SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* (STEC) IN CATTLE: FACTORS
AFFECTING FECAL SHEDDING OF *E. COLI* O157:H7 AND DETECTION METHODS OF
NON-O157 STEC

by

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B.S., Texas A&M University, 2006
M.S., Texas A&M University, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Pathobiology/Diagnostic Medicine
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Abstract

Escherichia coli O157:H7 and over 380 non-O157 serotypes of Shiga toxin producing *E. coli* (STEC) are human food-borne pathogens that inhabit the hindgut of ruminants and are shed in the feces, which subsequently contaminate food products. Recent epidemiological data have shown that six non-O157 STEC (O26, O103, O111, O121, O45 and O145) account for majority of human STEC infections. Fecal shedding of STEC is influenced by a number of factors, including diets, supplements, and feed additives, because of their potential to alter hindgut ecosystem. Not much is known about the fecal shedding of non-O157 STEC in cattle because of lack of standardized detection methods. Fecal shedding of *E. coli* O157:H7 was studied to determine the effects of supplemental urea, monensin, an ionophore, and ractopamine, a beta-agonist. Cattle fed monensin at 44 mg/kg of feed had lower ($P = 0.05$) fecal O157:H7 prevalence than cattle fed 33 mg/kg. Supplemental urea (0.35 or 0.70% of the diet) and inclusion of ractopamine at 200 mg/animal/day had no effect on fecal shedding of *E. coli* O157:H7. In an experimental inoculation study, inclusion of corn starch to a distiller's grains (DG)-supplemented diet had no effect on fecal shedding of *E. coli* O157 suggesting that either the decreased starch content in the DG-supplemented diet is not a factor in the increased shedding of *E. coli* O157:H7 or inclusion of pure starch in the diet may not have achieved our intended goal to have starch flow into the hindgut similar to that of corn grain. A multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 was designed and applicability to detect the seven serogroups in cattle feces was evaluated. A multiplex PCR, designed to detect *E. coli* O104, feces showed presence of O104 in cattle feces (20.6%), but the isolated strains did not carry genes characteristic of the virulent strain responsible for the 2011 food-borne outbreak in Germany. Two preharvest interventions, a siderophore receptor and porin proteins-based vaccine and a Lactobacillus acidophilus-based direct-fed microbial, intended to control *E. coli* O157, had no effect on fecal shedding of O26 assessed by culture-based or PCR-based method.

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Acknowledgements

I would like to thank Dr. Nagaraja, my major advisor; I do not think I could have chosen a better advisor for my PhD program. I would also like to thank my committee (Dr.'s Renter, Bai and Drouillard), who's individual specialties made this research possible. I would also like to thank my outside chair Dr. Vediyappan for overseeing the final stages of my program.

I would like to thank my wife Casey for the support and love throughout my program. I would also like to acknowledge and thank all the student workers, graduate students and friends who made my work and our life in Manhattan enjoyable.

Dedication

I dedicated this dissertation to my daughter, Reese Geiger Paddock.

Chapter 1 - Shiga toxin-producing *Escherichia coli*: A review of detection, isolation, pathogenicity and prevalence in ruminant feces.

Introduction

The bacterium, *Escherichia coli,* has been studied for over 125 years. After a standardized serotyping scheme was introduced (Kauffmann, 1947), it was observed that certain serogroups *E. coli* (O26, O55 and O111) were more commonly associated with human disease (Ewing et al., 1963); while other serogroups were seemingly avirulent (Koya et al., 1954). As researchers began to identify the virulence factors and virulence mechanisms of pathogenic *E. coli*, a potent cytotoxin was identified in certain strains (O26, O111, O113 and O157; Karmali et al., 1983) which was differentiated from previously discovered toxins due to specific cytotoxic effects on Vero cells (Smith and Linggood, 1971; Konawalchuk et al. 1977). Analysis of the toxin showed it to be an AB-type toxin similar that of *Shigella* spp leading some researchers to refer to it as "Shiga-like toxin" (O'Brien and La Veck, 1983). Currently the toxin is referred to interchangeably as either Verotoxin (Vtx) or Shiga toxin (Stx); consequently *E. coli* producing the toxin is referred to as either Verotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC; this review will use the Stx and STEC nomenclature). Over 380 STEC serotypes have been identified (Karmali et al., 2010), however only a small subset of these produces the majority of human disease, which some researchers have further defined as enterohemorrhagic *E. coli* EHEC (Levine et al., 1987; Cleary, 2004). However, in studies examining the ecology of STEC in ruminant reservoirs it is not possible to definitively differentiate STEC from EHEC, but putative EHEC from ruminants may be differentiated based on additional virulence factors

commonly observed in EHEC. Additional STEC virulence classification systems (STEC groups A through E) have been devised based on a serotype's past association with human disease (Karmali et al., 2003); however due to *E. coli's* constantly evolving nature, recently demonstrated with the an outbreak of a hybrid strain of STEC O104:H4 in Europe (Bielaszewska et al., 2011), classifying STEC based on past strains may be problematic.

