehringer Mannheim. Bacto Salmonella O antisera poly A (serogroups A, B, D, E₁, E₂, E₃, E₄ and L)(No. 2534-47), poly B (serogroups C₁, C₂, F, G and H)(No. 2535-47), poly C (serogroups I, J, K, M, N, and O)(No. 2536-47), poly D (serogroups P, Q, R, S, T and U)(No. 2537-47) and factors O:2 (serogroup A) (No. 2814-47), O:4 (serogroup B)(No. 2659-47), O:8 (serogroup C)(No. 2817-47), O:9 (serogroup D)(No. 2818-47) and O:3,15 (serogroup E)(No. 2954-47) were purchased from Difco Laboratories. The membrane peroxidase substrate system used in this study is a proprietary formulation based on the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) and an unspecified enhancer and was a kind gift from Ricoh Kyosan Inc. (Tokyo, Japan).

Bacterial strains

The Salmonella serovars used in this study are listed in Table 5.2. They were isolated from a variety of food, feed and environmental samples collected from various manufacturers by Agriculture and Agri-Food Canada inspection staff and submitted for routine microbiological analysis to Laboratory Services Division (LSD), Ottawa, Agriculture and Agri-Food Canada. The bacteria were routinely grown by plating on nutrient agar (NA) or by shaking in buffered peptone water (BPW) at 37°C for 16-20h.

Polymyxin-CEIA

Polyester cloth (Dupont, Sontara 8100) was cut into 1 X 5 cm strips and saturated with ca. 1 ml of 5 mg/ml polymyxin B sulfate in 0.01M phosphate-buffered (pH 7.4)/0.85% NaCl (PBS). After incubation at 37°C for 16 h, each strip was washed with

Table 5.2. Salmonella isolates examined in this study

Organism	Serogroup
<u>S. paratyphi</u> A	A
<u>S. abony</u>	B
<u>S. agona</u>	B
<u>S. banana</u>	B
<u>S. typhimurium</u>	B
<u>S. braenderup</u>	C1
<u>S. infantis</u>	C1
<u>S. livingstone</u>	C1
<u>S. mbandaka</u>	C1
<u>S. blockley</u>	C2
<u>S. hadar</u>	C2
<u>S. haardt</u>	C3
<u>S. kentucky</u>	C3
<u>S. eastbourne</u>	D1
<u>S. enteritidis</u>	D1
<u>S. amsterdam</u>	E1
<u>S. london</u>	E1
<u>S. orion</u>	E1
<u>S. binza</u>	E2
<u>S. thomasville</u>	E3
<u>S. bleedon</u>	J
<u>S. blukwa</u>	K
<u>S. cerro</u>	K
<u>S. alachua</u>	O
<u>S. johannesburg</u>	R

a total of about 6 X 5 ml of PBS containing 0.05% (v/v) Tween 20 (PBST) on a filter under suction. Polymyxin-coated cloth strips were moistened with PBS and stored at 4°C until use.

For the polymyxin-CEIA, bacterial lipopolysaccharide (LPS) antigens were extracted from cell suspensions (broth cultures or colonies from plates suspended in PBS) by the addition of 0.1 volume of 5% (w/v) sodium cholate and heated at 100°C for 5 min. The optimized working dilutions for the various O antisera used in the polymyxin-CEIA were as follows: poly A, poly B, and poly D were diluted 1:800 in PBST containing 0.2% (w/v) blocking reagent (PBST-B); poly C, factor O:2, O:4 and O:8 were diluted 1:400 in PBST-B; and factors O:9 and O:3,15 were diluted 1:200 in PBST-B.

