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**DEVELOPMENT OF CLOTH-ENZYME IMMUNOASSAYS FOR THE
DETECTION OF *E. coli* O157 AND VEROTOXIN**

by

Ronald A. Booth, B.Sc.

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

Department of Biology

Carleton University

Ottawa, Ontario

November 4, 1996

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Chair, Department of Biology

Thesis Supervisor

Carleton University

January, 1997

Abstract

Two types of cloth-based enzyme immunoassay systems were developed for the detection of the food-borne pathogen *Escherichia coli* O157. As an adsorbent for *E. coli* LPS antigens present in test samples such as enrichment cultures of foods, the first system used an anti-*E. coli* O157 monoclonal antibody-coated Polymacron™, whereas the second system used polymyxin-coated polyester cloth. These tests permitted the immunoenzymatic detection of as little as 1-10 colony forming units spiked into food samples after overnight selective enrichment in double modified trypticase soy broth or modified *E. coli* broth containing novobiocin.

Two cloth based enzyme-immunoassays (CEIA) for the detection of verotoxins (VT) were developed which used anti-verotoxin polyclonal antibodies (anti-VT) adsorbed to polyester cloth to capture VT which was subsequently immunoenzymatically detected by polyclonal antibodies conjugated to horseradish peroxidase. A dot blot assay (VT-CEIA) using large sheets of polyester cloth for the assay of multiple samples could detect VT at 5.0 ng/mL.

The VT-immuno-concentration assay (VT-ICA) concentrated VT present in a sample by passing a larger volume of the sample through an anti-VT coated polyester cloth, and could immunoenzymatically detect 0.5 ng/mL of VT. Some cross reaction was seen between other members of the family Enterobacteriaceae, but we suggest that with the introduction of commercially available monoclonal or affinity purified polyclonal antibodies these rapid test could be commercialized.

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Table of Contents

Abstract	iii
Acknowledgments	iv
Table of Contents	v
List of Figures	viii
List of Tables	ix
Abbreviations	x
 Chapter 1. General Introduction	 1
Foodborne infections and intoxications	4
<i>Escherichia coli</i> .	4
Enteropathogenic <i>E. coli</i> .	7
Enterotoxigenic <i>E. coli</i> .	8
Enteroinvasive <i>E. coli</i> .	9
Enterohaemorrhagic <i>E. coli</i> .	9
Diseases caused by <i>E. coli</i> O157:H7 and other EHEC.	10
Virulence factors associated with <i>E. coli</i> O157:H7 and other EHEC.	12
Outbreaks of <i>E. coli</i> O157:H7 and other EHEC.	17
Animal reservoirs of <i>E. coli</i> O157:H7 and other EHEC.	19
Control of <i>E. coli</i> O157:H7 and other EHEC.	20
Rapid detection procedures.	22
 Chapter 2. Development of rapid and simple cloth-enzyme immunoassay procedures for the detection of <i>E. coli</i> O157 in food.	 34
Abstract	35
Introduction	35
Materials and Methods	37
Chemicals and immunoreagents.	37
Bacterial strains.	38
Food samples.	39
Extraction of LPS antigens.	40
O157 Polymacron™ enzyme immunoassay (Polymacron™ EIA).	41
O157 Polymyxin-cloth enzyme immunoassay (O157 Polymyxin-CEIA).	41
Results and Discussion	42

Specificity of the Polymacron™ EIA.	43
Detectability of the O157 Polymacron™ EIA.	46
Detection of <i>E. coli</i> O157 in various foods by the Polymacron™ EIA.	46
Detectability of the O157 Polymyxin-CEIA.	52
Detection of <i>E. coli</i> O157 in enrichment cultures of ground beef.	52
Minimum enrichment time for detection of <i>E. coli</i> O157 spiked into ground beef.	53
 Chapter 3. Evaluation of various enrichment conditions for the recovery of healthy and thermally injured <i>E. coli</i> O157:H7 cells spiked into ground beef.	 58
Abstract	59
Introduction	59
Materials and Methods	62
Chemicals and bacterial strains	62
Growth of <i>E. coli</i> O157:H7 and ground beef microflora under various conditions.	62
Recovery of healthy <i>E. coli</i> O157:H7 spiked into ground beef.	63
Thermal injury of <i>E. coli</i> O157:H7.	65
Recovery of thermally injured <i>E. coli</i> O157:H7 in pure broth.	65
Recovery of thermally injured <i>E. coli</i> O157:H7 spiked into ground beef.	66
Results and Discussion	66
Growth of <i>E. coli</i> O157:H7 and ground beef microflora under various conditions.	66
Recovery of healthy <i>E. coli</i> O157:H7 spiked into ground beef.	73
Thermal injury of <i>E. coli</i> O157:H7.	78
Recovery of thermally injured <i>E. coli</i> O157:H7 in pure broth.	79
Recovery of thermally injured <i>E. coli</i> O157:H7 spiked into ground beef.	81
 Chapter 4. Development of a rapid cloth-enzyme immunoassay system for the detection of Verotoxins produced by Enterohaemorrhagic <i>E. coli</i>.	 85
Abstract	86
Introduction	86
Materials and Methods	89

Chemicals and immunoreagents.	89
Bacterial strains.	90
Preparation of peroxidase labelled anti-VT.	90
Verotoxin extraction	91
Dot-blot verotoxin cloth-enzyme immunoassay (VT-CEIA).	91
Verotoxin immuno-concentration assay (VT-ICA).	92
Minimum growth period required for Verotoxin production.	94
Ground beef samples.	95
PCR for Verotoxin detection	95
Results and Discussion	96
Verotoxin extraction.	96
Specificity of the Verotoxin assays.	97
Dot-blot verotoxin cloth-enzyme immunoassay (VT-CEIA)	97
Verotoxin immuno-concentration assay (VT-ICA).	100
Minimum growth period required for Verotoxin production.	103
Ground beef samples.	104
Chapter 5. General Conclusion	109
Appendix I	114
References	115

List of Figures

Figure 1.1	Gram negative cell wall showing the relative positions of components involved	6
Figure 1.2	Steps involved in the first few rounds of the polymerase chain reaction.	24
Figure 1.3	Heterogeneous EIA procedures.	29
Figure 1.4	The structure of polymyxin B.	30
Figure 3.1	Growth of <i>E. coli</i> O157:H7 ATCC 35150 at 37 °C without shaking in various media.	68
Figure 3.2	Growth of <i>E. coli</i> O157:H7 ATCC 35150 at 42 °C without shaking in various media.	69
Figure 3.3	Growth of ground beef microflora at 37 °C without shaking in various media.	71
Figure 3.4	Growth of ground beef microflora at 42 °C without shaking in various media.	72
Figure 3.5	Growth of ground beef microflora after 24 h without shaking. Microflora was extracted from ground beef and placed directly into the growth media.	74
Figure 4.1	VT-ICA device as described in Methods.	93
Figure 4.2	Example of VT-CEIA immunoassay.	102
Figure 4.3	Detection of VT 1 and VT 2 using the PCR from 25 g ground beef samples spiked with <i>E. coli</i> O157:H7 ATCC 35150 and grown in mEC+n for 24 h at 42 °C.	107

List of Tables

Table 1.1	Microorganisms which cause food poisoning	3
Table 2.1	Reactivity of <i>E. coli</i> strains in the O157 Polymyxin CEIA and the Polymacron™ EIA.	44
Table 2.2	Reactivity of non- <i>E. coli</i> strains in the O157 Polymyxin CEIA and the Polymacron™ EIA.	45
Table 2.3	Detection of <i>E. coli</i> O157:H7 cells inoculated into various food commodities.	48
Table 2.4	Detection of <i>E. coli</i> O157:H7 cells inoculated into ground beef.	50
Table 2.5	Detectability of the O157 Polymyxin-CEIA.	51
Table 2.6	Detection of <i>E. coli</i> O157:H7 inoculated into 25 g ground beef samples	54
Table 2.7	Time of recovery of <i>E. coli</i> O157:H7 inoculated into ground beef samples.	55
Table 3.1	Detection of <i>E. coli</i> O157:H7 inoculated into 25 g ground beef samples using various enrichment methods.	77
Table 3.2	Recovery of heat injured <i>E. coli</i> O157:H7 inoculated into sterile TSB and incubated for various times followed by the addition of mEC+n and incubated for 24 h.	80
Table 3.3	Recovery of heat injured <i>E. coli</i> O157:H7 inoculated into 25 g ground beef samples.	82
Table 4.1	Qualitative results of dot-blot VT-CEIA assay to determine the most effective VT extraction procedure	98
Table 4.2	Reactivity of <i>E. coli</i> strains in the VT-CEIA and the VT-ICA.	99
Table 4.3	Reactivity of non- <i>E. coli</i> strains in the VT-CEIA and the VT-ICA.	101
Table 4.4	Reactivity of the VT-CEIA and PCR in testing 25 g ground beef samples inoculated with <i>E. coli</i> O157:H7.	106

Abbreviations

A	absorbance
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
CCP	critical control point
CEIA	cloth-enzyme immunoassay
cfu	colony forming unit
cGMP	cyclic guanosine monophosphate
dmTSB	double modified tryptic soy broth
EIA	enzyme immunoassay
g	gram
h	hour(s)
HACCP	Hazard Analysis Critical Control Point
HC	haemorrhagic colitis
HRP	horseradish peroxidase
HUS	haemolytic uraemic syndrome
L	litre
LPS	lipopolysaccharide
mg	milligram
mL	millilitre
µg	microgram

μL	microlitre
mEC+n	modified <i>E. coli</i> broth containing novobiocin
ng	nanogram
PBS	0.01M phosphate buffer containing 0.85% NaCl (pH 7.2)
PBST	PBS containing 0.05% Tween 20
PBST-B	PBST containing blocking reagent
PCR	polymerase chain reaction
TMB	3,3',5,5'-tetramethylbenzidine
TSB	tryptic soy broth
TTP	thrombotic thrombocytopenic purpura

Chapter 1

General Introduction

Foodborne pathogens have been of great concern to society since the mid 1800's when the first "Adulteration of Food and Drink Act" was passed in Britain (Varnam and Evans, 1991). This Act was passed after a large food poisoning outbreak caused by the accidental adulteration of peppermint lozenges with arsenic. Although not caused by microorganisms, the ability of food to cause wide spread poisoning and disease was recognised. Since then, governments have had legislation in place to ensure the microbial and chemical quality of food. One of the newer measures for guaranteeing the chemical and microbiological quality of food is the HACCP system (Hazard Analysis and Critical Control Point), which monitors the production of food from slaughter/harvest to distribution and can prevent contamination before it becomes a problem.

Acute infections of the gastrointestinal tract are among the most frequent of all illnesses, exceeded only by respiratory tract infections, such as the common cold (Ryan, 1994). Diarrhea is the most common manifestation of these infections; however, because it is usually self-limiting within hours or days, most of those afflicted do not seek medical attention. World wide, diarrhoeal disease caused by food and water borne pathogens remains one of the most important causes of morbidity and mortality among infants and children (Ryan, 1994). In the US the yearly cost of foodborne disease due to medical attention and loss of wages is estimated at \$3.5 billion (US dollars) (American Gastroenterological Association, 1994). Therefore, the need to control foodborne disease is obvious.

Table 1-1. List of some microorganisms which cause food poisoning, the disease caused and the mechanism of pathogenesis.

Microorganism	Distribution	Disease Caused	Pathogenic Mechanism
<i>Salmonella</i> Spp.	Worldwide	Dysentery	Mucosal invasion
<i>Shigella</i> Spp.	Worldwide	Dysentery	Mucosal invasion, Shiga toxin
<i>Staphylococcus aureus</i>	Worldwide	Nausea and vomiting	Enterotoxins
<i>Campylobacter jejuni</i>	Worldwide	Dysentery	Unknown
<i>Escherichia coli</i> EIEC	Worldwide	Dysentery	Mucosal invasion
<i>E. coli</i> ETEC	Worldwide	Dysentery	Enterotoxin(s)
<i>E. coli</i> EHEC	Worldwide	Haemorrhagic Colitis, HUS, and TTP	Adherence, Verotoxins, and Enterohaemolysin
<i>E. coli</i> EPEC	Worldwide	Watery diarrhoea	Adherence
<i>Vibrio cholerae</i>	Asia, Africa, South America, Louisiana, Texas	Watery diarrhoea	Enterotoxin
<i>Yersinia enterocolitica</i>	Worldwide	Enteric fever	Penetration, spread
Rotavirus	Worldwide	Watery diarrhoea	Mucosal destruction
<i>Giardia lamblia</i>	Worldwide	Watery diarrhoea	Mucosal irritation
<i>Cryptosporidium</i>	Worldwide	Watery diarrhoea	Possible toxin

Abbreviations: EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; HUS, haemolytic uraemic syndrome; TTP, thrombotic thrombocytopenic purpura. (Ryan, 1994)

Foodborne infections and intoxications. A number of microorganisms which cause food poisoning are presented in Table 1-1. There are two main types of food poisoning caused by the presence of microorganisms, intoxications and infections. Intoxication is caused by the ingestion of preformed toxins produced by the growth of microorganisms. Because the preformed toxin is what causes the symptoms, there is little time between the onset of symptoms and the consumption of the contaminated food, usually within 1 - 4 hours (Ryan, 1994). An example of food poisoning due to intoxication are the Enterotoxins produced by *Staphylococcus aureus* (Varnam and Evans, 1991). This type of food poisoning can be very serious because in many cases the toxin can not be inactivated by heating during the cooking process, which does destroys the microorganism that produced it. "An infection is caused by the entrance of pathogenic microorganisms in the body, colonization, and the reaction of tissues to their presence, development, or multiplication, or to the toxins they elaborate within the body" (Riemann and Bryan, 1979). There are two types of foodborne infection. One type results by the penetration of the mucosa and the multiplication of the bacterium within, as is done by *Shigella* (Riemann and Bryan, 1979). The other results when enterotoxins are released as the microorganism grows, as in the case of enterotoxigenic *E. coli* (Riemann and Bryan, 1979).

Escherichia coli. One organism which causes food poisoning by infection is *Escherichia coli*. *E. coli* belong to the family Enterobacteriaceae. This diverse family is characterized as Gram-negative rods which grow rapidly under both aerobic and anaerobic conditions, and can metabolize a variety of compounds. Many species are motile, having flagella

which extend 1 to 5 μm beyond the cell wall, and may contain surface pili. Most have extracellular slime layers and some species are encapsulated. None of the family form spores or demonstrate acid fastness. They are found free living in nature and as part of the indigenous gut microflora of animals and humans. *E. coli* is the most common member of the Enterobacteriaceae found in the normal animal and human colonic flora. Lactose is fermented by most strains, but may be delayed or absent. Indol is also produced. An important biochemical test for the detection and classification of *E. coli* is the IMViC test. This is a test for indole production ("I"), methyl red ("M" production of stable acid end-products from glucose fermentation), Voges-Proskauer ("Vi" production of acetoin from glucose metabolism), and utilization of citrate as a sole carbon source ("C") (Doyle and Padhye, 1989). About 95 % of all isolates are considered Biotype 1 and show a "+ + - -" pattern respectively in the IMViC test (Doyle and Padhye, 1989). The remaining 5 % are considered Biotype 2 and show a "- + - -" IMViC pattern respectively (Doyle and Padhye, 1989). However, the most efficient way of classifying the species is through serotyping. This procedure is based on antigenic differences in surface structures. Kauffmann (1947) developed the initial serotyping scheme and was based on the *E. coli* somatic (O), flagellar (H), and capsular (K) antigens. The O antigen is based of the antigenicity on the O-specific polysaccharide of the outer cell membrane lipopolysaccharides (LPS), which is found on all smooth (S forms) Enterobacteriaceae (Figure 1-1). *E. coli* is classified into serogroups based on the O antigen, of which there are over 170 (Doyle and Padhye, 1989). Within the serogroups there are serotypes which are based on the H antigen, a heat labile proteinaceous flagellar antigen. Currently, there are 56 H antigens, but more will probably be established

Figure 1-1. Gram negative cell wall showing the relative positions of the components involved (Fomsgaard, 1990).

Abbreviations: CM, cytoplasmic membrane; PG, peptidoglycan; LP, lipoprotein; OM, outer membrane; CP, capsular polysaccharides (not present on all bacteria); PL phospholipid.

(Doyle and Padhye, 1989). An example of a serotype would be written as *E. coli* O157:H7. The K antigens are acidic polysaccharide capsular antigens, of which there are nearly 80 different types (Doyle and Padhye, 1989). The K antigens are used when an O antigen can not be determined.

In the past, *E. coli* was largely considered an opportunistic pathogen and the food industry used it as a marker for bacterial contamination because it is indicative of faecal contamination. However, in the recent past, the role of *E. coli* as a specific pathogen both intestinally and extraintestinally has been well established. As intestinal pathogens, there are 4 groups of *E. coli* which cause disease. Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohaemorrhagic *E. coli* (EHEC) which are also known as verotoxigenic *E. coli* (VTEC).

Enteropathogenic E. coli. EPEC as defined at the National Institutes of Health workshop in 1982 are “diarrheagenic *E. coli* belonging to serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanisms have not been proven to be related to either heat-labile enterotoxins (LT) or heat-stable enterotoxins (ST) or to *Shigella*-like invasiveness” (Edelman and Levine, 1983). Since then a new group of *E. coli* have been identified which are not considered EPEC, they are the EHEC (to be discussed later). Initial colonization by EPEC is through small clumps or microcolonies which adhere to the enterocytes of the small bowel. This initial attachment is mediated by a bundle forming pilus (BFP) encoded by a plasmid (Ryan, 1994). Once attached, a more intimate adherence occurs through chromosomal factors which causes the loss of microvilli. This loss of

microvilli occurs through the rearrangement of the cytoskeleton into filamentous actin at the site of attachment (Ryan, 1994) . This phenomenon can be detected with the filamentous actin staining (FAS) test which is used to detect EPEC (Knutton *et al.*, 1989). Occasionally the bacterium enters the cell with the aid of a 94 kDa outer membrane protein 'intimin' which can cause a chronic disease, but antibiotics are curative (Ryan, 1994).

Enterotoxigenic E. coli. ETEC was first recognised as the cause of a cholera-like diarrhea not caused by cholera, and was later associated with travellers' diarrhea (Doyle and Padhye, 1989). ETEC, as implied by the name produce either LT or ST or both enterotoxins. The disease characterized by profuse watery diarrhea, abdominal cramps, malaise, and nausea may be present early in the course of the disease (Doyle and Padhye, 1989). Symptoms are usually mild and last for ca. 1 to 2 days, but can approach a full cholera-like disease (Varnam and Evans, 1991). The toxins produced are responsible for the symptoms of the disease. LTs are classic A-B type toxins, and act in a similar manner and are immunologically related to Cholera toxin. LT stimulates membrane-bound adenylate cyclase by catalysing the transfer of ADP-ribose from NAD to a regulatory protein which leads to the activation of the cyclase catalytic unit and increased cAMP levels (Gyles, 1991). This persistent elevation of cellular cAMP levels stimulate the hypersecretion of electrolytes and water, which manifests itself as watery diarrhea (Gyles, 1991). Since this toxin does no permanent damage to the cell, it is considered a cytotoxic toxin. ST is a heat stable toxin of 2000 Da which activates particulate guanylate cyclase in intestinal epithelial cells (Gyles, 1991). The elevated cGMP causes increased fluid secretion into the lumen of

the gut which causes the watery diarrhea characteristic of ETEC infections (Gyles, 1991; Ryan, 1994)

Enteroinvasive E. coli. EIEC resemble *Shigella* and are biochemically less reactive than other *E. coli*. They do not ferment lactose within 48 h, are non motile, and are anaerogenic (Doyle and Padhye, 1989). The disease caused is indistinguishable from shigellosis, with symptoms of chills fever, headache, myalgia (muscular pain), abdominal cramps, and diarrhea. The symptoms usually develop between 8 and 24 h after consumption (Doyle and Padhye, 1989). EIEC penetrate into the colonic mucosa which stimulates an intense acute inflammatory response resulting in ulceration of the mucosa (Doyle and Padhye, 1989).

