MONITORING PATHOGENIC ESCHERICHIA COLI IN ALBERTA BEEF CATTLE

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are human pathogens responsible for deadly outbreaks throughout the world. Cattle are the primary reservoir for these pathogens, secreting STEC in the feces. Bovine feces is the principal source of contamination of food products, however detection of STEC in feces has several inherent limitations. The objectives of this study were to develop and refine detection methods for STEC in cattle feces. Multiplex PCRs for detection of STEC serogroups and virulence genes were designed. Serogroup detection in feces significantly improved with enrichment (10 CFU/g feces). However, isolation of target serogroups from feces remains challenging, and additional refinements to immunomagnetic separation (IMS) techniques would be an asset. Novel techniques for detection and isolation of STEC across the farm-to-fork spectrum will continue to improve food safety monitoring and reduce risks to human health by decreasing circulation of contaminated products, aiding in diagnostics, and supporting outbreak management.

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Chapter III

Conrad CC, Stanford K, McAllister TA, Thomas J, Reuter T: **Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces**. *Journal of Microbiological Methods* 2014, **105**(0):22-30.

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A/E	attaching and effacing
ARP	actin related protein
ATP	adenosine triphosphate
bp	base pair
BPW	buffered peptone water
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
CT-SMAC	sorbitol MacConkey agar with cefixime and tellurite
DAEC	Diffuse-adhering *Escherichia coli*
DNA	deoxyribonucleic acid
eae	intimin encoding gene
EAEC	Enteroaggregative *Escherichia coli*
EAHEC	Enteroaggregative hemorrhagic *Escherichia coli*
EC	*Escherichia coli*
ECP	*Escherichia coli* common pilus
Efa	EHEC factor for adherence
Eha	EHEC autotransporter
EHEC	Enterohemorrhagic *Escherichia coli*
EhxA	EHEC hemolysin
EibG	*Escherichia coli* immunoglobin-binding protein
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Esp	*Escherichia coli* secreted protein
ETEC	Enterotoxigenic *Escherichia coli*
FDA	Food and Drug Administration
FSIS	Food Safety Inspection Service
Gb3	globotriaosylceramide
GHP	Good Hygiene Practices
GUD	β-glucuronidase
h	hour
HACCP	Hazard Analysis and Critical Control Point
HC	hemorrhagic colitis
HCP	hemorrhagic coli pilus
HGT	horizontal gene transfer
HUS	hemolytic uremic syndrome
IMS	immunomagnetic separation
IPTG	isopropyl-β-D-1-thiogalactopyranoside
IRTKS	insulin receptor tyronsine kinase substrate
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
LOD	limit of detection
Lpf	long polar fimbriae
MAC	MacConkey agar
mPCR	multiplex polymerase chain reaction
NLe	non-LEE encoded
N-WASP	neural Wiskott-Aldrich syndrome protein

OD	Optical Density
OD_{600}	Optical Density at 600nm
OI	O-island
PAI	pathogenicity island
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
qPCR	quantitative (real-time) polymerase chain reaction
RPM	revolution per minute
Saa	STEC autoagglutinating adhesin
SB	sodium borate
SMAC	sorbitol MacConkey agar
SOR	sorbitol
Stx	Shiga toxin
STEC	Shiga toxin-producing *Escherichia coli*
TE	Tris- EDTA/ Tris-Ethylenediaminetetraacetic acid
Tir	transmembrane intimin receptor
TccP	Tir-cytoskeleton coupling protein
TSA	trypticase soy agar
TSB	trypticase soy broth
T3SS	type III secretion system
U.S.	United States of America
USDA	United States Department of Agriculture
VBNC	viable but non-culturable
VTEC	verocytotoxin-producing *Escherichia coli*
Vtx	verotoxin/verocytotoxin
WGS	whole genome sequencing
w/v	weight/volume
wzx	O-antigen flippase gene
wzy	O-antigen polymerase gene
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

1.1 Introduction to *Escherichia coli*

Escherichia coli (*E. coli*) were first described in 1885 by German physician Theodor Escherich in healthy human feces and originally named "*Bacterium coli*" [1-3]. During the pre-molecular era, *E. coli* were distinguished from similar microbes based on their motility and metabolic profile such as the ability to ferment lactose. Belonging to the *Enterobacteriaceae* family, *E. coli* is a facultative anaerobic, rod-shaped, Gram-negative bacterium [4]. While most *E. coli* strains are beneficial to their hosts, others are pathogenic. *Escherichia coli* are residents of the gastrointestinal tract of warm-blooded organisms and are shed in the feces [5]. Commensal *E. coli* rarely cause disease except in immunocompromised hosts. However, several highly adapted *E. coli* clones have acquired virulence attributes allowing them to cause a broad spectrum of disease [6].

Since their discovery, *E. coli* has become one of the most comprehensively studied laboratory organisms. Descendants of the original strains isolated in the early 1900's remain as staples in microbiology, genetics, and molecular biology laboratories today [2]. Many traits of *E. coli* make it an ideal laboratory organism including the ease of growth and manipulation, amenability to genetic manipulation, and natural ability to acquire mobile genetic elements [7].

E. coli are characterized into somatic (O), flagellar (H), and capsular (K) antigen types. Serogroup (O-type) and serotype (O- and H-type) are defined by these antigens [8]. A total of 181 O antigens have been recognized, however some strains are "not typeable" (do not agglutinate), or do not possess a complete O antigen. Besides antisera-based tests, O-antigen genotypes can be determined by molecular serotyping assays [2]. Flagellar antigens are numbered 1 to 56 and also determined by traditional antisera-based methods. Non-motile strains can also be assigned an H-type by molecular methods [2].

There is a core genome shared between all *E. coli* strains of about 2,200 genes [3]. However, genomes of pathogenic strains of *E. coli* can be up to 1 Mb (~5000 genes) larger than non-pathogenic strains due to the acquisition of pathogenicity islands and mobile genetic elements (Figure 1.1) [8, 9]. As a result there is an abundance of genetic diversity and virulence profiles among strains. Disappearance of genes and acquisition of new genes has led to the emergence of novel pathogenic groups of *E. coli* [8]. Horizontal gene transfer (HGT) is one mechanism *E. coli* uses to accomplish exchange of genetic information within and beyond species barriers (e.g., *Shigella*) [9-11]. Mobile genetic elements that are transferred through HGT include plasmids, transposons, and integrons which are not only responsible for harboring virulence genes (Figure 1.1) [12], but also antibiotic resistance genes [13]. Additionally, HGT allows for genetic exchange between *E. coli* and bacteriophages. Some bacteriophages carry toxin genes (e.g., Shiga toxins) and have been proposed to be responsible for transferring genes among bacterial species such as the transfer of genes coding for Shiga toxins between *Shigella* spp. and *E. coli* (Figure 1.1) [14, 15].

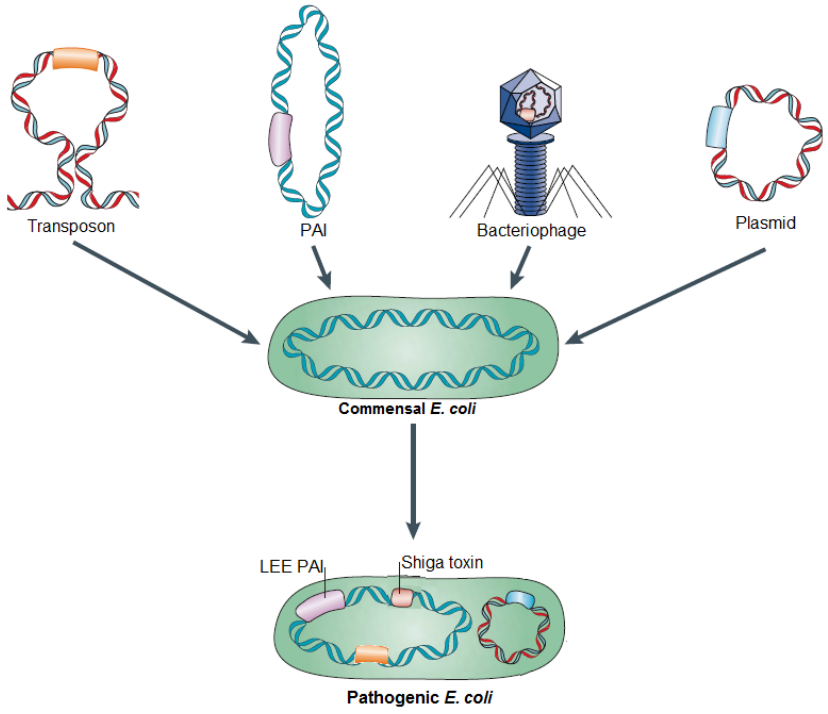

Figure 1.1. *E. coli* virulence factors can be encoded by transposons, pathogenicity islands (PAIs e.g. LEE), bacteriophage (e.g. Shiga toxins), and plasmids [Adapted from Kaper *et al*., 2004 [6]].

1.2 Classification of Pathogenic *E. coli*

Each year, the Centers for Disease Control and Prevention (CDC) estimates that roughly 1 in 6 Americans (48 million people) acquire a foodborne illness, 128,000 are hospitalized, and 3,000 die [16]. Estimating the number of illnesses, hospitalizations, and deaths is an important public health practice that plays a role in detection and prevention of diseases. However, most estimates are likely conservative due to under-diagnosis and underreporting.

Pathogenic *E. coli* are significant contributors to foodborne disease. There are six defined groups of intestinal *E. coli* pathogens: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [6, 17]. This review will focus on STEC (Shiga toxin-producing *E. coli*), a group which includes human pathogens (EHEC) and non-pathogens.

Upon their discovery, certain strains of *E. coli* were noted for their adverse effects on Vero cells (kidney cell line from monkeys) due to the production of a harmful cytotoxin, later known as verocytotoxin or verotoxin (Vtx). Protein analysis of the toxin showed homology to a toxin produced by *Shigella* spp., thereby becoming termed “Shiga-like toxin” or Shiga toxin (Stx) [8]. The strains are referred to interchangeably as Verotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC) [4]. The term enterohaemorrhagic *E. coli* (EHEC) refers to STEC strains with the same clinical, epidemiological, and virulence features as the pathogenic serotype O157:H7, such as bloody diarrhea [4, 18]. Consequently, all EHEC are pathogenic, while some STEC are not [4]. Furthermore, some STEC strains have only been found in animal hosts and have not been associated with human illness [19].

The first diagnosed outbreak of EHEC O157:H7 occurred in Oregon and Michigan in 1982, when the pathogen was isolated from individuals with bloody diarrhea and abdominal cramps [20]. The source of infection was linked to contaminated ground beef from a restaurant chain [21]. More than 40 people were diagnosed positive with clinical symptoms and at least 30 were hospitalized [20]. Only eight O157 isolates in culture collections date back farther than this outbreak consisting of one

from the USA, one from the UK, and six from Canada [21]. *E. coli* O157 is therefore considered to be a recently emerged pathogen as it harbors genetic elements which had not been previously documented [22].

An evolutionary model for O157 has been developed from genome analyses, consisting of a stepwise evolution from a non-toxigenic sorbitol-fermenting precursor related to *E. coli* O55:H7 (Figure 1.2) [18, 23]. The ancestor had the locus of enterocyte effacement (LEE) genes, responsible for intimate attachment of the bacteria to the intestinal epithelium. The evolutionary steps leading to the pathogen consisted of acquisition of the Shiga toxin 2 gene (S*tx2*), switching of the somatic antigen from O55 to O157, and the acquisition of a large virulence plasmid (pO157). Additionally, there was a loss of the ability to ferment sorbitol (SOR) and the gene encoding for β-glucuronidase (GUD), and acquisition of the Shiga toxin 1 gene (S*tx1*) from bacteriophage [18, 21].

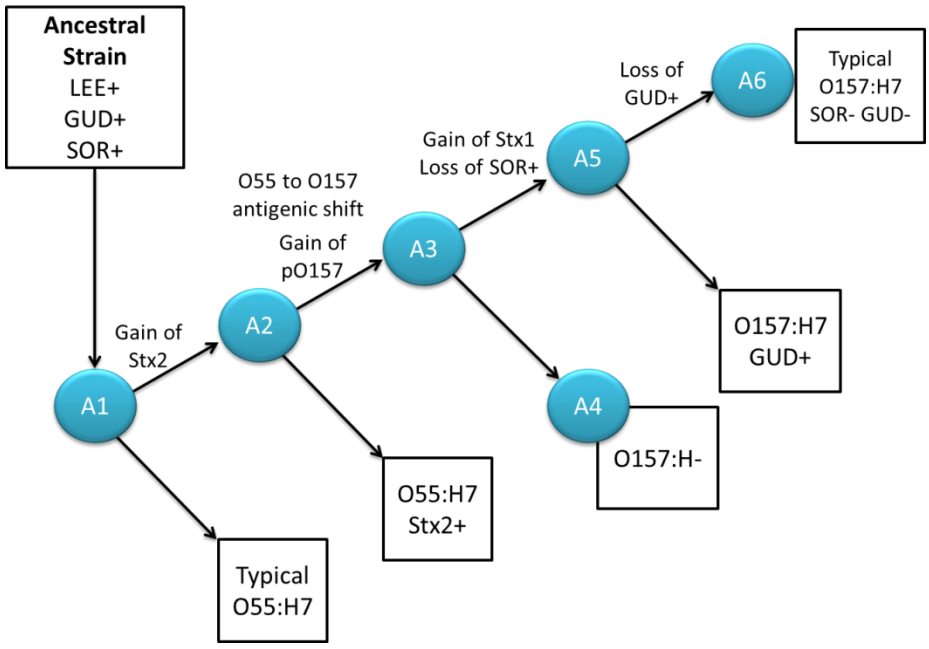

Figure 1.2. Evolutionary genomic changes in the emergence of E. coli O157:H7(A = ancestor) [Adapted from Wick *et al*., 2005 [23]].

1.3 Epidemiology

Human infections with STEC have been reported worldwide, causing an estimated 2.8 million illnesses, nearly 4000 cases of HUS, 270 cases of permanent renal disease, and 230 deaths annually [24]. In the U.S. alone, STEC cause an estimated 265,000 illnesses, 3,600 hospitalizations, and 30 deaths annually [25]. Classified as a notifiable disease in Canada since 1990, STEC are subjected to national reporting by the Public Health Agency of Canada [26]. In the U.S., O157:H7 was classified as a nationally notifiable disease in 1995, and a number of non-O157 serogroups (including O26, O45, O103, O111, O121, and O145) were added in 2000 [26, 27]. In 2000, less than 70% of U.S. clinical laboratories routinely tested for O157 [28]. The number of non-O157 infections reported between 2000 and 2005 increased from 171 to 501 cases, suggesting that this higher burden of illness was attributable to the increased testing for Stx and non-O157 serogroups [4, 29]. The U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) began testing for the top 6 non-O157 serogroups in beef trim in 2011 [30]. To date, Canada has not declared an official position on monitoring for non-O157 serogroups within the food production chain.

E. coli O157:H7 emerged as a human pathogen during the 1982 outbreaks in the U.S. [20, 21]. The first O157:H7 outbreak in the U.K. was in 1983, with the first isolations from other countries following a few years later [21]. Several large O157:H7 outbreaks have occurred since, including: Japan, in 1996 (~8000 cases, 106 HUS) due to contaminated white radish sprouts, and in the U.S., in 1993 (501 cases, 45 HUS, 3 deaths) due to undercooked ground beef [31, 32]. In the U.S., 24 multistate O157:H7 outbreaks were recorded between 1992 and 2002, with at least one per year [21]. Although STEC O157 infections account for a small percentage of total foodborne infections in the U.S., this pathogen is responsible for the highest case fatality rate (deaths per number of confirmed cases) across the population, and the highest annual mortality rate in children under 4 years [7, 33].

Several misconceptions arose following the initial O157 outbreaks, including the idea that O157 was the serogroup chiefly responsible for large STEC outbreaks [31]. Since the majority of laboratories have screened exclusively for O157 in the past, non-O157 outbreaks have very likely been attributed to O157 due to the presence of multiple serotypes within clinical and food specimens [5]. A documented example of multiple STEC present during outbreak investigations occurred in Australia. A rapid PCR technique had been developed that allowed for identification of serotype O111:H- in a number of patients and the identified food source, raw meat sausage. However, O157:H- was also found in both the sausage and a few clinical samples [5]. Therefore, had non-O157 serogroups not been sought, such an outbreak would have been solely ascribed to O157. Subsequent serological studies have found that the severity of HUS is associated with a higher number of O-group antigens in the patients serum, suggesting multiple STEC give rise to a more serious clinical condition [5].

Non-O157 serogroups can be just as deadly as O157. Some of the larger records for non-O157 outbreaks include: O111:NM in Australia in 1995 (158 cases, 23 HUS, 1 death), O111:NM in Oklahoma in 2008 (341 cases, 70 hospitalizations, 1 death), and O111 in New York in 2004 (213 cases) [4, 34]. Other notable international and clinically confirmed outbreaks include O26:H11 in the Czech Republic in 1988 (5 cases, 5 HUS, 1 death), O103:H25 in Norway in 2006 (18 cases, 10 HUS, 1 death), O26:H11 in Denmark in 2007 (20 cases), and O26 and O145 in Belgium in 2007 (13 cases, 5 HUS) [34].

Reports from public health surveillance studies suggest that sporadic cases of non-O157 infection occur more often than outbreaks [34]. Of 104 isolates obtained from STEC infections occurring in the U.S. between 1997 and 1999, 48 (46%) were non-O157 STEC, and 56 (54%) were O157 STEC [19]. There have been at least 22 non-O157 outbreaks in the U.S. since 1990 [4]. Between 1983 and 2002, the non-O157 serogroups most frequently associated with sporadic illness consisted

of O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%) [27]. The same serogroups were identified as most prevalent in a study of confirmed non-O157 STEC infections from FoodNet disease surveillance sites between 2000 and 2010, although O103 was found to be more predominant (22%) than O111 (19%) [35]. Global "hot spots" are thought to exist where non-O157 serogroups dominate over O157. For example, up to 80% of STEC illness in Germany is due to non-O157 STEC [19]. However, these data may be biased as the extent to which stool samples are sent for testing and provided by patients varies by country, as do the confirmatory tests used and extent of follow-up protocols [21]. Although the 2011 German outbreak strain O104:H4 was not a typical STEC, as the primary genetic material originated from an enteroaggregative hemorrhagic *E. coli* (EAHEC) strain, it did illustrate how readily *E. coli* pathotypes can evolve through the of exchange genetic material producing novel pathogenic strains [36]. The German outbreak was the largest number of STEC cases ever recorded [37]. Gastroenteritis was confirmed in nearly 3,000 patients along with 855 cases of hemolytic uremic syndrome (HUS), and 53 deaths [36, 37]. Initially the outbreak was falsely ascribed to cucumbers, but after more thorough evaluation, fenugreek sprouts were suggested to be the source of transmission [37].

1.4 Reservoirs and Transmission Vehicles

Ruminants are asymptomatic carriers and are considered the main reservoir of O157 and non-O157 STEC [18]. These bacteria do not cause disease in the host, but colonize the intestinal tract and recto-anal junction and are shed in feces [4, 5, 38]. Over 400 STEC serotypes have been isolated from cattle and 12 - 17% of these have been related to cases of human illness [4, 34]. Besides cattle, strains of STEC have been recovered from other ruminants including: sheep, goats, buffaloes, deer, and elk [17, 34, 39-42], with less frequent isolation from pigs, cats, dogs, horses, birds, and insects [4, 34, 40, 43]. Transmission of STEC between domestic animals and wildlife is possible due to shared water sources, geographic locations, and frequent contact with cattle and their feces [44].

Cattle feces is thought to be the principal source of contamination for food within the farm-to-fork production chain (Figure 1.3) [18]. Hides and intestinal content from cattle carcasses represent one source of contamination of meat products during slaughter [45, 46]. In the U.S., 41% of O157 outbreaks originate from contaminated ground beef [21]. Although information regarding non-O157 cases and outbreaks related to contaminated meat are scarce, a few have confirmed red meat as the source of transmission. The first definitive non-O157 outbreak associated with beef occurred in the U.S. in 2010 when 3 people became ill after purchasing ground beef from a local grocery store [47]. Worldwide, there have been 8 confirmed non-O157 outbreaks related to consumption of ruminant meat, six of which involved beef products. Together, these outbreaks were responsible for over 200 confirmed cases of illness, 45 cases of HUS, and 3 deaths [47]. Meat products other than beef implicated in causing human infection include fermented sausage, venison, lamb, horse, and buffalo meat [4, 34, 48].

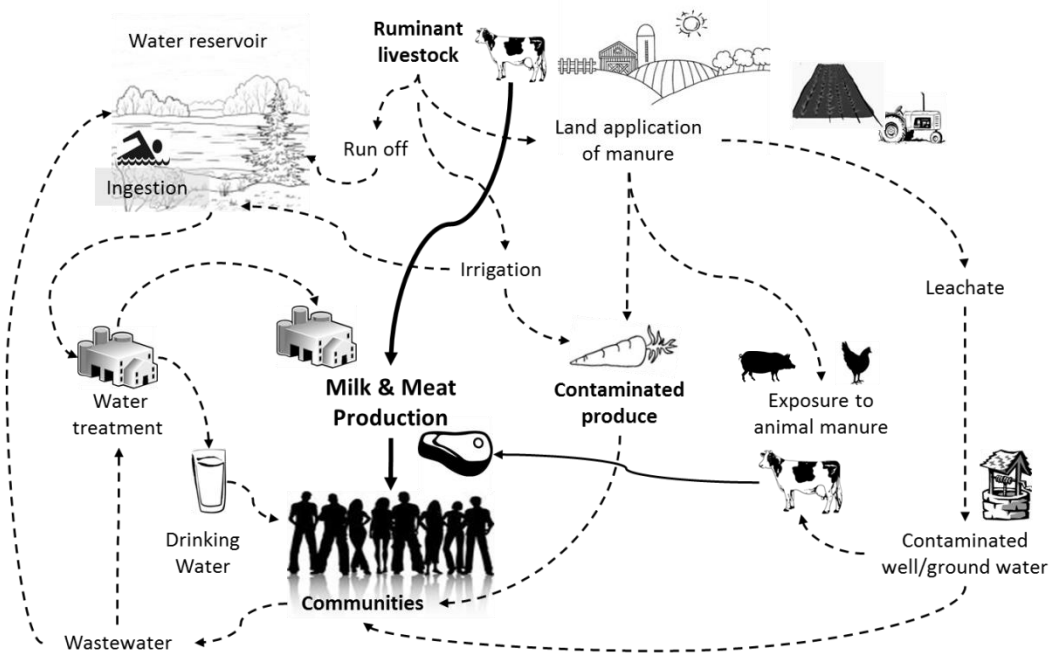

Figure 1.3. Flow of microbial contaminants from livestock operations to food, water, and the environment [Adapted from McAllister and Topp, 2012 [44]].