Two approaches were used in the serological analysis of Salmonella isolates by the polymyxin-CEIA:

I. Analysis of individual isolates with multiple antisera.

One ml of cholate-heat-treated Salmonella suspension was pipetted onto a polymyxin-coated cloth strip and incubated for 10 min at room temperature. The strip was then washed with PBST, and 5 µl of the different typing antisera were spotted at ca. 0.5 cm intervals, such that a single 1 X 5 cm strip could accommodate up to 7 samples. After 5 min incubation at room temperature, the strip was washed with PBST, and then saturated with 1 ml of anti-rabbit IgG-peroxidase diluted 1:1000 in PBST-B and incubated for 5 min at room temperature. The strip was then washed with PBST and incubated with 1 ml TMB membrane peroxidase substrate for 10 min at room temperature. Reactions

were graded qualitatively on the basis of whether they were positive (formation of a blue spot) or negative (no spot).

II. Simultaneous analysis of multiple isolates with individual antisera.

Five μl of different cholera-heat-treated Salmonella suspensions were spotted on a polymyxin-coated cloth strip at ca. 0.5 cm intervals, such that a 1 X 5 cm strip could accommodate up to 7 samples. After 10 min incubation at room temperature, the strip was washed with PBST, then saturated with 1 ml of diluted O antiserum and incubated for 10 min at room temperature. The strip was washed with PBST, and then saturated with 1 ml of anti-rabbit IgG-peroxidase conjugate diluted 1:1000 in PBST-B and incubated for 10 min at room temperature. The strip was then washed with PBST and incubated with 1 ml of TMB for 10 min at room temperature. Reactions were graded qualitatively as above.

Slide agglutination

For the purpose of comparison, Salmonella serovars were also analyzed by the slide agglutination technique according to the instructions provided by the manufacturer of the typing antisera (Difco). Briefly, one drop (30-40 μl) of 0.85% NaCl solution and one drop of antiserum were placed at each end of a glass slide. A loopful of growth from NA plates was transferred to the NaCl and the antiserum on the slide, and a smooth suspension of the cells was made. The slide was then rocked gently for 1 min.

Agglutination reactions were graded as follows:

4+, 100% agglutination; 3+, 75% agglutination; 2+, 50% agglutination; +, 25% agglutination; +/-, <25% agglutination; -, no agglutination.

RESULTS AND DISCUSSION

Serological screening with polyvalent O group-specific antisera

In the isolation of Salmonella from foods and other samples during their routine analysis, most diagnostic laboratories require only limited serological characterization of presumptive positive isolates to confirm that they are indeed Salmonella. Thus, polyvalent O antisera containing pools of antibodies to a number of different serogroups are commonly used to screen isolates, and also to reduce the number of individual antisera which must be used to achieve a complete serological analysis when this is desired. Therefore the performance of the polymyxin-CEIA in the serological screening of several Salmonella serovars was first examined using a panel of commercially available polyvalent antisera. Table 5.3 shows that the polymyxin-CEIA correctly identified 16 different Salmonella serovars based on their reactivity with the polyvalent antisera representing their respective serogroups. Analysis of these strains by the traditional slide agglutination technique using the polyvalent antisera confirmed the results obtained with the polymyxin-CEIA (Table 5.3).

Serotyping using O serogroup-specific antisera

In the previous section, polyvalent antisera recognizing a broad range of

Table 5.3. Screening *Salmonella* isolates using polyvalent antisera*

Organism	Antiserum							
	Poly A		Poly B		Poly C		Poly D	
	A	B	A	B	A	B	A	B
<i>S. abony</i>	+	2+	-	-	-	-	-	-
<i>S. agona</i>	+	+	-	-	-	-	-	-
<i>S. banana</i>	+	+	-	-	-	-	-	-
<i>S. typhimurium</i>	+	2+	-	-	-	-	-	-
<i>S. enteritidis</i>	+	3+	-	-	-	-	-	-
<i>S. orion</i>	+	2+	-	-	-	-	-	-
<i>S. braenderup</i>	-	-	+	+	-	-	-	-
<i>S. infantis</i>	-	-	+	+	-	-	-	-
<i>S. livingstone</i>	-	-	+	+	-	-	-	-
<i>S. mbandaka</i>	-	-	+	+	-	-	-	-
<i>S. hadar</i>	-	-	+	2+	-	-	-	-
<i>S. bleedon</i>	-	-	-	-	+	2+	-	-
<i>S. blukwa</i>	-	-	-	-	+	3+	-	-
<i>S. cerro</i>	-	-	-	-	+	3+	-	-
<i>S. alachua</i>	-	-	-	-	+	3+	-	-
<i>S. johannesburg</i>	-	-	-	-	-	-	+	3+