Enterohaemorrhagic E. coli. Verotoxic *E. coli* were first described in 1977 by Konowalchuk *et al.* (1977). The first isolate was made from a patient with severe abdominal cramps followed by grossly bloody diarrhea. Investigators at the Laboratory Centre for Disease Control, Ottawa identified six isolates of *E. coli* serotype O157:H7 while screening for enterotoxigenic and cytotoxic *E. coli* from patients with diarrhea between 1978 and 1982 (Johnson *et al.*, 1983). The isolation of *E. coli* O157:H7 from a number of sporadic cases of gastroenteritis suggested a link between *E. coli* O157:H7 and human disease, but could not be proven at that time. A conclusive connection however, was made in 1982 following two outbreaks of haemorrhagic colitis in which *E. coli* O157:H7 was isolated from patients and the food consumed by the affected individuals (Riley *et al.*, 1983). Because of the connection between *E. coli* O157:H7 and haemorrhagic colitis, the

organism was called enterohaemorrhagic *E. coli*. They are also called verotoxigenic *E. coli* (VTEC) because of their production of one or more verotoxins. Currently, there are over 50 different serotypes that are classified as EHEC, but the most frequently isolated serotype from patients is O157:H7 and therefore most efforts are aimed at detecting and controlling it (Karmali, 1989).

Normally, *E. coli* are identified in stools and foods by incubation at 44 - 45.5 °C, but *E. coli* O157:H7 does not grow well, or at all above 44 °C. Therefore, new techniques for the detection of *E. coli* O157:H7 were developed. Most biochemical reactions of *E. coli* O157:H7 are typical of *E. coli* biotype 1, but it does not ferment sorbitol within 24 h and does not produce β -glucuronidase (Doyle and Schoeni, 1984). Most human isolates of *E. coli* ferment sorbitol within 24 h and possess β -glucuronidase activity. These differences in metabolism are exploited in a selective agar, Sorbitol MacConkey Agar, and a biochemical test for β -glucuronidase activity using a flurogenic compound, 4-methyl-umbelliferone glucuronide (MUG). *E. coli* O157:H7 can easily be differentiated from other *E. coli*, but other EHEC do not have such metabolic differences and hence, can not be easily distinguished from other *E. coli*.

Diseases caused by E. coli O157:H7 and other EHEC. The most frequent disease caused by EHEC infection is haemorrhagic colitis (HC). HC begins with severe abdominal cramps approximately 3 to 5 days after exposure (Riley *et al.*, 1983). Within 24 h of abdominal pain watery diarrhea begins which becomes bloody and is described as “all blood and no stool” (Riley *et al.*, 1983). The duration of the illness is between 2 to 9 days, and is usually

much more severe in children and the elderly. The use of antibiotics has not been shown to be useful but more study is necessary, and in some cases increased the severity of symptoms (American Gastroenterological Association, 1994). Two less frequently encountered manifestations of EHEC infection are haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Patients with HUS typically have a number of findings including microangiopathic haemolytic anemia (intravascular coagulation of erythrocytes which results in mechanical damage of erythrocytes as they pass through abnormally narrow channels), thrombocytopenia (low circulating platelets), and acute nephropathy (kidney disfunction) (Doyle and Padhye, 1989). The most central aetiology of HUS is the glomerular endothelial injury. Injury to renal tissue is probably due to obstruction at the glomerular and arteriolar level due to swelling of the endothelial cells lining the capillaries (Taylor, 1990). Similar processes may occur in larger blood vessels in tissue other than the kidney such as the myocardium, pancreas and brain, as is the case with TTP (Taylor, 1990). Individuals experience substantial morbidity and often require dialysis and ultimately in severe cases, renal transplant. Death may also occur. TTP has clinical symptoms and pathology similar to HUS, but usually without the initial HC (Doyle and Padhye, 1989). In contrast to HUS, TTP has neurological involvement as the dominant feature. Patients often develop blood clots in the brain and death is a frequent outcome (Doyle and Padhye, 1989).

Another disease that has recently been associated with EHEC infection is sudden infant death syndrome (SIDS) (Bettelheim *et al.*, 1990; Bettiol, *et al.*, 1994). Toxigenic strains of *E. coli* producing LT or VT were found in the intestines of between 35 to 42 %

of infants who died of SIDS, whereas none of the control children sampled contained any toxigenic *E. coli*. It is possible that EHEC are the cause of SIDS, but at this time no definite connection has been made.

Virulence factors associated with E. coli O157:H7 and other EHEC. Four main virulence factors are possessed by EHEC, the production of one or more Verotoxins (VT), the production of an enterohaemolysin, the ability to cause an attaching and effacing lesion, and the production of LPS. The Verotoxic activity of EHEC was first demonstrated by Konowalchuk *et al.* (1977), when 10 isolates of *E. coli* were found to produce a toxin active against Vero cells (African Green Monkey kidney cells). A later study by O'Brien *et al.* (1983) demonstrated that the toxin produced by *E. coli* was physicochemically identical to Shiga toxin and was neutralized by antiserum against Shiga toxin, and hence, was named Shiga-like toxin (SLT). The initial study by Konowalchuk *et al.* (1977) showed that the toxic activity of two of the VT producing isolates were not neutralized by anti-Shiga toxin. This was evidence that two separate toxins were produced, and this other toxin was named VT 2. Eventually it was realised that the SLT identified by O'Brien *et al.* (1983) and the VT identified by Konowalchuk *et al.* (1977) were the same toxin. The toxins produced by EHEC are now called VT 1 and VT 2, but are still referred to SLT 1 and SLT 2 by some researchers. Neutralization studies have demonstrated that VTs belong to two different antigenic groups. Group 1 consists of VT 1, whose activity is neutralized by antiserum to Shiga toxin (Gyles, 1992). Group 2 consists of a number of antigenically closely related toxins and are referred to as VT 2 and VT 2 variants (Gyles, 1992). VT 1 and VT 2 share

approximately 60 % homology, but act in a similar manner (MacLeod, *et al.*, 1991; Taylor, 1995).

VTs are typical A-B type toxins, where the B subunit binds to cell surface receptors and internalizes the toxin while the A subunit contains the enzymatic activity. Each VT consists of 1 A subunit surrounded by 5 B subunits (Taylor, 1995). The B subunit binds to the terminal galactose $1\alpha \rightarrow 4\beta$ galactose disaccharide motif (Armstrong *et al.*, 1991). This motif is present in man as the Pk blood group antigen (CD 77) [globotriaosylceramide (Gb3)], which is present on B lymphocytes, renal tubular cells and glomerular endothelial cells (Taylor, 1995). The presence of CD 77 on glomerular endothelial cells is important because glomerular endothelial tissue injury is central in the pathogenesis of HUS. Although no proof exists that VT is absorbed by the colonic mucosa and transported to the kidney via the blood to cause tissue damage, it seems likely. One bit of evidence that VT is transported to the blood, is the fact that the number of P1 antigen present on red blood cells was reduced in patients who developed HUS after HC (Taylor *et al.*, 1990; Newberg *et al.*, 1993). Since P1 antigen on RBC binds the VT through the terminal digalactose motif (Bitzan *et al.*, 1994), there is less free VT present in the blood to cause disease, and HUS does not develop.

The genes for VT involved in human disease are encoded by bacteriophages (Smith *et al.*, 1983; Scotland *et al.*, 1983). The VT 1 operon consists of two open reading frames, one encodes the A subunit and the other encodes the smaller B subunit. The amino acid sequence of VT is identical to Shiga toxin except for one amino acid in the B subunit while VT 2 is not as closely related (Calderwood *et al.*, 1987). The nucleotide sequence of VT

2 was compared to that for VT 1 and it was found that 57 % and 60 % homology was present in the A and B subunits respectively (Jackson *et al.*, 1987). The genes for VT 1 are controlled by the *fur* regulatory system, and highest amounts of toxin are produced in media with reduced iron (Calderwood and Makalanos, 1987). However, the VT 2 genes are not iron regulated and are not under the control of the *fur* locus (MacLeod and Gyles, 1989). VT 1 is a highly cell-associated toxin, it appears to reside within the periplasm. The VT 1 A and B subunits' amino acid sequence contains hydrophobic signal sequences which direct the secretion of the proteins to the periplasm (O'Brien and Homes, 1987). VT 2 is less cell associated, and can be found in the culture medium. In the case of VT the B subunit appears to control the secretion of the toxin to the exterior of the cell, but control mechanisms in other VT 2 toxins have not been completely characterized (Gyles, 1992).

In eukaryotic cells the holotoxin is internalized through clatherin-coated pits. The coated vesicle fuses with lysosomes to form an endosome which transports the toxin to the trans-Golgi network (Sandvig *et al.*, 1994). Once inside the cell, the 32 kDa A subunit is proteolytically cleaved at a single disulphide bond to release a 27 kDa A₁ active fragment (Endo *et al.*, 1987). Neither the mechanism nor the location of the A subunit processing and translation to the cytosol are known at this time. The A₁ fragment contains the enzymatic activity of specifically cleaving the N-glycosidic bond at adenine 4324 in the 28S rRNA (Endo *et al.*, 1987). This effectively prevents protein elongation by blocking the binding of aminoacyl tRNA on the 60S ribosomal subunit (Endo *et al.*, 1987). Surprisingly, cell death does not occur due to the inhibition of protein synthesis, but occurs by apoptosis (Taylor, 1995). The exact reason for death by apoptosis is not known but may be due to the

synergistic activity of inflammatory cytokines and VT (Taylor, 1995). Recently, apoptosis was shown to be induced in epithelium when a pro-inflammatory stimulus was applied prior to a period of protein synthesis inhibition (Buchman *et al.*, 1993). When sublethal doses of LPS were applied before a sublethal concentration of protein synthesis inhibitor, cycloheximide, was added, the cells were readily killed by apoptosis (Buchman, *et al.*, 1993). A heat shock response could also work as the second signal for apoptosis, and interestingly, VT induces a heat shock response in human umbilical vein endothelium (Chant *et al.*, 1994). Apoptosis was also induced after exposure to tumour necrosis factor- α (TNF α) followed by cycloheximide (Polunovsky *et al.*, 1994). Therefore, an inflammatory response followed by exposure to VT may cause apoptosis in some cells. This mechanism of apoptosis induction could occur in patients with HUS, but more research is necessary to determine where in the body these two signals coincide, if at all.

The adherence of *E. coli* to intestinal tissue is central to the development of disease, without adherence and colonization of the colon, no toxins could be released and no lesion would be formed in the colon. EHEC cause a specific adherence and colonization pattern known as an attaching and effacing lesion, which is similar to that caused by EPEC, that cause watery diarrhea. The adhesins responsible for attachment of EHEC to intestinal epithelium have not fully been characterized, but are expected to be similar to the bundle-forming pili of EPEC. Current candidate adhesins, such as fimbriae and intimin, do not fully explain the adherence of all EHEC, and must be further investigated.

Another important virulence factor is the enterohaemolysin produced by EHEC. Almost 90 % of EHEC isolates produce one or more of the three enterohaemolysins (Beutin

et al., 1989). *E. coli* serogroup O26 produce two enterohaemolysins, Ehly1 and Ehly2, which are encoded by bacteriophages (Stroeher, *et al.*, 1993; Beutin, *et al.*, 1993). The mechanism of action of these two toxins is not known, but is known not to be an α -haemolytic mechanism (Stroeher, *et al.*, 1993). The exact role that Ehly1 and Ehly2 play in causing disease in humans requires further investigation. *E. coli* O157:H7 and some other EHEC are known to harbor a large 60 MDa virulence plasmid, pO157, which until recently its purpose in virulence was not known. Initially the plasmid was thought to encode adherence factors but this was determined not to be the case. The 60 MDa virulence plasmid encodes another haemolysin, Enterohaemolysin or EHEC-Hly (Schmidt, *et al.*, 1994). This haemolysin, however, is related to *E. coli* α -haemolysin, two open reading frames in the plasmid share approximately 60 % homology with the *hlyC* and *hlyA* genes of the *E. coli* α -haemolysin (α -*hly*) operon (Schmidt, *et al.*, 1995). The EHEC however show a different haemolytic pattern than normal α -haemolytic *E. coli*, in that haemolysis does not appear until 20 - 24 h of growth unlike α -haemolytic *E. coli* which is present after 4 h (Bettelheim, 1995). This difference can be attributed to a defective haemolysin secretion system (Schmidt *et al.*, 1995). When wild type secretion genes, *hlyB* and *hlyD* were used to transform *E. coli* O157:H7 EDL933, this defect was cured and an α -haemolytic phenotype resulted (Schmidt, *et al.*, 1995). Because the EHEC-Hly may act as an α -haemolysin, the effects of such a toxin can be used for modelling the interaction of EHEC-Hly with VT in the pathogenesis of EHEC. When sublytic amount of α -Hly were administered to cultured monocytes, a release of IL1 β was seen (Bhakdi, *et al.*, 1990). The release of IL1 β caused an increased production of Gb3 (VT receptor) on some cells, and

therefore may increase the susceptibility of specific cells to VT (van de Kar *et al.*, 1992; Schmidt, *et al.*, 1995).

Lipopolysaccharide (LPS) may also play a role in the pathogenesis of EHEC. LPS stimulate an inflammatory response which is separate from the toxic response but may act together with VT to increase the severity of HUS. During an inflammatory response a number of cytokines are released, such as $\text{TNF}\alpha$ and $\text{IL}1\beta$, which tend to increase the expression of some cell-surface receptors including Gb3 (van de Kar *et al.*, 1992). This increase in receptors may render some cells more vulnerable to VT (van de Kar *et al.*, 1992). Whether glomerular endothelium *in vivo* is stimulated by LPS or inflammatory cytokines to increase the production of receptors is unknown. Another way in which tissue damage could occur is through apoptosis of susceptible cells. As described above, LPS which induces the production of $\text{TNF}\alpha$ which in turn acts as the first signal for apoptosis and VT (the protein inhibitor) may act as the second signal. This process may be involved in the development of HUS but more research is necessary. It appears that all the virulence factors interact in to cause post HC diseases, such as HUS and TTP. The LPS as well as the enterohaemolysin may cause an inflammatory response which could act as the first signal in apoptosis which when followed by VT may cause cell death at specific sites. Whether these signals coincide at specific sites of pathology seen in HUS and TTP is unknown.

Outbreaks of E. coli O157:H7 and other EHEC. In 1982 the first reported outbreak of food poisoning due to *E. coli* O157:H7 occurred in the United States (Riley *et al.*, 1983).

Since then a number of outbreaks in Canada, the US and the UK have been reported. This increase in frequency may be in part due to the increased surveillance for *E. coli* O157:H7. Among the US outbreaks between 1982 and 1990, the average number of cases per outbreak was 56; among those cases 18 % were hospitalized, 3.6 % developed HUS and 1.9 % died (Griffin and Tauxe, 1991). Ground beef is most often the vehicle of transmission, but a number of other foods have been associated with outbreaks, such as unpasteurized milk, chicken, lamb, pork, and vegetables (Doyle and Schoeni, 1987; Doyle, 1991). The reason for ground beef being the major bovine vehicle of transmission of *E. coli* O157:H7 is because during the grinding procedure, bacteria which is normally present only on the surface of the meat are ground into the centre of the meat. The bacteria in the centre of the meat require a longer cooking time before they are inactivated, since there is a finite time for the centre of a hamburger patty to reach the inactivation temperature. Human disease has also been caused by EHEC other than serotype O157:H7, *E. coli* O26:H11 (Bopp et al., 1987; Scotland et al., 1990), O111:NM (Dorn *et al.*, 1989), O113:H21 (Johnson and Lior, 1987) as well as O5:NM have been isolated from patients with diarrhea, but *E. coli* O157:H7 is still the most frequent cause of haemorrhagic colitis. Non-O157 serotypes are more often isolated from cattle and food than O157:H7 (Griffin and Tauxe, 1991), and it is possible that a large number of cases of HC due to non-O157 EHEC are not detected because most labs look for the O157:H7 serotype only, and the non-O157:H7 EHEC do not have any specific biochemical differences from relation to non-EHEC.

Animal reservoirs of E. coli O157:H7 and other EHEC. After the initial outbreaks of *E. coli* O157:H7 were traced to ground beef, a bovine reservoir was suspected. Therefore, researchers sampled cattle from farms that were implicated in outbreaks. In Canada, *E. coli* O157:H7 was isolated from two calves on a farm where cows supplied raw milk implicated in an outbreak among children (Borczyk *et al.*, 1987). The rate of isolation of O157:H7 from beef cattle was 1.5 %, and in dairy cows was 0.5 % (Clarke *et al.*, 1988). One study in the UK by Wells *et al.* (1991), isolated 27 VT producing non-O157 serotypes from cattle. Eighty percent of the farms tested were positive for VT-producing *E. coli*, with 8.4 % of adult cows and 19.0 % of heifers and calves testing positive. A similar Canadian study showed that 10.5 % of beef cattle and 19.5 % of dairy cows contained VT-producing *E. coli* (Clarke *et al.*, 1988). Both *E. coli* O157:H7 and other VT-producing *E. coli* have been isolated from healthy and diseased cattle, although more often from young animals than in older animals. This data suggests that healthy cattle can carry EHEC and can transmit these bacteria to foods. Since healthy cattle are a reservoir, it is difficult to remove them before slaughtering because no visible signs of infection are present. This inability to remove infected cattle can pose a serious problem if a proper meat monitoring system is not in place. The most likely mode of contamination of beef is through faecal contamination during the slaughtering process, this was shown to true by the identification of a bacterium on beef tissue which had the same plasmid and toxin profile that was identified by rectal swab (Chapman *et al.*, 1993). Contamination of other food commodities may be due to cross contamination at the preparation stage or other animals may harbor EHEC within

their digestive tract. The contamination of fruits and vegetables is most likely through the fertilization of farmland with a bovine faecal slurry containing EHEC.