Milk and dairy products are also frequently implicated as sources of human infection (Figure 1.3) [49]. In 1994 an outbreak occurred from post-pasteurization contamination of milk, later identified as *E. coli* O104:H21 [4]. In 2007 there was an outbreak of STEC O145 and O26 associated with ice cream produced and sold on a dairy farm in Belgium [4, 50].

Contamination of vegetables has been increasingly associated with STEC outbreaks. Land application of manure and use of contaminated irrigation water may introduce these bacteria to produce crops [34]. Proper cooking and pasteurization procedures help to kill potential pathogens before consumption of food, however fresh produce is often consumed raw. Spinach, lettuce, and cilantro have been identified as sources of STEC [51]. In 1999, a contaminated salad bar infected 58 people at a camp in Texas with STEC O111:H8 [4]. Another outbreak was associated with *E. coli* O121:H19 on lettuce in 2006 when 73 people became ill after eating at a fast food chain [4]. Sprouts have also been implicated in several STEC outbreaks including the high-profile O104:H4 outbreak in Germany [52].

In 90 outbreaks from 1982 to 2006, food products (54%) were the most common vehicle of STEC transmission, followed by animal contact (e.g. farms, petting zoos, 8%), water (7%) and the environment (2%), with the source of infection being unidentified in 29% of outbreaks [53]. Direct and indirect contact with ruminant animals, such as on farms, agricultural shows, county fairs, and camps, has also resulted in outbreaks [21, 34]. The largest recorded outbreak due to direct animal contact occurred in England in 2009, leading to 93 infections including 17 cases of HUS [21].

Waterborne outbreaks have been associated with lakes, ponds, paddling and swimming pools, municipal and local drinking waters (springs and wells), and ice [21]. In extensive and intensive livestock production systems, land application of manure and manure catchment basins can be sources of STEC, which then can enter surface and/or groundwater during heavy rainfall and flooding events. Furthermore, if allowed access, livestock may directly defecate into streams and rivers.

Safety of recreational water is subsequently compromised, since STEC pathogens can survive in water for extended periods and the current can transport pathogens over long distances, increasing their potential to come in direct contact with humans [44, 54]. Heavy rain has been repeatedly associated with waterborne O157 outbreaks, including the Walkerton, Canada O157 outbreak in 2000 [21, 42]. This outbreak was a result of manure directly contaminating the town's water supply by flowing into an unsealed well, followed by inadequate chlorination during water treatment [44].

Secondary transmission from infected persons within families, day-care facilities, and health care institutions are also recognized as sources of infection [18, 55]. Some of the larger reported outbreaks include 26 cases (with 10 developing HUS) of O111 infection in a school in France (1992), and 13 cases of O111 infection in a nursing home in Australia (2003) [34]. There have also been numerous non-O157 outbreaks reported in nursery schools in Japan from 2005 to 2009, with over 470 cases being linked to person to person transmission [34].

1.5 Prevalence of STEC in Cattle

Cattle carriage is dynamic, and the prevalence of STEC shown to be highly variable, with periods of high prevalence and apparent absence [21, 56]. A survey of data collected from examination of cattle fecal samples at slaughter found O157:H7 and non-O157 STEC at prevalence rates ranging from 0.2% to 28% and 2% to 70%, respectively [34, 57]. Prevalence rates for non-O157 *E. coli* in feedlot cattle range from 5% to 56%, and from 5% to 45% in grazing cattle [57]. Such variance is likely due to differences in climate, ecology, farming practice, and variation in sampling and testing methodology [57].

Nearly 200 STEC serotypes have been reported in dairy cattle, and more than 260 from beef cattle [34]. One or more of the top 7 STEC serogroups were found on 44% of fecal swabs collected from individual cattle from 21 feedlots in the U.S. [58]. A study of commercial feedlot cattle in the

U.S. found all of the top 7 serogroups in fecal samples, with O157 (50%), O26 (20%), and O103 (12%) being the most prevalent [59]. Several other studies have also found a high prevalence of O26 at both the individual and pen level [60-62]. A study of pooled cattle fecal swabs collected from 21 feedlots found the prevalence of serogroups O157, O45, O26, O103, O121, O145, and O111 of 20, 14, 10, 9, 5, 1 and 0.5%, respectively [58]. Taken together, these studies suggest that cattle shed a variety of non-O157 serotypes [34].

About 80% of transmission of STEC (particularly O157) may arise from the 20% of animals that are most infectious, also known as super-shedders [21]. Super-shedders of STEC O157 are characterized by colonization of the mucosal epithelium of the terminal rectum. Some experimental and field studies suggest that cattle colonized at this site are not only associated with higher levels of STEC shedding, but also shed for a longer duration [63]. However, the mechanisms that lead to a super-shedder are largely unknown [63]. Although data is limited, it is likely that supper-shedders of non-O157 serogroups also exist [64].

Shedding of STEC by cattle has been suggested to be seasonal, with more shedding occurring in the warmer than colder months [34, 57, 65, 66]. Ogden *et al*. (2004) found that beef cattle shed higher concentrations of O157 during the summer [66]. Studies of shedding of non-O157 serogroups and detection of non-O157 STEC on cattle hides have found a higher prevalence in the fall [57, 64]. Seasonal shedding may be related to number of factors including ambient temperatures, age of cattle, or type of feed consumed as well as the strain or serotype of STEC [34]. Interestingly, Aslam *et al*. (2010) found that O157:H7 isolates obtained during winter and spring were closely related, while isolates obtained during summer and fall months were more distantly related. This suggests that certain genetic types of O157:H7 are more equipped to survive the winter months than others [65].

1.6 Non-O157 Serotypes

Although reported before the 1982 O157:H7 outbreak, the level of awareness of non-O157 STEC was and remains low since very few laboratories screen for them, aside from the program implemented by the USDA in 2011 [5]. Despite rarely being sought, these pathogens are estimated to cause more than 60% of the 265,000 STEC infections each year in the United States [67]. Many infections might go undetected due to limited testing, making estimations of the frequency of STEC cases difficult [5, 68]. To date, 380 STEC serotypes have been associated with human disease [18]. Of the non-O157 serogroups, six (O26, O45, O103, O111, O121, and O145) account for as much as 70% of non-O157 STEC infections [27].

Each of the top 6 non-O157 serogroups possesses one or more serotypes most commonly associated with human disease. The majority of O26 isolates belong to two serotypes, O26:H- and O26:H11, 89% of which have been isolated from infected humans or healthy cattle [5]. Two serotypes predominate within O103, namely O103:H- and O103:H2, accounting for 82% of STEC isolated within this serogroup. The predominate serotypes within O111 include O111:H- and O111:H8, however O111 STEC have been rarely isolated [5, 69]. The remaining serogroups are predominantly comprised of the following serotypes: O121:H19 or H7, O45:H2 or H-, and O145:H- [27]. Molecular studies have demonstrated that the non-motile (H-) strains often possess the motile genotype. This suggests that the motile and non-motile strains actually belong to one clone, and the close relationship between these two strains of the same serotype has been confirmed by pulsed-field gel electrophoresis typing [5, 70].

1.7 Reducing STEC Contamination of Beef

Since the 1990's, a significant effort has been made to reduce the risk of STEC adulterating beef products. Following the 1992 O157:H7 US outbreak, regulation and control measures were

implemented to reduce the risk of this pathogen entering the red meat supply [46]. Millions of dollars have been invested by government agencies in research for prevention and control of this pathogen. These efforts have resulted in intervention strategies, particularly in the processing sector, that have significantly reduced the number of O157:H7 positive meat samples. Correspondingly, the number of O157:H7 related illnesses has also decreased [46]. The emphasis on intervention strategies has gained renewed attention with the addition of six non-O157 STEC serogroups to the list of reportable adulterants in beef in the United States. Past research has focussed primarily on control strategies for STEC O157 and may require reassessment for the control and prevention of non-O157 STEC [18].

Strategies at all stages of food production from farm-to-fork aim to reduce the risk of potential contamination of the food supply. Implementation of multiple measures to control pathogens enhances food safety and decreases risks to human health [46]. Several approaches exist to minimize risk of STEC contamination of food. These can be classified as either strategies which prevent STEC from entering the food supply, or control strategies to eliminate the pathogens within the food supply.

1.7.1 Prevention

Prevention strategies are meant to decrease the frequency with which cattle come into contact with pathogens. Shiga toxin-producing *E. coli* are introduced to the farm environment through feces from cattle and/or other ruminants and wildlife. These pathogens can survive for long periods in manure, soil, and water [71]. Consequently, there is considerable opportunity for STEC to be recycled through the farm environment and between cattle. On-farm prevention strategies target reduction of: i) contamination of cattle and their hides, ii) human illnesses from direct animal

contact, as well as iii) contamination of the environment, crops, and drinking, irrigation, and recreational waters [18].

Manure is the primary source of STEC within the farm environment. STEC have been detected for after 42 days in aerated manure heaps and up to 90 days in static heaps [72]. Survival in soil is also lengthy (50 – 75 days) [73] with run-off and leaching from manure amended soil introducing these pathogens into groundwater [44, 72]. Within the open environment, limited nutrients and energy sources, and low temperatures, can induce *E. coli* cells into a "survival mode" in which they enter a state of dormancy (viable but non-culturable) [71, 74]. These pathogens can revert from this dormant state when conditions become more favorable growth and thus remain a potential source of infection [75].

Exposure reduction strategies include maintaining environment and water quality, feed hygiene, and excluding wildlife. In addition, composting is an effective method to lower pathogen viability in manure [44]. During composting, temperatures rise (55 – 70°C) for prolonged periods thereby killing pathogens within the manure. Additionally, potential environmental contamination through leaching is diminished [44, 72]. Composting is a practice that can minimize the potential for subsequent transmission of STEC to the human food chain by effectively decreasing the number of pathogens within manure prior land application. Furthermore, composting decreases the potential for STEC to persist in the farm environment and reduces the chances of cattle becoming infected or re-infected with these pathogens [44].

1.7.2 Control

Many stages on the farm-to-fork continuum contribute to an increased risk of foodborne illness. Control mechanisms are required at these stages to reduce the associated public health threat [18]. For example, carcasses and meat products can become contaminated during processing, storage, and handling. No intervention method is 100% effective, therefore multiple controls or

hurdles is the most effective approach to pathogen reduction [46]. Several pre- and post-harvest strategies have been proposed.

1.7.2.1 Pre-Harvest Interventions

Prior to slaughter, exclusion strategies are utilized to prevent establishment or displace pathogenic bacteria within the bovine gastrointestinal tract [76]. Proposed methods include altering composition of the diet, probiotics, prebiotics, vaccines, and bacteriophages, all of which have been discussed in detail in recent reviews [76, 77]. However, these pre-harvest methods have predominantly targeted *E. coli* O157:H7, with the probiotic *Lactobacillus acidophilus* being the only repeatedly effective control measure for reducing O157:H7 shedding in cattle [76, 77]. The effectiveness of *L. acidophilus* in reducing non-O157 STEC has not been examined in vivo, but in vitro studies have found this probiotic to be ineffective at reducing non-O157 serogroups [78]. Two vaccines have shown promising preliminary results for serogroup O157:H7, namely Epitopix SRP® and Econiche®, however their cost/benefit outcomes during large commercial trials have been inconsistent [46]. Additionally, two O157-killing bacteriophage products are commercially available, from Elanco and Omnilytics. Similar vaccines and bacteriophage products are not available for the non-O157 serogroups.

1.7.2.2 Post-Harvest Interventions

During slaughter, carcasses and meat can become contaminated from stomach contents and fecal matter during processing. Contamination is also possible during storage, cutting and food. However, the primary source of carcass contamination is from the hide. Three factors have an essential impact on the risk of carcass contamination: the level of contamination of the hides, the proficiency with which hides are removed to minimize bacterial transfer to carcasses, and the

effectiveness of antimicrobial interventions [46]. Post-slaughter, cutting and further processing of the meat can also introduce microbial contaminants if sanitation is lost.

As the name suggests, direct antimicrobial strategies are designed to kill bacterial pathogens, and include physical, chemical, or biological treatments [18, 34, 46, 76]. The most common physical interventions include knife trimming, steam-vacuuming, and washing, which allow visible contamination to be removed from the carcass [46]. However, steam pasteurization is the most effective direct intervention method. Other antimicrobial interventions include organic acids and surface radiation [18].

Due to the natural variation among bacteria and their susceptibilities to interventions, pathogen contamination is most effectively reduced when multiple post-harvest interventions are utilized [46]. A study of non-O157 STEC prevalence on bovine carcasses in U.S. processing plants found that contamination of carcasses was reduced from 54 to 8% following multiple processing interventions (steam-vacuum, hot water, organic acids, and steam pasteurization) [45].

1.7.2.3 Training and Education

Not only can meat products become contaminated with pathogens, but improper handling, preserving, or cooking can encourage the growth of these microorganisms. Proper training for employees within the processing environment can significantly decrease the level of carcass contamination, especially during hide removal. Proper techniques are enforced through numerous industry efforts, such as best practices' documents, webinars, and workshops[46]. Furthermore, Good Hygiene Practices (GHP) and Hazard Analysis and Critical Control Point (HACCP) production processes are mandated, which provide a systematic approach to ensuring food safety [46].

Retailer and consumer education are also critical for the prevention of human STEC infections. Public health regulations in many countries outline minimal cooking temperatures and

good hygienic practices in many countries [18], including food handling and cooking practices for restaurants and commercial food processing facilities. Consumer food safety education includes emphasis on frequent hand washing, maintaining clean food preparation surfaces, separation of foods during storage and preparation to avoid cross-contamination, proper cooking temperatures to kill pathogens, and constant refrigeration of purchased and leftover foods [18].

1.8 Virulence Features of STEC

Elucidation of the crucial interactions between host proteins and STEC virulence factors is essential to the prevention of transmission and the development of effective novel therapeutics to target these pathogens [9]. Ultimately, it is these interactions that lead to disease in the human host. Numerous strategies are utilized by pathogenic STEC that lead to the development of disease, including host cell attachment, subverting host cell processes using secreted proteins, and evasion of host immune responses [9].

Many STEC virulence features are acquired by HGT. One form of this genetic exchange involves pathogenicity islands (PAIs), which are found on plasmids or incorporated into the chromosome in emerging pathogenic bacteria [9]. PAIs are flanked by mobile genetic elements including bacteriophages, insertion sequences, or transposons, and are usually located near tRNA genes [9]. Shiga toxins (Stx) are major phage-encoded virulence factors of STEC/EHEC [9]. Among Stx, two types have been identified (Stx1 and Stx2), each of which have several subtypes [7]. Stx2 is more prevalent than Stx1 in cases of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Stx is comprised of 2 functional subunits: the “A” or active component, and “B” the binding component.

EHEC do not possess a secretory mechanism for Stx. The release of Stx, although not described, has been linked to lambdoid phage-mediated cell lysis [79]. Lambdoid prophages can grow lytically (killing the bacterial host cell) or lysogenically in which the phage genome

is incorporated into and replicated within the host genome [42]. When the host cell perceives a signal, lytic growth is induced and Shiga toxins are released typically into the intestinal tract of the host.

Globotriaosylceramides (Gb3s) are membrane-bound molecular receptors for Stx, located on the surface of human intestinal Paneth cells and kidney epithelial cells (Figure 1.4) [9]. Cattle and other ruminants lack Gb3s in the intestinal tract, which may contribute to the asymptomatic nature of STEC/EHEC colonization within ruminants [9].

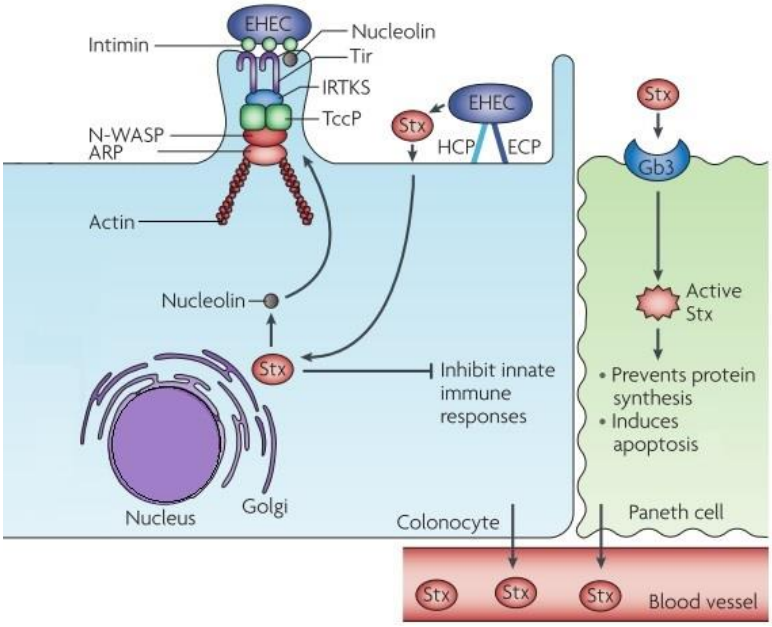

Figure 1.4. Pathogenic mechanisms of enterohemorrhagic *E. coli* (EHEC) [Adapted from Croxen and Finlay, 2010 [9]].

Internalization of the toxin is mediated by the B subunit of Stx, which interacts with the Gb3 receptors inducing plasma membrane invaginations [7]. Early endosomes carry the internalized Stx to the Golgi, where the A subunit is activated by a cleavage event. The catalytic A-subunit cleaves an adenine residue from the 28S rRNA (ribosomal RNA) of eukaryotic ribosomes, preventing protein synthesis, leading to necrosis and cell death [7, 9].

Pathogens expressing Stx toxin genes form characteristic attaching and effacing (A/E) lesions on intestinal epithelial cells. Microvilli are effaced by these pathogens, when they disrupt the host cell actin to form pedestal-like structures beneath the site of attachment. This phenotype is a result of the locus of enterocyte effacement (LEE), a highly regulated 35 kb PAI of non-*E. coli* origin. The size of LEE gene fragments varies from ~35 to 110 kb due to various flanking regions including insertion sequences, prophages, and novel effector genes [8]. The LEE encodes a type III secretion system, a mechanism that translocates effector proteins and secreted proteins (Esp) from the bacteria into the host cell cytoplasm working similarly to a syringe [8, 9, 80]. More specifically, the T3SS is composed of a cytosolic ATPase, inner and outer membrane rings, a periplasmic shaft, an extracellular needle protein, and a ~260 nm filament protruding from the needle generated by protein (EspA) subunits [7]. This filament functions in initial attachment to the host cell. Translocator proteins (EspB and EspD) form the translocation pore in the host cell membrane [7].

The outer membrane adhesin, intimin, is essential for the intimate attachment of bacteria to epithelial cells. The intimin encoding gene (*eae*) is found on the LEE, and its product is secreted into the bacterial outer membrane. Twenty-two variant types of intimin have been described [81]. The transmembrane intimin receptor (Tir) is a LEE-encoded effector translocated via the T3SS, anchored to the host cell plasma membrane and secured with intimin on the bacterial cell surface (Figure 1.4) [7]. In other words, these pathogens ensure intimate attachment by secreting their own adhesin into the host cell. Attachment is enhanced by the interaction of intimin with the surface-localized receptor nucleolin, expression of which is increased by Stx2 [9].

Tir-cytoskeleton coupling protein (TccP), translocated by the T3SS, is responsible for polymerization of the host cell actin and pedestal formation, along with neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin related protein (ARP) complex (Figure 1.4) [7, 80].

The TccP is linked to Tir through the host protein insulin receptor tyrosine kinase substrate (IRTKS), activating the N-WASP-ARP complex [9].

The LEE encodes seven effector molecules, small molecules which regulate the activity of specific proteins, but other effectors exist that are non-LEE encoded (Nle) [82]. The structural proteins of the T3SS in the LEE are highly conserved and thought to have been obtained by HGT, while effector proteins are much more variable and may have been obtained by distinct events [8].

Several adherence factors have been identified in STEC including long polar fimbirae (lpf), several other fimbriae types, and flagella [83]. EHEC attach to colonocytes through the *E. coli* common pilus (ECP) and the hemorrhagic coli pilus (HCP) (Figure 1.4) [9]. Additional adherence factors include the adhesins, EHEC factor for adherence (Efa-1) and *E. coli* immunoglobin-binding protein (EibG) [7, 83]. Many autotransporters have also been identified for their role in adhesion including STEC autoagglutinating adhesin (Saa) and EHEC autotransporters (Eha) [7, 83].

Nearly all disease-associated STEC strains contain a large plasmid encoding hemolysin (EhxA) [84, 85]. Hemolysins are responsible for lysing mammalian erythrocytes, but otherwise their role in STEC pathogenicity is relatively unknown [86]. Several subtypes of EhxA exist.

1.9 Pathogen Interactions & Competition

An infectious disease is understood as a product of the interactions of three main factors: the infectious agent, the host's immune response, and the environment. However, pathogens often do not act independently and their disease potential is often shifted by their relationships with other pathogens [87]. STEC utilize a type of pathogen interaction known as gene assortment. Genes are moved from one strain to another (via HGT) either within or among species (e.g., Shigella), mediated by a viral vector [9, 87]. *E. coli* strains are also known to act antagonistically through the production of colicins (antimicrobial proteins). Colicins, a type of bacteriocin, are highly specific molecules that

target other *E. coli* or enterobacteria [88]. Typically produced under stress, these molecules act by forming pores, hydrolyzing DNA, cleaving RNA, and/or inhibiting protein or peptidoglycan synthesis [88]. Genetic exchange, especially which enhances metabolic diversity, confers an advantage for survival in nutrient-limiting environments. Furthermore, production of and or resistance to colicins allows some strains of STEC to compete successfully against others [89]. In order for pathogenic STEC to have emerged and be maintained, natural selection suggests they must be more fit than competing non-pathogenic *E. coli* strains [89]. Similarly, such competition likely exists between pathogenic STEC.