* One loopful of *Salmonella* cells from the growth on NA plates was suspended in 2.5 ml of PBS and then processed in the polymyxin-CEIA according to serotyping method I, as described in Methods, using the typing antisera poly A, poly B, poly C and poly D. Alternatively, a loopful of cells was directly subjected to the slide agglutination procedure using the same antisera. A, serotyping by the polymyxin-CEIA (method I); B, serotyping by slide agglutination of *Salmonella* serovars using O serogroup-specific antisera.

serogroups were used in the screening of Salmonella serovars by the polymyxin-CEIA. In some instances it may be desirable to obtain a more complete characterization of the O antigens. Therefore, the applicability of the polymyxin-CEIA in the analysis of Salmonella serovars using O serogroup-specific antisera was studied. The results obtained in the analysis of 13 Salmonella serovars show that the polymyxin-CEIA was able to correctly identify the different serogroups on the basis of the reactivities with their respective antisera (Table 5.4). No cross-reactions were observed in any of the isolates with the heterologous antisera, confirming the specificity of this method. Identical homologous reactions were obtained by the slide agglutination technique (Table 5.4). More detailed O antigenic analyses of the isolates can be achieved by using single factor antisera in the polymyxin-CEIA.

Simultaneous analysis of multiple isolates

In the preceding experiments, each Salmonella serovar was applied to an individual polymyxin-coated cloth strip which was then probed simultaneously with different typing antisera. For some applications, such as the screening of larger numbers of isolates, it may be more convenient to adopt a format in which multiple isolates are screened simultaneously with individual antisera. I examined such an application of the polymyxin-CEIA in the analysis of 7 Salmonella strains using different polyvalent antisera, where all of the strains were applied to several replicate strips and each strip was then probed with a different antiserum. Fig. 5.1 shows that each strain reacted only with its homologous antiserum. Thus, using this method it was possible to simultaneously

Table 5.4. Serotyping *Salmonella* isolates using serogroup-specific antisera*

Organism	Antiserum									
	Group A		Group B		Group C		Group D		Group E	
	A	B	A	B	A	B	A	B	A	B
<i>S. paratyphi</i> A	+/-	+/-	-	-	-	-	-	-	-	-
<i>S. abony</i>	-	-	+	4+	-	-	-	-	-	-
<i>S. agona</i>	-	-	+	3+	-	-	-	-	-	-
<i>S. typhimurium</i>	-	-	+	4+	-	-	-	-	-	-
<i>S. blockley</i>	-	-	-	-	+	2+	-	-	-	-
<i>S. haardt</i>	-	-	-	-	+	2+	-	-	-	-
<i>S. kentucky</i>	-	-	-	-	+	2+	-	-	-	-
<i>S. eastbourne</i>	-	-	-	-	-	-	+	3+	-	-
<i>S. enteritidis</i>	-	-	-	-	-	-	+	3+	-	-
<i>S. amsterdam</i>	-	-	-	-	-	-	-	-	+	+
<i>S. binza</i>	-	-	-	-	-	-	-	-	+	+
<i>S. london</i>	-	-	-	-	-	-	-	-	+	2+
<i>S. thomasville</i>	-	-	-	-	-	-	-	-	+	2+

* One loopful of *Salmonella* cells from the growth on NA plates was suspended in 2.5 ml of PBS and then processed in the polymyxin-CEIA according to serotyping method I, as described in Methods, using the various serogroup-specific antisera. Alternatively, a loopful of cells was directly subjected to the slide agglutination procedure using these antisera. A, serotyping by the polymyxin-CEIA (method I); B, serotyping by slide agglutination.

screen multiple isolates. For general screening of isolates to identify them at the genus level, the polyvalent antisera can be pooled to give a broader coverage of different possible serogroups. Alternatively, a more detailed analysis of the isolates can be achieved by substituting more specific antisera for the polyvalent antisera. Very large numbers of isolates can be screened by simply using larger sheets of polymyxin-coated cloth capable of accommodating a greater number of samples.