Initially, only a few STEC serogroups were identified (O26, O111, O113 and O157; Karmali et al., 1983), but after large outbreaks of serotype O157:H7 (Riley et al., 1983; Bell et al., 1994) research was focused specifically on this serotype. Recently epidemiological data have shown that non-O157 STEC account for over 60 percent of STEC infections in humans (Scallan et al., 2011). Some researchers have linked this increase to better and increased testing in state health laboratories (Stigi et al., 2012); however only 30% of laboratories have implemented some form of non-O157 STEC testing. While the United States government declared O157:H7 an adulterant in raw ground beef and a reportable disease in 1994 and 1995 (USDA/FSIS, 2011), classification of non-O157 STEC as a reportable disease did not occur until 2005 and only the top 7 disease causing STEC serogroups have now been declared adulterants in non-intact beef cuts (USDA/FSIS, 2011). As a result, only recently has interest and funding for research into the epidemiology and prevention of non-O157 STEC occurred. The cost to the US economy through illness for O157:H7 is estimated to be 333.5 million dollars (Frenzen et al., 2005). However, this estimate only accounts for the O157 serogroup, since non-O157 STEC now account for a greater number of human illness and will now be subject to recalls, the cost to the US economy by STEC is likely to grow. Therefore implementing intervention strategies that mitigate the risk of human disease and economic loss caused by STEC will become paramount.

However, to design and evaluate intervention strategies, the ecology of non-O157 STEC in beef cattle must be researched and understood.

Ruminant animals are the primary reservoir for both non-O157 and O157 STEC where the organisms asymptomatically inhabit the gastrointestinal tract and are excreted in the feces, which subsequently contaminate meat, produce and water supplies, thereby contributing to human infections (Brooks et al., 2005; Karmali et al., 2010). The prevalence and ecology of O157 STEC in ruminants have been well studied and reviewed (Callaway et al., 2009). While multiple researchers world-wide have examined the prevalence of non-O157 STEC in ruminants, a limited number of experiments have attempted to investigate the ecology of non-O157 STEC in ruminants. However, due to the lack of a standardized and effective isolation and detection methods, coupled with the inherent variation in the shedding of *E. coli,* past research must be examined cautiously.

In order to study the ecology of STEC in ruminants, the organism must first be isolated from fecal samples. To isolate the O157:H7 STEC serotype, the procedure (Glyes, 2007; Vimont et al., 2006) has been standardized and optimized to the point in which it can be implanted in very large sample size studies. This procedure involves enriching fecal matter in a selective broth, followed by concentration of the O157 STEC from the enriched broth with immunomagnetic beads, which are then plated on modified MacConkey agar developed to exploit conserved resistance to cefixime and potassium tellurite and inability to ferment sorbitol within the O157:H7 serotype (CT-SMAC; Chapman et al., 1991; Zadik et al., 1993). However, CT-SMAC will not work to detect non-O157 STEC, primarily because majority of non-O157 STEC's ability to ferment sorbitol (Caprioli et al., 1997; Posse et al., 2007). Preliminary fecal sample screening methods, such as multiplex PCR (mPCR; Bai et al., 2012), have been developed to screen for

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STEC serogroup and virulence genes, but mPCR screening cannot confirm that serogroup and virulence genes exist in the same *E. coli*. Further processing of the sample to isolate the bacterium must be performed to determine if *E. coli* serogroups present harbor virulence genes. To isolate colonies, fecal samples must be enriched (Paddock et al., 2012), subjected to serogroup specific IMS procedures and plated on a selective and differential agar for STEC; however this agar has not been identified. Currently, a medium described by (Posse et al., (2007) and commercial (Rainbow, CHROMagar STEC) agars have been developed for non-O157 isolation and work relatively well to identify pure culture STEC serogroups (Hirvonen et al., 2012); however, when attempting to isolate STEC from a culture containing background flora, such as meat or fecal samples, the effectiveness has not been completely investigated and may be questionable. This review will discuss the current detection and isolation techniques, virulence characteristics of non-O157 STEC and non-O157 STEC prevalence in cattle feces.

Historical Overview

Escherichia coli was first described by Theodore Escherich (1885) as a commensal ubiquitous organism of healthy human feces. The organism, originally termed *Bacterium coli commune*, was re-named to *Escherichia coli* and further biochemically characterized by Castellania and Chalmers (1919) due to the *Bacterium* taxonomic group growing too large and diverse, however the two names were used interchangeably until 1958 when the Judicial Commission of the International Committee on Bacterial Nomenclature officially adopted *Escherichia coli* for bacteria matching the physical (Gram negative, short rod) and biochemical properties (facultative anaerobic; glucose, lactose, dulcitol, and adonitol fermenting, and indole producing). Early research into pathogenic *E. coli* involved investigations into infantile diarrhea,