1.10 Pathogenesis

Typically, the course of infection begins with onset of diarrhoea 2 – 12 days after ingestion of STEC [31]. Infection with O157:H7 typically causes 1 - 3 days of non-bloody diarrhoea, after which hemorrhagic colitis (HC) develops. The mechanisms by which EHEC/STEC induce diarrhoea are not fully understood but may include microvillus effacement, disrupted ion and glucose transport, inhibition of water absorption, disruption of epithelial tight junctions and gut inflammation [80]. Often, infected individuals will seek medical attention for bloody diarrhoea, however mild infections can occur and O157:H7 has been isolated from HUS (Hemolytic Uremic Syndrome) patients with no diarrhoea [31]. A small number of STEC infections progress into HUS. HUS is defined as an acute onset of renal impairment with oliguria reduced urine or non-passage of urine and high concentrations of serum urea and creatinine. Platelet counts are typically low resulting in microangiopathic haemolytic anemia as evidenced by fragmented red cells in a peripheral blood smear [21]. Thrombocytopenia (low platelets) is usually the first indication that HUS has developed, followed by anaemia and accumulation of metabolites in the blood. Irritability, lethargy, and confusion can be caused by fatigue, blood clots in the brain, oxygen depletion in the tissues, or direct neuronal effects of Shiga toxin. Stroke, seizures and coma occur in 10% of HUS patients, with the risk

of an infected child under 10 years old developing HUS being about 15% [31]. Other symptoms that help to differentiate STEC from other causes of colon inflammation, include fever before onset of bloody diarrhoea, severe abdominal pain, and painful defecation [31]. One study, of 5415 cases of sporadic diarrhoea in Canada revealed some slight differences in the clinical symptoms associated with non-O157 infection as compared to O157. These include a longer duration of diarrhoea, and less frequent bloody diarrhoea [21]. However, the frequency of abdominal pain, vomiting, and fever did not differ between non-O157 and O157 infections [19].

In a 4-year study of 30 U.S. patients with HUS, 17% were infected with non-O157 STEC [90]. The highest global rates of HUS are in Argentina at 12.2 cases per 100,000 in children under 5 years. Nearly all of these cases are due to non-O157 serotypes [19]. Almost half of all HUS cases in Germany and Austria have been attributed to non-O157 STEC, and reports from Belgium, Finland, Czech Republic, and Italy also suggest the rate of isolation of non-O157 STEC from HUS patients exceeds O157 [19]. However, testing for and reporting of these pathogens varies by country, and national notification schemes are changed periodically [21].

During treatment of human STEC infection, no method has proven more effective than supportive therapies such as control of fluid and electrolyte imbalance, dialysis, control of hypertension, and blood transfusion[21]. Release of Stx can be prompted by antibiotics, highlighting why they are not a recommended therapy [9, 31, 79]. Intravenous rehydration and maintenance fluid provides protection for the kidneys [31]. In the case of HUS patients, renal perfusion should be maintained, and deleterious fluid overload should be avoided. Renal shutdown may occur, in which case dialysis is inevitable. About 80% of HUS patients require blood transfusions, since the rapid development of anemia severely decreases the ability of blood to carry oxygen [31]. Approximately 5% of HUS cases result in death while 70% of patients recover completely [19, 91]. The remainder of HUS patients (25%) suffer long-term consequences related to permanent renal

injury [19, 91]. However, knowledge of HUS progression and severity in cases of non-O157 infection is limited since most documented HUS cases have been associated with O157 infection [19].

1.11 Economic Costs of Infection

The severity of food-borne outbreaks and the long-term consequences of infection can result in significant economic costs. For example, the estimated annual costs of diarrhoeal disease are €345 million in the Netherlands, AU$343 million in Australia, and CA$3.7 billion in Canada [92]. The cost of the Walkerton, ON O157 outbreak (2000) was estimated at CAD$64.5 million, excluding costs attributable to premature deaths [21]. In Scotland (1994), the medical costs, productivity loss, and outbreak control costs were estimated to be £3.2 million within the first year after the outbreak and £11.9 million over 30 years [21]. Medical costs and productivity loss in Australia's 1995 outbreak of O111 were an estimated AUS$5.6 million [21]. Non-health care costs are also pronounced, including class action and victim claim settlements, fines, recalls, and prosecution costs [21].

1.12 Methods for Detection and Differentiation of STEC in Bovine Feces

Since the emergence of serotype O157:H7 and the severe disease outbreaks that followed, most research and regulatory framework has focussed on this pathogen [93]. Methods for culture-based detection and isolation of O157 are well established, including developed media and sensitive antibody-based technologies [2]. The unique biochemical characteristics of O157:H7 allowed for rapid development of selective and differential media; however, such traits are not present among all non-O157 serogroup. This makes the development of similar selective media more problematic [5, 93]. Food safety regulatory agencies such as the U.S. Food and Drug Administration (FDA) and USDA have developed and validated detection methods for STEC. These methods are available online in the FDA's *Bacteriological Analytical Manual* and FSIS's *Microbiology Laboratory Guidebook* [93, 94].

Government and academic research is also focussed on improving methods for detection of STEC and other foodborne pathogens.

1.12.1 Fecal Sampling Methods

There are many inherent challenges associated with detection and isolation of pathogens from complex matrices such as feces including stressed/injured cells, background microflora, downstream analysis inhibitors, and non-homogenous distribution of target pathogens [44, 93]. Consequently, effective sampling and preparation methodologies prior to completion of analyses are critical [93, 95]. Fecal sample collection methods vary among studies. Usually it involves collection of fecal pats from pen floors, rectal fecal grabs, or rectal fecal swabs. Fecal grabs and rectal swabs are seldom undertaken in large scale or commercial feedlot studies as sampling requires restraint of animals in a squeeze chute. Sample collection requires the careful use of an aseptic technique to minimize contamination from fecal matter with the animal handling system or from the ground during collection of fecal pats.

1.12.2 Enrichment Methods

Culture-based methods are commonly mandated for pathogen detection and confirmation, which primarily includes enrichment, plating on selective media. Enrichment is often used to overcome the challenges associated with pathogen isolation and detection in complex matrices. Since very few cells (~10 CFU) are sufficient to cause disease in humans [71], and STEC are usually present at low concentrations, enrichment is necessary to increase cell numbers to levels that can be detected and isolated. The optimal enrichment conditions resuscitate injured or stressed cells, increase the target cell numbers, and dilute inhibitors and some background microflora [93]. Typically, if an enrichment step is not utilized, only STEC concentrations above 10^4 CFU g^{-1} of feces are detected by PCR[96].

Although many have been evaluated, no enrichment media has been proven superior for all sample types [2]. The most commonly used base media for STEC enrichment are trypticase soy broth (TSB), buffered peptone water (BPW), and *E. coli* broth (EC) [97]. Frequently these media are supplemented with antibiotics (e.g., novobiocin, cefixime) to suppress growth of background microflora [93, 98]. The enrichment broth, selective agent, and incubation conditions have given varied outcomes to the enrichment of non-O157 from feces [93]. Although TSB and BPW are often reported in enrichment protocols, including the USDA and FDA procedures respectively, there are no obvious elements in their formulation to make these broths exclusive for the enrichment of *E. coli* [93, 98]. In contrast, EC broth contains bile salts to inhibit non-*Enterobacteriaceae* strains, lactose which is easily fermented by STEC , and buffers to maintain the pH around 6.9 [98]. A comparison of modified TSB described by Possé *et al*. (2008) and EC broth demonstrated a one-log greater sensitivity for detection of STEC by PCR from EC broth enrichments [96]. A comparison of TSB and EC broths, and the effect of the antibiotic novobiocin, demonstrated that false negative results were obtained when TSB was used to enrich STEC from feces. Furthermore, the addition of novobiocin to either medium inhibited non-O157 STEC, and as a result the addition of some antibiotics is not recommended [99, 100].

Typically, enrichment temperatures range between 37°C and 42°C, with 37°C being the most common. Incubating at lower temperatures (37°C) allows for resuscitation of injured or stressed cells from complex matrices such as feces [93]. The optimal growth temperature for O157 and some non-O157 strains is ~40°C [101]. Higher enrichment temperatures (≥42°C) inhibit growth of many non-coliform bacteria, increasing the selectivity of enrichment [2, 93]. Incubation times typically range from 6 to 24 h [2]. Shorter incubation times have resulted in improved r detection when serially diluted STEC was added to bovine feces [96]. Since *E. coli* are facultative anaerobes , they do not require aeration and are often incubated statically [2].

Following enrichment, molecular detection methods are typically used to identify target serogroups. First, DNA is isolated from a subsample, either by boiling or extracted by a commercial kit. The isolated DNA is then screened for STEC serogroups and/or toxin genes using polymerase chain reaction (PCR). Initial serogroup screening by PCR reduces the number of samples that require further processing, while also being highly sensitive. However, PCR does not discriminate DNA from living or dead organisms, nor does it determine if toxin genes and serogroups genes came from the same origin. Therefore, living cells must be obtained for further analysis.

1.12.3 Isolation of *E. coli* Serogroups

Immunomagnetic separation (IMS) is a technique that uses antibody-coated magnetic beads specific for the O-antigens of STEC to isolate cells of target serogroups. Most often IMS is completed in enrichment broths. The IMS beads are incubated for several minutes in the enrichment suspension with agitation and then removed using magnets. The bead/bacteria complexes are then rinsed, and suspended in buffer before plating on agar media to obtain colonies. Using this method, target bacteria are separated from non-target cells, and thereby concentrated, while many of the background microorganisms are eliminated. Consequently, IMS improves target cell recovery compared to direct plating methods. The sensitivity of primary culture methods to isolate O157 from complex matrices was significantly enhanced with the advent of O157:H7 serotype-specific IMS [2]. Commercial IMS kits are available for O157 and the top 6 non-O157 serogroups, however, the sensitivity and binding-capacity of IMS beads varies among serogroups. For example, commercial O111 beads rarely yield O111 cells and are often more specific to O103 [69, 102].

Selective enrichment and IMS often fails to complete eliminate background microflora. Additionally, the lack of a standard selective and differential media for non-O157 STEC makes isolation of target colonies challenging. MacConkey agar containing sorbitol rather than lactose as a

carbon source (SMAC) is used for isolating *E. coli* O157:H7 [18]. Most strains of serotype O157:H7 cannot ferment sorbitol, so when plated on SMAC, colonies of O157:H7 appear colourless and 2-3 mm in diameter [21]. Unlike O157:H7, most *E. coli* appear as pink colonies, while growth of gram-positive microorganisms is inhibited by the crystal violet and bile salts present in the media [4, 31]. Therefore, SMAC medium cannot be used to differentiate sorbitol-fermenting *E. coli,* as is the case for most non-O157 STEC, from *E. coli* O157:H7 [18].

Several different culture media have been described to detect non-O157 STEC, for either individual or multiple serogroups [2, 4, 93]. A set of media based on a mixture of carbohydrate sources, β-D-galactosidase activity, and selective reagents for colour differentiation have been described [103]. The first medium of the set contains a MacConkey agar base, supplemented with sucrose, sorbose, bile salts, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isopropyl-β-D-1-thiogalactopyranoside (IPTG), novobiocin, and potassium tellurite. The second group of media is for confirmation of individual serogroups based on specific carbohydrate utilization and consists of a phenol red broth base supplemented with dulcitol, L-rhamnose, D-raffinose, or D-arabinose. The main limitation of this media is that it only differentiates four non-O157 serogroups (O26, O103, O111, and O145). Furthermore, subsequent studies have shown that colony colour, size, and texture can differ on this media as a function of incubation time, degree of colony crowding, or the matrix from which the cells were isolated [4]. Commercial agars used for screening the top 6 non-O157 STEC include CHROM and Rainbow agar. Gill *et al*. (2014) compared the growth of 96 STEC strains on eight agar media including MacConkey , Rainbow with or without supplements, CHROM, VTEC , and tryptone bile agar with or without supplements [104]. All strains were able to grow on MacConkey, Rainbow, and VTEC agar, while several strains failed to grow on any of the agars [104]. A new chromogenic agar medium has recently been developed by USMARC (US Meat Animal Research Center), to distinguish the top 6 non-O157 serogroups [105]. It has been extensively tested and

validated for both beef and fecal samples [2]. Additionally, there is extensive information on phenotypic screening of reference strains based on colony morphology [2, 105]. Although strains of the top 6 non-O157 STEC can be discriminated from background microflora, they cannot easily be distinguished from each other and would require further serogroup confirmation (PCR).

1.12.4 Confirmation of Serogroups

As outlined above, the selectivity of enrichment assays, IMS, and plating procedures for the non-O157 STEC is limited. Therefore, further testing is required in order to confirm colonies for the type of serogroup and/or virulence. Complete antibody-based O:H serotyping can only be completed at *E. coli* reference laboratories, such as the Foodborne and Diarrheal Diseases Laboratory of the CDC. Few laboratories are equipped with the capacity to fully serotype *E. coli* isolates since it is time consuming and requires expensive specialized reagents [27]. However, many other methods exist to confirm O-group including PCR, colony hybridization, microbead immunoassay, and latex agglutination [93, 106, 107]. Although colony hybridization allows for screening of all colonies on a plate, it is time consuming and logistically difficult for large numbers of plates [106]. Latex kits are rapid and easy to use but some antisera cross-reacts with other serogroups producing false positives [93]. Shiga toxin production can be confirmed by traditional cell cytotoxicity assays (Vero cell assay), that are sensitive, but labor intensive, time consuming, and costly [19, 93]. Other conformational assays include PCR for *stx* genes, Shiga toxin enzyme immunoassays, and Shiga toxin colony immunoblot [93].

Most commonly, isolates are screened by PCR for O-serogroup, Shiga toxin, and other related genes (intimin, enterohemolysin). PCR is fast (2-4 h) and highly sensitive (10-10^3 CFU/reaction) [93]. Although many assays (conventional and quantitative) have been developed [69, 102, 108-116], their performances (e.g. sensitivity, accuracy, efficiency) differ when evaluated

individually [93, 102]. Furthermore, few multiplex assays are able to detect all 7 clinically-relevant STEC in a single reaction [69, 102]. Both the FDA and USDA methods require qPCR to screen for *Stx* genes [93, 94, 117]. Furthermore, the USDA method requires four separate PCR reactions to screen for all O-groups and Stx genes.

With complete sequence data for O-antigen gene clusters continually becoming available, PCR assay development and sensitivity is evolving at a fast pace. The genes most often targeted for O-serogroup identification are *wzx* and *wzy* (Figure 1.5) which encode for the O-antigen flippase and O-antigen polymerase respectively, although other genes have been used [93, 118]. Virulence of STEC isolates is usually based on the presence of *stx*1, *stx*2, *eae*, and *ehx* genes, although many other genes are also potential indicators of virulence [82, 119]. *Stx*1 is highly conserved with only 3 variants, while 7 subtypes of *Stx*2 have been reported [81]. An 11-gene multiplex PCR was described by Bai *et al*. (2012) to detect the four main virulence factors in addition to the 7 clinically-relevant STEC [69].

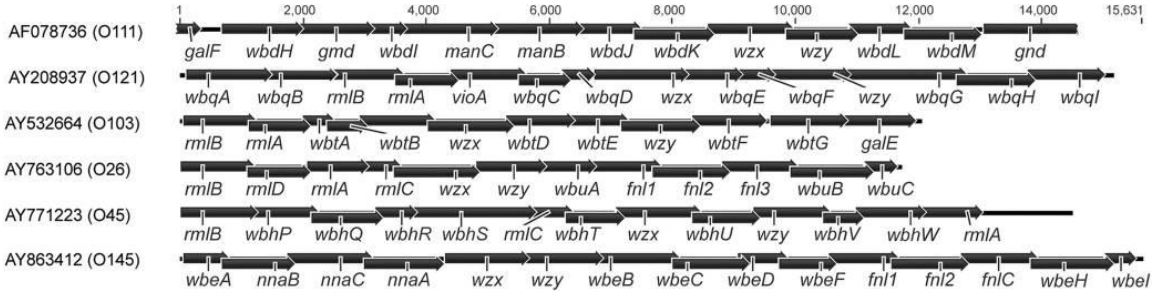

Figure 1.5. O-antigen gene clusters from *E. coli* serogroups O26, O45, O103, O111, O121, and O145. GenBank accession numbers are on the left next to serogroups in parentheses, and base pair length is located above the gene clusters [From Norman et al, 2012 [118]].

Limitations of applying PCR to environmental samples include inhibitors, and inability to differentiate living from dead cells. However, PCR can be applied to single colonies, and internal amplification controls can be used to address these concerns. The detection of virulence genes however, is not a guarantee of gene expression [93].

1.12.5 Further Evaluation and Subtyping Methods

Subtyping methods are important during outbreak investigations to determine relationships among strains and trace their sources. Pulsed-field gel electrophoresis (PFGE) is considered the most discriminatory subtyping method [4, 120]. This method requires the isolated DNA from a pure culture to be digested with restriction enzymes and separated by gel electrophoresis. The relationship of isolates to one another is determined by their banding pattern, and PFGE data is gathered and stored in the PulseNet system [4]. PCR-based subtyping methods include multiple locus variable-number tandem repeat analysis, multilocus sequence typing, amplified fragment length polymorphism, random amplification of polymorphic DNA, and octamer-based genome scanning and lineage-specific polymorphism assays [121]. Other subtyping methods include phage-typing, ribotyping, microarray, and detection of single nucleotide polymorphisms [4, 18, 121]. Phenotypic methods such as phage-typing have limited applicability since few laboratories are equipped for such analyses, and these time-consuming methods require costly specialized reagents. Furthermore, phage-typing has not been developed for non-O157 STEC [121]. The application of whole genome sequencing (WGS) technologies is becoming more widespread as they offer much greater resolution of closely related genotypes and higher phylogenetic accuracy than traditional typing methods. Increased speed and decreasing cost of acquisition of WGS technologies allows functionally annotated genomes within days or hours [122]. The discriminatory power, ease of standardization and reproducibility, data analysis and interpretation, availability, and cost of the aforementioned genotyping methods vary considerably [121].

1.13 Hypothesis

In order to monitor STEC in cattle prior to harvest and human consumption, and as mandated by the USDA-FSIS, reliable and sensitive screening methods are required. However, several limitations to current detection and isolation methods exist. First, molecular detection methods

require a positive control. However isolates of pathogenic strains, particularly those which are novel or emerging are difficult to obtain. Second, the sensitivity and accuracy of developed molecular methods are often not thoroughly evaluated. Cattle feces are a complex matrix, possessing unique challenges for detection and isolation procedures. This necessitates further development of feces-specific detection and isolation methods. Finally, it is unknown whether sample storage or enrichment can bias detection results due to within-species competition. The hypothesis of this study was that the methods for detection of the top 6 non-O157 and O157 STEC could be validated and developed further through careful evaluation of microbiological and molecular laboratory techniques. Specifically, this study aimed to improve preparation of bovine fecal samples (e.g. enrichment, DNA extraction) and advance sensitivity and accuracy of PCR detection methods. The main objectives were:

1. To design synthetic oligonucleotides for rapid molecular detection of relevant STEC serogroups that can be utilized as positive controls in the absence of natural DNA from the target organism.
2. To develop a sensitive and economically feasible protocol for detection and isolation of potential STEC pathogens from bovine feces, by first:
    a. Carefully evaluating current sample preparation procedures and STEC detection techniques including freeze drying, enrichment, and Polymerase Chain Reaction (PCR).
    b. Developing sensitive multiplex PCR assays for detection of: 1) the top 6 non-O157 and O157 STEC serogroups and 2) toxin and related virulence factor genes.
3. To determine if potential competition exists between multiple serogroup isolates (in one-on-one competitions) in an in vitro experiment using enrichment cultures, and to

predict if this competition could create an enrichment-bias during detection of STEC from feces.

CHAPTER II: Synthesis of O-serogroup specific positive controls and real-time PCR standards for nine clinically relevant non-O157 STECs

2.1 Introduction

Shiga-toxin producing *Escherichia coli* (STEC) are major foodborne pathogens that have been linked to outbreaks and sporadic cases of human illness worldwide. Infection may result in a variety of outcomes including diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and can lead to death in immunocompromised individuals [108, 123]. *E. coli* O157:H7 has been a recognized foodborne pathogen since the 1982 outbreaks in the United States. Due to the lack of regular testing for non-O157 STEC, many outbreaks may have been falsely ascribed to *E. coli* O157 generating a false perception that O157 are the only STEC of significance to human health [5]. Since the increase in testing for Shiga toxins in diarrheal cases and for STEC other than O157, it has been estimated that 20 to 50% of all STEC infections can be attributed to non-O157 strains in both Canada and the USA [4, 26].

Transmission of STEC occurs mainly through the fecal-oral route via contamination of food and water. Although STECs have been recovered from chicken, sheep and goat products, the major reservoir is cattle, with the potential to contaminate both milk and beef products [4]. The U.S. Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) has recently announced regulations concerning monitoring of non-O157 STEC in beef products. Canada has yet to adopt an official position, but due to the high degree of integration between Canadian and US beef industries, Canada will be directly affected [26].

Part of the reason non-O157 STEC are not regularly sought after is because no standard detection method is accepted. Pathogenic *E. coli* behave biochemically and ecologically like non-pathogenic *E. coli* making their detection very difficult [5, 26]. As a consequence of inadequate detection methods, the source of contamination in the 2011 non-O157 STEC outbreak in Germany

was initially misdiagnosed, consuming valuable time to identify the correct biological hazard and resulting in significant social and economic burden [124].

One distinguishing feature of STEC are their O antigens, highly variable polysaccharide components of the outer membrane. These antigens are detectable using serological methods based on the binding of antibodies to these cell surface structures. Phenotypic methods such as serotyping, however, have limited applicability since they require specialized reagents, are costly, time consuming, difficult to standardize among laboratories and available in only a few specialized laboratories [121]. In contrast, molecular methods such as polymerase chain reaction (PCR) can be employed to detect genes coding for strain-specific O antigens, and have the advantage of being less expensive, highly sensitive, with many laboratories being equipped for such analyses.

The genes that have been targeted to serogroup putative isolates include *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) [96]. Primers have been designed for PCR detection of these genes for nine of the non-O157 STEC most frequently associated with HC and HUS (O26, O45, O91, O103, O111, O113, O121, O128, and O145) [108]. However, PCR requires target DNA as a positive control, commonly extracted from defined target cell cultures, to confirm specificity and sensitivity of the assay. In some cases, biological isolates are rare and not commonly available, especially during the initial phase after discovery of a novel biological threat [125]. In addition, work with pathogenic organisms requires a biosafety level two or higher laboratory in order to grow the control culture. Single target control plasmids and gene fragments have been reported and tested as positive controls in PCR assays [125, 126]. Alternatively, by stacking multiple primers into a single plasmid, screening efficiency may be enhanced and more versatile.

The objectives of this study were to design and generate stacked synthetic controls for PCR detection of nine non-O157 STEC serotypes, in which no natural DNA template was required, and to

evaluate the synthetic control DNA fragments as standards for multiplex and real-time PCR detection assays.