Control of E. coli O157:H7 and other EHEC. *E. coli* O157:H7 is now recognized as an important foodborne pathogen and its absence from food must be assured due to the severity of the diseases caused. Most *E. coli* O157:H7 infections are due to the consumption of contaminated ground beef. Since cattle have been identified as a reservoir for *E. coli* O157:H7 and other EHEC the majority of control efforts are aimed at ground beef and beef products. A survey of retail meats revealed that many different types and cuts of meat may be contaminated at the retail level, but whether the products are contaminated at the retail level or during processing is not known (Doyle and Schoeni, 1987). The need for control at the processing level is of great importance to the consumers safety, because if the pathogen is removed at the processing level no cross contamination at the retail level is possible.

To control *E. coli* O157:H7 and other foodborne pathogens a food safety measure known as HACCP (Hazard Analysis Critical Control Point system) is now being implemented in North America and Europe. HACCP was developed 1959 for NASA by the Pilsbury Company to control and monitor every step of a food production process to work as a preventative safety measure rather than an inspection oriented monitoring system (Bauman, 1995). This preventative system could ensure a 100 % safe food supply, which was necessary for the space program. In 1971 the HACCP concept was made public at the National Conference of Food Protection (Bauman, 1995). By the mid 1980s a number of

companies had set up HACCP programs, and as recently as 1993 the Codex Committee on HACCP has recommended HACCP for international use (Bauman, 1995). There are seven basic principles involved in a HACCP system: 1) Conducting a hazard analysis and risk assessment, 2) Determining critical control points, 3) Establishing critical limits for CCPs, 4) Monitoring critical limits, 5) Correcting deviations from critical limits, 6) Establishing an effective record keeping system, and 7) Verifying that the HACCP program is working correctly (Webb and Marsden, 1995). HACCP will monitor for the presence of chemical and microbial hazards at all stages in the production of food, including growing, harvesting, processing, manufacturing, distributing, and merchandising (Stevenson, 1990). The use of a HACCP system in the manufacturing of food is extremely important since end product testing can not prevent contamination, it can only detect it. A HACCP system, by its very nature, can detect and eliminate contamination before the food is at the end product stage.

Due to the seriousness of the diseases caused by *E. coli* O157:H7, and the fact that a very low number of cells are necessary to cause HC (1-10 cfu), its absence in ground beef and other foods must be assured. HACCP is an ideal system for monitoring and controlling the presence of *E. coli* O157:H7 in ground beef. A HACCP system requires rapid testing at a number of sites throughout the production of meats, from slaughtering to distribution. Currently the accepted Health Canada method for the identification of *E. coli* O157:H7 requires numerous steps over a number of days (Warburton and Todd, 1995). For isolation from most foods, a 25 g sample is placed in 225 mL of selective enrichment broth and grown for 22-24 h at 42 °C. The enrichment broth is then screened using a rapid test and/or plated on selective agar to detect the presence of O157:H7. If the rapid test detected *E. coli*

O157:H7, but it was not recovered on the selective plates, a secondary enrichment must be carried out by placing 1 mL of the primary enrichment broth in 9 mL of the secondary enrichment broth and grown for 18 - 24 h at 35 °C. After this second enrichment the broth must again be plated on selective agar and grown for 18 - 24 h. *E. coli* O157:H7 suspect colonies are picked and a number of biochemical and serological reaction are assessed, including cellobiose, lysine decarboxylase, citrate, urea, the production of β -glucuronidase, Gram stain, and finally agglutination with the appropriate antisera. This method of detection and isolation of *E. coli* O157:H7, although sensitive, is not an effective way of measuring microbial contamination due to the time, expense and experienced personnel required for accurate results. The food industry requires rapid, sensitive, specific, and economical screening methods that do not require a highly skilled operator which can be applied to a HACCP system.

Rapid detection procedures. There are a number of rapid detection procedures available for the detection of bacteria, including *E. coli* O157:H7. These rapid methods normally fall into two categories (Eyles, 1990). The first category includes methods which are primarily aimed at reducing the labour required to isolate and identify a specific bacterium. These methods include automated colony counters and biochemical identification by automated systems (VITEK™ Automated Bacterial Identification System). The second category aims to reduce the time involved in making a positive identification. These include immunoassays, and detection of bacterial DNA or RNA by gene probes or the polymerase chain reaction (PCR) (Eyles, 1990).

Gene probes utilize unique DNA sequences to distinguish the target bacteria from other competing bacteria. A short, single stranded oligonucleotide complementary to a specific sequence in the target bacterium is used to “probe” the sample (Swaminathan and Feng, 1994). The sample is prepared by lysing the cells, denaturing the DNA using heat or alkali, and immobilizing the denatured DNA on a solid phase, such as a nylon membrane. After immobilization, the probe is added under specific conditions which allow only complementary DNA sequences to hybridize (Swaminathan and Feng, 1994). The probe is labelled either radioactively or chemically so the hybridized samples can be distinguished from the non-hybridized samples. Radioactively labelled probes were used in the past but chemical labels such as biotin and digoxigenin are used more often now because of the reduced risk. This method of detection has been used for *E. coli* O157:H7 and its virulence factors in a laboratory setting, but has not been widely used in food testing because of the complex and labour intensive procedure (Franko, *et al.*, 1991; Dytoc, *et al.*, 1993).

Another genetic based technique for the detection of bacteria is the PCR. The PCR specifically amplifies a unique DNA sequence in the target microorganism and can be used to indirectly detect extremely low numbers of microbes by detecting the amplified DNA (Swaminathan and Feng, 1994). In theory this method can detect a single organism in a sample. The PCR amplification technique is carried out in cycles, each cycle consisting of three steps (Figure 1-2) (Swaminathan and Feng, 1994). The first step consists of denaturation of the DNA using heat (95-100 °C). In the second step, specific primers that

Figure 1-2. 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 ◁ and ▷, respectively. The product 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).

are homologous to flanking segments of complementary strands on the target DNA allowed to anneal to the target DNA. Thirdly, Taq DNA polymerase extends the annealed primers using target DNA sequences as the template. By cycling these steps, a single piece of target DNA can generate 1.5×10^6 copies after 30 cycles (Swaminathan and Feng, 1994). PCR based assays have been developed for many pathogenic bacteria, including *E. coli* O157:H7, using virulence genes and ribosomal RNA as the primary target. Assays for the detection of VT genes (Read *et al.*, 1992; Pollard *et al.*, 1990), and the attaching and effacing genes (Louie *et al.*, 1994) have been developed and are effective in detecting EHEC in pure culture. Although the theoretical sensitivity of the PCR is one copy of a gene per sample this does not always occur (Read *et al.*, 1992). When sampling food which contains one viable pathogenic cell, it is extremely difficult to actually detect that single cell in a large sample. Therefore, an enrichment step is usually completed to ensure the detection of the pathogen. Also, when the sample is obtained from food, some chemicals present in food may interfere with the PCR reaction itself. The above reasons, along with the expense of the thermal cycler make the PCR better suited as confirmatory method of detection rather than a rapid screening method of detection.

Another method for detecting food pathogens is by using immunoassays. Immunoassays are based on the highly specific nature of antibody-antigen reactions. The interaction of antibodies with their specific antigen are governed by relatively weak non-covalent forces, including hydrophobic interaction, hydrogen bonds, van der Waals forces, and ionic interactions. An antibody recognizes the three dimensional structure (an epitope) of a specific molecule and binds to that and only that epitope. Since many structures in

nature are repeated from organism to organism there is the possibility of a cross reaction between two different antigens that possess an identical epitope. This cross reaction can be a problem, but with the advent of monoclonal antibodies and affinity purification of polyclonal antibodies the possibility of a cross reaction can be minimised.

There are several different types of immunoassays for the detection of *E. coli*, including latex agglutination, fluorescent antibody (FA), and enzyme immunoassays (EIA). Latex agglutination uses latex particles sensitised with antibodies specific for the target analyte (Swamniathan and Feng, 1994). A positive reaction is detected by the agglutination of the latex particles due to the presence of the antigen linking the particles together. Unipath Ltd. (Oxoid Division, Hamshire, England) developed an agglutination test for *E. coli* O157 and the Verotox-F agglutination test for VT. These assays are specific, but due to the use of live cells, the need for a pure culture, and the time involved in the VT assay (20 - 24 h agglutination time) they are not suited for HACCP testing. The use of fluorescein-labelled antibodies specific for *E. coli* O157 was combined with a direct epifluorescent filter technique (DEFT), which measures the number of viable cells, to enumerate the number of *E. coli* O157:H7 in milk and apple juice (Tortorello and Gendel, 1993). This technique is well suited for the detection and enumeration of living cells, but because of the need for a skilled operator and the time consuming process it is not widely used.

EIAs are however, suited for rapid detection. EIAs can be classified into two different categories: homogeneous and heterogeneous, which are further divided into competitive and non-competitive (Rittenburg, 1990). In homogeneous EIAs, the activity

of the enzyme is modulated by the antibody-antigen reaction, and separation of the reactants is not necessary. Heterogeneous EIAs, have an antigen capture mechanism on a solid phase and the activity of the enzyme is unaffected by the antibody-antigen reaction, while separation of the bound and unbound fractions is required. Heterogeneous EIAs are more applicable in rapid food testing and will be further discussed. Competitive EIAs measure the difference in absorbance generated between two different reactions in which the sample antigen concentration is inversely proportional to the measured absorbance (Figure 1-3a). In one reaction only the enzyme labelled antibody is added, while in the others, enzyme labelled antibody plus the unknown antigen is added. The difference in absorbance is inversely proportional to the concentration of the unknown antigen. Non-competitive EIAs measure the absorbance of a single reaction in which the antigen concentration is directly proportional to the absorbance read (Figure 1-3b). There are a number of solid phases used in heterogeneous EIAs and include: non-porous solid phases, such as plastic microtitre plates, microporous membranes, such as nylon and nitrocellulose, and recently, polyester cloth.

The choice of a solid phase matrix for an EIA is one of the most important considerations in the design of a diagnostic test. The nature of the solid phase will dictate the format and the operational characteristics of the test. When designing a test for use in a HACCP system, it should be rapid, cost-effective, simple and safe. Because of the need for daily representative CCP samples for *E. coli* O157:H7 or other pathogen detection, the test should be rapid to perform and should not add significantly to the cost of the food product. Also, the test should not require a highly skilled operator and should not generate

Figure 1-3. Heterogeneous EIA procedures.

Figure 1-4. The structure of polymyxin B.

R = 6-methyloctanoyl or 6-methylheptanoyl

DAB = diaminobutyric acid

biohazards (e.g. assay of live pathogens). Polyester cloth as a solid phase will help overcome many of the disadvantages inherent in other traditional solid phases. The hydrophobic surface of the non-woven polyester cloth allows for the immobilization of antibodies and other compounds by passive adsorption and create a high affinity solid phase for the immunoenzymatic detection of specific analytes. The macroporosity of polyester cloth presents a large surface area to permit rapid and efficient immunoreactions and washing procedures. Microtitre plates, which are normally used for EIAs, have a reduced surface area and increased diffusional distances in relation to polyester cloth and therefore, reduced reaction kinetics. The large sheet format of polyester cloth allows for the assay of multiple samples and simplifies washing by allowing all samples to be simultaneously and efficiently washed, unlike microtitre trays in which each well must be individually washed. Also, the simple visualization of positive result as a coloured spots on a white background are produced.

Antibody-coated polyester cloth was used for the detection of *Brucella* and *Salmonella* antigens by using a sandwich cloth-enzyme immunoassay (CEIA) system (Blais *et al.*, 1989a, c). Since then, the detection of Gram negative bacterial LPS has been accomplished by polymyxin coated cloth (Blais and Yamazaki, 1990a, b, 1991a; Blais *et al.*, 1993). Polymyxin B is a cyclic polypeptide produced by *Bacillus polymyxa* which contains a short peptide side chain acylated with eight or nine carbon fatty acids at its N-terminus (Figure 1-4). The antibiotic action of polymyxin B occurs through interaction with the LPS of the outer membrane of Gram negative bacteria. LPS of Gram negative bacteria are composed of three structural regions, the O polysaccharide, which consists of

repeating oligosaccharide units and determines the serological specificity of the molecule, the oligosaccharide core, and the lipid A, which anchors the LPS into the outer membrane (Figure 1-1). Outer membrane integrity is maintained through interactions between ionic, acidic phosphates and carbonyl groups on the LPS and a variety of cations, such as Mg^{2+} and Ca^{2+} . Polycationic antibiotics, such as polymyxin B block the interactions between the divalent cations and the LPS, and therefore disrupt the outer membrane.

The polymyxin B is able to bind to polyester cloth hydrophobically through the fatty acid chain and still retain its ability to bind the lipid A moiety of LPS. With the LPS bound to the polymyxin coated polyester cloth in this manner, the O antigen is available to interact with antibodies and so can be detected immunoenzymatically. The ability of polymyxin coated polyester cloth to bind LPS irrespective of its serological grouping, gives this capturing method the possibility of being applied to the analysis of many different Gram negative bacteria. Also, the relative inexpensiveness and ability to obtain polymyxin B in a pure homogeneous form makes it an ideal capturing agent for the development of inexpensive EIA assays.

In order to detect *E. coli* O157:H7 using either polymyxin coated polyester cloth or antibody coated cloth, suitable detector and capture antibodies need to be obtained. Previous antibody preparations against O157 LPS antigens suffered from cross reactivity between a number of bacteria such as *Escherichia hermannii*, *Citrobacter freundii*, *Yersinia enterocolitica*, *Brucella* spp., and the *Salmonella* N group (Padhye and Doyle, 1991). Newer preparations, however do not suffer from these cross reactions except with *Salmonella* N group, which have an identical O antigen structure (Dr. M. Perry, personal

communication). This reduced cross reactive, commercially available detector antibody was used to detect *E. coli* O157:H7 cells in pure culture and spiked into ground beef samples using both polymyxin CEIA and antibody CEIA (Chapter 2).

In Chapter 3 media used to enrich the numbers of *E. coli* O157:H7 spiked into ground beef samples was evaluated. Initially double modified tryptic soy broth (dmTSB) was used as the enrichment medium. This medium however, did not perform as well as expected and another medium, modified *E. coli* broth containing novobiocin (mEC+n), was evaluated using a number of conditions.

Because *E. coli* O157:H7 is only one of a number of EHEC that is capable of causing disease in humans and is found in ground beef another EIA was developed to detect a specific virulence factor common to all EHEC. The detection of VT produced by EHEC should be a more reliable manner to detect the true number EHEC present in food. A CEIA was developed using rabbit antisera raised against VT 1 and VT 2 bound to cloth as the capture agent. The bound VT was then immunoenzymatically detected with a rabbit anti-VT peroxidase conjugate (Chapter 4).

Chapter 2

**Development of rapid and simple cloth-enzyme immunoassay
procedures for the detection of *E. coli* O157 in food.**

Abstract

Two types of cloth-based enzyme immunoassay systems were developed for the detection of the food-borne pathogen *Escherichia coli* O157. The first system used an anti-*E. coli* O157 monoclonal antibody-coated Polymacron™. The inexpensive macroporous polyester cloth, Polymacron™, was used as a high surface area adsorbent of monoclonal antibodies for the rapid capture and subsequent immunoenzymatic detection of *E. coli* O157 lipopolysaccharide (LPS) antigens extracted from test samples. O157 antigens were captured on the cloth after heating at 100 °C in the presence of sodium cholate. The second system used polymyxin-coated polyester cloth as a different capturing method. A Polymyxin-cloth enzyme immunoassay system (O157 Polymyxin-CEIA) using inexpensive polymyxin B-coated polyester cloth for an adsorbent for *E. coli* LPS antigens present in test samples such as enrichment cultures of foods was developed. Captured LPS antigens in both assays were immunoenzymatically detected using commercially available anti-O157 polyclonal antibodies. These assays were sensitive and highly specific for strains bearing the O157 and related antigens, and permitted the detection of ca. 1-10 cfu spiked into food samples after overnight enrichment in double modified trypticase soy broth or modified *E. coli* broth + novobiocin. Also, the entire assay procedure for both the polymyxin and antibody coated cloth can be completed in less than 35 min.

Introduction

Verotoxin-producing *Escherichia coli* O157:H7 have caused serious outbreaks of foodborne illness worldwide associated with the consumption of contaminated ground beef

and some other food commodities (Doyle, 1991; Johnson *et al.*, 1995; Padhye and Dolye, 1991; Weagant *et al.*, 1995). To ensure the safety and quality of its products, the food industry will need to monitor for the absence of this pathogen on a routine basis. For this purpose, the food industry will require rapid, simple and inexpensive screening methods. Rapid methods currently available for detection of this pathogen in foods include the EHEC-TEK ELISA system (Organon Teknika, Durham, North Carolina), the Petrifilm™ Test Kit-HEC system (3M Health Care, St. Paul, Minnesota), the Micro-Screen™ *E. coli* O157:H7 Kit (Neogen Corporation, Lansing, Michigan) and the Dynabeads anti-*E. coli* O157 method (Dynal, Lake Success, New York). While effective, many of these tests have some or all of the following limitations, which are particularly relevant to food testing laboratories processing many samples on a routine basis: most have a high cost per test; some involve time-consuming reaction steps, and some require handling each device or well individually at every stage of the assay. Although some of the ELISA methods are automatable or semi-automatable, the specialized equipment required will add significantly to the costs of performing the tests. As well, HACCP systems require daily samples to be taken at critical control points (CCPs), for which cloth-based EIA is extremely effective

The use of macroporous polyester cloth as a solid phase for the immunoenzymatic assay of a variety of antigens (Blais *et al.*, 1989; Blais and Yamazaki, 1989a), and antibodies (Blais and Yamazaki, 1989c; Blais and Yamazaki, 1989b; Blais and Yamazaki, 1991b) has been previously described. Polyester cloth provides a high surface area for immunoreagent immobilization by passive adsorption, giving rapid immunoreactions in subsequent assay procedures. Because of its macroporosity, polyester cloth exhibits

excellent flow characteristics, providing ease of washing between immunoreaction steps to remove unreacted material. As well, the cloth sheet format can accommodate numerous samples simultaneously.

This chapter describes two simple cloth-enzyme immunoassay (CEIA) systems for the detection of *E. coli* O157 based on the use of Polymacron™ cloth, an inexpensive macroporous hydrophobic polyester fabric. Two different antigen capturing systems were investigated for their ability to capture *E. coli* LPS from food samples. First, a monoclonal antibody, specific for the O157 antigen, hydrophobically bound to the cloth was used to capture the O157 LPS antigens. Monoclonal antibodies have the advantage of being very specific and is not affected by the presence of other Gram negative bacteria in the sample, but because of the expense of monoclonal antibodies and the storage problems associated with antibodies a separate LPS capturing method was used. The second capturing system used polymyxin B as the LPS capturing agent, which is inexpensive and highly stable under various condition, but will capture LPS from any Gram negative bacteria present. Using a dot blot format which facilitates the assay of multiple samples, we demonstrate the applicability of these assay systems as an effective screening tool for enrichment cultures of food samples.

Materials and Methods

Chemicals and immunoreagents. The following were obtained from Sigma Chemical Co. (St. Louis, MO): polymyxin B sulphate (No. P-1004), sodium cholate (C-1254), anti-rabbit IgG-peroxidase conjugate (No. A-6154), lactose (No. I3625), acriflavin hydrochloride (No.