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also known as "summer diarrhea", which was a leading cause of infant morbidity and mortality in industrialized nations during the late 19th and early 20th century (Robins-Browne, 1987) and is still a problem in developing countries (Trabulsi et al., 2002). Seasonality and communal infection patterns observed with summer diarrhea lead early researchers to hypothesis it was caused by a bacterial agent (Robins-Browne, 1987). Bray (1945) is regarded (Donnenberg and Kaper 1992; Jenkins et al., 2007; Robins-Browne 1987) as the definitive publication demonstrating a positive association between *E. coli* and summer diarrhea in infants. However, evidence suggesting *E. coli*'s gastro-intestinal pathogenicity was demonstrated earlier by Lesage (1897) when serum from patients experiencing diarrhea agglutinated *E. coli* from different diarrheic patients, but not *E. coli* of healthy individuals. This research was also the first to postulate that there may be both pathogenic and non-pathogenic strains of *E. coli.* Adam (1923; 1927), Goldschmidt (1933), and Dulaney and Michelson (1935) all cultured sero-distinct *E. coli* strains from infants experiencing diarrhea, but it was not until Bray (1945), who identified an antigenically homogenous strain of *E. coli* from infants with diarrhea, which was subsequently confirmed by Giles et al., (1949) and Taler et al., (1949), did the scientific community accepted certain *E. coli* strains were pathogenic and the causative agent of summer diarrhea in infants. At this time a standardized *E. coli* serotyping scheme, based the serotyping scheme for *Salmonella enterica*, was developed (Kauffmann, 1947) and researchers observed that the same serogroups were regularly isolated from infants experiencing diarrhea regardless of location (Neter et al., 1955).

Up to the early 1950, pathogenic *E. coli* were only considered to cause gastrointestinal disease in infants, the ability of certain *E. coli* strains to cause gastrointestinal disease in adults was not proven until Kirby et al., (1950); who performed experimental inoculations of *E. coli*

isolated from diarrhetic infants on adult volunteers resulting in diarrhea, but when *E. coli* from healthy babies was inoculated into adult volunteers no symptoms were observed (Koya et al., 1954). The term "Enteropathogenic *E. coli*" (EPEC) was subsequently coined by Neter et al., (1955) to describe *E. coli* strains causing gastrointestinal disease. Research then began to focus on mechanisms of pathogenicity, primarily by identifying cytotoxic substances first in animal ligated-illeal loop models and later *in vitro* cell cultures. Taylor and Bettelheim (1966) were the first to demonstrate a heat-liable enterotoxigenic (LT) substance from EPEC produced diarrheic effects when applied to a ligated-illeal loop assay, while *E. coli* from healthy individuals lacked these substances. This was confirmed by Smith and Gyles (1970) who demonstrated that heat stable forms of the enterotoxin (HT) also existed. Enteropathogenic *E. coli* strains producing HT and LT were re-termed enterotoxigenic *E. coli* (ETEC) and produce watery diarrhea commonly referred to as "Traveler's Diarrhea". It was observed that some EPEC were negative for LT and HT therefore research continued and identified an additional toxin that was biochemically and antigenically different LT and HT (Smith and Linggood, 1971). It was soon observed that this new toxin could be easily distinguished from HT and LT by observing its greater cytotoxic effects on Vero cells (kidney cells from African green monkey; Smith and Linggood, 1971) and was therefore termed Verotoxin (Vtx; Konowalchuck et al., 1977). The structure of Vtx was observed to be closely related to the *Shigella* spp toxin (O'Brien and La Veck, 1983) and therefore some began referring to Vtx as Shiga toxin (Stx). The Vtx and Stx nomenclature are used interchangeably; strains of *E. coli* the toxin gene or producing toxin are commonly referred to as either Verotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC), during this review the Stx and STEC nomenclature will be used.

Since these early discoveries over 380 serotypes of STEC have been identified (Karmali et al., 2010) belonging to 162 O groups (Bettelheim, 2007); however only a small subset of these cause the majority of human illness. While the infamous O157:H7 serotype has received much of the public and research attention due to early well publicized outbreaks (Riley et al., 1983; Bell et al., 1994), other major STEC serogroups (O26, O103, O111, O128 and O145) were simultaneously causing less publicized outbreaks (Bettelheim, 2007). Recently epidemiological data have shown that non-O157 STEC account for over 60 percent of STEC infections in humans (Scallan et al., 2011); however only 30% of laboratories test for non-O157 STEC (Stigi et al., 2012). The top human disease causing STEC serogroups in the US are O157, O26, O103, O111, O121, O45, O145 O124 O118, O69 and O128 (Scallan et al., 2011). Epidemiological investigations into outbreaks of these serogroups have commonly identified ruminants as the source of contamination (Brooks et al., 2005). Research studying the prevalence of non-O157 STEC in ruminants have also commonly isolated these serogroups (Bettelheim, 2007); however due to variations in detection and isolation methods, it is difficult to compare results across studies and to access the effectiveness of each studies ability to detect and isolate non-O157 STEC.