2.2 Methods

2.2.1 O serogroup specific oligonucleotides as positive controls for PCR assays

Three synthetic positive oligonucleotides (controls) were designed using Geneious bioinformatics software (Biomatters Ltd, New Zealand). Each of the controls contained sequences for amplification of three different O serogroups (Table 2.1), producing 140, 150, and 160 bp amplicons, respectively. Controls were made up of long oligonucleotides (100-120 bp) synthesized by Eurofins MWG Operon (Canada), coding for specific forward, reverse and probe sequences (Table 2.2). In addition, they contained a 10 or 20 bp complimentary sequence for size distinction. Complementary forward and reverse oligonucleotides were hybridized in a PCR-like cycle, in which the polymerase generated a 200 bp double stranded product (Figure 2.1). Hybridization mixtures (25 µl total volume) contained 9µl HotStar master mix (Qiagen, Canada) and 8 µl each of the forward and reverse oligonucleotides at a concentration of 10 µM. Hybridization conditions were 95°C for 5 min, 94°C for 15 sec, 52°C for 30 min, with a final extension at 72°C for 7.5 min.

Table 2.1. Synthetic positive controls and O serogroup specific primers used in this study (primer sequences from Lin *et al*., 2011)

Positive Control	O Serogroup Primers	Sequence (5'-3')	Amplicon Size (bp)
A	O26- F	ttttatctggcgtgctatcg	140
	O26-R	cggggttgctatagactgaa	
	O111-F	caatccaatttgcatcttca	150
	O111-R	accgcaaatgcgataataac	
	O91-F	catgctgctcattcttctca	160
	O91-R	tggagtttgcaacaaacaaa	
B	O45-F	tacgatttcacaagcttcca	140
	O45-R	tgcaatcgcataaggaaata	
	O128-F	tcgatcgtcttgttcaggtt	150
	O128-R	gaatgcaatgggcaattaac	
	O121-F	tggatggcattcctcagtat	160
	O121-R	agcaagccaaaacactcaac	
C	O103-F	gggcttgtattgttgtaccg	140
	O103-R	agtggcaaacagccaactac	
	O145-F	tgttcctgtctgttgcttca	150
	O145-R	atcgctgaataagcaccact	
	O113-F	tgaccttacttcctgcgaat	160
	O113-R	agcaccacgataggattgaa	

Table 2.2. Staggered oligonucleotide sequences used to synthesize positive controls

Control		Oligonucleotide Sequence (5'-3')[a]
A	F	ttttatctggcgtgctatcgcaatccaatttgcatcttcacatgctgctcattcttctcatggcactcttgcttcgcctgtggaggatgttccgcatggaaaatggtttgctgcgacgct
	R	tggagtttgcaacaaacaaaACGTAGTCAGaccgcaaatgcgataataacACGTAGTCAGcggggttgctatagactgaaAGCGTCGCAGCAAACCATTT
B	F	tacgatttcacaagcttccatcgatcgtcttgttcaggtttggatggcattcctcagtattcgcgggctcccttattgtggggttgcacaattggcctcctttaacacgggcgtggttgga
	R	agcaagccaaaacactcaacACGTAGTCAGgaatgcaatgggcaattaacACGTAGTCAGtgcaatcgcataaggaaataTCCAACCACGCCCGTGTTAA
C	F	gggcttgtattgttgtaccgtgttcctgtctgttgcttcatgaccttacttcctgcgaattcggggattttctgcggatttgggctgccactgatgggatcctgggaggaggctgcaaaa
	R	agcaccacgataggattgaaACGTAGTCAGatcgctgaataagcaccactACGTAGTCAGagtggcaaacagccaactacTTTTGCAGCCTCCTCCCAGG

[a] Primer pairs: Blue - 140 bp amplicons; Green - 150 bp amplicons; Purple - 160 bp amplicons; Black, underlined - inserts; Black upper case, no underline - Complimentary sequences; Grey - probe binding recognition sites.

Hybridization was followed by amplification in 25 µl reactions containing 16 µl HotStar master mix, 8 µl hybridization product, and 0.5 µl each of the outer forward (O26-F, O45-F, and O103-F) and reverse (O91-R, O121-R, and O113-R) primers (200 nM final concentration). PCR conditions were 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 45 sec, and 72°C for 90 sec, followed by a final extension at 72°C for 5 min. Hybridization and amplification were performed on a mastercycler (Eppendorf, Canada). The amplified controls were run on a 2% (w/v) agarose gel, excised and gel-extracted using a Gel Extraction kit (Qiagen). Each of the entire control (200 bp product) containing internal O serogroup specific targets were amplified and electrophoresed on a 2.2% (w/v) agarose gel stained with ethidium bromide, and visualized using a gel imaging system (AlphaImager, AlphaInnotech, USA).

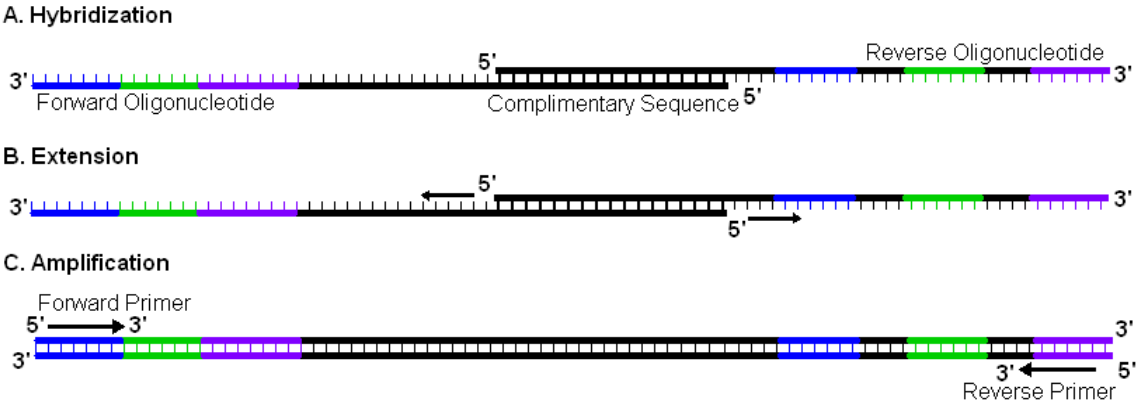

Figure 2.1. Experimental procedure for the preparation of synthetic positive controls: A) Hybridization of forward and reverse oligonucleotides at 20 bp complimentary sequence; B) Extension of oligonucleotides by polymerase generates a double stranded molecule; C) Amplification of double stranded molecule by PCR and outer primer sets (Colors indicate O serogroup-specific primer binding sites).

2.2.2 Synthetic controls as standards for real time PCR

Following gel-extraction and amplification, the 200 bp PCR products were cloned using a Qiagen PCR Cloning Kit into a pDrive cloning vector and transformed into Qiagen EZ competent cells following the manufacturer's instructions. After overnight incubation, blue/white screening identified positive transformed colonies. Luria broth (LB) was inoculated with a single white colony and grown overnight at 37^{o}C propagating target plasmids which were confirmed by PCR and gel electrophoresis. Plasmids were subsequently purified by QIAprep Spin Miniprep Kit (Qiagen). Plasmids were quantified by PicoGreen using a NanoDrop 3300 fluorospectrometer (Thermo Scientific, USA). The resulting plasmid DNA was diluted to generate standards for real-time PCR ranging from 10 to 10^{8} copies, and tested in both iQ SYBR Green and TaqMan assays on a realplex Mastercycler (Eppendorf). The real-time PCR mixtures contained 22 µl iQ SYBR Green Supermix (Biorad, Canada), 0.5 µl each primer (200 nM final concentration), and 2 µl of synthetic standard DNA. The conditions for SYBR Green real-time PCR were: 3 min at 95°C, 40 cycles of 95°C for 15 sec, 52°C for 60 sec, and 72°C for 15 sec, followed by a final extension at 72°C for 7 min. The TaqMan assay was conducted using a serogroup specific probe (6FAM-tggcactcttgcttcgcctg-BHQ1; Eurofins MWG Operon, Canada). Reaction mixtures contained 1× QuantiTect Multiplex PCR NoROX master

mix, 0.4 µM of each primer, 0.2 µM of probe, and water to a final volume of 50 µl. A temperature gradient was used to determine the optimal annealing temperature. PCR conditions were 15 min at 95^{o}C, 45 cycles of 94^{o}C for 1 min, 52-66^{o}C for 1 min, and a final extension at 72^{o}C for 5 min. Amplification by real-time PCR assays was confirmed by gel electrophoresis.

2.2.3 Evaluation of synthetic controls in conventional and multiplex PCR assays

The O serotype specific PCR assays were tested with DNA from pure cultures of each of the nine non-O157 STEC. For each serotype, four different strains from different sources (human, bovine, ovine) were grown overnight in LB at 37^{o}C. After centrifugation, DNA was extracted from cell pellets using the QIAamp DNA Stool Mini Kit (Qiagen). PCR reaction mixtures contained 200 nM of each primer, 12.5 µl HotStar master mix, 2.5 µl CoralLoad concentrate, 7 µl of nuclease-free water, and 1 µl of DNA template. Synthetic controls were included, as well as a negative control containing water instead of template DNA. PCR conditions were identical as those used to amplify the synthetic controls. Amplicons were electrophoresed on a 2% (w/v) agarose gel.

To demonstrate the utility of synthetic DNA as an internal control, PCR reactions were spiked with varying dilutions of synthetic control template. Dilutions of 10^{-1} to 10^{-6} of synthetic control DNA (10 ng/µl) were prepared in nuclease-free water. Reaction mixtures and PCR conditions were identical to those used for pure cultures of non-O157 STEC, with the additional inclusion of 1 µl of the appropriate dilution of synthetic control DNA.

Multiplex reactions were prepared containing three non-O157 DNA templates in varying concentrations. Reaction mixtures contained 100 µl Qiagen Multiplex master mix, 40 µl 10× primer mix (containing a final concentration of 0.4 µM of each primer), 34 µl of water. The three DNA templates were combined in concentrations ranging from 5 ng to 0.5 ng total DNA. The PCR

conditions were 95°C for 15 min, 35 cycles of 94°C for 30 sec, 52°C for 90 sec, 72°C for 90 sec, and a final extension at 72°C for 10 min. Multiplex PCR products were electrophoresed on a 2% (w/v) agarose gel.

2.3 Results

2.3.1 O serogroup specific oligonucleotides as positive controls

The forward and reverse oligonucleotides hybridized efficiently to generate the 200 bp synthetic controls. The hybridization product generated a template for PCR amplification by using both outer primers framing the entire product. Gel electrophoresis confirmed amplification of the 200 bp target PCR product. The 200 bp synthetic controls were successfully purified by gel extraction thereby removing non-specific amplification products and primer dimers. The entire 200 bp controls were re-amplified following gel-extraction and visualized by gel electrophoresis. The synthetic controls were designed such that each specific O serogroup control produced a different amplicon size (140, 150 and 160 bp respectively), allowing visualization of serotypes by gel electrophoresis following conventional PCR (Figure 2.2).

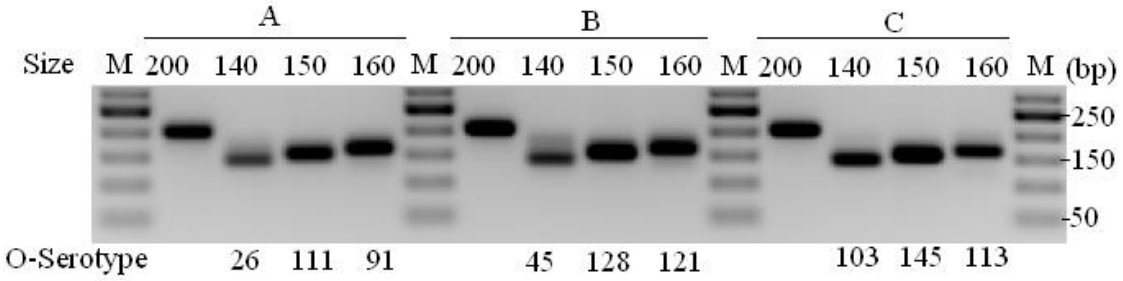

Figure 2.2. PCR amplification of the entire 200 bp positive controls (A, B, C) and internal O serogroup-specific targets of 140 (O26, O45, O103), 150 (O111, O128, O145) and 160 bp (O91, O121, O113), resolved on a 2.2% (w/v) agarose gel. Size marker (M) 50 bp ladder.

2.3.2 Synthetic controls in conventional and multiplex PCR assays

The extracted DNA from a total of 36 cultures of nine different STEC serotypes were amplified and visualized by gel electrophoresis. Each serotype specific assay amplified distinct bands with the given primers from pure cultures of STEC template DNA, as shown for O145 and O113 in Figure 2.3 A. The synthetic positive controls in combination with the associated primer set amplified bands with a distinct size from native amplicons.

Native DNA template spiked with synthetic DNA was amplified by PCR as shown in Figure 2.3 B. For spiking experiments, reactions containing a total of 1 pg synthetic control DNA produced the most distinct bands for both the native template and synthetic controls. Reactions containing concentrations ≥10 pg of synthetic DNA inhibited the amplification of natural DNA template (data not shown).

The standard amplicons account for a 26 bp to 131 bp difference in fragment size in comparison to the amplified native fragment (Table 1). Three multiplex assays were designed to cover the 9 O serotypes, each specific for three of the serotypes as shown in Figure 2.3 C for O103, O145, and O113 (corresponding to 169, 245, and 291 bp respectively). Three bands could clearly be distinguished for all template concentrations. In addition, utilization of a synthetic control produced a band visibly different in size.

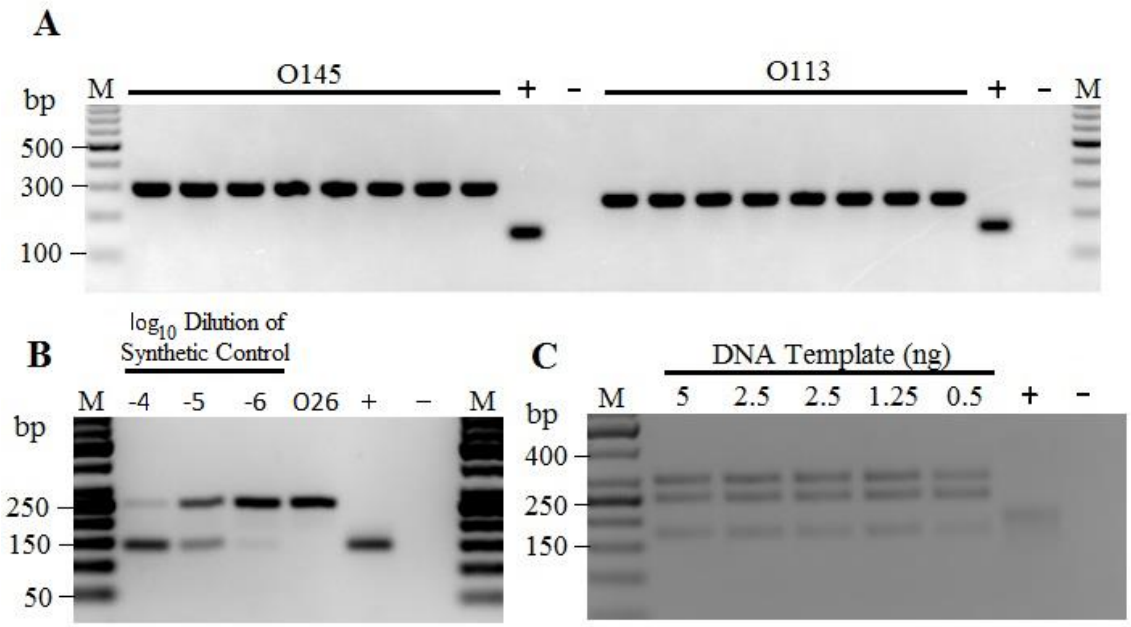

Figure 2.3. PCR amplification of DNA from different STEC strains: **A**) four samples of O145 and O113 in duplicate; **B**) O26 spiked with synthetic positive control DNA in varying concentrations (O26 = only); **C**) multiplex of O103, O145, and O113 in varying concentrations; synthetic positive control (+); negative control (-) and size marker (M); resolved on 2% (w/v) agarose gels.

To verify the assay, synthetic DNA standards were tested quantitatively by two real-time PCR assays. The R^2 values ranged from 0.991 to 0.999, confirming the reliability of synthetic standards for quantification by real-time PCR. An annealing temperature of 55^{o}C resulted in optimum amplification curves for the TaqMan assay.

2.4 Discussion

Our study demonstrates for the first time a synthetic positive control enabling the use of conventional and/or multiplex real-time PCR for rapid screening of nine non-O157 STEC serogroups. Molecular techniques such as PCR are well developed, but positive controls are essential to verify the efficiency and reliability of the assay. Often a true positive control may be difficult to obtain; the organism containing the target of interest may be difficult to culture, possess unknown

growth conditions, or the organism itself may be unknown [126]. In the case of pathogens, obtaining a positive control may be undesirable due to biosafety concerns. Oligonucleotides, like those used to synthesize the positive controls, can be ordered from a variety of providers with molecular supplies. Therefore, construction of synthetic controls is simple and convenient, and enables PCR detection without requiring native DNA templates. The assay offers potential for high standardization and synthetic controls do not pose distribution limits.

The O serogroup specific primers used to design the synthetic positive controls and standards in this study were previously found to accurately identify all 9 clinically relevant non-O157 STEC strains [108]. Nevertheless, the PCR protocols used in this study were further validated using native STEC DNA.

Presence of PCR inhibitors in a sample can result in false negative results. Synthetic controls are advantageous for spiking DNA templates from complex matrixes which can reveal false negatives due to PCR inhibitors.

The SYBR Green real-time PCR assay using synthetic standards proved highly accurate, and provides a PCR option for single serotype screening. The TaqMan assay on the other hand, provided the option for multiplex screening applications in which multiple *E. coli* serogroups can be simultaneously identified and quantified. The size differences among the serotype amplicons enabled recognition of contamination. On the other hand, the size differences are small enough not to affect the real-time PCR efficiency between synthetic standard and native fragment amplification. Outbreaks caused by STEC may be the result of several serotypes, therefore combining multiple serogroups into single controls and multiplex assays could provide high-output screening procedures.

In conclusion, the simplicity of the current protocol provides standards for the enhanced, reliable and rapid detection of pathogenic non-O157 Shiga-toxin producing *Escherichia coli*, without

the need biocontainment protocols. Stacking multiple primers into single controls enables more efficient and user-friendly detection of targets as compared to individual controls. For diagnostic analysis, rapid initial screening for STEC by serotype-specific PCR has several advantages over agglutination techniques, and may reduce the impact of foodborne outbreaks by increasing the speed of isolate identification and sources of contamination [108]. Furthermore, the design of synthetic standards can be expanded to any known set of genetic information for PCR primer and probe assays in order to bypass limitations making viable control isolates unattainable.

CHAPTER III: Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces

3.1 Introduction

As production of food and agricultural products in large centralized facilities increases, so does the risk of transmission of pathogenic microorganisms. Shiga toxin-producing *Escherichia coli* (STEC) are food borne pathogens responsible for mild to severe illness in humans, sometimes leading to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [57]. For decades, one STEC serotype (O157:H7) was considered to be a major public health concern [5]. More recently it has been estimated that 20-50% of STEC infections are caused by non-O157 serogroups, equivalent to 37,000 illnesses annually in the U.S. [19, 27]. Six non-O157 serogroups (O26, O45, O103, O111, O121, O145) have been identified as a public health concern due to their frequent association with HC and HUS [19, 27]. The U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) now includes the "big-6" non-O157 serogroups as adulterants in beef trim [30]. Cattle have been identified as the principal reservoir for STEC, and contaminated beef products have been implicated as sources of human infection [5, 26, 57]. The association between STEC serotypes found in cattle and those causing human illness is not well understood [118].

Food safety monitoring systems are vital, but are only as sensitive, reliable, and feasible as their applied methodologies. Determining STEC prevalence in samples of cattle origin is complex. Effective methods are available for detection of O157, but the same methods cannot be applied to the non-O157 serogroups due to the complexity and diversity of these pathogens and the lack of standardized isolation and culturing methods [5, 118]. Genes for synthesis of the outer membrane O-antigens (*wzx*, *wzy*) can be used to discriminate STEC based on serogroup in molecular assays [108]. Several PCR assays have been developed for such serogroup-based detection in food and carcass

samples [93, 108, 112, 117, 127]. However, few studies have thoroughly evaluated PCR for its sensitivity and accuracy of detecting STEC serogroups in cattle feces [69, 96]. Cattle feces are a complex matrix, with unique challenges such as the presence of high concentrations of PCR inhibitors [93].

The abundance and proximity of *E. coli* and *stx*-encoding bacteriophage across diverse environments allows for dynamic exchange of mobile genetic elements resulting in ever evolving STEC genomes and pathogenicity [79, 119]. Due to globalization and industrial centralization of the primary and secondary food production chain, the transfer of food related pathogens and their mobile genetic elements across global distances becomes more probable [63]. The potential for emerging and novel pathogens to enter the food supply is invariably present. Therefore, monitoring systems are imperative to secure food safety and minimize associated human health risks. In order to gain a better understanding of the potential impact of non-O157 STEC in cattle and the associated human health risks, reliable, sensitive and economical serogroup detection methods are required. The main objective of this study was to validate and/or further develop detection methods for O157 and non-O157 STEC in cattle feces. Initially, we evaluated a current PCR assay [108] for detection of potential STEC serogroups in feces, and subsequently enhanced the breadth of this assay by developing a novel multiplex PCR (mPCR) assay. Both fresh and autoclaved feces (to eliminate potentially competing background microflora) were used to evaluate the effects of enrichment on PCR detection of STEC. Conventional culturing methods were compared to optical density (OD) measurements, and quantitative PCR (qPCR) serogroup gene copy numbers to allow for estimations of cell numbers between the different measurements.

3.2 Methods

3.2.1 Determining limit of detection

3.2.1.1 Pure culture optical density, colony forming units, gene copy numbers

The procedures used for determining the limit of detection and ability of the assay to detect STEC associated serogroups in feces is outlined in Figure 3.1. Limit of detection was determined using two serotypes of *E. coli*, O157:H7 (EDL933; Public Health Agency of Canada) and O26:H11 (source: human; Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, Canada), with *E. coli* ATCC 25922 (Public Health Agency of Canada) used as a control. Cells were streaked on nutrient agar or tryptic soy agar and grown overnight at 37°C. A single colony was selected, transferred to 10 mL Luria Bertani (LB) broth and grown for 12 h at 37°C with shaking (150 RPM) in triplicate for each serotype. The optical density (OD) of 1.5 mL culture solution was measured by spectrophotometry at 600 nm using a Genesys 20 spectrophotometer (Thermo Scientific) to define OD in relation to CFU/mL as described below.