A-8251), Tween 20 (No. P-9416), and CM-cellulose (low viscosity) (No. C-8758). Rabbit anti-*E. coli* O157 antiserum (No. 2970-47), casamino acids (No. 0230-01), tryptone (No. 0123-17-3), and bile salts #3 (No. 0130-01) were obtained from Difco Laboratories (Detroit, MI). Peroxidase-labelled affinity purified-anti-*E. coli* O157:H7 antibody (No. 04-95-90) was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Trypticase soy broth (TSB) was obtained from BDH Inc. (Darmstadt, Germany). Polymacron™ sheets were donated by Kalyx Biosciences (No. PMU-2101, Kalyx Biosciences Inc., Nepean, Ontario), as was the proprietary anti-*E. coli* O157 murine monoclonal antibody (immunoglobulin G). The membrane peroxidase substrate system used in this study is a proprietary formulation based on the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) with an unspecified membrane enhancer, and was a kind gift from Ricoh Kyosan Inc. (Tokyo, Japan).

Bacterial strains. A total of 35 *Escherichia coli* strains from the Health of Animals Laboratory and Laboratory Services Division culture collections, Agriculture and Agri-Food Canada, were examined in this study (Table 2-1). A number of Gram-positive and Gram-negative non-*E. coli* organisms from the Laboratory Services Division culture collection were also examined (Table 2-2). Unless otherwise stated, *E. coli* strains were routinely grown in TSB by shaking at 37 °C for 16 - 20 h. Growth conditions for the non-*E. coli* bacteria were grown as previously described (Blais et al., 1993). Viable counts were estimated by plating serial dilutions of cell suspensions on trypticase soy agar (TSA) and incubating the plates at 37 °C for 16 - 20 h.

Food samples. Ground beef samples were purchased from a local retailer. To study the recovery of cells inoculated in the samples, 25 g of ground beef were aseptically transferred to a stomacher bag containing 225 mL of sterile double modified TSB (dmTSB) (trypticase soy broth, 30 g/L; casamino acids, 10 g/L; K_2HPO_4 , 1.5 g/L; bile salts #3, 1.5 g/L; and, acriflavine hydrochloride, 10 mg/L) (Padhye and Dolye, 1991) or sterile modified EC with novobiocin broth (mEC + n) (tryptone, 20 g/L; bile salts #3, 1.12 g/L; lactose, 5 g/L; K_2HPO_4 , 4 g/L; KH_2PO_4 , 1.5 g/L; NaCl, 5 g/L; and novobiocin, 0.02 g/L) (Okrend *et al.*, 1990), and stomached for 120 s. Twenty mL portions of the resulting mixture were aseptically transferred to sterile 125 mL erlenmeyer flasks and inoculated with 200 μ L containing varying concentrations of *E. coli* O157:H7 cfu in TSB. The inoculated meat-broth cultures were then grown by shaking at 37 °C for 24 h, and LPS antigens were extracted for the assays as described below.

To determine the minimum enrichment period for the assay, 25 g samples of ground beef were homogenized in stomacher bags containing 225 mL of dmTSB as before. Each mixture was then aseptically transferred to a sterile 1000 mL erlenmeyer flask and inoculated with 40 μ L of varying concentrations of *E. coli* O157:H7 cfu in TSB, followed by shaking at 37 °C. Samples (1 mL) were withdrawn through a sterile foam Filtatip (Filtaflex Ltd, Almonte, Ontario) for the assay after 0, 4, 8, and 24 h incubation.

To study the recovery and assay of *E. coli* O157:H7 cells inoculated directly into bulk samples of ground beef with fat contents varying from 18 to 28%, 25 g samples of ground beef were placed in sterile stomacher bags and inoculated in the centre with 200 μ L containing varying concentrations of *E. coli* O157:H7 cells in TSB. The cells were then

blended into the meat by gently hand massaging the contents of the bags. Each sample was then blended with 225 mL of either dmTSB or mEC + n by stomaching for 120 s. Enrichment was carried out by incubation at 37 °C for 24 h.

Various ground meat, milk and cheese samples (listed in Table 2-3) were purchased from local retailers and used fresh. To study the recovery and assay of *E. coli* O157:H7 cells in the presence of the various foods, 30 g samples of different types of ground meats, milk or cheese were aseptically transferred to a stomacher bag containing 270 mL of either sterile dmTSB, or sterile mEC + n, and stomached for 120 s. Twenty-five millilitre portions of the resulting mixtures were inoculated with 200 µL containing varying concentrations of *E. coli* O157:H7 cells in TSB. The samples were then enriched by incubation at 37 °C for 24 h.

Extraction of LPS antigens. The LPS antigens of bacterial cells in pure broth culture and enrichment cultures of foods were extracted by adding 0.1 volume of 5% sodium cholate, for the O157 Polymyxin-CEIA, and 2 volumes of 0.75% sodium cholate to the samples for the Polymacron™ EIA followed by heating at 100 °C for 10 minutes. The samples were then cooled to room temperature and applied to the appropriate assay. For cell suspensions in broth, the treated samples were used directly in the assay. Food-broth mixtures were withdrawn through Filtatips (Filtaflex Ltd, Almonte, Ontario) to remove particulate matter before heat-cholate treatment and the clear supernatant was used directly in the assay.

O157 Polymacron™ enzyme immunoassay (Polymacron™ EIA). Polymacron™ sheets, precut EIA-grade polyester cloth with a printed grid, were coated with capture antibody by overnight incubation with an anti-*E. coli* O157 murine monoclonal antibody (immunoglobulin G) diluted 1:100 in 0.01 M phosphate-buffered (pH 7.2)/0.15 M NaCl (PBS). The sheets were then washed 4 times with PBS containing 0.05% (v/v) Tween 20 (PBST) on a filter under suction. All incubations were carried out at room temperature. Five μ L of 0.75% cholera-heat-treated samples were pipetted within the grids of an antibody coated Polymacron™ sheet and incubated for 10 min, followed by washing with PBST as before. Bound O157 antigens were detected by sequential 10 min incubations with anti-*E. coli* O157 antibody-peroxidase conjugate diluted 1:2000 in PBST and TMB membrane peroxidase substrate, with a PBST wash in between. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

O157 Polymyxin-cloth enzyme immunoassay (O157 Polymyxin-CEIA). Polymyxin cloth sheets (6 X 8 cm) were prepared as previously described (Blais *et al.*, 1993). Briefly, the cloths were washed in 20 % ethanol for 10 min and thoroughly rinsed in distilled water. For coating, the sheets were saturated with polymyxin B in distilled water (5 mg/mL) and incubated at 37 °C for 18 - 20 h and washed with distilled water. The cloths were then blocked by soaking in a 1% (w/v) solution of CM-cellulose at room temperature for 1 h. The blocked sheets were washed 4 times with PBST on a filter under suction. All incubations were carried out at room temperature. Five μ L of 5% cholera-heat-treated samples were pipetted on a polymyxin-cloth sheet at ca. 1 cm intervals and incubated for

10 min, followed by washing with PBST as before. To detect bound O157 LPS antigens, the sheet was incubated with 4 mL of detector antibody solution for 10 min. Two sources of detector antibodies were examined at their optimized concentrations: 1) Peroxidase-labelled affinity purified anti-*E. coli* O157:H7 antibody (KPL conjugate) diluted 1:4000 in PBST, or 2) Rabbit anti-*E. coli* O157 antibody (Difco antibody) diluted 1:200 in PBST, followed by a 10 min incubation with anti-rabbit IgG-peroxidase conjugate diluted 1:1000 in PBST. The sheets were washed with PBST after each immunoreaction, followed by incubation with 4 mL of TMB membrane peroxidase substrate system for 10 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

Results and Discussion

Lipopolysaccharide (LPS), a major cell surface antigen of *E. coli* and other Gram-negative bacteria, is composed of three regions: a repeating polysaccharide region constituting the somatic or O antigen, the core oligosaccharide and a lipid A region. The sugar composition, linkages, sequence and attached groups of the O antigen determine the serological character of the LPS molecule (e.g., the O157 antigen). Since LPS antigens are highly specific for a particular bacterium, they can be used as a way of identifying a particular bacterium. The present EIA systems capture LPS for detection of bacteria. The captured LPS is bound to a solid phase and is detected by specific antibodies raised against the LPS antigen of the target bacterium. In this study, we examined two LPS capture methods, a monoclonal antibody adsorbed to the surface of polyester cloth and polymyxin B coated cloth. The two different LPS capture methods were developed because each has

its own advantages and disadvantages. Monoclonal antibodies are highly specific for a particular LPS and therefore do not suffer from interference from competing LPS, but antibodies are expensive to produce, must be stored at refrigerated temperatures and have a limited lifespan. Polymyxin B is very inexpensive, is stable under various conditions and does not suffer from batch to batch variation as antibodies can. Since polymyxin binds to LPS through interaction with the lipid A portion it captures LPS from any Gram negative bacteria present. In the Polymacron EIA, a monoclonal antibody specific for the O157 somatic O antigen bound to the cloth captures the LPS. Since there are a number of repeated segments in the O antigen, detector antibodies also specific for the O157 O region can bind to other repeated segments within the O antigen.

Specificity of the Polymacron™ EIA. To determine whether bacteria routinely found in foods and environmental samples would cross react with the O157 antigens, we tested broth cultures of several *E. coli* strains, including a number of strains bearing the O157 antigen, and a variety of other Gram-positive and Gram-negative bacteria in the Polymacron™ EIA system. All of the *E. coli* strains bearing the O157 antigen produced positive results in the Polymacron™ EIA whereas all of the *E. coli* strains lacking this antigen were negative (Table 2-1). With the exception of *Salmonella urbana* (group N), all of the non-*E. coli* bacteria tested were negative in the assay (Table 2-2). Cross-reaction of the anti-O157 antibodies with *Salmonella* group N bacteria was expected, since it is known that their O

Table 2-1. Reactivity of *E. coli* strains in the O157 Polymyxin-CEIA using different detector antibodies, and the Polymacron™ EIA using the KPL detector antibody. ^a

Serotype/strain	Verotoxin Type	O157 Polymyxin CEIA		Polymacron™ EIA
		Difco	KPL	KPL
0157:H7 (ATCC 35150)	1 & 2	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT34	2	+	+	+
0157:H7 PT34	2	+	+	+
0157:H7 PT32	1 & 2	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT34	2	+	+	+
0157:H7 PT34	2	+	+	+
0157:H7 PT14	2	+	+	+
0157:H7 PT67	1 & 2	+	+	+
0157:H7 PT23	1	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT23	1 & 2	+	+	+
0157:H7 PT14	2	+	+	+
0157:NM	2	+	+	+
0157:H12	VT Neg.	+	+	+
0157:H19	VT Neg.	+	+	+
(ATCC 11775)	VT Neg.	-	-	-
0111:H11	1	-	-	-
0111:H11	1	-	-	-
0111:H11	1	-	-	-
0111:H8	1	-	-	-
0111:H8	1	-	-	-
0111:NM	1 & 2	-	-	-
0111:NM	1	-	-	-
0111:NM	1	-	-	-
0111:NM	1	-	-	-
026:H11	1	-	-	-
026:H11	1	-	-	-
026:H11	1	-	-	-
05:NM	1	-	-	-
05:NM	1	-	-	-

^a Various *E. coli* strains suspended in TSB at ca. 10⁸ cfu/mL were subjected to the two assays using either the KPL conjugate or Difco antibody as the source of detector antibodies as described in Materials and Methods. +, positive reaction; -, negative.

Table 2-2. Reactivity of non-*E. coli* strains in the O157 Polymyxin-CEIA using different detector antibodies, and the Polymacron™ EIA using the KPL detector antibody. ^a

Strain	O157 Polymyxin CEIA		Polymacron^T EIA
	Difco	KPL	KPL
<i>Bacillus cereus</i>	-	-	-
<i>Bacillus subtilis</i>	-	-	-
<i>Citrobacter freundii</i>	-	-	-
<i>Enterobacter aerogenes</i>	-	-	-
<i>Enterobacter cloacae</i>	-	-	-
<i>Enterococcus faecalis</i>	-	-	-
<i>Escherichia hermanii</i>	-	-	-
<i>Klebsiella pneumoniae</i>	-	-	-
<i>Listeria innocua</i>	-	-	-
<i>Listeria ivanovii</i>	-	-	-
<i>Listeria welshimeri</i>	-	-	-
<i>Proteus vulgaris</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-
<i>Salmonella typhimurium</i> LT2	-	-	-
<i>Salmonella urbana</i>	+	+	+
<i>Serratia marcescens</i>	-	-	-
<i>Shigella sonnei</i>	-	-	-
<i>Xanthamonas maltophila</i>	-	-	-
<i>Yersinia enterocolitica</i> (serotype O:9) ^b	-	-	-

^a Various bacterial strains suspended at ca. 10⁸ cfu/mL were subjected to the two assays using either source of detector antibodies as described in Materials and Methods.

^b A total of five isolates of this serotype were tested, all of which gave negative results using either source of detector antibodies.

antigen structure is identical to that of *E. coli* O157 (Padhye and Doyle, 1991, M. Perry, personal communication). Thus, the Polymacron™ EIA was specific for bacteria bearing the O157 and related antigens.

Detectability of the O157 Polymacron™ EIA. In order for an assay to be effective, it must detect a specific bacterium in a food enrichment sample where the target may represent less than 0.1 % of the total number of bacteria present. Therefore the assay should detect the target bacterium at a relatively low level. The detectability of this system in the assay of an *E. coli* O157 strain (ATCC 35150) was examined by assaying serial dilutions of a culture in dmTSB. In this manner, it was determined that the minimum cell density of a broth suspension giving a positive result in the assay was ca. 10^5 cfu/mL, or ca. 400 cholate-extracted cfu equivalents applied per 5 μ L spot. This result was reproduced in three separate trials. The assay exhibited a similar detectability when the cells were grown and serially diluted in mEC + n (not shown). Thus, it was surmised that the detectability of the assay might be sufficient to allow the detection of *E. coli* O157 in enrichment cultures of food samples, provided that the cells could be enriched to a level of at least 10^5 cfu/mL or greater.

Detection of E. coli O157 in various foods by the Polymacron™ EIA. The previous work which demonstrated the Polymacron™ EIA should be able to specifically detect *E. coli* O157:H7 in food enrichment samples was completed using pure cultures of *E. coli* O157:H7 and other Gram positive and Gram negative bacteria. Therefore, it was necessary

to determine if the presence of different food commodities would affect the specificity or detectability of the assay. To demonstrate the applicability of the Polymacron™ EIA, various concentrations of *E. coli* O157 cells were inoculated into different food types. Fresh samples of various types of ground meats and dairy products were homogenized in the enrichment media dmTSB (Padhye and Doyle, 1991) and mEC + n (Johnson *et al.*, 1995), and portions of the meat-broth mixture were then inoculated with different numbers of *E. coli* O157:H7 cells. The mixtures were incubated at 37 °C for 24 h, and then assayed by the O157 Polymacron™ EIA. Each experiment was carried out in triplicate. Table 2-3 shows that small numbers of *E. coli* O157:H7 cells were successfully detected by the assay after enrichment in both dmTSB and mEC + n in the presence of the different meat and dairy samples. Uninoculated mixtures were negative. Total aerobic counts performed on each of the food samples prior to inoculation indicated the presence of high levels of indigenous bacteria for all except the pasteurized milk, thus demonstrating the ability of this combined enrichment and assay procedure to detect small numbers of target organism in the presence of a very large background of non-target bacteria. With the meats, stronger reactions (darker spots) were consistently obtained with samples enriched in mEC + n, whereas no such qualitative differences between the results obtained with samples grown in either dmTSB or mEC + n were observed for the dairy samples. This suggests that higher *E. coli* O157:H7 cell densities were achieved after selective enrichment in mEC + n than in dmTSB for the inoculated meat samples. The lack of difference between the intensity of the spots produced by dmTSB and mEC+n in the dairy samples is most likely due to the lack of competing microflora.

Table 2-3. Detection of *E. coli* O157:H7 cells inoculated into various food commodities ^a.

Food ^b	Enrichment Broth	Inoculum (cfu/g) ^c	No. Positive/total
Regular ground beef (1.6 x 10 ⁶)	dmTSB	0	0/3
	"	7.2	3/3
	mEC+n	0	0/3
	"	7.2	3/3
Ground chicken (6.8 x 10 ⁶)	dmTSB	0	0/3
	"	7.2	3/3
	mEC+n	0	0/3
	"	18	3/3
Ground turkey (1.4 x 10 ⁷)	dmTSB	0	0/3
	"	7.2	3/3
	mEC+n	0	0/3
	"	7.2	3/3
Ground lamb (2.7 x 10 ⁸)	dmTSB	0	0/3
	"	7.6	3/3
	mEC+n	0	0/3
	"	7.6	3/3
Ground pork (2.6 x 10 ⁷)	dmTSB	0	0/3
	"	7.6	3/3
	mEC+n	0	0/3
	"	7.6	3/3
Pasteurized milk, 3.4 % M.F. (735)	dmTSB	0	0/3
	"	7.2	3/3
	mEC+n	0	0/3
	"	7.2	3/3
Cheddar cheese, 32 % M.F. (2.3 x 10 ⁹)	dmTSB	0	0/3
	"	7.2	3/3
	mEC+n	0	0/3
	"	7.2	3/3

^a Different numbers of *E. coli* O157:H7 (ATCC 35150) cells were inoculated into various food-enrichment broth mixtures (2.5 g food + 22.5 mL broth) and enriched for 24 h as described in Materials and Methods. The samples were then assayed by the O157 Polymyxin-CEIA and the Polymacron™ EIA.

^b Total aerobic counts (cfu/g) for each food before inoculation are indicated in brackets.

^c No. of colony forming units inoculated per gram of sample

^d Number of replicate samples giving positive results per total number tested.

The fat content of a specific food sample may affect the ability of the assay to detect the target bacterium or may affect the growth of the target. The effect of inoculating different levels of *E. coli* O157:H7 cells directly into 25 g samples of ground beef with varying fat content on their recovery and subsequent assay was studied. Table 2-4 shows that on average as few as 0.2 cfu per g of ground beef in a 25 g sample were detectable in all of the samples by the O157 Polymacron™ EIA after enrichment in either dmTSB or mEC + n. The fat content of the samples tested, from regular to lean ground beef, had no appreciable effect on this result. Again, stronger reactions were generally observed with samples enriched in mEC + n. It was also possible to detect in three replicate experiments as few as ca. 0.2 *E. coli* O157:H7 cfu per gram of regular ground beef after storage of inoculated 25 g samples at -20 °C for 5 days (not shown).

There are a number of drawbacks associated with using antibodies as the method for capturing LPS antigens. The amount of antibody needed to coat the cloth is quite high which adds to the already substantial cost of antibodies, and antibodies often vary in quality from batch to batch. Long term stability of antibodies can also be unpredictable, especially if they are not refrigerated, such as during transport. On the other hand, polymyxin is less costly and more stable than antibodies. Polymyxin B has been used previously to capture the LPS of *Salmonella* in foods (Blais and Yamazaki, 1990a,b; Blais and Yamazaki, 1991; Blais *et al.*, 1993). Polymyxin B is a polypeptide antibiotic which binds to bacterial LPS through interaction with the lipid A region (Blais and Yamazaki, 1990a). Thus, *E. coli* LPS should be captured in the same manner as *Salmonella* LPS was previously. Although polymyxin is quite stable, inexpensive and does not suffer from batch to batch variability,

Table 2-4. Detection of *E. coli* O157:H7 cells inoculated into ground beef ^a.