Fecal Detection and Isolation Methodologies

While the fecal detection and isolation methods for the STEC O157 are well defined and accepted, similar procedures have not been fully developed for non-O157 STEC. Due to the lack of standardized isolation and detection methods for non-O157 STEC, clinical laboratories have made limited attempts to detect STEC, most often only screening fecal matter for the Stx (Stigi et al., 2012). Not only are non-O157 STEC fecal isolation methods required for defining human

clinical cases, these methods are also required to study the ecology of the organism in its primary reservoir and source of human infection, cattle. For studies examining the prevalence of non-O157 STEC in cattle feces, few have used the same methodologies and even fewer have used all of the most current technologies (enrichment, screening, IMS and selective/differential agar). This section will focus on detection and isolation methodologies developed specifically to study non-O157 STEC in cattle feces.

Fecal Sampling methods

Variation in methodologies begins with the collection of a fecal sample; researchers have generally collected samples in one of four ways: pen floor fecal pat, rectal fecal grab, rectal fecal swab or recto-anal mucosal swab. It has not been examined if any one method is potentially more sensitive than the other for the detection of non-O157 STEC; however differences among fecal sampling methods have been examined for isolation of STEC O157. Cultures from recto-anal mucosal swabs were observed to have a greater sensitivity than fecal samples for the detection of STEC O157 (Rice et al., 2003; Greenquist et al., 2005); probably due to the colonization of the recto-anal mucosal by STEC O157 (Naylor et al., 2003; Walker et al., 2010). In contrast, others (Khaitsa et al., 2005; Niu et al., 2008) have found fecal grab samples to be more sensitive in the detection of STEC O157 compared to recto-anal mucosal swabs. It is possible that non-O157 STEC colonizes the same location and therefore sample collection techniques would be expected to similarly affect non-O157 STEC prevalence; however this has not been examined. Sample collection technique is more likely to be dictated by the experimental design and facilities available. Rectal fecal grab, rectal swabs and recto-anal

mucosal swabs must be collected when the animal is restrained in a squeeze chute. While this is an advantage in longitudinal studies since the prevalence in individual animals may be followed, it may not be practical for large or commercial feedlot studies. Also by moving the animals through the same chute system it is conceivable that any treatment effects being investigated may be confounded due to the spread of fecal matter throughout the chute system and cross-contamination of animals of different treatment groups. Pen floor fecal samples are logistically easier, avoid removing the animals from their pens, and minimize cross-contamination. However, records of which fecal pat came from which animal are not easily documented, therefore individual animal prevalence throughout a longitudinal study cannot be easily ascertained. Furthermore, care must be taken when collecting pen floor fecal pats in order to avoid ground contamination and repeated sampling of the same animal on the same sampling day.

Enrichment method

The collected fecal samples are commonly suspended in a broth either to allow for easier direct streaking onto isolation agar or for an enrichment step prior to IMS and streaking onto isolation agar. While the enrichment step is optional, if it is not utilized, only fecal samples with high concentrations ($> 10^5$ CFU/g) of the organism can be detected (Paddock et al., 2012). If an enrichment step is included the detection limit can be enhanced to approximately 10^2 CFU/g of pre-enrichment STEC (Paddock et al,. 2012); however those detection limits are based on a screening of the enrichment broth with a multiplex PCR, isolation techniques are less sensitive. A variety of enrichment procedures have been used with different broths, inoculation ratios and incubation conditions. The inoculation ratio of fecal sample placed in broth has ranged from 1.0

g of feces in 10.0 ml of broth up to 25.0 g of feces in 225 ml of broth. Across most studies, a dilution factor of 1 part feces to10 parts broth was utilized; however extremes were observed such as 0.05 g in 10.0 ml of broth (Pearce et al., 2004). Evans et al. (2011) evaluated the effectiveness of different ratios finding that 1.0 g of feces in 10.0 ml of broth compared to 10.0 g of feces in 225 ml of broth resulted in a greater numbers of fecal samples positive for targeted isolates. Evans et al. (2011) also observed that higher prevalence of target isolates were observed when samples were run in duplicate. Furthermore, collection of larger fecal samples (10.0 g) may be difficult. When swabs are used the weight of the sample cannot be determined but the swab is generally placed in 10.0 ml of broth (Cobbold and Desmarchelier, 2000; Cobbold et al., 2004; Fukushima and Seki, 2006).

For the enrichment media, a variety of broths have been utilized both non-selective and selective. Buffered peptone water (BPW) and trypticase soy broth (TSB) has been used as a non-selective recovery broth (Pearce et al., 2004; Shaw et al., 2004; Cookson et al., 2006; Fukuchima and Seki 2006; Pearce et al., 2006; Evans et al., 201; Khandaghi et al., 2011); while these broths may increase the viability of any STEC present they would may also increase the viability of background microflora. Kobayashi et al., (2001) used a modified version of *E. coli* broth in which the bile salts were removed due to reports that bile salts inhibit recovery of injured STEC cells (Kobyayshi et al., 2001); however bile salts are the selective component of *E. coli* broth and by removing them they were using a broth similar to TSB with the similar non-selective issues. A selective enrichment step using MacConkey broth has been utilized by Pradel et al., (2000) and Renter et al., (2005), which would prevent growth of enteric Gram positive bacteria but not select against non-STEC Gram negative bacteria. Others (Cobbold and Desmarchelier, 2000; Cobbold et al., 2004; Jeon et al., 2006; Sasaki et al., 2011) have utilized