Overnight culture (350 µL) was dispensed into 34.65 mL of LB (37°C), and incubated for 8 h at 37°C with shaking (150 RPM). Every 2 h, a 1.5 mL subsample of each culture was removed, centrifuged at 15,900 x g for 5 min, and the pellet stored at -20°C for later DNA extraction and analysis of gene copy number by qPCR (described below, 3.2.1.3). Additionally, OD was measured and serial dilutions (10^{-1} to 10^{-7}) were made for each culture at each 2 h time point. Selected dilutions were plated on MacConkey (MAC; 25922 and O26:H11) or Sorbitol MacConkey agar with Cefixime and Tellurite (CT-SMAC; O157:H7), and incubated at 37°C overnight. Colony forming units (CFU) were enumerated and a standard curve for each serotype was prepared by plotting OD, CFU, and gene copy number against time (Figure 3.2).

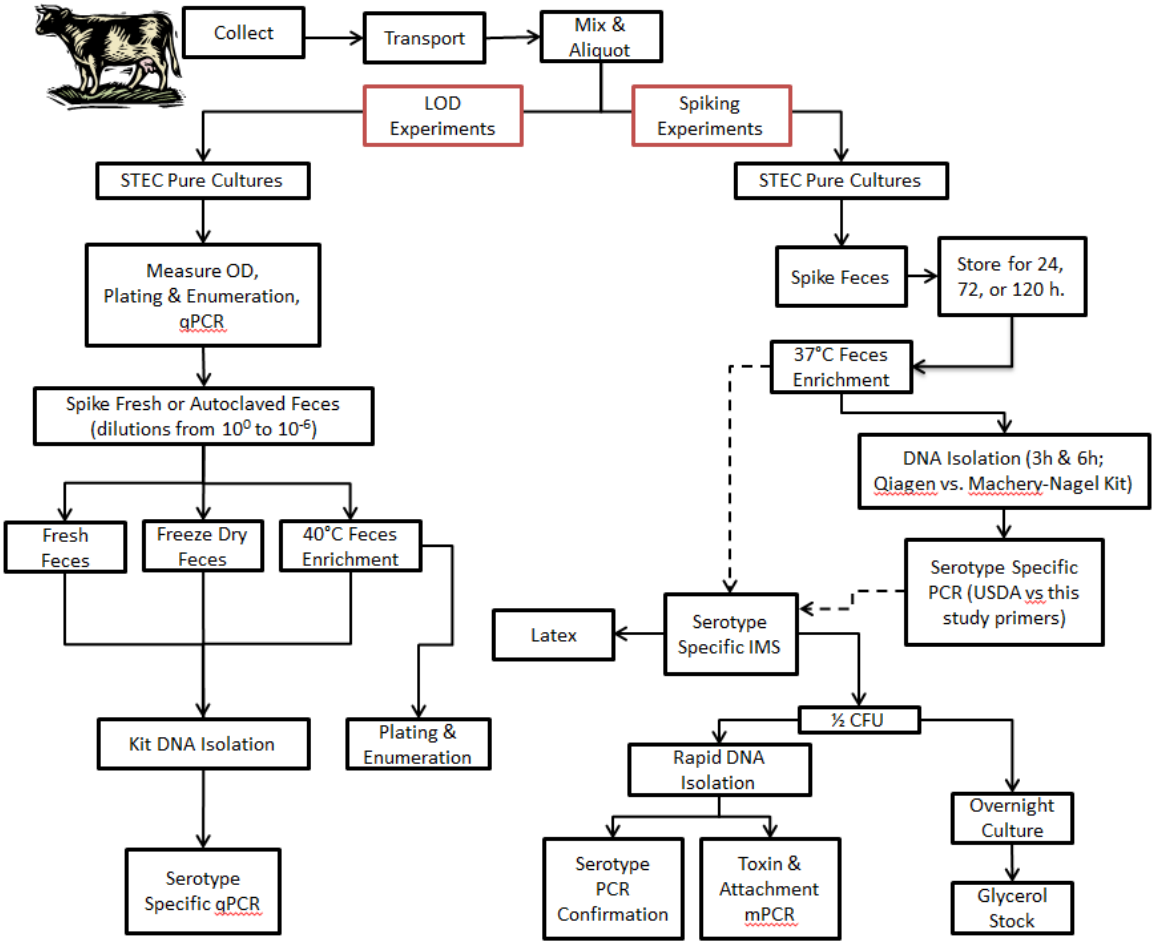

Figure 3.1. Schematic diagram of methodology used in this study. The limits of detection (LOD) experiments were completed with serotypes O157:H7 and O26:H11. The spiking experiments were completed with the seven regulated serogroups (O26, O45, O103, O111, O121, O145, and O157).

3.2.1.2 Comparison of fresh, freeze dried, and enriched feces for detection of STEC

Fresh feces were collected from a feedlot in southern Alberta. The feces were pooled from two pens containing 150 cattle fed finishing feedlot diet. In each pen, samples were taken from the center of fresh pats and pooled. A total of 7.5 kg of feces was transported on ice to the lab, where it was thoroughly mixed using a mixer (KitchenAid). Two kilograms of homogenized feces was autoclaved (a 45 min cycle, 121°C). Fresh and autoclaved feces were weighed into separate stomacher bags (20 g ± 1 g) and spiked with 2 mL of either O26:H11 or O157:H7 culture diluted over a range of 10^0 to 10^{-6}. Negative controls were also included (spiked with 2 mL of phosphate buffered

saline). Samples were homogenized for 2 min in a Stomacher 400 Circulator (Seward Laboratory Systems, USA). Spiked feces were split into three aliquots to compare efficiency of (A) DNA isolation from fresh feces, (B) DNA isolation from freeze-dried feces, and (C) DNA isolation from feces following enrichment. For DNA isolation from fresh feces, 200 mg (±5 mg) of feces was weighed into 2 mL microcentrifuge tubes and stored at -20°C until DNA extraction. For dried feces, ~4 g of feces was transferred to pre-weighed falcon tubes, frozen at -20°C, and freeze-dried in a VirTis Freezemobile 25XL (SP Industries, USA). Freeze dried samples were ball ground for 3 min at a frequency of 25 Hz using a Qiagen Tissue Lyser II (Qiagen), and DNA was extracted from 200 mg (±5 mg). For enrichment, 2 g (±0.01 g) of spiked feces was mixed with 36 mL EC broth in a 50 mL falcon tube. Enrichments were grown for 6 h with shaking at 90 RPM at 40°C in a Gyromax 939 waterbath (Amerex Instruments, USA). Each hour, 1 mL of enrichment suspension was centrifuged (15,900 x g, 5 min), the supernatant removed and the pellet frozen at -20°C for subsequent DNA extraction. Similarly, each hour 100 µL of enrichment suspension from autoclaved fecal samples was plated on either MAC (O26) or CT-SMAC (O157). Plates were incubated at 37°C overnight and enumerated. All DNA extractions were completed using the QIAamp DNA Stool Kit (Qiagen).

3.2.1.3 Quantitative polymerase chain reaction (qPCR)

Extracted DNA samples were quantified by PicoGreen using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific). Serogroups O26 and O157 were quantified by qPCR by detection of serogroup specific *wzx/wzy* gene fragments (O26 primers and probe designed by Lin *et al*., 2011; O157 primers and probe sequence are presented in Table 3.1).

Table 3.1. Primer and probe sequences for O-serogroup (*wzy*/*wzx* gene) and virulence genes designed in this study

		Sequence (5'-3')	Amplicon Size (bp)
Serogroup	O157	ggctgggaatgcatcggcct	1083
		tgtcagagcagcaccaagactgg	
	O145	gcgggtgttgcccgttctgt	766
		acggcattccgctgcgagtt	
	O121	ggttggatgggtggaacctt	595
		agcaagccaaaacactcaaca	
	O111	cgcaagacaaggcaaaacaga	450
		acaagagtgctctgggcttc	
	O103	atcttcttgcggctgcagtt	340
		aaaggcgcattagtgtctgc	
	O45	gatctgtggagccgagatgg	250
		tttgagacgagcctggcttt	
	O26	attgcagcgcctatttcagc	200
		attagaagcgcgttcatccct	
	O157	aggggttgtatgctcgttgt	121
		tggaacaccttcaacttgctct	
		ggacaagacggagaacaaaatgactca [TQ3~TF3][1]	
Gene	*repA*	agctgacacgtctttccctg	573
		gtctgaccgtctggtctgg	
	ehxA	ctgtgccattctctgagcca	481
		tctgggaaaagccggaacag	
	eae	ggacccggcacaagcataag	385
		ccacctgcagcaacaasagg	
	*Stx*2	actgtctgaaactgctcctgtg	307
		cgctgcagctgtattactttcc	
	*Stx*1	ggatgatctcagtgggcgtt	216
		gatgccattctggcaactcg	

Reaction mixtures contained 1X QuantiTect Multiplex PCR NoROX Master Mix (Qiagen), 0.4 µM each primer, 0.2 µM probe, 1 µL template DNA, and nuclease-free water in a final volume of 25 µL. Each qPCR was compared to standards containing 10 to 10^8 copies/µl and positive and negative controls in duplicate. Therefore, the limit of quantification was set at the lowest standard concentration of 10 copies/µl. Standards were prepared by PCR cloning as described previously [128]. Thermocycling conditions included an initial activation step of 95°C for 15 min, followed by 45 cycles at 94°C for 60 sec and at 60°C for 60 sec, and a final extension at 72°C for 5 min. A Mastercycler ep Realplex thermocycler (Eppendorf) was used for all qPCR. In addition, the fresh feces control samples were screened by PCR for 7 STEC serogroups using the primers designed by Lin *et al.* (2011).

3.2.2 Feces spiking with "big" six STEC comparing DNA extraction methods, length of refrigerated storage, and duration of enrichment

3.2.2.1 Sample preparation

Feces spiking experiments were completed in three separate trials (60 samples total). Approximately 2500 g of fresh cattle feces was collected from pen floors at the Lethbridge Research Center (Alberta, Canada) research feedlot. Feces were collected and homogenized as described previously (3.2.1.2), and subdivided into stomacher bags (90-100 g). Cultures of the STEC serotypes (O26:H11, O45:H2, O103:H2, O111:NM, O121:H19, O145:NM, and O157:H7) provided from the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, Canada, were inoculated into 10 mL EC broth from glycerol stocks, and incubated at 37°C overnight. Optical density (600 nm) of a 10^{-1} dilution of each overnight culture was measured and compared to the standard curves (3.2.1.1; Figure 3.2) for determination of approximate CFUs. The seven STEC cultures were prepared in mixtures, each containing 1 to 4 serotypes in 10 mL EC broth. All possible serotype combinations were included. Each serotype was present at final concentrations of 10^2, 10^3, or 10^5 CFU/mL. Stomacher bags containing 90 g feces were spiked with 10 mL of culture mixture and stomached at 230 RPM for 1 min. Samples were then stored at 4°C for 24, 72 or 120 h prior to further analysis. Following refrigeration, 270 mL EC broth was added to each stomacher bag and mixed at 230 RPM for 1 min. Then, 10 mL of each enrichment suspension was transferred to sterile culture tubes and incubated for 6 h at 37°C. After 3 and 6 h of enrichment, 1 mL of enrichment suspension was transferred into a microcentrifuge tube, and pelleted at 18,400 x g for 10 min. Supernatant was removed, and pellet was stored at -20°C until DNA extraction. Remaining enrichment suspensions and pure culture controls were stored at 4°C until completion of IMS (Part 3.2.2.3). DNA was extracted using either the QIAamp Stool Kit (Qiagen) or the NucleoSpin Tissue Kit (Machery-Nagel).

3.2.2.2 PCRs

Different PCR assays were compared including (A) screening for all serogroups in single reactions with primers designed by Lin et al. (2011), (B) single reactions for each serogroup using primers designed in this study, or (C) screening for 7 serogroups (O26, O45, O103, O111, O121, O145, O157) in a single multiplex PCR using primers designed in this study. Primers were designed using Geneious 5.5 (Biomatters Ltd.) and the sequences are given in Table 3.1. Single target PCRs contained 1X HotStarTaq Plus Master Mix (Qiagen), 0.2 µM each primer, 1X CoralLoad PCR buffer, 2 µL DNA template, and nuclease-free water for a final volume of 25 µL. Thermocycling conditions included an initial activation step of 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, at 60°C for 45 sec, at 72°C for 90 sec, and a final extension at 72°C for 5 min. Multiplex PCRs contained 50 nM of O121 primers, 40 nM of O157, O145, O111, and O103 primers, 25 nM of O26 and O45 primers, 1X QuantiFast master mix, 2 µL DNA template, and nuclease-free water in a final volume of 25 µL. Thermocycling conditions included an initial activation step at 95°C for 5 min, followed by 45 cycles at 95°C for 45 sec, and at 66°C for 60 sec. Each PCR contained positive and negative controls. Conventional PCRs were carried out on a Veriti™ Dx Thermal Cycler (Applied Biosystems). Conventional PCR products were run on 2% (w/v) agarose gels containing GelRed (Biotium) stain and TAE or sodium borate (SB) buffer [129].

3.2.2.3 IMS for colony isolation and confirmation

Immunomagnetic bead separation (IMS) was completed for the 6 non-O157 serogroups and O157 using RapidChek CONFIRM STEC IMS Kits (Romer Labs Technology Inc.) according to the manufacturer's protocol. IMS was completed for 40 of the 60 spiked feces samples. Isolated cells were confirmed by PCR to determine the serogroups targeted by IMS within each enriched sample. IMS was also tested for fecal samples stored at 4°C for 24 and 120 h prior to enrichment. An aliquot of 50 µL of bead-bacteria complex was plated on MAC and incubated at 37°C for 18-24 h. The

following day, 3-5 colonies were tested for the presence of O-antigen using serogroup-specific latex kits (Staten Serum Institute) (completed for serogroups O26, O103 and O111 only). For all serogroups, three colonies per plate were selected, labeled, and morphological characteristics (size, colour) were recorded. A fraction of each colony was suspended in 20 µL 1 X TE buffer (10mM Tris, pH 8.0) for rapid DNA isolation. The suspension was heated to 95°C for 5 min, and 2 µL was used as PCR template for confirmation of serogroup. For colonies confirmed positive for a target serogroup, the remainder of the colony was extracted from the plate, re-grown overnight in TSB (Tryptic Soy Broth) at 37 °C and glycerol stocks of the culture were made. Selecting targeted colonies was difficult due to co-isolation of non-target bacteria during IMS. Therefore, for each of the target non-O157 serogroups (O26, O45, O103, O111, O121, and O145) 11, 17, 11, 10, 38, and 5 plates whereas the number of plates corresponded to the number of fecal samples with a positive serogroup PCR result), respectively, were examined to confirm the presence of the target serogroup on each plate following the IMS procedure and incubation period. All colonies on a plate were suspended together in 750 µL of 1 X TE buffer. A 50 µL aliquot of total colony suspension was transferred into a microcentrifuge tube and further diluted with 100 µL 1 X TE. The mixture was vortexed and subjected to rapid DNA isolation. Serogroup-specific PCRs were then completed on each colony suspension sample to confirm the presence or absence of the targeted serogroup.

3.2.2.4 Application of serogroup and toxin screening methods on feces samples from commercial cattle

A total of ~400 g of feces were collected and pooled from the floors of four compartments within transport trailers after hauling cattle in southern Alberta on two separate occasions (n=67). Two to three samples were collected from trucks hauling cattle from the same location. Samples were pre-mixed by hand within the sampling bag, transported to the laboratory in a portable cooler and stored at 4°C overnight. The following day, samples were thoroughly mixed by hand a second

time and a subsample (15 g) was mixed with 135 mL EC broth in a stomacher bag at 230 RPM for 1 min. Part of the suspension (10 mL) was transferred to a sterile culture tube and enriched at 37°C for 6 h followed by DNA extraction using the NucleoSpin Tissue Kit (Macherey-Nagel). Serogroups were screened using the 7-serogroup mPCR, cells were isolated by IMS, and 3-5 individual colonies were confirmed by PCR after rapid DNA isolation from a fraction of one colony. Upon positive PCR confirmation, the remaining CFU was grown and glycerol stocks were prepared. Subsequently, virulence genes including Shiga toxins and pathogenicity island/locus of enterocyte effacement (LEE) related genes: *ehxA, eae, stx1*, and *stx2* (primers listed in table 3.1) were screened by mPCR. The plasmid replication initiation gene, *repA,* was selected for an internal PCR control as it is present in both LEE+ and LEE- strains. Final reaction mixtures contained 20 nM each primer, 1X QuantiFast master mix, nuclease-free water, and 2 µL DNA template in a 25 µL total reaction volume. Thermocycling conditions were as described above for multiplex PCRs (3.2.2.2).

3.3 Results

3.3.1 Determining limit of detection

3.3.1.1 Pure culture optical density, colony forming units, gene copy numbers

The OD, CFUs, and serogroup-specific DNA copy numbers were determined for pure cultures of O157:H7 and 26:H11 (Figure 3.2). Average OD ranged from 0.01 to 0.78 and 0.01 to 0.97 from 0 to 8 h for O157:H7 and O26:H11, respectively. Average CFU/mL for O157:H7 ranged from 3.6×10^6 at time 0 to 1.5×10^9 at 8 h. Average CFU/mL for O26:H11 ranged from 4.7×10^6 to 2.6×10^9 from 0 to 8 h, respectively. Over the 8 h, serogroup DNA copy numbers increased from 9.7×10^2 to 3.4×10^5 for O157:H7 and from 1.6×10^3 to 3.3×10^5 for O26:H11 per µl of extracted DNA. This equates to 9.7×10^5 to 3.4×10^8 copies and 1.6×10^5 to 3.3×10^8 copies per mL for O157:H7 and O26:H11,

respectively. Extracted DNA was quantified prior to PCR, and average DNA yield ranged from 2.2 x 10^2 to 7.2 x 10^2 ng/mL and 1.3 x 10^3 to 1.7 x 10^3 ng/mL from 2 to 8 h of growth for O157:H7 and O26:H11, respectively.

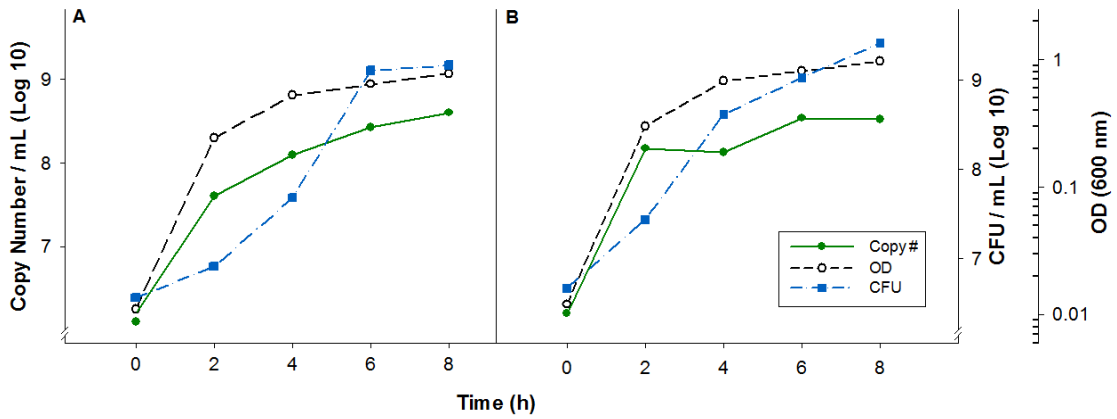

Figure 3.2. Optical density, colony forming units, and serogroup-specific DNA copy number from pure cultures (n=3) of *E. coli* O157:H7 (A) and O26:H11 (B) over an enrichment period of 6 h at 37°C.

3.3.1.2 Comparison of fresh, freeze dried, and enriched feces for detection of STEC by qPCR

The pooled fresh feces used in this study naturally contained 3 of the 6 non-O157 serogroups (O26, O45, O103) and O157 according to PCR using the Lin *et al*. (2011) primers (data not shown). Serogroup-specific DNA copy numbers for most serogroups increased by up to 4 $\log_{10}$ over the 6 h of enrichment or O157:H7 in both fresh and autoclaved feces (Figure 3.3).

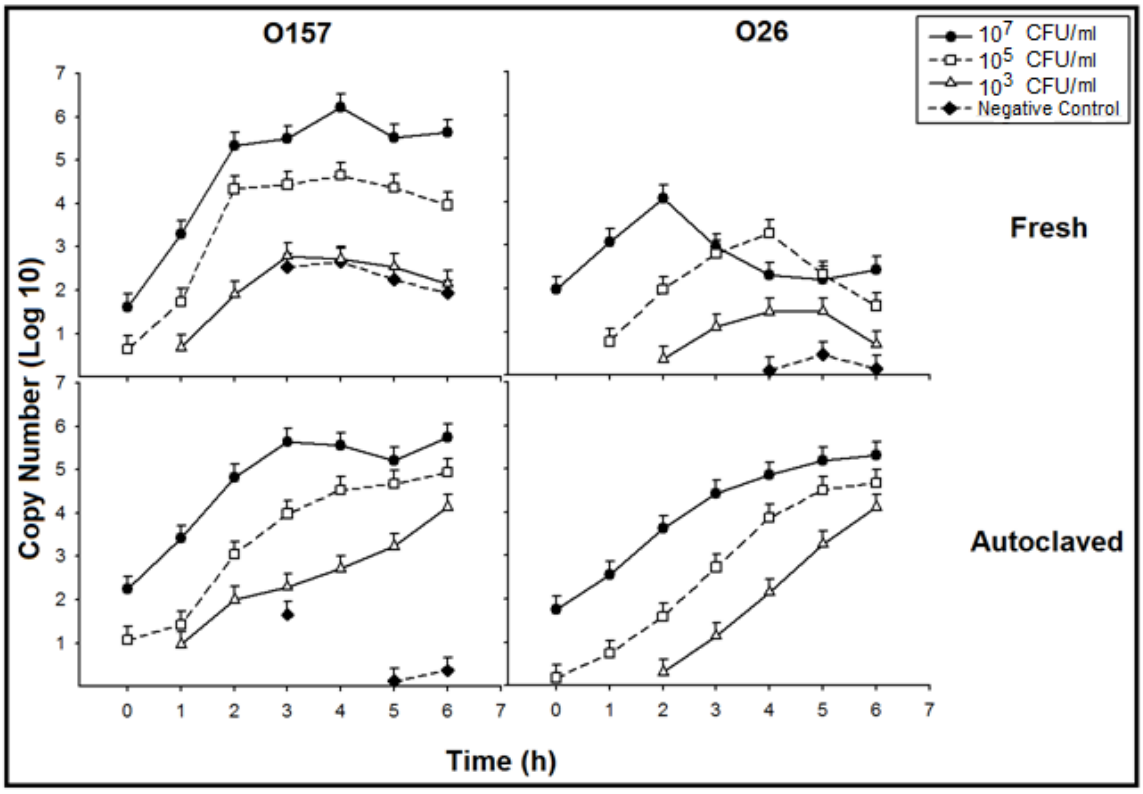

Figure 3.3. Serogroup-specific DNA copy number (per µL) of O157 and O26 spiked into fresh and autoclaved feces and enriched for 6 h. At time = 0, feces was inoculated with an aliquot of one of 7 concentrations ranging from 10^2 to 10^8 CFU/mL (3 representative concentrations are shown), resulting in 10^1 to 10^7 CFU/g feces respectively.