Ground beef sample ^b	Enrichment broth	Inoculum (cfu/g) ^c	No. Positive/total ^d
Regular, 28 % fat (5.2 x 10 ⁶)	dmTSB	0	0/3
	"	0.3	3/3
	"	3.2	3/3
	mEC+n	0	0/3
	"	0.7	3/3
	"	7.0	3/3
Medium, 23 % fat (6 x 10 ⁶)	dmTSB	0	0/3
	"	0.3	3/3
	"	3.0	3/3
	mEC+n	0	0/3
	"	0.2	3/3
	"	2.0	3/3
Lean, 18 % fat (3.5 x 10 ⁶)	dmTSB	0	0/3
	"	0.2	3/3
	"	2.0	3/3
	mEC+n	0	0/3
	"	0.4	3/3
	"	4.0	3/3

^a Different numbers of *E. coli* O157:H7 (ATCC 35150) cells were inoculated into 25 g of ground beef and enriched in either dmTSB or mEC + n broth for 24 h as described in Materials and Methods. The samples were then assayed by the O157 Polymyxin-CEIA and the PolymacronTM EIA.

^b The fat content for each sample is given in % (w/w). Total aerobic counts (cfu/g) for each ground beef sample before inoculation are indicated in brackets.

^c No. of colony forming units inoculated per gram of sample.

^d Number of replicate samples giving positive results per total number tested.

Table 2-5. Detectability of the O157 Polymyxin-CEIA using various strains. ^a

Organism	Serotype	Detector antibody	Detectability (cfu/mL x 10⁵)
<i>Escherichia coli</i>	O157:H7 (ATCC 35150)	Difco	2.6
	O157:H7 (ATCC 35150)	KPL	0.52
	O157:NM	KPL	2.1
	O157:H12	KPL	1.3
	O157:H19	KPL	1.3
<i>Salmonella urbana</i>	O:30	KPL	1.6

^a Bacteria were suspended in TSB at different concentrations and assayed by the O157 Polymyxin-CEIA as before. The detectability is expressed as the minimum cell density giving a positive reaction in the assay.

it does not only capture *E. coli* O157 LPS, as the monoclonal antibody does, it captures all types of LPS in the sample. Utilizing specific *E. coli* O157 detector antibodies, the bound O157 LPS should be detectable.

Detectability of the O157 Polymyxin-CEIA. The detectability of the Polymyxin-CEIA for various *E. coli* O157 strains and the cross-reacting *S. urbana* strain was examined. When the KPL conjugate and Difco antibody were compared as sources of detector antibodies in the assay of different concentrations of an *E. coli* O157:H7 strain, the former gave about a 5-fold greater detectability (i.e., minimum no. of cells detectable) than the latter (Table 5). This experiment was performed in duplicate, and the results were reproducible. Other *E. coli* O157 strains of different H antigen composition assayed using the KPL conjugate gave detectabilities within the range of $1.3 - 2.1 \times 10^5$ cfu/mL, as did the *S. urbana* strain (Table 2-5). These detectability limits should be sufficient to allow the detection of *E. coli* O157 in enrichment cultures of foods.

Detection of E. coli O157 in enrichment cultures of ground beef. The preceding results demonstrate the ability of the O157 Polymyxin-CEIA to detect *E. coli* O157 cells in pure culture. To demonstrate the applicability of this assay in the detection of low initial numbers of *E. coli* O157:H7 cells in ground beef, a 25 g sample was homogenized with 225 mL of dmTSB in a stomacher and then divided into 20 mL portions, which were inoculated with different numbers of fresh cells. The mixtures were incubated at 37 °C for 24 h, and then assayed by the O157 Polymyxin-CEIA using the KPL conjugate as detector. Each

experiment was carried out in triplicate. As few as ca. 1 *E. coli* cfu inoculated in meat could be detected by the O157 Polymyxin-CEIA after enrichment (Table 2-6). The uninoculated samples were negative. Total aerobic counts indicated the presence of ca. 10^5 cfu/g of indigenous bacteria in the uninoculated meat prior to enrichment, thus demonstrating the ability of this combined enrichment and assay procedure to detect very small numbers of target organism in the presence of a very large background of non-target bacteria. In these experiments, the failure to detect cells in some of the replicates with the lower inocula reflects the statistical probability of a sample receiving even a single cell at these levels of inoculation.

Minimum enrichment time for detection of E. coli O157 spiked into ground beef. In order to ascertain whether the overall procedure could be shortened by curtailing the enrichment period, 25 g samples of ground beef were mixed with 225 mL of dmTSB in a stomacher and inoculated with different numbers of fresh *E. coli* O157:H7 cells. As a control, 225 mL samples of dmTSB devoid of meat were similarly inoculated. Each experiment was carried out in duplicate. The mixtures were incubated at 37 °C, and 1 mL portions withdrawn after 0, 4, 8, and 24 h for testing in the O157 Polymyxin-CEIA. For the inoculated meat samples, a minimum of ca. 1-10 cfu were detected after an 8 h enrichment, and all samples inoculated with as few as ca. 1 cfu were positive after 24 h (Table 2-7). At these levels of inocula, the control dmTSB cultures devoid of meat failed to give positive results in the assay before a 24 h enrichment period. The reason for this is not known,

Table 2-6. Detection of *E. coli* O157 inoculated into 25 g ground beef samples. ^a

Sample	Inoculated (cfu)			
	Positive/total ^b			
	93	9.3	0.9	0
Ground beef-dmTSB	3/3	3/3	2/3	0/1
dmTSB alone	3/3	3/3	3/3	0/1

^a Different numbers of *E. coli* O157:H7 (ATCC 35150) cells were inoculated into broth alone or meat-broth mixtures and enriched overnight as described in Materials and Methods. The samples were then subjected to the O157 Polymyxin-CEIA using the KPL conjugate as detector.

^b No. of positives per total number of replicate samples tested.

Table 2-7. Time of recovery of *E. coli* O157 inoculated into ground beef samples. ^a

Sample	Inoculum (cfu)	Incubation (hours)			
		Positive/total ^b			
		0	4	8	24
Ground beef-dmTSB	0	0/1	0/1	0/1	0/1
	0.8	0/2	0/2	1/2	2/2
	8.2	0/2	0/2	2/2	2/2
dmTSB alone	0	0/1	0/1	0/1	0/1
	0.8	0/2	0/2	0/2	2/2
	8.2	0/2	0/2	0/2	2/2

^a Different numbers of *E. coli* O157:H7 (ATCC 35150) cells were inoculated into broth alone or meat-broth mixtures and enriched for various periods of time as described in Methods. Samples withdrawn after 0, 4, 8 and 24 h incubation were then subjected to the O157 Polymyxin-CEIA using the KPL conjugate as detector.

^b No. of positives per total number of replicate samples tested.

though it is surmised that the presence of meat components in the meat-broth mixtures mitigated any possible effects of selective agents from dmTSB on the kinetics of cell growth. These results indicate that low numbers of fresh, uninjured cells in a meat sample can be detected after only 8 h enrichment; however, since injured cells present in samples may have a prolonged lag phase, it is advisable to carry out a minimum overnight enrichment prior to performing the assay where the presence of such cells is suspected.

These studies have demonstrated the applicability of the O157 Polymyxin-CEIA and the Polymacron™ EIA as screening tools for *E. coli* O157 in enrichment cultures of food samples, detecting as few as ca. 1 cfu in a 25 g meat sample after overnight enrichment. Also, other EIAs have reported cross reaction with a number of bacteria such as *Escherichia hermannii*, *Citrobacter freundii*, *Yersinia enterocolitica*, *Brucella* spp., and *Salmonella* N group (Padhye and Doyle, 1991), while the two cloth based assays developed only cross react with the *Salmonella* N group bacteria. These rapid and inexpensive assays utilize a simple dot blot format permitting the simultaneous analysis of multiple samples, and should be ideally suited for use in the food testing or industry laboratory where large numbers of samples must be screened on a routine basis. Since these tests do not detect virulence factors *per se* (e.g., verotoxins), positive results are considered presumptive, and will need to be confirmed by more definitive tests (e.g., biochemical, serological, etc.). Nonetheless, the assays will be extremely useful for the rapid and economical identification

of negative samples which can be immediately released for market. On the basis of these and previous studies, we may anticipate other useful applications for the Polymyxin-CEIA concept in the assay of Gram-negative bacteria for clinical, agricultural and environmental testing, as well as the use of PolymacronTM as a solid phase for other EIA systems. The PolymacronTM EIA has been commercialized by a local biotechnology company Kalyx Biosciences under the name of *E. coli* O157 RapitestTM, and is currently being evaluated by Health Canada, Health Protection Branch for inclusion in the Compendium of Methods for Analysis.

On the basis of sensitivity and specificity, there was no difference between the PolymacronTM EIA and the Polymyxin-CEIA, but the advantages of polymyxin as an LPS capturing agent over antibodies makes the Polymyxin-CEIA a preferred capturing method.

Chapter 3

Evaluation of various enrichment conditions for the recovery of healthy and thermally injured *E. coli* O157:H7 cells spiked into ground beef.

Abstract

A study was undertaken to determine the best method for recovery of *Escherichia coli* O157:H7 from ground beef samples. *E. coli* O157:H7 and 4 other naturally occurring food isolates, as well as microflora extracted from ground beef were grown in TSB, dmTSB, and mEC+n at 37 and 42 °C. At 37 °C TSB produced the best growth for both *E. coli* O157:H7 and the ground beef microflora, while at 42 °C, mEC+n produced the best growth for *E. coli* O157:H7 and inhibited the ground beef microflora almost completely. When a number of recovery conditions using dmTSB and mEC+n were tested using a 24 h incubation, growth in mEC+n at 42 °C more consistently recovered healthy *E. coli* O157:H7 spiked into 25 g ground beef samples than did dmTSB at 37 °C, 100 % for 3-10 cfu inoculated, and 82 % for 3-10 cfu inoculated respectively. Thermally injured *E. coli* O157:H7 cells were used to simulate the possible injured state of naturally contaminated beef samples, and were also recovered by placing a 25 g ground beef sample directly into 225 mL of mEC+n and enriching at 42 °C for 24 h.

Introduction

For the last decade enterohaemorrhagic *Escherichia coli* (EHEC), predominantly serotype O157:H7, has been regarded as a major cause of food poisoning. This group of *E. coli* elaborate a number of toxins which cause many of the symptoms of the diseases. The haemorrhagic colitis caused by *E. coli* O157:H7's enterohaemolysin, causes severe stomach cramps and bloody diarrhoea for up to 9 days (Doyle, 1991). The other diseases associated with EHEC are haemolytic uraemic syndrome (HUS) and thrombo

thrombocytopenic purpura (TTP) which are caused primarily by the verotoxins (VT) produced and can be fatal to young children and immunocompromised individuals (Doyle, 1991). The first recorded illness was in 1982 in the states of Oregon and Michigan (Riley *et al.*, 1983). Both outbreaks were linked epidemiologically to the consumption of ground beef sandwiches, and *E. coli* O157:H7 was isolated from a raw ground beef patty from the same lot the sandwiches came from. The major source of infection is from ground beef, but it has been associated with unpasteurized milk, chicken, lamb, pork, and vegetables (Doyle and Schoeni, 1987; Doyle, 1991). These organisms may initially be present in low numbers (< 1 cfu/g of sample) in implicated foods containing high levels of competing microflora. Therefore, to effectively detect the presence of *E. coli* in foods, the number of *E. coli* O157:H7 organisms must be increased to a detectable level without increasing the number of background organisms. Since *E. coli* O157:H7 does not grow well under the usual conditions for isolation of *E. coli*, new methods for its isolation had to be developed (Doyle and Schoeni, 1987).

All rapid methods for the detection of *E. coli* O157:H7 and other bacteria in samples with initially low numbers require an enrichment step prior to detection. The enrichment media used include selective agents, such as antibiotics to selectively enrich the *E. coli* present in the sample. Currently, there are a number of isolation procedures for *E. coli* O157:H7 from foods which increases the number of O157:H7 cells without increasing the competing bacteria too greatly. Health Canada recommends the enrichment of food samples in modified trypticase soy broth containing novobiocin (mTSB+n) followed by biochemical and serological identification (Warburton and Todd, 1995). While the Food

Safety and Inspection Service (FSIS) of the US uses enrichment in modified EC broth containing novobiocin (mEC+n) followed by a direct blot ELISA for rapid presumptive positive identification (Okrend and Rose, 1989). A third enrichment broth, double modified TSB (dmTSB), was developed by Okrend *et al.* (1990), and is said to enhance the production of the somatic O157 antigen as well as increase the growth of *E. coli* O157:H7 relative to mTSB. The increase in the somatic antigen could effectively increase the sensitivity of an EIA which uses LPS as its target, such as the O157 Polymyxin-CEIA.

All of the methods above recommend placing the sample directly into a selective broth. Since the initial state of the *E. coli* O157:H7 cells may be injured from processing or cold storage, the immediate addition of a selective broth may kill any injured cells present. It therefore, maybe necessary to restore the injured cells to a healthy state by the addition of a non-selective broth before selective enrichment. We will therefore use thermally injured *E. coli* O157:H7 cells in the evaluation of enrichment media and conditions to approximate the possible injured state of cells present in a naturally contaminated food sample and determine if a non-selective pre-enrichment is necessary.

We have evaluated the growth of several *E. coli* O157:H7 cultures and ground beef microflora in mEC+n and dmTSB as well as a non-selective broth, TSB. A number of enrichment conditions employing both dmTSB and mEC+n were used to recover healthy *E. coli* O157:H7 inoculated into 25 g ground beef samples. The different isolates of *E. coli* O157:H7, as well as the ground beef microflora were grown at 37 and 42 °C both with and without shaking to determine the optimal growth conditions for O157:H7 while retarding the growth of competing microorganisms. The media and enrichment condition which gave

the best recovery of *E. coli* O157:H7 and inhibited the background microflora was used to determine how thermally injured cells were recovered in pure broth and ground beef samples. The ability of the media to recover O157:H7 and the ground beef microflora was monitored by the change in cell density using a spectrophotometer.

Materials and Methods

Chemicals and bacterial strains. Acriflavine hydrochloride (No. A-8251) and lactose (No. l-3625) were obtained from Sigma Chemical Co. (St. Louis, MO). The casamino acids (No. 0230-01), tryptone (No. 0123-17-3), bile salts #3 (No. 0130-01), Sorbitol MacConkey agar (SMAC) (No. 0079-17-7), Eosin Methylene Blue agar (EMB) (No. 0076-01-8) and the Rabbit anti-*E. coli* O157 antiserum (No. 2970-47) were purchased from Difco Laboratories (Detroit, MI). Trypticase soy broth (TSB) was obtained from BDH Inc. (Darmstadt, Germany). *E. coli* O157:H7 ATCC 35150 and three other *E. coli* O157:H7 isolates from the Laboratory Services Division, Agriculture-Agri-Food Canada culture collection were used in the experiments. Unless otherwise stated, *E. coli* O157:H7 was grown in TSB by shaking at 37 °C for 16 - 20 h.

Growth of *E. coli* O157:H7 and ground beef microflora under various conditions. To determine the effect of different media and growth temperatures on a pure cultures of *E. coli* O157:H7, 250 µL of an overnight culture was used to inoculate 50 mL of sterile TSB, double modified TSB (dmTSB) (trypticase soy broth, 30 g/L; casamino acids, 10 g/L; K₂HPO₄, 1.5 g/L; bile salts #3, 1.5 g/L; and, acriflavine hydrochloride, 10 mg/L) (Padhye

and Dolye, 1991) or modified EC broth containing novobiocin (mEC+n) (tryptone, 20 g/L; bile salts #3, 1.12 g/L; lactose, 5 g/L; K_2HPO_4 , 4 g/L; KH_2PO_4 , 1.5 g/L; NaCl, 5 g/L; and novobiocin, 0.02 g/L) (Okrend *et al.*, 1990). The inoculated media was grown at either 37 or 42 °C and 500 µL samples were withdrawn at 8 h, and after 24 h. The samples were mixed with 1 volume of 1 % sodium azide to stop further growth, and the A_{600} was recorded. Ground beef microflora was extracted and subjected to the different growth conditions as above. The ground beef microflora was extracted by placing 10 g of regular ground beef in 90 mL of sterile TSB and stomached for 120 s. Five hundred microlitres of the mixture was removed through a sterile Filtatip (Filtaflex Ltd, Almonte, Ontario) and was added directly to the 50 mL growth flasks (as described above) which were grown for 24 h. The A_{600} was read after 24 h as above. One millilitre of the extracted microflora was also added to 5 mL of sterile TSB and grown for 18 - 20 h at 37 °C with shaking. Two hundred and fifty microlitres of this overnight culture was used to inoculate each 50 mL selective broth which were grown for 24 h. Samples were removed after 8 h, and after 24 h. An O157 Polymyxin CEIA (Chapter 2; Booth *et al.*, 1996) was performed on each of the meat samples to be sure that there was no *E. coli* O157:H7 present which would alter the growth characteristics.

Recovery of healthy E. coli O157:H7 spiked into ground beef. Ground beef samples were purchased from a local retailer and used fresh on the day of purchase. Twenty five gram samples of ground beef were placed in sterile stomacher bags and inoculated in the centre with 100 µL of 0.01 M phosphate-buffered (pH 7.2)/0.15 M NaCl (PBS) containing varying

concentrations of *E. coli* O157:H7 cells. The cells were then blended into the meat by gently hand massaging the contents of the bags. Samples without pre-incubation were blended with 225 mL of either dmTSB or mEC+n by stomaching for 120 s and enriched for 24 h at 37 or 42 °C without shaking. Pre-incubated 25 g samples were inoculated as before, mixed by hand with 10 mL of TSB and incubated at 37 °C for 6 h. After pre-incubation, 215 mL of mEC+n broth was added, thoroughly mixed by stomaching for 120 s and incubated at 37 °C for a further 18 h. All enriched sample were then assayed for the presence of *E. coli* O157 by using the O157 Polymyxin CEIA and the Polymacron™ EIA (Blais *et al.*, 1996).

To confirm the presence of *E. coli* O157:H7 in a sample after enrichment, the sample was serially diluted and plated on Sorbitol MacConkey agar (SMAC). One hundred microlitres of a range of dilutions from 10^{-1} to 10^{-6} were spread plated for each sample on separate SMAC plates. Four colonies from each SMAC plate which exhibited typical *E. coli* O157:H7 morphology (colourless) were chosen and restreaked on SMAC and Eosin Methylene Blue agar (EMB). The colonies which exhibited both colourlessness on SMAC and typical *E. coli* morphology on EMB were sent to the VITEK for biochemical identification. Colonies which were confirmed *E. coli* by the VITEK were tested in pure culture by the O157 Polymyxin CEIA to show the presence of the O157 antigen. The O157 Polymyxin assay positive samples were then checked against *E. coli* O157 antiserum for final confirmation.