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selective media such as *E. coli* broth and enhanced it's selectivity with the addition of antibiotics (novobiocin at 20 mg/l), however this addition is controversial due to the documented inhibition of some STEC strains by this antibiotics (Kanki et al., 2011; Vimont et al., 2007). Joris et al., 2011 utilized a procedure developed and validated by Posse et al., (2008) in which TSB was modified by the addition of novobiocin (8 mg / l), vancomycin (16 mg / l), rifampicine (2 mg / l), bile salts (1.5 g / l) and potassium tellurite (1.0 mg / l). A limitation this broth (Posse et al., 2008) would be it was validated with a limited number STEC strains; therefore it is not known how many strains would be excluded when it was used in a larger population. Paddock et al., (2012) compared modified TSB described in Posse et al., (2008) and EC broth and observed that EC broth had a one log greater detection limit when the enrichment broth was tested with a multiplex PCR.

Incubation conditions have also varied, even when using the same broth. Temperatures, ranging from 37°C to 42°C or higher, have been used with the most common temperature being 37°C. It has been suggested (Gonthier et al., 2001; Vimont et al., 2006) the common use of 37°C originates from a publication utilizing the original outbreak strain of STEC O157 (Doyle and Schoeni et al., 1984). In other studies, utilizing many strains, the optimal growth temperature for STEC O157 was reported to be 40°C (Gonthier et al., 2001; Nauta et al., 1999). Few studies have examined optimal growth temperatures in non-O157 STEC; Gontheir et al., (2001), utilizing small sample sizes, have observed optimal temperatures of 41°C. Paddock et al., (2012) and Vimont et al., (2007a) also compared different enrichment temperature for smaller sample sizes of STEC strains and observed higher temperatures (42°C) resulted in better detection limits. No studies have examined optimal growth or enrichment temperatures for a larger collection of STEC strains. The length of incubation has also varied but can generally be

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classified into either shorter (6 h) or longer (18-24 h) incubations lengths. Paddock et al., (2012) and Tutenel et al., (2003) documented shorter, 6 h, compared to longer, 24 h, incubation lengths resulted in the better detection limits when fresh bovine feces was experimentally inoculated with serial dilutions of STEC. Enrichment tubes maybe held static or gently agitated during the incubation. In general, the logistically simpler, static incubations are used; however some studies have applied agitation (Renter et al., 2005) during the incubation. No studied has directly compared the recovery rates between static or agitation incubations.

At this point enriched fecal samples may or not be screened for STEC serogroup specific genes and virulence genes by PCR. Generally, a sub-sample of the enriched fecal sample is removed, boiled and centrifuged to lyse the bacterial cells releasing the DNA into the supernate. The DNA must then be extracted and purified from the supernate via any number of commercial kits. A single or multiplex PCR (Bai et al., 2012) may then be run on the purified DNA to determine if any genes of interest are present. The advantage to screening fecal sample is the number of samples that need to be further process may be reduced. While decreasing the number of samples that need to be further processed can greatly decrease cost and logistical concerns, there are disadvantages and concerns with screening samples by PCR. First while it is generally accepted that PCR is more sensitive than culture based methods, however this may not necessarily always hold true. The PCR screening must be completed quickly as to know which samples will need to be further processed. Also if more than one gene are examined (i.e. a STEC serogroup and *stx*) this method cannot determine if those genes were in the same isolate. Also this method relies on gene specific primers, if base pair mutations occur in the primer region of the target genes the PCR may no longer detect that gene.

Immunomagnetic separation is commonly performed on enriched fecal broths. The procedure consists of mixing a small aliquot of enriched fecal broth with metallic beads coated with antigens specific to a serogroup. The fecal broth bead mixture is gently mixed and incubated during which time bacteria with the appropriate surface serogroup will associate bead surface antigens. The bacterial/bead complexes are removed with magnets, washed and plated on agar plates (described below) for colony growth. While the advantages to the method are obvious in that a target bacterium are concentrated while at the same time background or non-target bacteria are removed thereby increasing selectivity and sensitivity (Olsvik et al., 1994), there are just as important disadvantage. First each bead is specific to a target serogroup; therefore this procedure and subsequent plating must be repeated by the number of serogroups that are targeted. Currently these beads are only produced by a couple of commercial companies around the world and shortages are common at this point in time. Also these beads are only commercially available for 5 serogroups (O26, O103, O111, O145 and O157), although blank beads are available and the procedure to coat beads in a serogroup of the researchers choosing has been published.