This chapter has demonstrated the applicability of the polymyxin-CEIA in the partial identification of Salmonella isolates using O antisera. Although the slide agglutination technique was rapid when applied to the analysis of very small numbers of isolates, the polymyxin-CEIA was much simpler to perform and more rapid overall when processing larger numbers of isolates. Furthermore, the interpretation of test results is greatly facilitated in the polymyxin-CEIA by the formation of readily distinguishable coloured spots for the positive samples. On the other hand, agglutination tests are sometimes difficult to interpret and somewhat subjective, since the analyst must determine the degree of agglutination that has occurred in order to score the reactions. The timing of the reactions is crucial to obtaining a reliable test result by the agglutination technique, since even negative samples may exhibit non-specific agglutination once the prescribed time is past. Such considerations are not as critical in the polymyxin-CEIA, since the immunoreactions normally reach equilibrium within 5-10 min, and do not change substantially thereafter. Another advantage of the polymyxin-CEIA is that the dot blot result may be stored for future reference, thus providing a

Fig. 5.1. Simultaneous analysis of multiple Salmonella isolates by the polymyxin-CEIA.

Five μ l of cholate-heat-treated broth cultures of different Salmonella isolates were spotted at ca. 0.5 cm intervals on polymyxin-coated cloth strips, which were then processed in the polymyxin-CEIA according to serotyping method II, as described in Methods. Bound Salmonella antigens on the polymyxin-coated cloth were detected by incubating the strips with either the poly A, poly B, poly C or poly D typing antisera. The isolates tested were as follows: spot 1, S. abony; spot 2, S. blockley; spot 3, S. enteritidis; spot 4, S. bleedon; spot 5, S. livingstone; spot 6, S. blukwa; spot 7, S. Johannesburg.

permanent record. Some other disadvantages of the agglutination technique include the possibility that cells might fail to react with the antisera if the O antigens are covered by capsular polysaccharides, and the need to disinfect all of the equipment used in carrying out the procedure. These problems are avoided in the present method since the cholera-heat treatment used to extract and expose the O antigens also kills the cells. Finally, the polymyxin-CEIA approach is much more economical, since the inherently greater sensitivity of this immunoenzymatic method permits the use of the antisera in highly diluted form. This is especially important, since high quality batches of antisera are sometimes difficult to obtain, and it is extremely desirable to prolong the use of reliable batches on hand. Provided that suitable O antisera are available, the polymyxin-CEIA approach to serological analyses should be applicable to other gram-negative pathogens, such as pathogenic Escherichia coli, Yersinia spp., Campylobacter spp., and others.

The results have been presented in the following published paper:

Wang, Haiyan, Burton W. Blais and Hiroshi Yamazaki (1994) A polymyxin-cloth enzyme immunoassay system for Salmonella O serogroup identification. Journal of Rapid Methods and Automation in Microbiology 3:105-113.

GENERAL CONCLUSIONS

The dramatic increase in the number of salmonellosis cases in recent years makes control measures for this pathogen more important than ever. The detection of Salmonella plays a key role in controlling this pathogen in the food supply. The application of the Hazard Analysis and Critical Control Point (HACCP) plan for ensuring a safe food supply will require fast, simple and economical detection methods since more frequent monitoring of this food pathogen will be necessary.