Thermal injury of E. coli O157:H7. A Log phase culture of *E. coli* O157:H7 (ATCC 35150) was used in all heat injury experiments. *E. coli* O157:H7 cells were harvested by centrifugation of a 20 mL culture at 9000 x g for 10 min. The cell pellet was then resuspended in 10 mL of sterile PBS. The resuspended cells were placed in a pre-warmed 125 mL erlenmeyer flask and placed in a precision circulating water bath at 55 °C for heat injury. The flask was gently shaken for 10 min, removed and the injured cells were serially diluted in sterile PBS. Plate counts to determine the number of viable and injured cells present before and after the heat treatment were performed by the drop plate method on TSA and TSA containing 3 % NaCl. The percent injury was calculated by dividing the number of cfu/mL on TSA containing 3 % NaCl by the number of cfu/mL on TSA and multiplying by 100.

Recovery of thermally injured E. coli O157:H7 in pure broth. *E. coli* O157:H7 cells were heat injured as described above and inoculated into pure broth. Between 1 and 10 cfu were inoculated into 5 mL of sterile mEC+n or TSB and grown for 24 h at either 37 or 42 °C. The tubes with no visible growth were checked for viable cells by plating 100 µL of the broth on TSA. In other experiments, a pre-incubation step in TSB was included before the addition of mEC+n to aid in the recovery of injured O157 cells. Approximately 10 cfu in 100 µL of PBS were added to tubes containing 400 µL of TSB which were pre-incubated for 0, 1, 2, 4 or 6 h at either 37 or 42 °C. After the pre-incubation, 4.5 mL of mEC+n were added to each tube and incubation was continued for a further period to equal 24 h of total incubation time. To determine at what time during pre-incubation the cells were recovered

from and injured state to healthiness, 100 μ L aliquotes of the TSB were removed after 0, 1, 2, 4, and 6 h and spread plated on TSA and TSA containing 3 % NaCl.

Recovery of thermally injured E. coli O157:H7 spiked into ground beef. Ground beef samples were purchased from a local retailer and used fresh. To study the recovery of thermally injured *E. coli* O157:H7 cells spiked into ground beef, 100 μ L of PBS containing various numbers of injured cells were inoculated into 25 g meat samples. For samples with no pre-incubation, the inoculated 25 g meat balls were aseptically transferred to stomacher bags and 225 mL of mEC+n were added. The contents were then stomached for 120 s. Samples were then incubated for 24 h at 42 °C. After the incubation period, the enriched samples were assayed using the O157 Polymyxin CEIA and the Polymacron™ EIA. Some samples were enriched using a pre-incubation step. The *E. coli* O157:H7 inoculated 25 g meat balls were aseptically transferred to stomacher bags containing 25 mL of sterile TSB. The contents were mixed by hand so that a uniform mixture was formed. They were then placed at 42 °C for 6 h. After the 6 h pre-incubation, 200 mL of mEC+n were added, and the samples were further incubated at 42 °C for 18 h. Each enriched sample was then assayed using the O157 Polymyxin CEIA and the Polymacron EIA.

Results and Discussion

Growth of E. coli O157:H7 and ground beef microflora under various conditions. In order to examine the growth of *E. coli* O157:H7 and normal meat microflora quantitatively, growth charts using a number of *E. coli* O157:H7 cultures, media and temperatures were

constructed. Currently, the Food Safety and Inspection Service, of the US, method for recovery of *E. coli* O157:H7 from ground beef consists of enrichment in mEC+n broth at 35-37 °C followed by a direct blot ELISA (Johnson *et al.*, 1994). The Government of Canada, Health Protection Branch procedure for the isolation of *E. coli* O157:H7 from foods involves enrichment in mTSB (Tryptic soy broth, 30 g/L; bile salts #3, 1.5 g/L; K₂HPO₄, 1.5 g/L) with novobiocin (100 mg/L) at 42 °C for 24 h (Warburton and Todd, 1995). We used dmTSB in the place of mTSB because dmTSB was developed to increase the production of the somatic O157 antigen and to promote rapid growth of *E. coli* O157:H7 (Padhye and Doyle, 1991). The addition of acriflavine hydrochloride increased the production of the O157 antigen by a yet unknown mechanism without affecting the growth of *E. coli* O157:H7 (Padhye and Doyle, 1991). Casamino acids were added to increase the growth rate of *E. coli* O157:H7 (Padhye and Doyle, 1991). The concentration of bile salts remained unchanged from mTSB to dmTSB. Therefore, since the O157 Polymyxin CEIA and the Polymacron™ EIA use the somatic O157 antigen for detection, it would be more appropriate to examine dmTSB. The mEC+n was evaluated because it was recommended by the FSIS for O157:H7 recovery from ground beef as well as was also reported to give increased recovery over dmTSB of naturally contaminated meat samples (Johnson *et al.*, 1995). Okrend *et al.* (1990) decreased the concentration of bile salts in EC broth from 0.15 % to 0.112 % to allow for the full recovery of *E. coli* O157:H7 at elevated temperatures and the addition of novobiocin was to inhibit the growth of Gram positive organisms.

E. coli O157:H7 (ATCC 35150), as well as 4 other *E. coli* O157:H7 natural food

Figure 3-1. Growth of *E. coli* O157:H7 ATCC 35150 at 37 °C with and without shaking in various media. Growth of other *E. coli* O157:H7 cultures were similar to the above data (data not shown)

Figure 3-2. Growth of *E. coli* O157:H7 ATCC 35150 at 42 °C with and without shaking in various media. Growth of other *E. coli* O157:H7 cultures were similar to above data (data not shown).

isolates (data not shown) were grown in TSB, dmTSB and mEC+n at 37 and 42 °C for 24 h. Samples were removed after 8 and 24 h and the absorbance read to produce the growth charts. Thirty seven Celsius with shaking in TSB produced the most rapid growth (Figure 3-1). The two selective broths, dmTSB and mEC+n, produced very similar growth with slightly faster growth in the dmTSB, which could be expected due to the presence of the casamino acids (Padhye and Doyle, 1991). However, at 42 °C mEC+n produced the best growth without shaking and growth equal to TSB with shaking (Figure 3-2). The dmTSB produced less growth at 24 h than the TSB and mEC+n both with and without shaking. The mEC+n produced a steady growth rate and after 24 had the highest cell density observed (higher than TSB at 37 °C). Presumably, the increased growth at 42 °C is due to the decrease in bile salts concentration, dmTSB contains 0.15 % while mEC+n contains 0.112 %. Incubation of the three media at 37 and 42 °C produced similar results, with slightly higher growth when shaken.

We next examined the growth of ground beef microflora and compared it with that of *E. coli* O157:H7 under the various conditions. The growth of indigenous microflora was studied because to selectively effectively enrich the numbers of *E. coli* O157:H7, the behaviour of competing microflora in the enrichment broth must also be investigated. The growth of the meat microflora was somewhat inconsistent, which is not surprising from a mixed culture of Gram positive and Gram negative bacteria. When the samples were placed in the selective broth after a 24 h growth period in TSB (which decreased the initial lag phase from 8 h to 1 h so measurements could be made) the samples at 37 °C in TSB produced the best growth both with and without shaking (Figure 3-3). This time the

Figure 3-3. Growth of ground beef microflora at 37 °C with and without shaking in various media.

Figure 3-4. Growth of ground beef microflora at 42 °C with and without shaking in various media.

dmTSB and the mEC+n produced vastly different growth characteristics. The dmTSB did inhibit the growth somewhat, but the mEC+n inhibited the growth of the microflora much more completely. This is most likely due to the addition of novobiocin. At 42 °C (Figure 3-4), after 8 h all the media seemed to almost completely inhibit the microflora, but after 24 h it was evident that the mEC+n totally inhibited growth while dmTSB allowed some growth. The shaken samples produced similar results to the statically incubated samples, but a greater amount of growth was seen. When the ground beef microflora was placed directly into the selective media without pre-growth in TSB similar results were obtained (Figure 3-5). This showed that the pre-growth in TSB did not alter the growth curves. Comparing the data received from the growth of *E. coli* O157:H7 and the ground beef microflora, mEC+n incubated at 42 °C without shaking appears to be the best media and condition for the recovery of *E. coli* O157:H7. mEC+n at 42 °C without shaking almost completely inhibits background microflora while allowing the O157:H7 present to grow to a relatively high cell density.

Recovery of healthy E. coli O157:H7 spiked into ground beef. A number of enrichment methods for *E. coli* O157:H7 were tested and are presented in Table 3-1. All incubations were carried out without shaking since it is logistically difficult to shake large numbers of samples at once. Our initial work was done with dmTSB since it was reported to increase the expression of the somatic O157 antigen and would therefore increase the signal in our EIA tests (Chapter 2). The dmTSB gave good results but we found that the spots produced were quite light for samples with a low inoculum and that some of the samples were not

Figure 3-5. Growth of ground beef microflora after 24 h with and without shaking. Microflora was extracted from ground beef and placed directly into the growth media.

being detected. Only 82 % of the samples with an initial inoculum of 3-10 cfu were detected as positive by either the O157 Polymyxin CEIA or the Polymacron™ EIA. According to the Poisson Distribution, with an estimated inoculum of 3 cfu there is a 95 % chance that at least 1 cfu will actually be inoculated into the sample. We therefore decided to use an estimated inoculum of 3 cfu as the lowest statistically valid inoculum. Since, with an inoculum lower than 3 cfu there is a high probability that an inoculated sample produced a negative result in the assays due to the lack of at least 1 cfu of *E. coli* actually being inoculated into the sample and not due to the lack of its growth.

mEC+n was reported by Johnson *et al.* (1994) to more often recover *E. coli* O157:H7 from naturally contaminated beef samples and in previous work (Chapter 2; Blais *et al.*, 1996) gave more intensely coloured spots in the Polymacron™ EIA and the O157 Polymyxin CEIA than did dmTSB. This would suggest a higher number of *E. coli* O157:H7 cells present after enrichment. We therefore turned our attention to mEC+n as an enrichment media. Initially we enriched the samples at 37 °C for 24 h as recommended by the FSIS. It was more efficient than the dmTSB at recovering inoculated samples, and gave a 94 % recovery, but some of the spots produced were still very light. The reasons for not detecting O157 in some of the samples could be due to the inoculated cells dying upon inoculation or the inability of the media to enrich the inoculum to a detectable level. To determine which of the two possibilities was responsible, 10^{-4} to 10^{-6} dilutions of the samples were plated on SMAC. After 16 - 20 h at 37 °C suspect O157:H7 colonies were chosen and restreaked on EMB plates. Any colonies that exhibited an *E. coli* phenotype were run on the VITEK bacterial identification system using the Gram negative

identification card. The VITEK is an automated, biochemical identification system using a 30 well Gram negative identification card for the identification of *E. coli* (the biochemical reactions used are presented in Appendix I). Of the 9 inoculated samples that were O157 negative by assay, none were positive for *E. coli* O157:H7 by the plating method. The three samples that were inoculated and were positive by assay, only one was confirmed to contain *E. coli* O157:H7. Since the samples must be diluted before plating, it is possible for the sample to contain *E. coli* O157:H7 but not be detected due to masking by background bacteria. For example if 100 μ L of the 10^{-4} dilution was plated and less than 10^5 target organisms are present, the target will not be detected. This level of sensitivity is slightly greater than that of the CEIAs developed in Chapter 2, and should be able to detect the *E. coli* O157:H7 present.

In an attempt to optimize the recovery of inoculated samples and to increase the number of O157 cells present after enrichment, two other enrichment methods were developed. One method was to pre-incubate the sample in the presence of 10 mL of TSB for 6 h at 37 °C, followed by the addition of 215 mL of mEC+n and further incubation at 37 °C. This did increase the percent recovery for samples with an inoculum of <3 cfu/25 g sample to 81 % and the recovery of 10 - 100 cfu to 100 %. Inoculum in the range of 3-10 cfu were not tested. The intensity of the spots produced in the assays were more intense than with dmTSB or mEC+n enriched at 37 °C without pre-incubation. This would suggest that a greater number of *E. coli* O157:H7 cells are present after enrichment. The second method was to incubate the samples in mEC+n at 42 °C for 24 h, since our growth data for *E. coli* O157:H7 in mEC+n suggested that it would be a good temperature for its recovery

Table 3-1. Detection of *E. coli* O157 inoculated into 25 g ground beef samples using various enrichment methods

Enrichment method	Inoculum (cfu/25g sample)	No. Positive/total		% Recovery
		Polymacron EIA	O157-Polymyxin-CEIA	
dmTSB at 37, static	0	3/32	3/32	
	<3	2/2	2/2	100
	3-10	18/22	18/22	82
	10-100	28/29	28/29	97
	>100	2/2	2/2	100
mEC+n at 37, static	0	2/56	2/56	
	<3	17/27	17/27	63
	3-10	17/18	17/18	94
	10-100	51/51	51/51	100
	>100	21/21	21/21	100
6 h Pre-enrichment at 37, static; 18 h enrichment in mEC+n at 37, static	0	0/19	0/19	
	<3	17/21	17/21	81
	3-10	ND	ND	ND
	10-100	21/21	21/21	100
	>100	18/18	18/18	100
mEC+n at 42, static	0	0/22	0/22	
	<3	14/15	14/15	93
	3-10	9/9	9/9	100
	10-100	24/24	24/24	100
	>100	11/11	11/11	100

Total number of replicates tested = 420

from ground beef. This produced excellent results, 100 % of samples inoculated with 3-10 and 10-100 cfu/25 g sample were recovered as well as 93 % of <3 cfu inoculated. Also, the spots produced by this method in the O157 Polymyxin CEIA and the Polymacron™ EIA were as intense as those produced by pre-incubation. A summary of the above data is presented in Table 3-1.

Thermal injury of E. coli O157:H7. Since the studies completed for testing recovery of artificially inoculated *E. coli* O157:H7 in mEC+n have used only healthy cells, we decided to use thermally injured cells to more accurately represent the possible injured state the cells might be in at the time of sampling. To injure the cells, we heated at 55 °C for 10 min in a precision circulating water bath. Log phase cells were used since stationary phase cells exhibit a number of physiological characteristics which makes them highly resistant to stresses such as heat shock, H₂O₂ and NaCl. (Hengge-Aronis, 1993; Jackson *et al.*, 1995). The cells were heated as described earlier and were plated before and after heat treatment on TSA and TSA containing 3 % NaCl. The extra NaCl added to TSA was used as a selective agent to differentiate the uninjured cells from the injured cells. The sub-lethally injured cells should not grow in the presence of high salt, while the uninjured cells should grow. It was assumed that on TSA all viable cells were recovered and therefore was used to determine the number of injured and uninjured cells. Before heating, both TSA and TSA with 3 % NaCl produced similar cfu counts. While after heating only between 0.2 and 5 % of the cells were recovered on TSA with 3 % NaCl as compared with TSA. The cells that did grow on the TSA + 3 % NaCl were assumed to be healthy uninjured cells. The

difference in the counts after heat treatment on TSA and TSA with 3 % NaCl was determined to be the injury rate (Chen *et al.*, 1993). We observed approximately a 95 - 99.8 % injury rate after heat treatment at 55 °C for 10 min. These injured cells were then used as inoculum in other experiments.

Recovery of thermally injured E. coli O157:H7 in pure broth. To determine if mEC+n was able to recover thermally injured *E. coli* O157:H7, a low number of cfu (1-100) were inoculated into 5 mL of sterile mEC+n and TSB and grown for 24 h at 37 or 42 °C. The TSB tubes were highly turbid after 24 h, while the mEC+n tubes were clear. Plate counts of the mEC+n tubes on TSA showed no growth which confirms that the injured cells did not survive in the selective broth. Growth in the TSB tubes demonstrated that the injured cells could be recovered to healthiness in a non-selective broth. Therefore, a pre-incubation step in a non-selective broth was included before the addition of the selective broth. Heat injured cells were added to 400 µL of TSB and incubated for 0, 1, 2, 4, and 6 h at 37 and 42 °C. Table 3-2 shows that after 2 h of incubation in TSB before the addition of mEC+n, the cells were revitalized enough for growth in the highly selective mEC+n. To determine the minimum time to fully recover all the sub-lethally injured *E. coli* O157:H7 present in a sample, plate counts were made during the pre-incubation stage. One hundred microlitres of the TSB-cell suspension was plated on TSA and TSA containing 3 % NaCl at 0, 1, 2, 4, and 6 h after inoculation. No growth was seen on the TSA + 3 % NaCl plates until 4 h of growth at either 37 or 42 °C (data not shown). Yet, after 6 h of growth, the cells began to multiply, therefore a pre-incubation period of 4 - 6 hours is necessary to completely recover

Table 3-2. Recovery of heat injured *E. coli* O157:H7 inoculated into 400 μ L of sterile TSB and incubated for a number of hours followed by addition of 4.5 mL of mEC+n broth and incubation for a total of 24 h.

Incubation Temperature	Total Inoculum (cfu)	Incubation Time (No. Positive/Total) ^a				
		0 h	1 h	2 h	4 h	6 h
37 °C	27	0/2	2/2	2/2	2/2	2/2
	2.7	0/2	0/2	2/2	2/2	2/2
42 °C	27	0/2	2/2	2/2	2/2	2/2
	2.7	0/2	1/2	2/2	2/2	2/2

^a Sample was considered positive if turbidity was present after 24 h of total growth time.

the low number of thermally injured cells inoculated into the TSB. A longer pre-incubation would allow background flora in a food sample to multiply and possibly inhibit the growth of the *E. coli* O157:H7 present.

Recovery of thermally injured E. coli O157:H7 spiked into ground beef. We demonstrated that for recovery of thermally injured *E. coli* O157:H7 in pure broth, a revitalization time in TSB was necessary. Therefore, for the recovery of thermally injured O157:H7 inoculated into ground beef, a pre-incubation step in TSB was included. This pre-incubation step should recover the injured cells to a healthy state before the addition of the highly selective mEC+n. The inoculated 25 g ground beef samples were placed in stomacher bags containing 25 mL of TSB, hand mixed to allow the TSB access to the entire sample and were then incubated at 42 °C for 6 h. Forty two Celsius was chosen for both the pre-incubation and enrichment temperature since it allows for the recovery of injured O157:H7 cells, as demonstrated above, but also inhibits the growth of the ground beef microflora (Figures 3-2 and 3-4). A 6 h pre-incubation step may allow the background microflora to multiply as shown by the plating results above, but a 42 °C pre-incubation temperature should slow the background from increasing too much and possibly overwhelm the initially low numbers of *E. coli* O157:H7 present. After pre-incubation, 200 mL of mEC+n was added, thoroughly mixed and placed at 42 °C for a further 18 h. Identical samples were also prepared without a pre-incubation step and were also incubated at 42 °C. After enrichment the samples were assayed using the O157 Polymyxin CEIA and the Polymacron™ EIA (Table 3-3). When 5 cfu/25 g sample were added, both with and

Table 3-3. Recovery of heat injured *E. coli* O157:H7 inoculated into 25 g ground beef samples.