Isolation Media

Whether it is the bacteria/bead complex or enriched fecal broth, to obtain isolates these materials must be plated on agar for colony growth and selection. Even after selective enrichment and an IMS step there are still non-target organisms present. Some researchers have plated enriched fecal broth or IMS beads on simple non-selective agars, such as blood agar (Beutin et al. 1989) or semi-selective medium such as MacConkey agar (Paddock et al., 2012). When random colonies are then chosen from these agar plates and tested for STEC, their success

is dependent on simply the ratio of STEC to non-target bacteria. Although, some researchers have added a DNA hybridization step (Jenkins et al., 2002) at this point to help them identify STEC from the mixed colony population (discussed below). Ideally, we should have a selective and differential agar for STEC similar to CT-SMAC for the O157 STEC serogroup. Most of the selective agars, some available commercially, were developed to target major STEC serogroups; however even within a STEC serogroup resistance and fermentation characteristics may widely vary (Posse et al., 2007). When it is taken into account that each major serogroup will undergo IMS with serogroup specific beads, it may be more advantageous to develop serogroup specific agars. For the STEC agars that have been developed, their efficacy when testing pure cultures or mixed populations of known STEC serogroups is often questionable; further exacerbating any discrepancies observed in pure culture tests happens when fecal samples are analyzed because the fermentation characteristics of the ever present background flora cannot be predicted and may phenotypically resemble a target serogroup. While the serogroup specific agar approach has been utilized in the case of O157 for many years, only recently has the approach been applied to the O26 serogroup. A Japanese research group (Hiramatsu et al., 2002) developed an agar based on the O26 serogroups Rhamnose fermentation deficiency and resistance to cefixime and potassium tellurite. However when researchers examined 102 disease causing strains of O26, only 89 were able to grow on the agar and 7 of the 89 were able to ferment the rhamnose sugar (Bielaszewska et al., 2005). Posse et al., (2007) developed a general STEC agar and procedure targeting the top 6 non-O157 serogroups. This procedure relies on antibiotic resistance (novobiocin and potassium tellurite) and fermentation characteristics of the STEC serogroups. However this procedure was only validated with 10 to 15 strains per serogroup. Furthermore after the initial plating, secondary plating on agars specific to each serogroup must be performed.

Commercial agars such as CHROMagar STEC (CHROMagar, Paris, France) and Rainbow

STEC (Biolog Inc., Hayward, CA), likely work off principles similar to Posse et al., (2007), but

do not disclose their composition. To date, no publication has compared these media for their

ability to recover STEC from fecal samples. Regardless of the selectiveness or differentiating

ability of an agar, colonies must be subsequently tested for *stx* and serogrouped.

Detection Methods

Once colonies are grown on an agar plate, even if suspect colonies are differentiated by a

type of agar plate utilized, further testing is required to confirm both virulence and serogroup.

Multiple methods exist for the confirmation testing, each with its own advantages and

disadvantages. The most comprehensive method in which all colonies grown on a plate can be

tested is known as colon hybridization (Paton and Paton, 1998). During this process colonies are

replicated on a nylon membrane and DNA oglio-nucleotides specific to either *stx* or a specific

serogroup. Once positive colonies are identified they can be taken from the original plate and

preserved. While the advantage to this method is that all colonies present on a plate are screened

during a single procedure, however the procedure is time consuming and would be difficult for

large numbers of plates. All other confirmation methods involve sub-culturing a certain number

of colonies then testing those colonies. However the number of colonies that are sub-cultured

varies widely (ranging from 1 to 50 to not stated) and publications rarely describe how they

arrived at the number of colonies they are sub-culturing. Subcultured colonies are commonly

screened via PCR for *stx* or serogroup specific DNA. These PCR reactions have been combined

into a one-step multiplex PCR (Bai et al., 2012) to detect top virulence (including stx) and

serogroup genes (O26, O45, O103, O111, O121, O145 and O157). While this method is quicker

and easier than a hybridization method, it is limited in that a separate reaction must be performed for every colony. Another method to detect Stx is to grow suspect colonies in a broth, centrifuge and filter sterilize the resulting supernate, then add this supernatant to Vero cells to observe cytotoxicity. The gold standard for serogroup determination is agglutination with a standardized set of antisera; however this is only practical for serogroup reference labs that specialize in this method as a service. Individual serogroup antisera can be obtained, but this method is more subjective than the PCR method for serogroup determination. A less subjective antisera-based method (latex agglutination) that is faster than PCR is available commercially for the O157 serogroup, however it has yet to become commercially available for any other serogroup.

Virulence Genes and Pathogenicity

Shiga toxin-producing *E. coli* by definition carry single or multiple prophages harboring a *stx* gene. Over 380 serotypes of STEC have been identified (Karmali et al., 2010); however only a subset of these are capable of causing human disease and are typically referred to as EHEC. Shiga toxins are A-B type toxins (O'Brien et al., 1992), with a B-pentamer subunit that binds to the Gb3 host cell receptor (Lingwood, 1987; Lingwood, 1996) and an A-monomer subunit that internalizes into the host cell, which then alters ribosomes, blocking protein synthesis (Donohue-Rolfe et al., 1989). There are two isomers of Stx, referred to as Stx1 and Stx2. Both Stx1 and Stx2 are associated with prophages (O'Brien et al., 1989; Strockbine, 1986) and share 56% amino acid homology (Jackson et al., 1987). Shiga toxin 1 has been shown to preferentially bind to the host Gb3 receptors in lung tissues and is cleared relatively quickly from the host serum (Rutjes et al., 2002). Shiga toxin 2 preferentially binds to host Gb3 receptors in the kidneys and has a longer half-life in the serum (Rutjes et al., 2002); consequently Stx2 has been shown to be