This trend was not seen for serotype O26:H11 in fresh feces. After 3 h of enrichment, numbers of this serotype were at 10 CFU/g feces, but remained below the limit of PCR quantification of 10 copies. After 6 h of enrichment, 10 CFU/g feces could be detected with confidence and was well above the limit of quantification (only three representative concentrations are shown in Figure 3.3; remaining data in Appendix I).

Freeze drying of feces prior to DNA isolation increased the sensitivity of STEC detection by PCR by 1 $\log_{10}$ compared to detection from fresh feces (Figure 3.4). The lowest concentration of cells detected, above the limit of quantification of 10 copies/µl, in fresh feces was spiked with an aliquot of 10^6 CFU/mL (resulting in 10^5 CFU/g feces). The lowest concentration of cells detected above 10

copies/µl in freeze dried feces was inoculated with an aliquot of 10^5 CFU/mL (resulting in 10^4 CFU/g feces). Below the limit of quantification of 10 copies/µl, feces inoculated with 10^5 CFU/mL (resulting in 10^4 CFU/g) and 10^4 CFU/mL (resulting in 10^3 CFU/g) could be detected in fresh and freeze dried feces respectively.

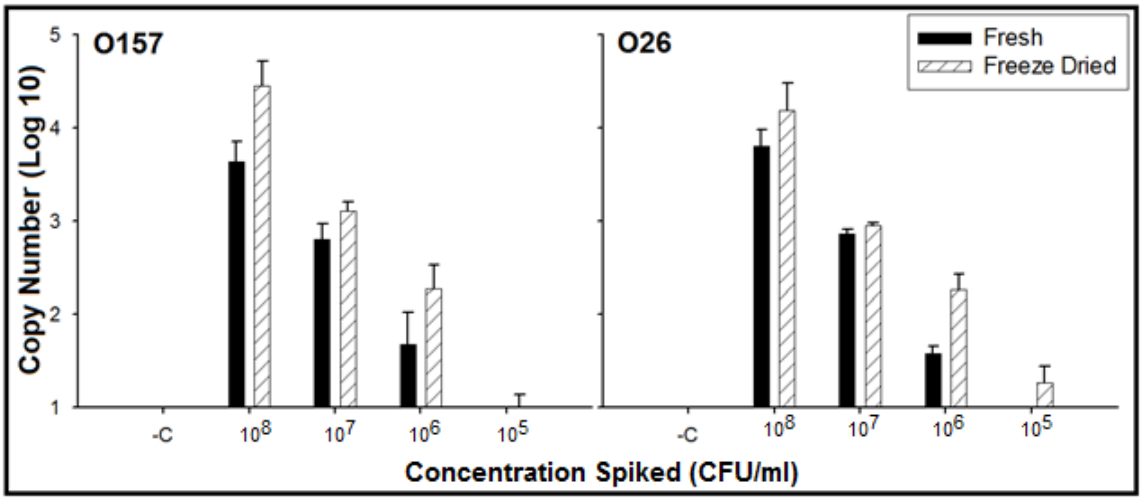

Figure 3.4. Serogroup-specific DNA copy number (per µL) in feces spiked with varying concentrations (10^2 to 10^8 CFU/mL; resulting in 10^1 to 10^7 CFU/g feces respectively) of O157 and O26 with or without freeze drying prior to DNA isolation. Concentrations below 10^5 CFU/ml were not detected. Negative control (-C) is shown.

3.3.2 Feces spiking with "big" six non-O157 STEC

3.3.2.1 Comparing DNA extraction methods, length of refrigerated storage, and length of enrichment

Comparing storage periods prior to enrichment ranging from 24 to 120 h, the correct identification of serogroups in spiked samples was most consistent with shorter storage times. Out of 42 spiked targets in 20 feces samples, 41 (98%) were correctly identified by PCR after 24 h, 32 (76%) after 72 h, and 21 (50%) after 120 h. Longer storage time resulted in reduced ability of PCR to identify serotypes added to feces. The recovery of serotypes by IMS was also less successful with samples that were incubated for a longer period of time. Serotypes were more consistently identified by PCR when DNA was extracted with the Machery-Nagel kit as compared to the Qiagen kit (results

not shown). Latex kits were found to be unable to confirm non-O157 O-serogroups (O26, O103, and O111) isolated from feces. Visually determining a positive latex result was difficult and subjective. Additionally, latex kits provided false positives (as compared to serogroup-specific PCR) for each serogroup tested (results not shown).

3.3.2.2 Comparison of PCR assays

The primers designed in this study were used in a mPCR assay to detect each serogroup (Figure 3.5). The corresponding amplicon sizes were 200, 250, 340, 450, 595, 766, and 1083 bp for serogroups O26, O45, O103, O111, O121, O145, and O157, respectively. Described PCR conditions resulted in the correct and specific target size for each serogroup both individually (data not shown) and in mixtures of genomic DNA containing multiple serogroups (Figure 3.5).

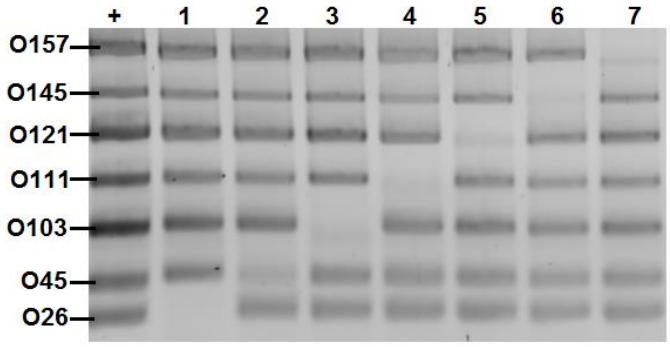

Figure 3.5. Amplification of seven DNA combinations (lanes 1-7) using a 7-serogroup multiplex PCR is shown. Each lane contains DNA from six STEC. Where a band is absent, DNA from that serogroup was not present in the DNA mixture. A positive control contained DNA from all seven STEC.

The accuracy of the primers described by Lin *et al*. (2011) for detection of the 6 regulated non-O157 serogroups in feces ranged from 30.4% to 100% (Table 3.2). In contrast, the primers designed in this study were 100% accurate at detection of all 6 non-O157 serogroups spiked into feces. Single PCR reactions using the Lin *et al*. (2011) primers for serogroups O45 and O111 were unable to detect cell concentrations below 10^4 CFU/g (Figure 3.6). Furthermore, the primers for serogroups O121 and O145 inconsistently detected cells below 10^4 CFU/g (not shown), while

serogroups O26 and O103 could be detected at 10 CFU/g. In contrast, the PCR assays using primers designed in this study consistently detected all serogroups at levels as low as 10 CFU/g in both single and multiplex reactions (Figure 3.6). Additionally, the O-serogroup mPCRs detected serogroups naturally present in feces whereas the Lin *et al*. primers did not (bands with no concentration indicated) (Figure 3.6). To further demonstrate that these serogroups were naturally present in the feces, cells were isolated from enrichment broths by IMS and confirmed by PCR.

Table 3.2. Comparison of PCR serogroup detection using primers described in this study versus those from Lin et al., 2011.

Assay	Spiked Samples	O111	O121	O145	O45	O26	O103
Lin *et al*., 2011	Positives	**7**/22	**13**/17	**13**/18	**7**/23	**16**/17	**14**/14
	% Accuracy	31.8	76.5	72.2	30.4	94.1	100
This study	Positives	**10**/10	**8**/8	**7**/7	**6**/6	**5**/5	**6**/6
	% Accuracy	100	100	100	100	100	100

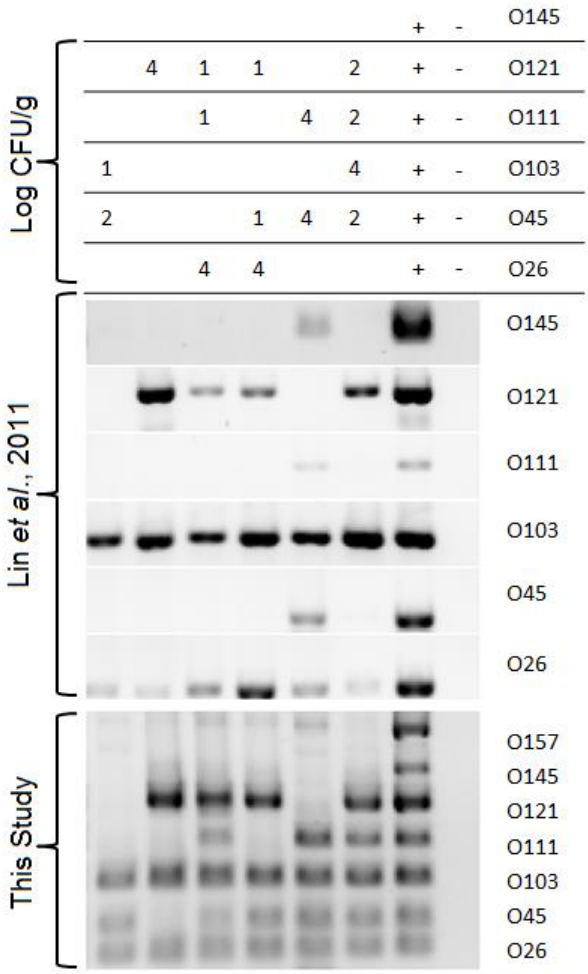

Figure 3.6. Comparison of Lin *et al*. (2011) primers with those designed in this study for serogroup detection in spiked feces samples. Concentrations (log CFU/g feces) of spiked serogroups is given in the table above corresponding to each sample lane in the gel images. Where no concentration is indicated, the sample was not spiked with the respective serogroup, and a positive PCR result indicates the serogroup was naturally present in the feces. Positive (+) and negative controls (-) are shown.

3.3.3 IMS for colony isolation and confirmation

3.3.3.1 Spiked feces

IMS was more effective on spiked samples than feces naturally containing target serogroups (confirmed by PCR) (Table 3.3). IMS isolated colonies from spiked feces were subsequently through PCR with of all serogroups being obtained. A total of 13, 16, 9, 16, 13, and 14 spiked samples, for serogroups O26, O45, O103, O111, O121, and O145, respectively, were subjected to IMS. At least 1

isolate was obtained from 67% (54) of spiked samples. Isolates of serogroup O103 were obtained from 100% of spiked samples, while O26, O145, O45, O111 and O121 were obtained from 92%, 71%, 69%, 44%, and 39% of spiked samples, respectively.

Table 3.3. Non-O157 isolates obtained by IMS from spiked and natural feces. Fecal samples were spiked with multiple serogroups. The numbers of isolates collected are indicated for each serogroup, as well as the number of samples (natural and spiked) from which isolates were obtained.

Feces	Number of	O26	O45	O103	O111	O121	O145
Spiked (n=40)	Samples Spiked	13	16	9	16	13	14
	Samples with ≥1 PCR-positive colony (%)	12 (92.3)	11 (68.8)	9 (100)	7 (43.8)	5 (38.5)	10 (71.4)
	PCR confirmed isolates collected	22	44	22	20	15	27
Natural (n=67)	Samples PCR Positive	66	65	52	11	45	17
	Samples with ≥1 PCR-positive colony (%)	14 (21.2)	16 (24.6)	5 (9.6)	1 (9.1)	4 (8.9)	1 (5.9)
	Presumptive positive isolates collected from IMS plates	19	20	9	2	6	1
	PCR positive plates for target serogroup after IMS / number of plates evaluated	11/11	15/17	10/11	2/10	19/38	3/5

3.3.3.2 Natural feces

A positive serogroup-PCR result was achieved from 66, 65, 52, 11, 45, and 17 samples, respectively, for serogroups O26, O45, O103, O111, O121, and O145 (for a total of 256 PCR-positives) (Table 3.3). Isolates were obtained from 16% (41) of these PCR-confirmed serogroup targets. Serogroups O26 and O45 were most frequently detected by mPCR and were subsequently most frequently isolated by IMS. However, by screening multiple colonies per sample, we collected at least 1 PCR-confirmed isolate per serogroup from the 67 fecal samples. This resulted in a total of 62 non-O157 isolates being obtained. Colony morphology (Appendix II) did not aid in serogroup

identification as the same serogroup was found to produce colonies of varying size and colour. To confirm that IMS was correctly retrieving target serogroups, single-serogroup PCRs were completed on DNA from the total suspension of all colonies on each plate following the IMS procedure and incubation period. The number of plates screened per serogroup was based on the number of positive fecal sample enrichments during initial PCR screening. Between 50-100% of plates were confirmed to contain the correct serogroup by PCR, except for O111, which was found on only 2 of 10 plates (Table 3.3). Of the 5 available plates following IMS for O145, 3 plates were PCR positive.

A total of 70 isolates, 57 non-O157 and 13 O157, were screened by PCR for toxin and pathogenicity island/LEE related genes (Table 3.4). Sixty-five isolates (93%) contained one or both Shiga toxin genes. *Stx1* was found in 80% of isolates and in order of frequency (highest to lowest) from serogroups O45 (16), O26 (12), O157 (11), O103 (8), O121 (6), O111 (2), and O145 (1). *Stx2* was present in 53% of isolates, serogroups O157 (10), O26 (9), O45 (7), O103 (6), O121 (3), O111 (1), and O145 (1). Of the non-O157 isolates, 26 (46%) possessed *stx1* alone (serogroups O26, O45, O103, O111, and O121), 9 (16%) contained *stx2* only (O26, O45, O103), and both toxin genes were present in 20 (35%) non-O157 isolates (all 6 serogroups). For O157, 9 (69%) possessed both *stx1* and *stx2*, 1 (8%) contained *stx1* alone, and 1 (8%) contained solely *stx2*. The remaining genes *eae* and *ehxA* were present in 96% (55/57) and 82% (47/57) of non-O157 isolates respectively, and 92% (12/13) and 85% (11/13) of O157 isolates.

Table 3.4. The number of serogroup isolates obtained from feces and the corresponding number of isolates confirmed by PCR to possess the virulence genes *stx1*, *stx2*, *eae*, and *ehxA* [PCR positive (+), negative (-)].

Serogroup	# Isolates	*Stx1*+ & *Stx2*+	*Stx1*+ & *Stx2*-	*Stx1*- & *Stx2*+	*Stx1*- & *Stx2*-	*eae* +	*ehxA* +
O26	19	4	8	5	2	18	12
O45	20	6	10	3	1	19	18
O103	9	5	3	1	0	9	9
O111	2	1	1	0	0	2	2
O121	6	3	3	0	0	6	5
O145	1	1	0	0	0	1	1
O157	13	9	1	1	2	12	11
Total	**70**	**29**	**26**	**10**	**5**	**67**	**58**

3.4 Discussion

In current assays, the detection and isolation of STEC most commonly involves enrichment, IMS, plating on selective or non-selective media for phenotypic identification, and latex agglutination with O-serogroup specific antiserum (primarily for O157) [93]. Molecular assays utilizing the sensitivity of PCR to screen for genes coding O-antigen synthesis, Shiga toxins, and flagellar gene targets are also often utilized [96]. Serogroup O157 can be distinguished from other serogroups based on its inability to ferment sorbitol [108]. Unlike O157, however, the other 6 non-O157 *E. coli* serogroups lack any unique phenotypic or biochemical characteristic, making the development of selective media challenging [5]. Several commercial and non-commercial chromogenic agars have been developed [93], but each appears to be with substantial limitations. For example, a media which only differentiates 4 (O26, O103, O111, O145) of the 6 non-O157 serogroups [103], or the inability to conclusively rely on colony colour since the morphology and phenotype may vary between strains of the same serogroup, and with proximity to background microflora colonies [94]. Similarly, an optimal enrichment medium for non-O157 serogroups has not been established. Therefore, culture-based detection methods for the top 6 non-O157 serogroups are time consuming, expensive, have logistical constraints when processing large numbers of samples, and may not be fully reliable due to limited sensitivity [96]. Consequently, a number of studies have adopted PCR,

conventional or real-time based approaches, to screen for potentially pathogenic serogroups by detection of O-antigen synthesis genes (*wzx*, *wzy*) [108]. It is important to note, that serogroup does not indicate pathogenicity, and further screening of virulence genes is required. Numerous primer sets and PCR assays exist, but with an increasing number of complete *E. coli* O-antigen gene cluster sequences (93 to date) available [93], more specific primers for PCR detection can be developed. Our results show that primer sets differ substantially in their ability to accurately detect low copy numbers of targeted sequences isolated from feces. We were able to use the same serogroup-specific gene targets as described by Lin *et al*. (2011) but modified the primer recognition sites, thereby increasing both the accuracy and sensitivity of the assay.

Initially, this study evaluated the advantages of different sample preparation approaches. The limits of detection after cell enrichment or freeze drying were compared in fresh feces for O157 and across non-O157 serotypes. Although up to 10^5 CFU/g could be detected in fresh feces, it has been proposed that as few as 10 cells are required to cause illness in humans [4]. Freeze drying increased sensitivity of detection by one $\log_{10}$, reducing the limit of detection to 10^4 CFU/g. Freeze drying provides some advantages including the need for fewer supplies and equipment, the ability to compare cell detection among samples based on the % dry matter content. One limitation however, is that individual isolates cannot be obtained from a freeze dried sample. Freeze drying of feces without enrichment would be sufficient for detection of supershedding cattle, releasing $\geq 10^4$ CFU/g feces, which may be responsible for the majority of pathogenic bacteria shed , even though they account for less than 10% of the cattle in a herd [130, 131]. The freeze drying method, however, would not be sensitive enough for detection of STEC in the majority of cattle, which often shed less than 10^2 CFU/g feces [63].

The most sensitive detection at $\leq$10 CFU/g, was achieved after 6 h enrichment as it increased bacterial growth and diluted potential PCR inhibitors in feces [96]. Additionally, length of sample storage and the yield, purity and integrity of DNA all impact assay sensitivity [110]. As our

results demonstrate, commercial DNA extraction kits have divergent isolation capabilities. For collection and source tracking of viable isolates by IMS, enrichment assays are typically required; however, certain serogroups may achieve more efficient growth and cell replication under enrichment conditions than others. We suspect this was the case when O26:H11 was spiked into fresh feces (Figure 3.3) with several other serogroups present (O26, O45, O103, and O157) and competing for vital resources. Autoclaved feces were used as a comparable matrix to fresh feces, but with the background microorganisms eliminated. Serogroup O26 cell-related copy numbers solely increased slightly in the presence of competitive microflora in fresh feces, followed by a decrease over the course of enrichment. This contrasts with autoclaved feces where copy numbers increased 3-4 log. Research data suggest, that certain *E. coli* strains naturally found in cattle feces can produce colicins (proteins that specifically target *E. coli*), and are effective at out-competing and displacing established *E. coli* O157:H7 in adult cattle [132]. We argue that serotype O26:H11 may have been out-competed by other, more vibrant fecal microflora under enrichment conditions. Such differential growth among serogroups during enrichment may lead to incorrect interpretation of data during food safety monitoring. Furthermore, quantitative PCR data following enrichment may not be representative of the original feces sample, making screening by less-costly conventional PCR methods appealing.

The efficiency of IMS kits relies on antibody specific binding capacity to retrieve target cells from enrichment broths, and may vary among serogroup and/or antibodies. For serogroups O26, O45, and O103, 88% or more of IMS plates contained the target serogroup confirmed by PCR from the total growth on the plate. Identifying a single positive colony, however, was often unsuccessful due to the absence of any distinct morphology. The overall success rate of identifying pathogens across all serogroups on IMS plates was 8%. After IMS, serogroup O121 was present on plates no more than 50% of the time. As only 5 plates for O145 were available during completion of this analysis, we cannot conclude the efficiency of this IMS assay. For serogroup O111 however, only 20%

(2 of 10) of the plates tested contained the target serogroup. This is consistent with the findings of Bai *et al*. (2012) who discovered O111 IMS beads did not yield any O111 colonies, but readily selected for O103 [69]. Accordingly, Paddock *et al*. (2012) reported two-thirds of PCR-positive samples were negative by culture based methods [96]. Small discrepancies between molecular and culture methods may be expected, since PCR can detect non-viable cells and viable cells may be unevenly distributed within a fecal sample. However, our results suggest that PCR based detection is sensitive and accurate while current non-O157 isolation methods such as IMS and latex kits do not have the specificity of those previously developed for O157. Furthermore, O111 is the second most prevalent non-O157 serogroup in cases of human illness [27], and therefore further refinements to isolation methods are required.

To date, no serogroup-based detection method exists that differentiates disease-causing STEC from harmless *E. coli*. Therefore, we designed an additional multiplex PCR to detect STEC virulence genes including Shiga toxins and pathogenicity island/locus of enterocyte effacement (LEE) related genes. In our study, we obtained a high number of STEC (65 of 70 isolates) in comparison to similar studies using other PCR assays [69, 96, 133]. Bai *et al*. (2012) reported that in a total of 27 non-O157 isolates, no *stx* genes were detected. Our feces samples were pooled rather than from individual animals which may have improved our chances of detecting target serogroups. The procedure used in this study for enrichment and isolation, combined with multiplex PCR screening appears to provide a more sensitive detection (10 CFU/g feces) assay compared to others, including the Bai *et al*., assay (10^2 CFU/g).

Since feces contain a number of additional *E. coli* strains and species of bacteria which potentially contain one or all of the genes *ehxA, eae, stx1* and *stx2*, we instead screened individual serogroup-confirmed colonies obtained by IMS to determine the potential pathogenicity of each isolate. A limitation of this approach is the lack of specificity of current IMS kits. However, food safety evaluation using mPCR for toxin/LEE related genes would be far superior for initial screening than

serogroup-based methods, since toxin genes are a more reliable indicator of pathogenic *E. coli.* The nature of horizontal gene transfer and exchange of mobile genetic elements in these pathogens allows for novel genetic configurations to arise, sometimes increasing pathogenicity regardless of serogroup. With over 100 *E. coli* serogroups, and 373 STEC serotypes available to exchange genetic material [57], the majority of emerging pathogens would go undetected using the current "big 6"/O157 serogroup-based assays, including O104, an enterohaemorrhagic *E. coli* with Shiga toxin and enteroaggregative *E. coli* genetic material responsible for the deadly 2011 outbreak in Germany [134]. Furthermore, serogroup-based initial screening is less conclusive for food safety evaluation because many strains of the "big 6" serogroups do not contain toxin/LEE related genes and are therefore harmless [118].