Sample Enrichment Procedure	Inoculum (cfu/25 g Sample)	O157 Polymyxin CE1A (No. Positive/Total)
Pre-enrichment ^a	0	0/2
	5	4/4
No Pre-enrichment ^b	0	0/2
	5	4/4

^a25 g sample mixed with 25 mL of sterile TSB and incubated at 42 °C for 6 h., after which 200 mL of mEC+n broth was added and the sample was incubated for a further 18 h.

^b25 g sample placed directly in 225 mL of mEC+n broth, stomached and incubated for 24 h at 42 °C.

without a pre-incubation step, positive results in the O157 Polymyxin CEIA and the Polymacron™ EIA were observed. When 0.5 cfu/25 g sample were added 4 of 4 inoculated samples with pre-incubation were positive while only 2 of 4 inoculated sample without pre-incubation were positive. This result does not show that pre-incubation is more efficient at recovery because the probability of actually inoculating 1 cfu/sample is very low, but it does show that both of the enrichment procedures are efficient at recovery of low number of thermally injured cells. Since thermally injured cells could not be recovered in pure selective broth, it is surmised that the added nutrients from the ground beef counteracted some of the affects of the selective agents in the mEC+n. An identical experiment with 25 mL of milk in 225 mL of mEC+n broth gave similar results, which shows that the added nutrition of the food sample and the lessening of the selective pressure allows for the recovery of even injured *E. coli* O157:H7.

This study has demonstrated the ability of mEC+n, and dmTSB to recover *E. coli* O157:H7 spiked into ground beef. The growth studies of *E. coli* O157:H7 and ground beef microflora demonstrate that mEC+n broth provided a better growth environment than dmTSB at 42 °C, as well as almost totally inhibited the ground beef microflora. In comparing the results of different methods to recover *E. coli* O157:H7 spiked into ground beef, enrichment in mEC+n at 42 °C gave the best recovery. This data however, does not take into account that the cells present may be injured. Therefore, thermally injured cells

were used and were also recovered using this method. Taking all the data into account we recommend the use of mEC+n broth incubated at 42 °C for the recovery of *E. coli* O157:H7 from ground beef samples.

Chapter 4

**Development of a rapid cloth-enzyme immunoassay system for
the detection Verotoxins produced by Enterohaemorrhagic
Escherichia coli.**

Abstract

Cloth based enzyme-immunoassays (CEIA) for the detection of verotoxins (VT) produced by enterohaemorrhagic *Escherichia coli* (EHEC) were developed. These assays used anti-verotoxin polyclonal antibodies (anti-VT) bound to polyester cloth to capture the VT which was subsequently immunoenzymatically detected by polyclonal antibodies conjugated to horseradish peroxidase. A dot blot assay (VT-CEIA) using large sheets of polyester cloth was developed for assay of multiple samples. The VT-CEIA detected purified VT reliably at a concentration of 5 ng/mL. This level of sensitivity allows for VT detection from pure EHEC cultures grown in TSB for 6 h. To increase the sensitivity of detection, an immunoconcentration assay (VT-ICA) was developed which increased the sensitivity ten fold over the VT-CEIA assay. The VT-ICA concentrates the VT present in a sample by passing a larger volume of the sample through an anti-VT coated polyester cloth, and immunoenzymatically detects the bound VT. Although not tested, the VT-ICA should permit direct detection of VT in clinical samples, such as stools. Some cross reaction was seen between other members of the family Enterobacteriaceae, but we suggest that with the introduction of commercially available monoclonal or affinity purified polyclonal antibodies these rapid test could be commercialized.

Introduction

Verotoxin producing *Escherichia coli*, also known as enterohaemorrhagic *E. coli* (EHEC) are important human pathogens, with *E. coli* O157:H7 being the most prevalent. EHEC organisms have been shown to cause haemorrhagic colitis (HC), and in rare cases

haemolytic uraemic syndrome (HUS) when consumed in contaminated foods, most notably undercooked ground beef. EHEC have also been found in other foods such as unpasteurized milk, chicken, lamb, pork, and vegetables (Doyle and Schoeni, 1987; Doyle, 1991). HC caused by EHEC persists for 4 - 9 days and consists of bloody diarrhea with severe abdominal cramps, fever is low grade when present (Doyle, 1991). HUS is characterized by a combination of acute renal failure, thrombocytopenia, and microangiopathic haemolytic anemia and is often preceded by diarrhoeal disease (Gyles, 1992). There is strong evidence which associates the presence of VT-producing *E. coli* with disease in patients as well as that HUS and haemorrhagic colitis are two different expressions of the same toxemia produced by VT (Gyles, 1992). There are two antigenically distinct VT. VT 1 is almost identical to Shiga toxin, except for one amino acid, and is neutralized by antiserum for Shiga toxin (O'Brien *et al.*, 1982; Calderwood *et al.*, 1986; Scotland *et al.*, 1988). VT 2 is a group of antigenically related toxins, which share 60 % homology with VT 1 and are not neutralized by antibodies to Shiga toxin or VT 1 (MacLeod, *et al.*, 1991; Taylor, 1995). The genes for VT 1 and VT 2 associated with human disease are present on temperate bacteriophages, unlike the gene for Shiga toxin which is chromosomally located (Scotland, *et al.*, 1983; O'Brien and Homes, 1987). VT 1 and 2 are A-B type toxins, where the B subunit attaches to cellular receptors and internalizes the toxin, while the A subunit inhibits protein synthesis in a manner resembling the plant toxin Ricin (Endo *et al.*, 1987).

To monitor for the absence of the most prevalent of these pathogens a number of methods for the detection of *E. coli* O157:H7 in foods have been developed. Although *E.*

coli O157:H7 is the serotype that is most commonly associated with human disease, there have been a number of other serotypes isolated from patients. Over 50 different serotypes of EHEC have been recovered from humans and cattle (Karmali, 1989). The presence of VT and some other virulence factors seems to be the cause of the symptoms and not simply the presence of the O157:H7 serotype. Therefore, the detection of only one of the many EHEC serotypes is not an effective way of determining the presence of EHEC in foods or infected patients. For this reason the detection of the VT in food and clinical samples will be a more accurate test for the presence of EHEC organisms.

We have previously described a rapid enzyme immunoassay utilizing antibodies bound to polyester cloth for the detection of *E. coli* O157 lipopolysaccharide (LPS) antigens (Chapter 2; Blais *et al.*, in press), as well as *Salmonella* LPS and *Brucella* antigens (Blais and Yamazaki, 1989; Blais and Yamazaki, 1991b). The assay used a monoclonal antibody bound to polyester cloth as an inexpensive, high surface area solid phase for the capture of O157 LPS. The bound LPS was then detected using a commercially available anti-O157-HRP antibody conjugate. A simple dot blot format employing large sheets of polyester cloth coated with the monoclonal antibody facilitated the assay of numerous samples simultaneously. This technique should prove useful for the detection of VT. Using a cloth-enzyme immunoassay for the detection of VT would greatly decrease the time involved in determining the toxicity and toxin type of an EHEC culture over the current methods such as the Vero cell assay. The Vero cell assay uses a eukaryotic cell line of Vero cells to determine if a culture is verotoxic, and the toxin type is determined by neutralization studies using anti-VT 1 and anti-VT 2 antibodies. The entire procedure can take over 48

h, and due to the need for a cell culture, a highly skilled worker is necessary which increases the cost considerably.

This chapter describes the development a rapid cloth-based enzyme immunoassays for the detection of VT. These assays utilize VT specific polyclonal antibodies bound to polyester cloth as the capture method, and an anti-VT-HRP conjugate for the detection of the bound VT. Since there are two main types of VT (VT 1 and VT 2) that are antigenically distinct, two separate tests were developed.

Materials and Methods

Chemicals and immunoreagents. The following were obtained from Sigma Chemical Co. (St. Louis, MO): sodium cholate (C-1254), lactose (No. 13625), Tween 20 (No. P-9416), horseradish peroxidase (P-6782), and acriflavin hydrochloride (No. A-8251). Casamino acids (No. 0230-01), and tryptone (No. 0123-17-3) were obtained from Difco Laboratories (Detroit, MI). Trypticase soy broth (TSB) was obtained from BDH Inc. (Darmstadt, Germany). Biorad supplied the blocking agent (No. 121692B). The rabbit anti-VT 1 and anti-VT 2 serum was kindly donated by Dr. Roger P. Johnson, Health of Animals Laboratory, Guelph, ON. The membrane peroxidase substrate system used in this study is a proprietary formulation based on the chromogen 3,3',5,5'-tetramethylbenzidine (TMB) with an unspecified membrane enhancer, and was a kind gift from Ricoh Kyosan Inc. (Tokyo, Japan).

Bacterial strains. A total of 18 *Escherichia coli* serovars from the Health of Animals Laboratory and Laboratory Services Division culture collections, Agriculture and Agri-Food Canada, were examined in this study. A number of Gram-positive and Gram-negative non-*E. coli* organisms from the Laboratory Services Division culture collection were also examined. Unless otherwise stated, *E. coli* strains were routinely grown in TSB by shaking at 37 °C for 16 - 20 h. Growth conditions for the non-*E. coli* bacteria were as previously described (Blais et al., 1993). Viable counts were estimated by plating serial dilutions of cell suspensions on trypticase soy agar (TSA) and incubating the plates at 37 °C for 16 - 20 h.

Preparation of peroxidase labelled anti-VT. The rabbit antisera was partially purified by ammonium sulphate precipitation. The protein concentration of the sample was adjusted to 2.5 - 3.0 % (w/v) protein by the addition of PBS. Once adjusted, saturated ammonium sulphate was slowly added with stirring to give a final saturation of 50%. The solution was stirred for a further hour at 4 °C, after which it was centrifuged at 4000 X g for 15 min at 4 °C and the supernatant was dialysed over night against PBS. A second precipitation using 33% ammonium sulphate saturation was completed on the dialysed supernatant as before. The resulting solution was dialysed against PBS at 4 °C overnight and stored in PBS at -20 °C until needed.

A modified version of the method developed by Nakane and Kawaoi (1974) was used for conjugation of horseradish peroxidase (HRP) with rabbit-anti-VT. Ten milligrams of HRP was dissolved in a mixture of 2 mL of 0.3 M Na₂CO₃ pH 8.1 and 2 mL of 0.08 M

NaIO₄, and was gently mixed for 30 min at room temperature. Two millilitres of 0.16 M ethylene glycol was added and the solution was stirred for a further hour at room temperature. The product was then dialysed against 4 L of 0.01 M Na₂CO₃ pH 9.5 overnight at 4 °C, with at least one change of the dialysis solution. One millilitre of anti-VT 1 and anti-VT 2 ammonium sulphate precipitated anti-VT serum was also dialysed against 4 L of 0.01 M Na₂CO₃ pH 9.5 at 4 °C. The antibody solution and the periodate-treated peroxidase solution were mixed in a 1:1 (mg:mg) ratio and allowed to react overnight at room temperature with gentle shaking. The next day, 5 mg of NaBH₄ was added slowly at 4 °C, and left to react for 3 h. The resulting product was extensively dialysed against PBS at 4 °C, and stored at -20 °C until needed.

Verotoxin extraction. A number of agents at different temperatures were tested for their ability to release both VT 1 and VT 2 from an EHEC (*E. coli* O157:H7 ATCC 35150, VT 1 and 2) overnight culture. One millilitre of cultures grown in TSB broth at 37 °C for 18 - 20 h was mixed with 0.1 volume of the surfactant (bile salts #3, EDTA, sodium cholate, SDS, Triton X-100 and lysozyme) and heated in a heat block at various temperatures for 10 min. To determine the most effective method of VT extraction, the treated samples were tested by following the VT dot blot assay technique (as described below). Results were scored qualitatively according to the relative size and intensity of the spots produced.

Dot-blot verotoxin cloth-enzyme immunoassay (VT-CEIA). DuPont Sontara 8100 polyester cloth sheets (5.5 cm X 8.0 cm) were soaked in 20 % ethanol for 10 min and

washed under suction with distilled water. The cloth sheets were coated with capture antibody by overnight incubation in 4 mL of either ammonium sulphate precipitated anti-VT 1 or anti-VT 2 rabbit antisera diluted 1:100 in PBS at 37 °C. The sheets were then washed 4 times with PBS containing 0.05% (v/v) Tween 20 (PBST) on a filter under suction. To block the cloths, they were incubated at room temperature in a solution of 1 % (w/v) Biorad blocking agent in PBS for 1 h, followed by washing as before. The coated and blocked cloths were stored at 4 °C until ready for use.

To perform the VT-CEIA, 5 µL of the 1% bile salts treated EHEC overnight culture were pipetted onto the coated cloth and allowed to incubate for 10 min at room temperature. The cloth was then washed 4 times with 10 mL of PBST using a vacuum filtration apparatus. Anti-VT-HRP conjugate diluted 1:100 in PBST-B 0.5% (PBST + 0.5 % blocking agent) was pipetted onto the cloth so that the entire cloth was saturated with liquid. After a 10 min incubation period, the cloth was washed as before. TMB membrane peroxidase substrate was used to saturate the cloth for 10 min and visualize the results.

Verotoxin immunoconcentration assay (VT-ICA). Polyester cloth was cut into 6 mm squares and coated with capture antibody as before. One square of anti-VT cloth was placed in the tip of a 1 mL syringe so that liquid must flow through the cloth plug (Figure 4-1). One millilitre of bile salts treated sample was loaded into the syringe and allowed to flow through the cloth plug over a 30 - 45 min period. The volume of sample which passed through the immunoconcentration apparatus was controlled by placing a 1-10 µL pipette tip, with a piece of polyester cloth placed in the barrel, on the end of the 1 mL syringe.

Figure 4-1. VT-ICA device as described in Methods.

Non-specifically bound sample was removed by repeatedly drawing PBST into the syringe and expelling it through the ICA cloth. This was done 4 times per wash. Approximately 100 μ L of anti-VT-HRP diluted 1:100 X in PBST-B 1% (PBST + 1% Blocking agent) was then drawn into the syringe and allowed to interact with the bound VT for 20 min. After the incubation period, the plug was washed as before. Results were visualized by one of two ways. One, qualitatively by incubating the cloth plug in TMB by drawing it into the syringe and allowing it to react for 10 min followed by visual inspection, or two, quantitatively by removing the plug to a test tube containing 0.5 mL of TMB soluble substrate and the A_{450} of the solution read after 10 min.

Minimum growth period required for Verotoxin production. To determine the amount of time a culture must be grown before sufficient VT is produced to be detected, an EHEC culture was grown in broth and sampled over time. *E. coli* O157:H7 ATCC 35150 from a plate stored at 4 °C was inoculated into a number of 1.7 mL microfuge tubes containing 0.5 mL of TSB or sterile dmTSB (Padhye and Doyle, 1991) and were incubated at 37 °C for 8 h. Tubes were removed after 0, 2, 4, 6, and 8 h and tested for VT. In other experiments, 20 μ L of actively growing *E. coli* O157:H7 cells or a loop of cells from a fresh plate were added to 5 mL of TSB, dmTSB and mEC+n and grown for 8 h at 37 and 42 °C. Samples were removed after 0, 2, 4, 6, and 8 h and tested for VT. In both experiments the presence of the VT were detected qualitatively using the dot blot VT-CEIA (as above).

Ground beef samples. Ground beef was purchased from a local retailer and used fresh on the day of purchase. Twenty five gram samples of ground beef were inoculated with 100 μ L of PBS containing 10 - 100 cfu of *E. coli* O157:H7 ATCC 35150. The samples were then placed in 225 mL of sterile mEC+n and stomached for 120 s. They were then incubated at 42 °C for 24 h without shaking. After enrichment, 1 mL of the samples was removed through a sterile Filtatip (Filtaflex Ltd, Almonte, Ontario) and bile salt-heat treated as described above. The treated samples were then applied to the VT-CEIA.

PCR for Verotoxin detection. A modified version of the method developed by Read *et al.* (1992) was used to detect both VT 1 and VT 2 genes in a pure culture or a ground beef enrichment sample using one set of generic primers. From broth cultures, 1 mL of an overnight (18 - 20 h) culture was centrifuged at 13,000 $\times$ g for 2 min and the supernatant decanted. The pellet was suspended in PBS and recentrifuged. The supernatant was again decanted and the pellet was suspended in 0.5 mL of distilled water, heated at 100 °C for 10 min and centrifuged as before. When ground beef samples were tested, a pre-filtering step of the enrichment culture was included to remove the large particulates, the above method was then followed. The PCR reaction was performed using Boehringer Mannheim PCR reagents. Five microlitres of the samples supernatant was added to a reaction mixture containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 0.01 % gelatin; 200 μ M each of dATP, dCTP, dGTP and dTTP; 1 μ M of each primer [(5' - 3') ES 151 gag cga aat aat tta tat gt; ES 149 cga aat ccc ctc tgt att tgc c]; 0.31 Units AmpliTaq DNA polymerase; and 1.5 mM $MgCl_2$ for a final volume of 25 μ L. The reaction mixture was overlaid with mineral oil and

processed through 35 cycles in a Perkin Elmer 480 Thermal Cycler. The samples were loaded at 72 °C with the cycle consisting of 94 °C for 1 min, annealing of primers at 49 °C for 1 min and primer extension by DNA polymerase at 72 °C for 30 s. To ensure complete strand extension, the reaction mixture was incubated at 72 °C for 5 min for the last cycle. The amplified 323 bp fragment was visualized by standard submarine gel electrophoresis of 10 µL of the final reaction mixture on a 2 % agarose gel stained with ethidium bromide.

Results and Discussion

Verotoxin extraction. VT 1 and VT 2 are located in different parts of the cell. VT 1 is a highly cell associated toxin. Analysis of the translated amino acid sequences indicates that the A and B subunit contain hydrophobic signal sequences at the amino terminus which are synthesized as precursor forms of periplasmic proteins (O'Brien and Homes, 1987). VT 2 is secreted and hence, could be found in a much higher titre in the growth medium (Strockbine *et al.*, 1986). For these reasons a single method which extracts the maximum amount of both VT 1 and VT 2 from a sample was needed.

VT extraction using a final concentration of 1% bile salts #3 at 55 °C for 10 min was determined to be the method that released the greatest amount of toxin from a given culture. A temperature of 55 °C was chosen because it gave consistent results for the extraction of both VT 1 and VT 2. Slightly higher temperatures of 60 to 65 °C extracted the VT 1 present in the sample well but the VT 2 immunoreactivity was greatly reduced. Temperatures above 65 °C seemed to lessen the immunoreactivity of all the VT present in the sample. This agrees with the fact that the activity of the toxins is destroyed after

heating at 80 °C for 10 min (Yutsudo *et al.*, 1987). Due to limited quantities of the detector antibodies, the results were based upon qualitative observations of the size and darkness of the spots produced (Table 4-1)

Specificity of the Verotoxin assays. Broth cultures of several Gram positive and Gram negative bacteria were tested using both VT 1 and VT 2 detector antibodies in the dot blot VT-CEIA. All the verotoxic *E. coli* strains tested reacted as expected in the assays (Table 4-2). None of the Gram positive strains produced positive results but some of the Gram negative strains did. *Enterobacter aerogenes* and *cloacae*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* produced positive reactions in one or both VT 1 or VT 2 tests (Table 4-3). The two *Enterobacter* strains and *Enterococcus faecalis* produced very faint positive reactions in the VT 2 CEIA only. The reason for this cross reaction with some species of the Enterobacteriaceae family and *Pseudomonas aeruginosa* is unknown but is likely due to the serum was not being purified other than removing some extraneous protein, and hence the antibodies normally present in serum against other bacteria were co-purified and detected those bacteria when used. Attempts were made to remove the cross reaction by centrifuging the samples for 10 min at 10 000 x g. This lessened the cross reaction but did not remove it entirely.