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400 times more lethal in mouse model (Tesh et al., 1993). Furthermore, Stx2 is more frequently

associated with severe human disease compared to Stx1 (Boerlin et al., 1999; Ostroff et al.,

1989). The DNA sequence of *stx*1 is highly conserved (Paton et al., 1995) with only a few

variants (Stx1$_a$, Stx1$_c$ and Stx1$_d$; Burk et al., 2003; Paton et al., 1995). Alternatively over 23 *stx*2

variants have been identified in the literature (Stx2, Stx2$_v$, Stx2$_{va}$, Stx2$_{vh-a}$, Stx2$_{vh-b}$, Stx2$_{vh-c}$,

Stx2$_{v-ox392}$, Stx2$_{v-ox393}$, Stx2$_c$, Stx2$_{2d-ox3a}$, Stx2$_{ox3b}$, Stx2$_{d-ount}$, Stx2$_{O118}$, Stx2$_{O111}$, Stx2$_{O113}$, Stx2$_{O48}$,

Stx2$_e$, Stx2$_{ev}$, Stx2$_{NV206}$, Stx2$_f$, Stx2$_g$, Stx2$_h$, and Stx2$_i$) which may occur alone or in combination

(Bertin et al., 2001; Gannon et al., 1990; Ito et al., 1990; Johnson et al., 1991; Leung et al., 2003;

Meyer, 1992; Paton et al., 1995; Paton and Paton, 1998; Paton, 1993; Pierard et al., 1998;

Schmidt et al., 2000; Schmitt et al., 1991; Strockbine, 1986; Weinstein et al., 1989). Due to a

variety of stx2 variant's nomenclatures that have been employed throughout history some of

these names are likely referring to the same or very similar variants. Scheutz et al., (2012) has

set forth a Stx nomenclature system to help resolve these issues in which variants are named in a

sequential alphabetical order (Stx2$_a$, Stx2$_b$, Stx2$_c$, etc.). As seen in figure 1, serogroups can vary

widely between stx variants; therefore under the historical system in which some variants were

named after the serogroup in which they were detected may greatly confound research efforts.

Virulence can vary widely between variants with some Stx2 variants (Stx2$_a$, Stx2$_d$ and

elastase-cleaved Stx2$_d$) being up to 25 times more virulent compared to other Stx2 variants

(Stx2$_c$ and Stx2$_b$; Fuller et al., 2011). Sequence analysis comparing variants that are commonly

associated with severe human disease to variants which are not has revealed small amino acid

differences within in either the α-subunit or β-subunit (Ito et al., 1990; Schmitt et al., 1991)

contributing to these variations. For example, Stx2$_c$ has α-subunit is identical to Stx2, however

the β-subunit is closer to the $Stx2_{vh-a}$ β-subunit (Schmitt et al., 1991). The changes in the $Stx2_c$

β-subunit have been shown to decrease its affinity for the Gb3 receptor (Lindgren et al., 1994).

While past researchers (Beutin et al., 2001; Brett et al., 2003) have examined the *stx* subtype

within different serogroups from cattle, these studies were performed prior to the standardized

stx nomenclature scheme and therefore it difficult to determine associations to current *stx*

variants. However it was observed that a wide range of *stx* variants exists and can multiple

variants can be found with in a serogroup (Figure).

Multiple copies of *stx* harboring prophage can be present in a single bacterium although these

are not maintained efficiently (Fogg et al., 2011). Loss of the prophage from STEC has also

been document in human infections (Bielaszewska et al., 2007) and upon sub-culturing of

ruminant isolates (Joris et al., 2011). While *stx* is responsible for the severe disease caused by

STEC, some STEC do not cause disease signifying other virulence and colonization factors may

be required for disease.

STEC form a close attachment to the host intestine through effector proteins that target the

microvilla epithelial cells, inducing actin re-arrangement and pedestal formation (Knutton et al.,

1989) resulting in effacement of the microvilli (Moon et al., 1979) creating areas known as

attachment and effacement lesions (A/E; Moon et al., 1983). The first of these proteins to be

discovered was intimin (Jerse et al., 1991) which is a bacterial cell surface protein that assists in

the attachment of bacterial cells and the host cells. The intimin protein is encoded by a gene

termed *E. coli* attaching and effacing (*eae;* Jerse et al., 1990) located on a chromosomally

encoded pathogenic island termed the locus of enterocyte effacement (LEE). Presently 33

variants of the *eae* have been described (Horcajo et al., 2011). The LEE pathogenicity island and

specially *eae* is commonly used as a marker for EHEC. While much of the research into *eae* and LEE was carried out with O157:H7 strains, it has been documented that non-O157 STEC strains have the same *eae* variants and similar LEE pathogenicity islands. Posse et al., (2007) examined 84 STEC strains and that 97.6 % were positive for *eae*. When the *eae* variants were determined, an association between variant and serotype was documented (Table 1).