3.5 Conclusion

Advances in molecular biology and other technological progress hold the potential to improve the utility of detection assays for potentially pathogenic microorganisms within the food chain. Enhanced detection of STEC can decrease the market circulation of products compromising human health, aid in diagnostics, and support outbreak management. Our multiplex PCR assay to detect seven potentially pathogenic serogroups has been verified for a number of advantages over previous methods including greater sensitivity and specificity. Economically important, our method is less costly than quantitative assays and does not require qPCR equipment and probes. Combining a multiplex PCR in one assay decreases the costs even further. Present assays, however, do not target and/or unveil the principal genetic source which can induce disease. Although DNA-based detection methods are reliable, culture methods are needed for complete confirmation, but isolation of viable STEC is limited by the culturing methods available today.

CHAPTER IV: STEC with different toxin gene profiles compete during enrichment

4.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are human pathogens responsible for foodborne outbreaks and sporadic cases of illness throughout the world. Complications from STEC infection may result in diarrhoea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and death. National and international surveillance systems have been designed to track STEC infections and outbreaks in many countries [26]. Although, *E. coli* O157:H7 has been subjected to national reporting in Canada and the U.S. for nearly two decades, the most prevalent non-O157 serogroups (O26, O45, O103, O111, O121, and O145) have only recently gained attention [26]. Cattle are asymptomatic carriers of O157 and non-O157 STEC, transmitting the pathogens in their feces. Cattle feces can be the source of contamination of carcasses during meat processing, as well as vegetable crops during manure application. Therefore, improper food processing, handling, washing, and cooking procedures can all increase the risk of human STEC infection [18].

To determine if STEC are present, dilution plating methods are often used to isolate, identify, and describe pathogenic strains. As STEC may be present at low numbers within the total bacterial population, enrichment and selective media may be required for their isolation. Enrichment is necessary to increase the probability of identifying pathogens by increasing their numbers to a level that can be detected in both culture and molecular-based assays (at least 100 cells/g food or feces) [69, 96, 103]. Standard detection procedures and selective media have been developed for STEC serogroup O157; however these same methods lack specificity to differentiate non-O157 STEC. Although culture methods are required to isolate and confirm STEC contamination, there is no gold standard for the cultivation of non-O157 STEC. Isolation of relevant strains from complex matrices such as food or feces can be impaired due to high levels of background microbiota, growth inhibitors,

and a lack of specificity of selective media. Additionally, samples hosting multiple pathogenic strains complicate detection measures and a relevant strain may not be identified [5, 135]. Several enrichment protocols for STEC have been reported in the literature, each utilizing different combinations of media, supplements, incubation temperatures, and assessment times. So far, no enrichment media or protocol has been proven appropriate for all serogroups or sample types (e.g. food, feces, water) [2].

Enrichment culture results in competition between microbes for available nutrients [135]. Media are usually designed to favor target organisms, however conditions may not favor every serotype or serogroup equally, resulting in a culture bias [135]. Culture bias or culture fitness has been described between strains of the same species or subgroup for *Listeria monocytogenes* and *Salmonella* [135]. Studies using *Salmonella* strains have reported dissimilar recovery characteristics in enrichment media leading to isolation of certain strains over others [135, 136]. Similarly, variants naturally present in populations of *Escherichia coli* O157:H7 show major differences in stress resistance which can affect the phenotypes isolated after a 6 h enrichment [137].

It is unknown whether a culture bias arises during enrichment of STEC, which could result in certain strains being more (or less) likely to be isolated than others. Over 400 STEC serotypes have been isolated from cattle, with 12 - 17% of these also being associated with human illness [4, 34]. However, the inability to detect certain serotypes may result in an underestimation of human illnesses associated with non-O157 STEC. Additionally, a culture bias could also contribute to an underestimation of disease-associated serotypes if the pathogens are out-competed by other *E. coli* or non-*E. coli* bacteria during enrichment. Furthermore, *E. coli* are able to survive and grow outside their hosts, as documented by their growth and prolonged survival in water and soil [71, 73]. However, knowledge regarding the abiotic and biotic factors controlling their ability to survive, such as intra-species competition and production of colicins is limited [138]. Additionally, the principles

governing competitive growth of these bacteria are not well understood and controlled *in vitro* experiments are scarce.

Ongoing studies in our laboratory indicate that numerous STEC serogroups are usually found concurrently within pooled cattle feces following 6 h enrichment in EC broth. A previous study found that increasing the length of storage time prior to enrichment of fecal samples significantly decreased the number of serogroups detected (Chapter III). This indicates that the *E. coli* population in stored cattle feces, even without enrichment, is constantly changing. Additionally, in the same study, numbers of STEC O26 inoculated into fresh feces did not increase over the course of enrichment (as measured by qPCR estimate of copy number), suggesting that this strain lacks the ability to effectively compete with other bacteria in fecal samples during enrichment.

Many factors contribute to fitness in mixed culture enrichments including nutrient composition, culture conditions, and nature of competing microbiota [135]. Accordingly, the objectives of this study were to determine if serogroup- or strain-specific competition (culture bias) exists between STEC during controlled enrichment of pure cultures. Secondly, this study aimed to determine whether this competition changed over the course of 6 subsequent enrichments. Lastly, this study examined whether toxin gene profiles (Stx1, Stx2, or both), and presence of related virulence genes (*eae*, *ehxA*), influenced an STEC strain's ability to compete during enrichment.

4.2 Methods

4.2.1 Bacterial Cultures, Media, and Culture Conditions

All STEC isolates used in this study were obtained by collection and processing of cattle feces from transport trailers in southern Alberta as described previously (Chapter III). Isolates were selected for this study based on virulence gene profile (Table 4.1). Trial 1 isolates possessed both

Stx1 and Stx2, while trial 2 isolates had only one Stx gene. Non-O157 STEC were streaked from glycerol stocks onto MacConkey agar (MAC), and O157 isolates were streaked onto sorbitol MacConkey agar containing cefixime and tellurite (CT-SMAC; Dalynn Biologicals). Plates were incubated overnight (~16 h) at 37°C. A single colony was selected from each overnight plate and inoculated into 10 ml *E. coli* (EC) broth (EMD). Overnight broth cultures were incubated statically at 37°C. A flow chart representation of the methodology is given in Figure 4.1.

Table 4.1. STEC isolates used in this study and respective virulence gene profile

Serogroup	Trial	*Stx1*	*Stx2*	*eae*	*ehxA*	*repA*
O26	1	✓		✓	✓	✓
O26	2	✓		✓		✓
O45	1		✓	✓	✓	✓
O45	2	✓		✓	✓	✓
O103	1	✓	✓	✓	✓	
O103	2	✓		✓	✓	✓
O111	1	✓	✓	✓	✓	✓
O111	2	✓		✓	✓	✓
O121	1	✓	✓	✓	✓	✓
O121	2	✓		✓	✓	✓
O145	1	✓	✓	✓	✓	✓
O145	2	✓		✓	✓	✓
O157	1	✓	✓	✓	✓	✓
O157	2		✓	✓	✓	✓

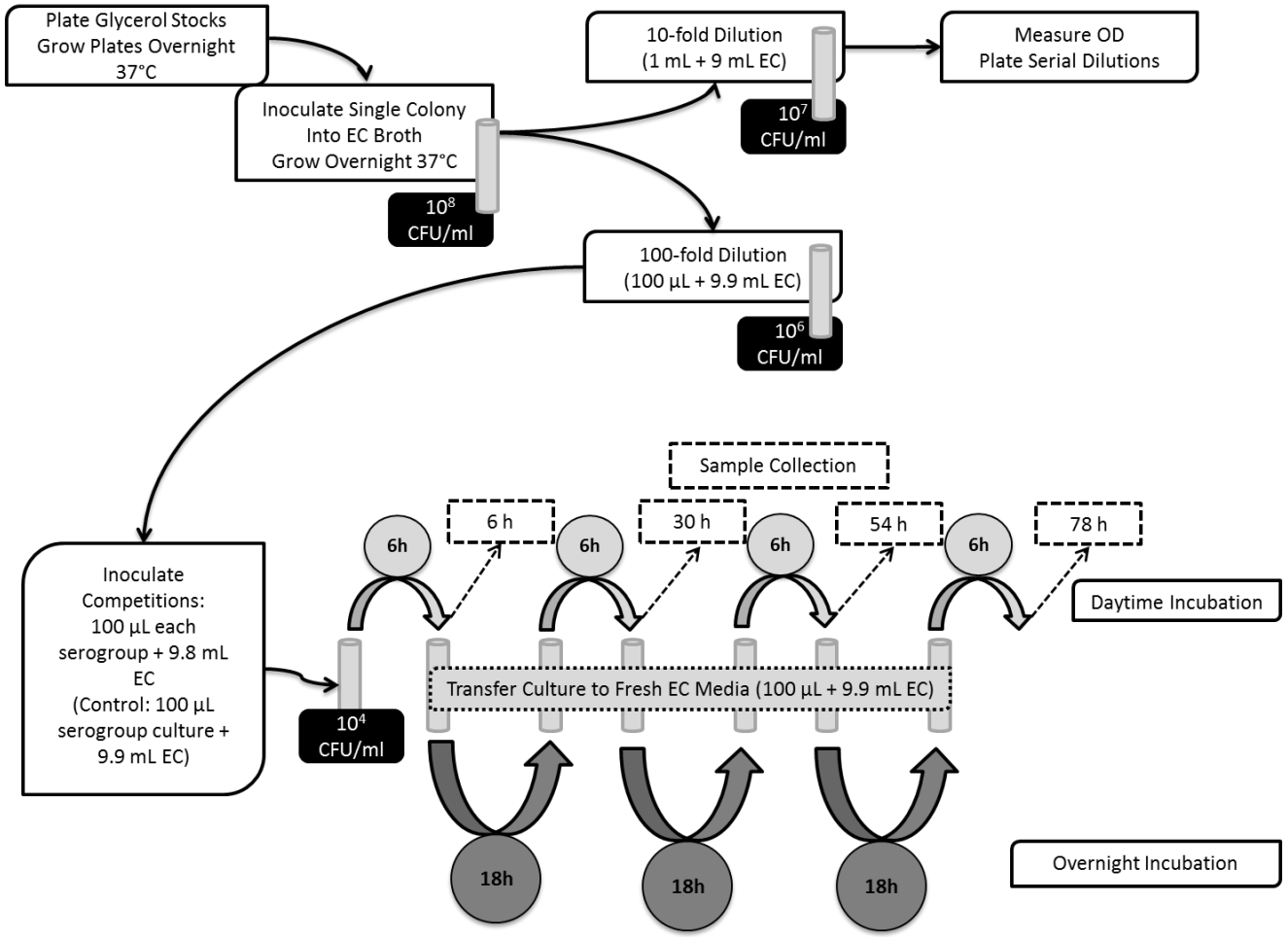

Figure 4.1. Schematic diagram of methodology used in this study. Competitions were completed in both 100% EC, and 50% diluted EC broth.

4.2.2 Dual-Serogroup Competitions and Preparation of Enrichment Cultures

In a Latin square design, each of the group 1 isolates (Table 4.1) was plotted against each of the other serogroup isolates within that group in two-serogroup competitions. Similarly, each isolate within group 2 competed against each of the other serogroup isolates within group 2. Finally, select isolates from group 1 and group 2 were assessed in trial 3. Every competition was completed in both EC broth and diluted (50%) EC broth (1:1 broth: deionized water).

Each overnight culture was diluted 10-fold in EC and optical density (OD_{600}) was measured photometrically using the Genesys 20 spectrophotometer (Thermo Scientific). Additional 10-fold serial dilutions were prepared in phosphate-buffered saline (PBS) and 100 µl of 10^{-5} and 10^{-6} dilutions were plated on CT-SMAC or MAC. Plates were incubated overnight at 37°C.

For inoculation of dual-serogroup competitions (Figure 4.1), each overnight culture was diluted 100-fold in EC (except for isolate O121-1 which had a lower OD, and was adjusted by 10-fold dilution prior to inoculation). Then, 100 µl of each diluted culture was added to 9.8 ml EC or 50% EC broth. For controls, 100 µl of diluted pure culture was added to 9.9 ml broth. Each competition (including pure-culture controls) contained 10^4 CFU/ml of each strain at time 0.

Cultures were incubated statically for 6 h at 37°C, at which time 1 ml of culture was removed. The 1 ml sample was centrifuged at 11,000 × g for 10 min. Supernatant was removed and cell pellets were stored at -20°C until DNA isolation. Extraction of DNA was completed using the NucleoSpin Tissue Kit (Macherey-Nagel) for the first set of competitions (group 1 isolates). For subsequent competitions, 200 µl of culture was removed, pelleted as above and supernatant removed. These cells were re-suspended in 50 µl 1 xTE (10mM Tris, pH 8.0) and heat-lysed at 95°C for 5 minutes in order to minimize costs related to extraction.

An additional 100 µl of the mixed 6 h culture was transferred to 9.9 ml fresh broth, and incubated statically overnight at 37°C (Figure 4.1). This process was repeated over four consecutive days, and three nights. The intermediate samples were taken after 30 h and 54 h of total enrichment time. The final sample was taken 78 h from the first inoculation, and after 7 alternating incubations of 6 h and ~18 h.

4.2.3 Detection of Serogroups by Quantitative PCR

E. coli were quantified by real-time polymerase chain reaction (qPCR) detection of serogroup specific *wzx/wzy* gene fragments (primer and probe sequences given in Table 4.2). Reaction mixtures contained 1X QuantiFast Multiplex PCR NoROX Master Mix (Qiagen), 0.5 µM each primer, 0.2 µM probe, 2 µL template DNA, and nuclease-free water in a final volume of 25 µL. Each qPCR was compared to standards containing 10 to 10^8 copies and positive and negative controls in duplicate. Standards were prepared by PCR cloning as described previously (Chapter II). Thermocycling conditions included an initial activation step at 95°C for 5 min, followed by 45 cycles at 95°C for 30 sec and at 60°C for 30 sec. A Mastercycler ep Realplex thermocycler (Eppendorf) was used for all qPCR.

4.2.4 Statistical analyses

Copy numbers were log transformed prior to analyses using the mixed model procedure of SAS, with serogroup, media (EC or diluted EC), repA profile of challenger and opponent and Stx gene profile of challenger and opponent as independent variables and isolate as a repeated variable. Competition “winners” were defined as the isolate with the higher serogroup-specific copy number by qPCR. Winners vs losers were then analyzed using the Glimmix procedure of SAS in a binary distribution with replicate as a random variable and isolate, serogroup, media, repA and Stx gene profiles as independent variables.

Table 4.2. Primer and probe sequences used in this study

Serogroup	Name[1]	Sequence (5'-3')	Amplicon Size (bp)
O26	O26-F-50	ATTGCAGCGCCTATTTCAGC	200
	O26-R-50	ATTAGAAGCGCGTTCATCCCT	
	O26-P-52	GTTGAAACACCCGTAATGGCCACT [TQ3~TF3]	
O45	O45-F-50	GATCTGTGGAGCCGAGATGG	250
	O45-R-50	TTTGAGACGAGCCTGGCTTT	
	O45-P-52	TGCTGCAAGTGGGCTGTCCA [TQ2~TF2]	
O103	O103-F-50	ATCTTCTTGCGGCTGCAGTT	190
	O103-R-52	ATGGGGGTGATTGGAGCGTT	
	O103-P-52	ACAGCTTGCCAATATCCGGCATCC [TQ3~TF3]	
O111	O111-F-50	CGCAAGACAAGGCAAAACAGA	243
	O111-R-52	ACTGGTGGCGTCTCACTTAGTT	
	O111-P-52	AGGCAAGGGACATAAGAAGCCAGT [TQ2~TF2]	
O121	O121-F-52	GGCATTCCTCAGTATCTTCTTGTTAAG	250
	O121-R-50	AGCAAGCCAAAACACTCAACA	
	O121-P[2]	TTAACACGGGCGTGGTTGGA [TQ3~TF3]	
O145	O145-F-51	GCGGGTGTTGCCCGTTCTGT	216
	O145-R-52	ACCTGGCATGCTCCCCTCCT	
	O145-P-52	ACCGCCTGGAGTTGGGGCTT [TQ2~TF2]	
O157	O157-F2	AGGGGTTGTATGCTCGTTGT	121
	O157-R2	TGGAACACCTTCAACTTGCTCT	
	O157-P	GGACAAGACGGAGAACAAAATGACTCA [TQ3~TF3]	

[1] Primers and probes were designed in this study except where indicated otherwise

[2]Lin *et al*., 2011 [108].

4.3 Results

All overnight cultures reached 10^8 CFU/ml. Optical densities for 10-fold dilutions of overnight pure cultures ranged from 0.104 to 0.158, with the exception of O121-1 (Table 4.3). When the glycerol stock of O121-1 was first plated, colonies appeared beige (indicating non-lactose fermenting). However, following the first overnight incubation in EC broth and re-plating of serial dilutions, approximately 15% of O121-1 colonies appeared pink indicating a shift toward lactose fermentation. PCR was used to confirm that both pink and beige colonies were of serogroup O121 and were not the result of contamination with a different serogroup.

Table 4.3. Optical densities (600 nm), CFU/ml (log_{10}), and copy numbers/ml (log_{10}) of STEC isolates used in each trial

Isolate	Trial	OD_{600}[1]	Log_{10} CFU/mL[2]	Log_{10} Copy Number/mL[2]
O26-1	1	0.119	7	7
O26-2	2	0.158	7	7
O26-2	3	0.134	7	7
O45-1	1	0.109	7	7
O45-1	3	0.111	7	7
O45-2	2	0.155	7	7
O103-1	1	0.124	7	7
O103-1	3	0.132	7	7
O103-2	2	0.138	7	7
O103-2	3	0.124	7	7
O111-1	1	0.114	7	7
O111-1	3	0.126	7	7
O111-2	2	0.150	7	7
O111-2	3	0.145	7	7
O121-1	1	0.028	6	6
O121-1	3	0.033	6	6
O121-2	2	0.135	7	7
O121-2	3	0.120	7	7
O145-1	1	0.115	7	7
O145-1	3	0.140	7	7
O145-2	2	0.134	7	7
O145-2	3	0.138	7	7
O157-1	1	0.104	7	7
O157-1	3	0.111	7	7
O157-2	2	0.131	7	7
O157-2	3	0.148	7	7

[1] OD was measured on 10-fold dilutions of overnight cultures

[2] Values are estimates based on standard curves generated in a previous study (Chapter III)

After the first 6 h incubation, all isolates reached between 10^6 - 10^7 copies per 2 µl extracted DNA (relation of copy number/ml to CFU/ml described in Chapter III) across all competitions, with the exception of O121-2 (Figure 4.2). Although the initial OD reading was similar to all other isolates, O121-2 reached 10^4 copies per 2 µl extracted DNA in all competitions (Figure 4.2), a difference of 2 $\log_{10}$ or more compared to all other serogroups.

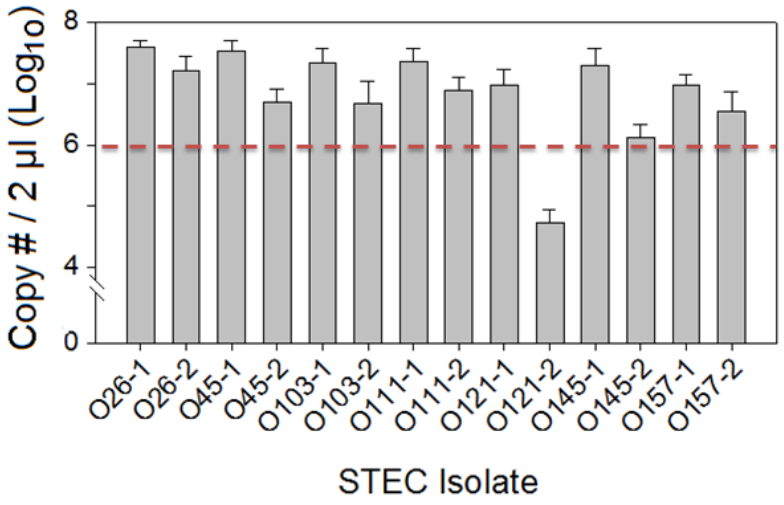

Figure 4.2. Average serogroup-specific DNA copy numbers of STEC following the first 6 h incubation of serogroup competitions (red dotted line indicates 10^6 copies per 2µl DNA).

There were 57 individual competitions in each media (114 in total; Figure 4.3). Differences in copy number greater than 1 $\log_{10}$ were apparent after 30 h of consecutive enrichments for some competitions. In most cases, these differences increased with subsequent enrichments (examined at 54 h and 78 h), diverging by more than 3 $\log_{10}$. For the remaining enrichments, the copy numbers of the two strains were similar with less than 1 $\log_{10}$ difference between 30 h to 78 h of incubation.

The winning isolates from each competition, after 78 h of sequential enrichments, for the three trials are given in Figure 4.3. The serogroup-specific copy numbers between competing isolates differed from less than 1 $\log_{10}$ to 8 $\log_{10}$ copies per 2 µl DNA.