Dot-blot verotoxin cloth-enzyme immunoassay (VT-CEIA). The polyester cloth solid phase used in the VT-CEIA is a macroporous, hydrophobic cloth fabric. The macroporosity of the cloth presents a large surface area for rapid and efficient reactions.

Table 4-1. Qualitative results of dot blot VT-CEIA assay to determine the most effective VT extraction procedure. All samples were mixed with 1/10 volume of the surfactant and then heated for 10 min. Results are based on qualitative observation of the intensity and the size of the spots produced (ND, not done)

Extraction Method	Final Concentration	Extraction Temperature (°C)					
		37	50	55	65	80	100
Bile Salts #3	0.1%	+	+	ND	ND	ND	++
	0.5%	+++	+++	ND	ND	ND	+
	1.0%	+++	+++	++++	+++	++	+
EDTA	10 mM	+/-	+/-	ND	ND	ND	+
Cholate	0.1%	+	+	ND	ND	ND	+
	0.5%	+	+	ND	ND	ND	++
	1.0%	++	++	ND	ND	ND	+
Triton X-100	1.0%	+	++	ND	ND	ND	+
	5.0%	+	++	ND	ND	ND	+
SDS	0.1%	+	+	ND	ND	ND	-
	0.5%	+	+	ND	ND	ND	-
	1.0%	+	+	ND	ND	ND	-
Lysozyme	0.5 mg/mL	++	ND	ND	ND	ND	ND
Polymyxin	2 mg/mL	++	ND	ND	ND	ND	ND
No treatment		+/-	ND	ND	ND	ND	ND

Table 4-2. Reactivity of *E. coli* strains in the VT-CEIA and the VT-ICA using both VT 1 and VT 2 detector antibodies ^a

Serotype/strain	Verotoxin Type	Reactivity	
		VT 1	VT 2
0157:H7 (ATCC 35150)	1 & 2	+	+
0157:H7 PT34	2	-	+
0157:H7 PT32	1 & 2	+	+
0157:H7 PT14	2	-	+
0157:H7 PT67	1 & 2	+	+
0157:H7 PT23	1	+	-
0157:H7 PT23	1 & 2	+	+
0157:H7 PT14	2	-	+
0157:NM	2	-	+
0157:H12	VT Neg.	-	-
0157:H19	VT Neg.	-	-
(ATCC 11775)	VT Neg.	-	-
0111:H11	1	+	-
0111:H8	1	+	-
0111:NM	1 & 2	+	+
0111:NM	1	+	-
026:H11	1	+	-
05:NM	1	+	-

^a Various *E. coli* strains suspended in TSB at ca. 10^8 cfu/mL were subjected to the two VT-CEIA assays as described in Materials and Methods. +, positive reaction; -, negative.

The ease with which the cloth passes liquids increases the efficiency at removing unreacted materials as well as facilitates washing procedures. The use of sheets of cloth accommodates the assay of multiple samples in a dot blot format, and provides simple visualization of test results as coloured spots on a white background (Figure 4-2).

Anti-VT 1 or anti-VT 2 antiserum was immobilized on the cloth to detect VT in pure culture or as purified VT 1 and VT 2. When the VT-CEIA was tested using pure overnight (ca. 10^9 cfu/mL) EHEC cultures, VT 1 and VT 2 were easily detected. Therefore, we decided to determine the absolute sensitivity of the assay. Purified VT 1 and VT 2 were diluted in PBS for assay. The VT-CEIA was capable of detecting 5 ng/mL of purified toxin. This is within the same range as other VT tests, the latex agglutination test available for VT detection which routinely detects in the range of 1.5 to 5.0 ng/mL. This is sufficient for detecting VT in a pure culture but is not for detection in a clinical sample. However, it may be possible to detect VT in a ground beef enrichment culture.

Verotoxin immuno-concentration assay (VT-ICA). For a VT assay to be useful in the detection of EHEC cultures in clinical samples, it should be able to detect the VT in very small quantities. A typical stool sample obtained from a patient with EHEC infection during the first week of symptoms may have approximately 0.1 to 1.0 ng/mL of VT (Dr. Roger P. Johnson, personal communication). Therefore, the dot blot VT-CEIA would not be useful for such an application. A way of increasing the sensitivity of the assay had to be found. The solution was not to increase the sensitivity of the assay but was to increase

Table 4-3. Reactivity of non-*E. coli* strains in the VT-CEIA and the VT-ICA using VT 1 and VT 2 detector antibodies. ^a

Strain	Reactivity	
	VT 1	VT 2
<i>Bacillus cereus</i>	-	-
<i>Bacillus subtilis</i>	-	-
<i>Citrobacter freundii</i>	-	-
<i>Enterobacter aerogenes</i>	-	+ ^b
<i>Enterobacter cloacae</i>	-	+ ^b
<i>Enterococcus faecalis</i>	-	+ ^b
<i>Escherichia hermanii</i>	-	-
<i>Klebsiella pneumoniae</i>	+	+
<i>Listeria innocua</i>	-	-
<i>Listeria ivanovii</i>	-	-
<i>Listeria welshimeri</i>	-	-
<i>Proteus vulgaris</i>	-	-
<i>Pseudomonas aeruginosa</i>	+	+
<i>Salmonella typhimurium</i> LT2	-	-
<i>Salmonella urbana</i>	-	-
<i>Serratia marcescens</i>	-	-
<i>Shigella sonnei</i>	-	-
<i>Xanthamonas maltophila</i>	-	-
<i>Yersinia enterocolitica</i> (serotype O:9)	-	-

^a Various bacterial strains suspended at ca. 10^8 cfu/mL were subjected to the two assays using either source of detector antibodies as described in Materials and Methods.

^b These cultures produced very faint cross reactivity in the VT 2 CEIA.

Figure 4-2. Example of VT-CEIA immunoassay. Limit of detection of VT 1 and VT 2 using purified VT 1 and VT 2 at a number of concentrations. Cloth **A**, VT 1; Cloth **B**, VT 2. From the left, spot a, 1000 ng/mL; b, 100 ng/mL; c, 20 ng/mL; d, 10 ng/mL; e, 5 ng/mL; and f, 2.5 ng/mL.

the amount of analite bound to the cloth. Because the solid phase is a macroporous cloth, a large volume of sample could be passed through the cloth without fear of clogging. A previous study (Boyd and Yamazaki, 1995) showed that it was possible to increase the signal received in an immunoassay by passing a larger sample volume through an antibody coated cloth. We therefore decided to create an immunoconcentration assay for VT.

An anti-VT 1 or anti-VT 2 coated 6 mm square of cloth was placed in the tip of a 1 mL syringe so that when the sample is loaded it must flow through the cloth. One millilitre of an overnight culture was tested in the VT-ICA by allowing it to flow through the cloth over 30 - 45 min and it produced promising results. Using the same technique, purified VT was used to determine the maximum sensitivity. Using a 1 mL sample of VT diluted in PBS, the VT-ICA detected 0.5 ng/mL of VT 1 and 1.0 ng/mL of VT 2. Although not tested, this level of sensitivity should be able to detect VT in clinical samples provided that the disease is in its early stages.

Minimum growth period required for Verotoxin production. All rapid methods require some method of conformation after a suspected positive sample is found. The presently accepted method for conformation involves plating the sample on a selective agar with biochemical and serological identification to follow. This selective agar produces an isolated colony which can be used in the VT-CEIA. To shorten the time required for detection after isolation on a plate, the minimum growth period for VT production was determined. Two initial growth states were examined. The first was inoculation using an *E. coli* O157:H7 (ATCC 35150) culture stored on a TSA plate at 4 °C. A colony from the

plate was placed in 0.5 mL of TSB, dmTSB, and grown at 37 °C for 8 h. The production of VT 1 and VT 2 was checked after 0, 2, 4, 6 and 8 h. VT 1 was detected from the beginning of the experiment, which demonstrates that VT 1 is present in a non-actively growing cell. VT 2 on the other hand was not detected until 6 h of growth at 37 °C and only in the TSB. It was not detected in the dmTSB even after 8 h of growth, which is likely due to the selective agents slowing the growth. The second method used to measure the production of VT used an actively growing culture. Actively growing cells, either from a plate or a broth culture were added to 5 mL of TSB, dmTSB and mEC+n and were grown at 37 and 42 °C for 8 h. Samples were withdrawn after 0, 2, 4, 6 and 8 h, and tested using the dot blot VT-CEIA. Since the initial inoculum was diluted much more than with the previous experiment, the VT 1 was not detected at time 0. Yet after 2 h very light positive results were seen for both VT 1 and VT 2, with the maximum intensity after 4 - 6 h of growth in all the media. This shows that after 6 h of growth, from either a non-vegetative culture or an actively growing culture, VT can be detected.

Ground beef samples. Most rapid methods detect a specific bacterium present in a sample, which may or may not be pathogenic. A more efficient method of surveillance would be to detect the virulence factors associated with a group of pathogenic bacteria, such as VT for EHEC. We therefore decided to assay for the VT produced by EHEC directly in a food enrichment culture. To determine if it was possible to detect VT present in ground beef, samples were spiked with *E. coli* O157:H7 (ATCC 35150). Twenty five gram ground beef samples were enriched in mEC+n for 24 h and assayed using the O157 Polymyxin CEIA

(Chapter 2; Booth *et al.*, 1996), the dot blot VT-CEIA for VT 1 and VT 2 as well as a PCR system which detects both VT 1 and VT 2 genes through common sequences. According to the O157 Polymyxin CEIA all samples inoculated with *E. coli* O157:H7 were positive for O157 after enrichment and all uninoculated samples were negative. The VT-CEIA however did not produce such good results (Table 4-4). All the samples spiked with O157:H7 produced positive results, but the samples with an initially low inoculum (< 20 cfu) produced very light and hard to interpret results. The uninoculated samples tested with the VT 1 VT-CEIA produced negative results, while the VT 2 assay produced very light results similar to the low inoculum samples. The light positive results for the VT 2 TV-CEIA could have been due to a naturally contaminated sample with a non-O157 EHEC strain. Therefore, the PCR was used to detect the presence of VT genes. The PCR gave positive results for all the inoculated samples and negative results for the uninoculated samples (Figure 4-3). The reason for the uninoculated samples producing positive results in the VT 2 assay is most likely due to a cross reaction with some other bacterium present in the ground beef sample. Table 4-3 shows that the VT 2 VT-CEIA does mildly cross react with a number of Enterobacteriaceae which are normally present in ground beef and would survive in the mEC+n broth.

This chapter has demonstrated the ability of the enzyme immunoassays to detect VT produced by EHEC. The VT-CEIA was able to detect VT 1 and VT 2 in pure culture, but due to cross reaction with other Enterobacteriaceae were not able to specifically detect VT 2 in a ground beef enrichment sample. However, the assay is still very useful using a

Table 4-4. Reactivity of the VT-CEIA and the PCR in testing 25 g ground beef samples spiked with various numbers of *E. coli* O157:H7 ATCC 35150 cells.

Inoculum (cfu) per 25 g Sample	Reactivity in the VT-CEIA ^a		323 bp PCR product produced ^a
	VT 1	VT 2	
0	0/6	6/6	0/6
3-100	6/6	6/6	6/6
>100	6/6	6/6	6/6

^a Number of positive samples per total number of samples tested.

Figure 4-3. Detection of VT 1 and VT 2 using the PCR from 25 g ground beef samples spiked with *E. coli* O157:H7 ATCC 35150 and grown in mEC+n for 24 h at 42 °C. Ten microlitres of the amplified reaction mixture was run on a 2 % agarose gel. From the left: lane L, 123 bp ladder; lane 1, *E. coli* O157:H7 ATCC 35150 (VT 1 and VT 2 positive); lanes 2, 3, and 4, 0 cfu inoculum; lanes 5, 6, and 7, 18 cfu inoculum; lanes 8, 9, and 10, 180 cfu inoculum.

purified culture. Currently, when a suspect positive food sample is identified it must be plated on selective agar to isolate the offending bacterium. When an isolated colony is obtained, the VT-CEIA can be used to rapidly detect whether it is verotoxic and determine the toxin type. With a detection level of 0.5 to 1.0 ng/mL the VT-ICA system developed should be useful in the assay of clinical samples although no tests were done except on pure cultures and purified VT. Nevertheless these assays should be useful in the detection of VT. The antibodies used in this study were crude rabbit antisera, as could be seen by the cross reactions observed. When monoclonal antibodies or an affinity purified polyclonal anti-VT antibodies which have less cross reactivity are available these assays should be in a position to be commercialized

Chapter 5

General Conclusions

Since the first outbreak of *E. coli* O157:H7 food poisoning in 1982, the number of cases has risen drastically in North America and the United Kingdom. Due to the rise in the number of reported cases and the severity of the diseases caused, the United States has declared *E. coli* O157:H7 a food adulterant, and any food found to contain *E. coli* O157:H7 must be destroyed or reprocessed. Therefore, methods to detect this pathogen in food must be developed. The Hazard Analysis and Critical Control Point system (HACCP) which is used by a number of retail food producers throughout the world require, rapid, sensitive and economical methods to detect *E. coli* O157:H7 and other pathogens. The rapid assays developed in this thesis should be applicable for implementation in a HACCP system.

The use of enzyme immunoassays for the rapid detection of microbial pathogens has become a popular method due to their relative simplicity, sensitivity and inexpensiveness. Many of these EIAs use microtitre trays as the solid phase which require numerous wash steps and have a limited surface area for immunoreaction. Polyester cloth has previously been shown to be an excellent solid phase for EIAs using either antibodies or polymyxin B as an antigen capturing agent (Blais and Yamazaki, 1989c; Blais *et al.*, 1989; Blais *et al.*, 1993; Blais *et al.*, 1996; Booth *et al.*, 1996). As demonstrated in these and other studies, the macroporous polyester cloth permits the simultaneous processing and washing of all samples with a single washing procedure, as well as presents a large surface area for immunoreactions which accelerates the immunoreaction time over that of traditional EIAs. Polymyxin B coated cloth as an LPS capturing agent provides a stable, and inexpensive ligand for LPS which can be obtained in a pure form. The dot-blot format of the cloth-enzyme immunoassay (CEIA) utilizing large sheets of cloth provides a simple

means of assaying multiple samples simultaneously. The rapid, simple and economical nature of the CEIA will permit the application in HACCP testing for the detection of *E. coli* O157:H7 and other bacterial pathogens in foods.

In Chapter 1, a polymyxin B CEIA and a CEIA utilizing Polymacron™ cloth coated with a monoclonal antibody specific for the *E. coli* O157 somatic antigen were developed and compared. A commercially available antibody-peroxidase conjugate specific for the O157 somatic antigen was used for detection of bound LPS. Cross reactivity was only seen with the *Salmonella* N group bacteria, which was expected because both *E. coli* O157 and *Salmonella* N group LPS are structurally identical in their O antigen (M. Perry, personal communication). Both assays were able to detect low numbers of *E. coli* O157:H7 (3 - 10 cfu) spiked into 25 g ground beef samples after a 24 h enrichment period. The tests were also able to detect *E. coli* O157:H7 spiked into other foods, such as ground chicken, ground pork, ground lamb, milk, and cheese. During the comparison of the two assays, the sensitivity and specificity were comparable, but the Polymyxin CEIA is preferred because of a number of reasons. Polymyxin B as an LPS capturing agent is more stable during storage, can be obtained in a pure form, and is less expensive than antibodies. Also the ability of polymyxin B to capture LPS irrespective of its serology makes it suitable for CEIA of all Gram negative bacteria.

There are a number of selective enrichment media used for the recovery of *E. coli* O157:H7 from foods, including double modified tryptic soy broth (dmTSB) and modified *E. coli* broth with novobiocin (mEC+n). Since the CEIAs developed for detection of *E. coli* O157 require 24 h enrichment before detection, different media were investigated for their

ability to recover *E. coli* O157:H7 spiked into ground beef. The recovery of healthy and thermally injured *E. coli* O157:H7, as well as normal ground beef microflora was evaluated using non-selective TSB, and selective dmTSB and mEC+n. Pure cultures of 3 food isolates of *E. coli* O157:H7 and strain ATCC 35150 grown at 37 and 42 °C in the three media showed that all three media produced approximately equal growth at 37 °C after 24 h. While at 42 °C, mEC+n produced substantially more growth than TSB and dmTSB. When the ground beef microflora were tested at 37 and 42 °C, TSB produced the best growth and mEC+n produced the least. Since a selective media should selectively increase the numbers of the target bacterium, the use of mEC+n incubated at 42 °C was determined to be the best growth condition. In order to simulate the state of *E. coli* O157:H7 in a naturally contaminated ground beef sample, thermally injured *E. coli* O157:H7 cells were used. These injured cells were inoculated into ground beef and enriched using mEC+n for 24 h at 42 °C. mEC+n was able to recover even low numbers (1 - 5 cfu) of thermally injured *E. coli* O157:H7 inoculated into 25 g ground beef samples which were then detected using the O157 Polymyxin CEIA.

Since there are a number of EHEC organisms other than serotype O157:H7 that are known to cause human disease, an effective method for the detection of all EHEC is needed. Because all EHEC produce verotoxins, the detection of VT in a culture or a food sample would be an effective method to detect EHEC. Therefore, two rapid and simple EIAs were developed for detection of VT. A VT-CEIA using a dot-blot format similar to the O157 CEIAs was developed. This test was rapid, and was able to detect VT in a pure culture at a level of 5.0 ng/mL. Some cross reactions were seen between other members

of the Enterobacteriaceae family. An immuno-concentration assay for the detection of VT was developed which increased the sensitivity to between 0.5 and 1 ng/mL, but the cross reaction between other bacteria was still a problem. The concept behind the VT-CEIA is well established and the VT-ICA appears to work as expected in increasing the sensitivity over that of the dot-blot CEIA. With more specific antisera for verotoxin, these tests should be able to effectively detect VT in food samples as well as clinical stool samples.

The combined enrichment and detection procedure for *E. coli* O157 is rapid, simple and inexpensive and should prove to very useful is HACCP testing. A modified version of the PolymacronTM EIA is marketed by a local biotechnology company, showing the usefulness of this type of test in the food industry. The verotoxin tests developed are not ready for commercialization at this time, but with higher specificity antibodies to VT 1 and VT 2 these test should be marketable.

Appendix I

For Gram negative identification

There are 30 wells in the Card which contains 29 biochemical broths and 1 growth control broth. The GNI Card has uniquely reproduced aerobic and microaerophilic conditions 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

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