Enzyme immunoassay is becoming increasingly popular as rapid method for the detection of various harmful agents including salmonellae, since immunoreagents are relatively easy to prepare and the simple assay procedures provide a high degree of sensitivity. A polymyxin-cloth enzyme immunoassay (polymyxin-CEIA) was developed which utilizes polyester cloth coated with polymyxin B as an adsorbent for capturing and subsequent immunoenzymatic assay of LPS antigens (Blais, 1990; Blais and Yamazaki, 1990a,b; 1991). As demonstrated in these and other studies, the macroporous polyester cloth acts as a solid phase which promotes fast immunoreactions, and the use of polymyxin B as an LPS capturing agent provides a stable and inexpensive affinity ligand. The dot blot format of the polymyxin-CEIA which utilizes large sheets of cloth accommodating multiple samples provides a simple means for their simultaneous assay. The simplicity, rapidity and economy of the polymyxin-CEIA will permit wide applications for Salmonella detection and other food safety measures, some of which were examined in this thesis.

In the preliminary investigations of this thesis, I have demonstrated that cholate is better than deoxycholate as LPS extracting reagent, not only because it remains in a

liquid state at concentration upto 20% (w/v), which makes it more convenient to use, but also because it gave higher sensitivity than deoxycholate. I have also shown that polymyxin B is the best non-antibody LPS capturing agent among several cationic polypeptides and one protein, since it gave higher sensitivity and is less expensive than other agents tested.

I have demonstrated the applicability of an anti-core LPS monoclonal antibody in polymyxin-CEIA for detection of various Salmonella serovars, since most salmonellae are believed to share a common core LPS region (Tsang *et al.*, 1991). The control of cross-reactivity with non-salmonellae in the assay is difficult using polyclonal detector antibodies because the presence of antigenic factors common to other Enterobacteriaceae can not always be eliminated. On the other hand, monoclonal antibodies are highly specific, and could provide a good alternative to polyclonal antibodies. However, the production of different O-antigen specific monoclonal antibodies to allow for the detection of all Salmonella serotypes is cumbersome and impractical. In order to detect a wide range of salmonellae, many individual factor specific monoclonal antibodies would have to be produced and pooled for the assay. The results of my studies have proved for the first time that the core region LPS is present in many different Salmonella serovars and that they are accessible to the detector antibodies when LPS is bound to the polymyxin coated cloth. The fact that this monoclonal antibody failed to react with serogroup O salmonellae confirms that some degree of variability may exist in the Salmonella outer core oligosaccharide structure (Tsang *et al.*, 1991). I have shown that this monoclonal anti-core LPS antibody supplemented with single factor antiserum O:35

reacted with all of the Salmonella strains and none of the non-Salmonella strains tested in polymyxin-CEIA and the assay has been applied in the enrichment cultures of several food types. The production of a murine anti-factor O:35 monoclonal antibody will further simplify the assay procedure since only anti-mouse IgG-enzyme conjugate will be needed. The use of this monoclonal antibody eliminated cross-reactivity with non-salmonellae and allowed much easier quality control of the procedure. This will be most useful in developing commercial kits of the polymyxin-CEIA technology.

I have developed an assay procedure which allowed the specific detection of group D salmonellae (which includes S. enteritidis) when an anti-group D Salmonella antiserum was used as detector antibody in polymyxin-CEIA. The procedure involving pre-enrichment of egg-samples in a small volume of enrichment broth and their subsequent analysis by the polymyxin-CEIA, requires less than 2 days to detect as few as 1-2 cfu of S. enteritidis in a whole egg. The use of low enrichment broth to egg ratio (a ratio of 2:1 of broth:egg) was proved to be suitable for the recovery and detection of S. enteritidis cells even when injured. The low volume of enrichment broth used makes this procedure more convenient and economical, especially for screening large numbers of eggs in the food industry and government testing laboratories.

I have also demonstrated the suitability of the highly sensitive and specific polymerase chain reaction (PCR) targeting the Salmonella genus, for confirmation of the positive results obtained by the polymyxin-CEIA. Since the positive results obtained by the polymyxin-CEIA are considered presumptive, positive samples require further confirmation, which is traditionally done by cultural diagnosis procedure. This takes

several extra days (upto 4 days) to complete and therefore reduces the advantage of any rapid screening method. PCR is based on a totally different principle than polymyxin-CEIA, and thus provides an excellent rapid confirmation test, although its technical complexity and cost limit its use as a screening tool for large numbers of samples. The majority of food samples will be negative and need not be confirmed by PCR. As an example, I used PCR to confirm the presence of S. enteritidis in egg samples initially screened by the polymyxin-CEIA. This strategy enabled the confirmation of polymyxin-CEIA results within several hours and should be applicable for general Salmonella detection in other types of food samples, although the clean-up of different food samples before PCR might be necessary.