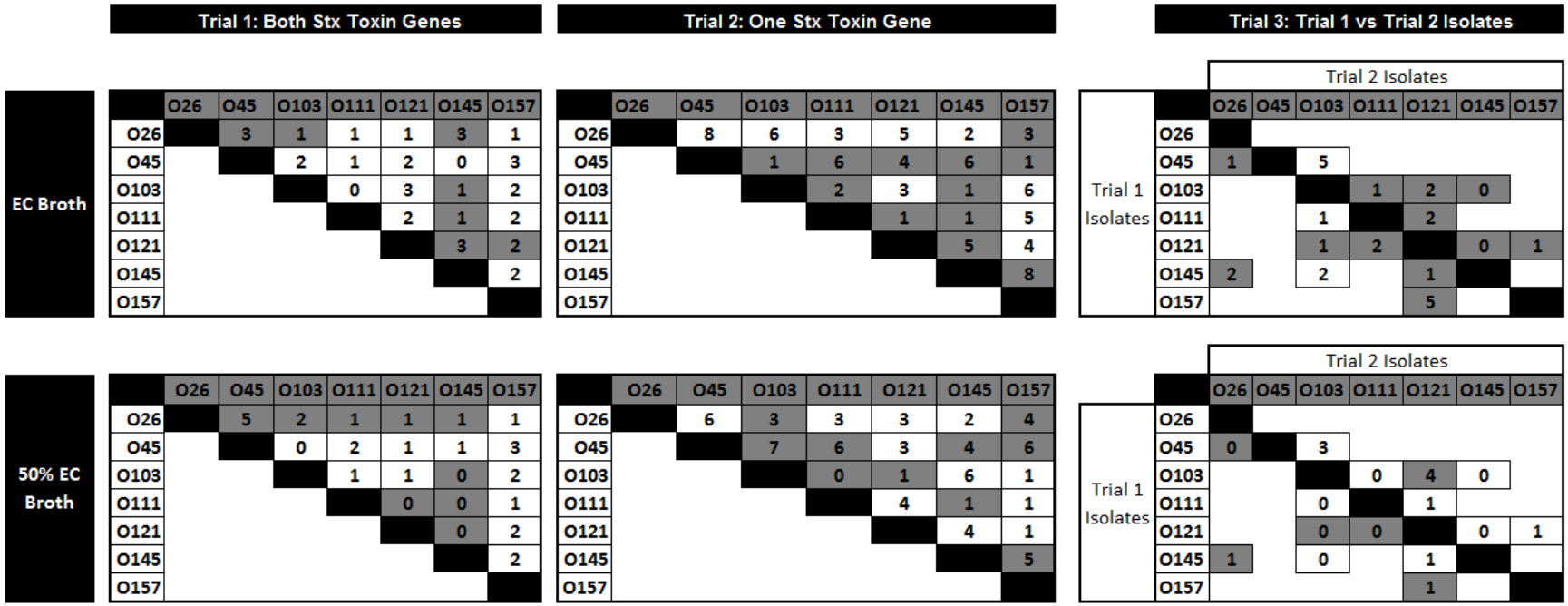

Figure 4.3. Competition matrices for trials 1, 2, and 3 after 78 h of alternating day and night enrichments. The dominant serogroup isolate in each competition is colour coated as either: grey, if the "winning" serogroup is listed above (horizontally) or white, if the "winning" serogroup is listed to the left (vertically). For trial 3, all of the trial 1 isolates are white (vertical list), and the trial 2 isolates are grey (horizontal list). The numbers indicate the log difference (e.g. 2 = 10^2) between the serogroup-specific DNA copy numbers within each competition. Zero (0) indicates less than one $\log_{10}$ difference between the copy numbers of the two isolates.

For the majority of competitions, the winning strain was the same in both concentrated and diluted media. In cases where the dominant serogroup differed between media, one of the serogroups was often O121. However, O121-1 and O121-2 dominance differed depending on the type of media. The isolates with the highest number of competitions won (as percentage) were O45-1, O26-2, and O145-1 (Figure 4.4). However, only O45-1 and O26-2 out competed O26-1, O121-1, O45-2, and O157-1 (P <0.05). Serogroup *per se* did not influence the outcome of competitions. Rather, the number of competitions won was only significantly impacted by the individual isolate (P <0.01).

For trial 1, isolate won nearly the same percentage of competitions irrespective of media, with the exception of O121-1 which won 60% more competitions in diluted EC broth (Fig 4.4 A). Of trial 1 isolates, O157-1 performed the poorest, winning less than 20% of competitions. For trial 2, isolate O26-2 won the greatest number of competitions, and O45-2 won the least (Figure 4.4 B). The percentage of competitions won was more variable between the two media for trial 2.

Shiga toxin profile of isolates had a significant impact on copy number (P <0.01), while serogroup, media, and other virulence genes had no significant impact in all trials. Isolates possessing solely Stx1 competing against isolates with solely Stx2 had significantly lower copy number than Stx2 isolates competed against Stx1 ($P<0.01$). If both isolates shared similar Stx gene profiles (e.g. Stx1 vs Stx1, Stx2 vs Stx2, both toxin genes vs both toxin genes), copy numbers for each were superior to Stx1 matched against Stx2 only isolates ($P< 0.001$), but did not significantly differ from copy numbers when other combinations of Stx genes were present (e.g. Stx1 vs both, Stx2 vs both). Isolates with solely Stx1 won 80% of competitions against isolates with both toxin genes (trial 3) in EC broth, while isolates with both toxin genes won 60% of competitions against Stx1 isolates in the diluted EC broth (Figure 4.4 C). Two of the isolates contained Stx2 only: O45-1 and O157-2. The O45-1 isolate won 100% of competitions in both media when competing against isolates with both Shiga toxin genes.

However, when O157-2 competed against isolates with Stx1 only, it won 50% of competitions in both media (Figure 4.4 D).

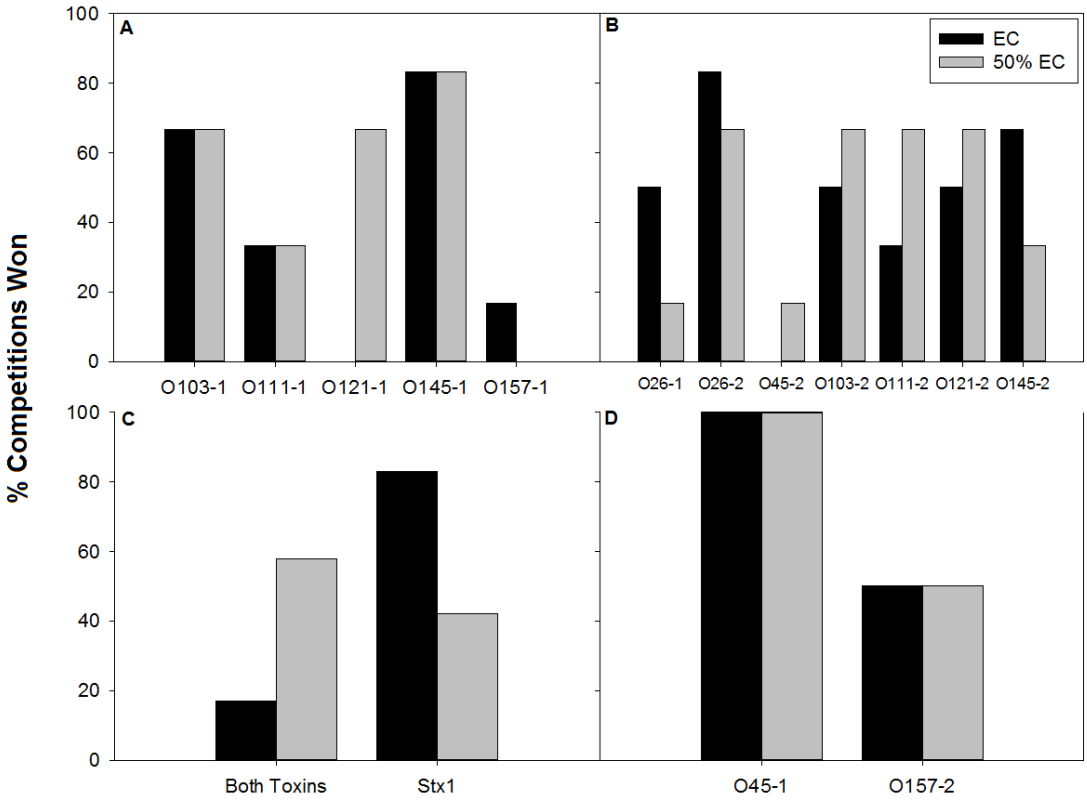

Figure 4.4. Percent of competitions won in each media for **A**) trial 1 isolates (mostly both toxin genes), **B**) trial 2 isolates (mostly only Stx1 genes), **C**) trial 3 (trial 1 vs trial 2 isolates), **D**) isolates with only Stx2 (O45-1 versus trial 1 isolates, O157-2 versus trial 2 isolates).

4.4 Discussion

Enrichment is often an essential step during isolation of STEC in cases of disease outbreaks and during routine STEC monitoring to increase the threshold of detection. However, no standardized enrichment medium or protocol has been developed for the detection of non-O157 STEC [97]. Among current protocols, various broths and enrichment procedures have been reported. Enrichment protocols typically utilize temperatures between 35°C and 42°C, incubation durations of 6 to 24 h [97, 98]. While higher enrichment temperatures may be more selective for some STEC

strains, longer incubations are sometimes applied simply for practical reasons in the laboratory such as allowing incubations to run overnight to avoid removing cultures form the incubator during non-typical work hours [98]. Frequently utilized broths include *E. coli* (EC) broth, trypticase™ soy broth (TSB), and buffered peptone water (BPW). Any enrichment should provide a balance between recovery of the desired organism while avoiding the overgrowth of competing organisms [135]. Consequently, enrichment broths are frequently modified by the addition of antibiotics to suppress the growth of background microflora. However, O157 and non-O157 STEC are a diverse group, and the addition of antibiotics has been shown to inhibit the growth of some STEC strains [97, 99].

Whether different enrichment protocols selectively promote growth of certain STEC strains over others (produce an enrichment bias) has been largely unexplored. Reports of detected prevalence of STEC (O157 and non-O157 serogroups) in cattle feces have ranged from 0.2% to 70% [34, 57]. Such variance could be due to differences in climate, ecology, farming practice, and variation in sampling [57]. However, such differences could also, in part, be a reflection of culture biases in the enrichment methodologies used to isolate these pathogens. Similarly, only a small percentage of STEC serotypes found in cattle have been associated with human disease, which could be reflective of low screening diligence for non-O157 serogroups. However, enrichment bias during screening could also potentially influence the successful isolation of some non-O157 serotypes.

Strain-specific differences in enrichment cultures have been reported for a number of bacterial species. Different versions of a selective medium for *Salmonella* gave dissimilar patterns of strain dominance in both pure culture and fecal enrichment cultures [135]. No serotype was most fit, but strains of certain serogroups were more likely to dominate than others. In addition, the probability of detecting a specific strain of *Salmonella* was not related to the starting concentration prior to enrichment [135]. Another study found a greater diversity of alleles involved in catabolic pathways in populations of soil bacteria by direct plating compared to enrichment [139].

Enrichment severely distorts the apparent diversity or taxonomic profile within populations due to culturing biases [139, 140]. Similarly, three agar media (Rainbow, Chromagar, and Blood agar) were compared for recovery of STEC from over 4,000 enrichments of a variety of sample types (feces, plant, soil, and water). Out of 880 positive samples, no more than 56 were positive on all three media [141]. Additionally, 377, 112, and 110 samples were positive on only one of the three media, suggesting a potential culture bias [141]. Utilization of multiple enrichment media has been suggested to aid in detection and isolation of target *Salmonella* strains [135], and could also be an effective approach for identifying and isolating non-O157 STEC.

The present study utilized one enrichment medium to examine potential intra-species competition that could bias STEC serogroup detection following enrichment. The duration of a 6 h enrichment as described in this study did not produce a significant culture bias from intra-species competition. However, this is a simplified competition compared to that which would likely occur during enrichment of complex samples such as food and feces. Following enrichment of complex samples, the distribution of strains could change depending on the nature of the microbiota present, production of bacteriocins (colicins), presence of bacteriophage, differences in relative growth rates, and presence of growth inhibitors [140, 142, 143]. From the one enrichment medium examined in this study, increasing the length of enrichment from 6 h (or use of multiple enrichment steps) could alter the STEC serogroups detected due to intra-species competition. Furthermore, it is assumed that laboratory-cultured bacteria behave like those in nature, however, bacterial strains have been reported to rapidly mutate during laboratory culture such as the O121 in the present study which switched to lactose fermentation. Therefore, to decrease the likelihood of laboratory domestication, which could also produce a biased culture, shorter and fewer incubations are recommended [144]. Enrichment protocols other than the one in this study (different media, duration, and/or temperatures) would likely change the dominance order of STEC from the current study.

Additionally, different concentrations and combinations of multiple strains (more than two) should be examined to more realistically reflect the natural variation present in feces.

Competitive trends were examined between STEC strains belonging to serogroups O157 and the top 6 non-O157. The results within our limited test group of 14 strains belonging to the 7 serogroups do not suggest that any of the "Top 7" serogroups would dominate other serogroups during enrichment. Conversely, our results indicate that intra-species STEC competition is strain-specific. Furthermore, the initial 6 h enrichment did not produce significant differences between copy numbers of the competing strains within the same medium (diluted or undiluted), suggesting that intra-species competition was not revealed in this time frame although with subsequent enrichments (30, 54, and 78 h) some produced biased cultures.

The individual toxin gene profile may have impacted the ability of STEC strains to grow together in the same medium. Overall, strains possessing one Stx toxin gene won a greater number of competitions compared to those containing both toxin genes. The majority of single toxin isolates contained Stx1 only; however two isolates possessed solely Stx2, with one of these isolates winning 100% of its competitions. In undiluted enrichment broth, isolates with solely Stx1 won the greater number of competitions compared to isolates with both Stx genes. However, when the nutrients within the media were diluted by 50%, isolates with both toxin genes won nearly 20% more competitions than Stx1-only isolates. This suggests that STEC strains may differ in their competitive ability when nutrient concentrations are limited, although statistical analyses did not show a significant influence of media, likely as models which included isolate by media interactions failed to converge due to lack of replication.

The mechanisms and evolutionary forces governing the emergence of new STEC types are not well understood, despite substantial efforts. Phylogenetic analyses have revealed that the gain and loss of virulence elements has occurred several times and in parallel in separate lineages [122].

Such convergent evolution suggests the accumulation of specific combinations of virulence factors offers a selective advantage to the bacterium [122]. Competitive advantages of *E. coli* possessing Shiga toxin genes have been reported. As one study demonstrates, Shiga toxins may act as a bacterial defense against Eukaryotic predators [145]. Shiga toxins have also been implicated in the promotion of adherence and colonization of O157:H7 to human intestinal epithelial cells in comparison to Stx-negative strains [146]. However, selective advantages are more likely to operate in the bovine reservoir or the external environment than in humans since they are considered 'accidental hosts' and STEC/EHEC lineages are unlikely to have evolved specifically to cause infections in humans [122].

Further studies are necessary to fully determine the influence of nutrient concentration on competitive ability of STEC with different Stx gene profiles. These studies should include comparisons with Stx-negative strains also. A review of 90 STEC outbreaks from around the world, occurring between 1982 and 2006, found the vehicle of transmission was unknown in nearly 30% of outbreaks, suggesting that traceability of these pathogens possess limitations [53]. One such limitation could be a culture bias during enrichment procedures. Although this study demonstrates that 6 h enrichment in EC broth does not produce a biased culture for the STEC evaluated, no conclusions can be drawn as to whether or not a stacked and/or exponential enrichment bias occurs within samples until the time of analysis (including enrichment). Future studies should examine STEC culture bias during enrichment of environmental samples (food, feces), each with diverse native microbiota, spiked with known concentrations of additional STEC. Additionally, future studies should compare different enrichment media, since no standard media exist. The U.S. Department of Agriculture Food Safety and Inspection service (USDA-FSIS) and U.S. Food and Drug Administration (FDA) procedures utilize TSB and BPW, respectively. STEC enrichment bias using these broths has not been examined; however production of such a bias would critically influence trace-back investigations. Such bias during outbreak investigations could have several outcomes, including the relevant pathogen being

missed and the transmission vehicle being unidentified, or the outbreak being attributed to the wrong pathogen/strain due to competition during enrichment. If the organism responsible for an outbreak is unknown, enrichment could greatly hinder the ability to identify the pathogen [140]. With continued emergence of novel pathogens, advances in STEC detection protocols can potentially improve food safety monitoring systems from farm-to-fork, decreasing circulation of compromised food products, minimizing risks to human health.

4.5 Conclusion

Currently, knowledge regarding enrichment bias during detection and isolation of STEC serogroups is limited. Research is needed to determine the influence of enrichment procedures on detection and enumeration, and outbreak investigations for pathogenic *E. coli* from fecal samples. Additional research may aid in development of an improved selective media for STEC, which could standardize enrichment procedures, and minimize errors during pathogen screening. Results of this study suggest the described enrichment protocol within a confined 6 h timeframe does not produce an intra-species culture bias for the STEC tested. Further development of enrichment assays is crucial to evaluate other possible competition (background microflora, bacteriophage, growth inhibitors) for downstream diagnostics.

CHAPTER V: Summary & General Conclusions

Shiga toxin-producing *E. coli* can cause human illnesses resulting in life threatening complications. A great number of these illnesses can be linked to cattle and their edible products, which has led the USDA to implement screening regulations for raw beef. As a result, fast and reliable detection protocols for these pathogens are required to maintain food safety and decrease circulation of contaminated food products. However, screening for STEC, particularly in feces, has many inherent limitations. The objectives of this study were to further develop detection methods for STEC in cattle feces. First, a simple and rapid method to design synthetic PCR controls for STEC serogroups was developed, without requiring natural template DNA from the target organism. Second, this study describes a protocol that greatly improves the limit of detection for these pathogens, which includes enrichment and multiplex PCR. Development of primers with greater specificity to target DNA enhanced the sensitivity to 10 CFU/g feces and accuracy of PCR screening for the top 7 STEC by 1 $\log_{10}$ as compared to a similar assay [69]. Furthermore, multiplex assays for STEC serogroups and toxin and related virulence genes were designed, decreasing costs and the time required to obtain results. Lastly, competition was examined in dual-serogroup enrichments, demonstrating that 6 h enrichment does not produce an intra-species culture bias.

Limitations for detection and isolation of STEC still remain. First of all, fundamental components of detection methodologies are enrichment and plating. Typically, recovery of viable colonies of target STEC is a requirement for food safety screening and outbreak investigations. However, no standard media exist for this diverse group of pathogens. A robust and credible enrichment medium for detection of multiple STEC serogroups, and isolation from different matrices (food, feces, etc.) would be valuable. Enrichment is an imperative screening component since these pathogens are often present at concentrations below the level of detection for current methods. Further refinements of antibody-based assays are crucial, since substantial cross reactivity between serogroups have been reported which limits the isolation of target cells.

Although the aforementioned developments to STEC detection and isolation would significantly improve current methods, another restriction still remains: serogroup does not indicate pathogenicity. Even with enhanced methods for detection of serogroups, there is no differentiation between pathogenic and non-pathogenic strains of the same O-type as isolated colonies have to be screened for virulence genes. Additionally, this diverse group of pathogens is always evolving, exchanging genetic material, and resulting in novel STEC with O-serogroup antigens not commonly associated with human infections (e.g. O104:H4 Germany outbreak). Therefore, solely screening for a fixed number of common O-types could lead emerging pathogens to potentially go undetected. The most significant contribution to current detection methods would be the development of a rapid, sensitive, and high-throughput approach that differentiates pathogens from non-pathogens of the same serogroup. Such novel technology would allow rapid detection of emerging pathogens regardless of serogroup. Technologies are always improving, increasing in sensitivity, speed, and accuracy. Droplet digital PCR, for example, has a much lower limit of detection than qPCR (1 copy/µl). Such technologies could potentially be incorporated to existing protocols to further improve STEC detection.

In conclusion, the assays developed in this study are rapid, sensitive, and specific methods for screening for the top 7 STEC serogroups in bovine feces. Still, without enrichment, no technologies to date can detect STEC within typical ratios of target to analyte due to lack of sensitivity. Enhanced detection of STEC can continue to maintain food safety by decreasing circulation of products compromising human health, aid in diagnostics, and support outbreak management.

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APPENDIX I

O157 and O26 serogroup-specific DNA copy numbers. Serogroup-specific DNA copy number (per µL) of O157 and O26 spiked into fresh and autoclaved feces and enriched for 6 h. At time = 0, feces was inoculated with an aliquot of one of 7 concentrations ranging from 10^2 to 10^8 CFU/mL (four concentrations are shown), resulting in 10^1 to 10^7 CFU/g feces respectively.

		Fresh Feces				Autoclaved Feces			
		Inoculum (CFU/mL)				Inoculum (CFU/mL)			
	Time (h)	10^8	10^6	10^4	10^2	10^8	10^6	10^4	10^2
O26	0	6.27E+02	8.89E+00	9.50E-01	0.00E+00	2.93E+02	5.46E+00	1.70E-01	0.00E+00
	1	4.93E+03	8.54E+01	2.85E+00	0.00E+00	2.83E+03	5.33E+01	6.30E-01	0.00E+00
	2	1.28E+04	1.19E+03	1.59E+01	0.00E+00	2.21E+04	5.82E+02	6.37E+00	0.00E+00
	3	6.93E+02	4.21E+03	1.11E+02	2.60E+00	5.91E+04	5.12E+03	7.78E+01	1.82E+00
	4	3.08E+02	3.70E+02	3.09E+02	3.38E+00	1.82E+05	3.48E+04	1.03E+03	7.20E+00
	5	2.94E+02	7.32E+01	4.60E+02	4.00E+00	1.75E+05	7.33E+04	9.40E+03	1.10E+02
	6	1.01E+03	6.92E+01	4.03E+01	5.90E+00	1.67E+05	1.07E+05	2.51E+04	1.10E+03
O157	0	2.12E+02	9.61E+00	4.66E+01	1.15E+00	2.29E+03	5.00E+00	0.00E+00	3.00E-01
	1	1.22E+03	3.42E+02	0.00E+00	1.90E+00	1.11E+04	1.90E+02	3.10E+00	0.00E+00
	2	2.21E+05	2.19E+04	2.33E+02	9.04E+01	2.43E+05	1.00E+04	2.51E+02	0.00E+00
	3	1.71E+06	2.01E+05	2.09E+03	4.42E+02	8.19E+05	5.40E+04	1.52E+03	1.20E+02
	4	5.03E+06	3.82E+05	4.65E+03	2.92E+02	9.23E+05	1.90E+05	8.57E+03	1.75E+02
	5	4.91E+05	1.52E+05	2.08E+03	1.63E+02	5.81E+05	1.03E+05	8.53E+03	6.70E+01
	6	7.31E+05	1.32E+05	8.17E+02	1.36E+02	6.94E+05	2.56E+05	5.96E+04	8.98E+02

APPENDIX II

STEC CFU morphology. Morphology of CFUs screened by PCR following IMS for each target serogroup. Serogroup confirmed CFUs (by PCR) indicated in bold out of the total number of CFUs tested with that morphology.

	Serogroup					
Morphology	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Pink, Small[1]	**2**/27	**10**/96	**4**/35	**1**/17	**2**/44	0/14
Pink, Medium[2]	**10**/82	**5**/19	**4**/42	0/17	0/45	**1**/27
Pink, Large[3]	na	0/5	0/7	**1**/1	na	na
Clear, Small[1]	na	**1**/16	**1**/19	na	na	na
Clear, Medium[2]	na	**4**/16	0/13	0/1	**2**/4	na
Beige, Small[1]	**2**/20	0/31	0/11	0/9	**1**/8	na
Beige, Medium[2]	**5**/68	0/4	0/9	0/6	**1**/19	0/10
Beige, Large[3]	na	na	0/1	na	na	na

[1] Small: ~1-2 mm in diameter
[2] Medium: ~3 mm in diameter
[3] Large: ≥4 mm in diameter
na: not applicable. No colonies with that morphology.