I have also used polymyxin-CEIA as a simple O-serogroup identification method in the final stage of cultural diagnosis procedure for Salmonella identification. The slide agglutination technique commonly used for Salmonella O antigen identification involves individual handling of bacteria and consumes large amounts of typing antisera. It is therefore labor-intensive, time-consuming and uneconomical, especially when large numbers of bacteria need to be tested. The polymyxin-CEIA allows simultaneous processing of multiple samples, therefore it allowed simple O-serogroup identification. The polymyxin-CEIA was found to be much less cumbersome and time-consuming. It consumes much less typing antisera than slide agglutination, since highly diluted antisera (eg. 1/500) were used in this polymyxin-CEIA. Good quality typing antisera are difficult to produce and batch-batch variations often occur, therefore it is important to minimize the amount of antisera used in the test. These advantages become even more obvious

when large numbers of strains are tested. In addition, the interpretation of test results is much easier in polymyxin-CEIA than in slide agglutination.

Since polymyxin B has a general affinity for the LPS antigens of gram-negative bacteria through the lipid A portion of the molecule, polymyxin-cloth should also allow the capture of LPS from other gram-negative bacteria. The polymyxin-CEIA may therefore be applied to the detection of other important food-borne gram-negative pathogens such as E. coli O157 and Campylobacter sp.. Investigations are now under way to explore these possibilities.

APPENDIX I

ENTEROTUBE II

Identification system for Enterobacteriaceae

Enterotube II permits the inoculation of all media and the subsequent performance of 15 standard biochemical tests from a single bacterial colony: glucose, gas production from glucose, lysine decarboxylase, ornithine decarboxylase, H₂S, indole, adonitol, lactose, arabinose, sorbitol, Voges-Proskauer, dulcitol, phenylalanine deaminase, urea and citrate.

The resulting combination of reactions, together with the Computer Coding System, allow the identification of Enterobacteriaceae.

ENTEROTUBE II

Culture Number or Patient Name

Date

Organism Identified

APPENDIX II

VITEK GNI CARD

For gram-negative identification

There are 30 wells in the CARD which contain 29 biochemical broths and 1 growth control broth. The GNI CARD has uniquely reproduced aerobic and microaerophilic conditions suited for each test provided. The media used are listed below.

Well No.	Media	Well No.	Media
AEROBIC		MICROAEROPHILIC	
1	DP 300	16	Raffinose
2	Glucose	17	Sorbitol
3	Growth Control	18	Sucrose
4	Acetamide	19	Inositol
5	Esculin	20	Adonitol
6	Plant Indican	21	p-Coumaric
7	Urea	22	H ₂ S
8	Citrate	23	ONPG
9	Malonate	24	Rhamnose
10	TDA	25	Arabinose
11	Polymyxin B	26	Glucose
12	Lactose	27	Arginine
13	Maltose	28	Lysine
14	Mannitol	29	Base Control
15	Xylose	30	Ornithine
		31	Oxidase reaction

APPENDIX III

DIG DNA Labeling and Detection

DNA is labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (DIG-dUTP). After hybridization to the target DNA the hybrids are detected by EIA using an anti-DIG-alkaline phosphatase conjugate and subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolylphosphate (X-phosphate) and nitro blue tetrazolium salt (NBT).

Linear denatured DNA

+ random hexanucleotides

+ dATP, dCTP, dGTP, dTTP + Dig-dUTP
+ Klenow enzyme →

Synthesis of labeled DNA

Filter-bound homologous DNA
+ labeled DNA

Hybridization

+ anti-digoxigenin-AP

Antibody hapten complex

+ X-phosphate + NBT

Color reaction

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