The LEE also encodes a variety of effector proteins as well as a type III secretion system (T3SS) that injects the effector proteins directly into host cells. One effector protein is a translocated intimin receptor (*tir*) host cells that acts as a receptor for *eae*. A-typical EPEC are hypothesized to be closely related to STEC as they harbor *eae* and LEE with the same association between serotype and *eae* variant (Sekse et al., 2011). It is further hypothesized that aEPEC are STEC that have lost the stx prophage (Bielaszewska, et al., 2007; Joris et al., 2011) or alternatively STEC are aEPEC that have gained a *stx* prophage (Sekse et al., 2011).

STEC may possess accessory putative virulence factors such as the enterohemolysin, also known as enterohemorrhagic *E. coli* hemolysin (EHEC-Hly; Beutin et al., 1989), which is encoded by *ehx* gene on a plasma termed pO157 (Schmidt et al., 1995). This hemolysin produces a small turbid zone of hemolysis on blood agar after 18-24 h. It is commonly associated with O157 and non-O157 STEC (Djordjevic et al., 2004) and is thought to enhance the virulence of STEC through synergistic cell disruption effects with Stx. Posse et al., (2007) examined 84 STEC strains and observed that 91.7% were positive for *ehx*.

The *fli*C gene is responsible for the H-type of *E. coli* and has also been associated with disease causing strains (Beutin et al., 2004). While some H-types have a clear and well known association to disease causing serotypes, such as the H7 in the O157:H7 serotype, associations with disease and non-O157 serotypes is less clear. Some associations have been documented

(Table 2); however H-negative clinical STEC strains have also been historically associated with these O-groups. The discrepancy may arise from the method of H-typing that was performed. When classic antigen-based typing is performed, the flagella must be present on the surface of the bacteria; however when molecular methods targeting polymorphisms in the *fli*C gene are utilized, only the gene must be present (Prager et al., 2003). When researchers have re-examined culture collections of STEC which were previously evaluated with H-type antigen-based testing with molecular H-type testing, an H-type was observed which matched the expected H-type of flagella producing strains. (Mellmann et al., 2008).

Genetic transfer has been shown to be a major factor of bacterial evolution (Ochman et al., (2000) responsible for the Stx prophages that convert *E. coli* to STEC. Ogura et al., 2009 performed genomic analysis on published sequences of 4 STEC and 21 other non-STEC *E. coli* finding that an independent, parallel evolution lead to the many serogroups of EHEC; probably due to each EHEC serogroup having similar propensities for taking up bacterial phages, plasmids, and other integrative elements ubiquitous in the environment. With a past record of acquiring genetic virulence elements, such events are likely to occur in the future creating new STEC strains or hybrid STEC will emerge in the future.

Prevalence and Ecology of STEC in Ruminants

Ruminant animals are asymptomatic carriers of STEC and are considered to be the primary reservoir (Karmali et al., 2010). Human STEC infections typically occur via consumption of contaminated meat or produce; contamination is thought to originate with ruminant fecal matter (Karmali et al., 2010). Ecological and epidemiological studies of STEC in ruminants are important tools for determining the interventions strategies to reduce or prevent STEC

contamination and subsequent outbreaks. These studies rely on our ability to determine STEC

prevalence in ruminants which commonly involves isolating STEC from bovine feces which is

complicated by the greater than 10^{12} CFU/g background microflora. Multiple isolation and

detections procedures for non-O157 STEC have been utilized but a standardized, efficacious

procedure has not been agreed upon. Many studies have examined the prevalence of STEC in

cattle feces; however this review will focus on studies which utilized all of the most up-to-date

techniques including: fecal enrichment, screening of fecal enrichment by PCR, concentration of

target organisms with IMS beads and plating the beads on a selective agar before screening of

individual isolates.

Barlow and Mellor, (2010) collected 300 fecal samples from both grain-fed and grass-fed

cattle from 25 different abattoirs in Australia over an 8 month period. Twenty-five grams of

fecal sample were diluted into 250 ml of trypticase soy broth (TSB) and incubated at 37°C for 18

h. A sub-sample of the enriched fecal broth was tested with PCR for *stx* and *eae*; samples

positive for both genes were subsequently tested by PCR for the top 8 disease causing STEC

serogroups. For serogroups with commercially available IMS beads (O26, O103, O111, O145

and O157), IMS was performed and beads were plated onto chromocult-TBX agar (O103, O111

and O145), rhamnose MacConkey agar (O26) and sorbitol MacConkey agar (O157) with

cefixime and potassium tellurite. For O121, O91 and O45 enriched fecal broth was plated on

Chormocult-TBX agar (Merck KGaA, Darmstadt, Germany). and colony hybridization was

performed with probes targeting those serogroup (O121, O91 and O45). Following each

procedure, up to 50 isolates were streaked individually and tested again by PCR. Forty-two

percent (128 out of 300) of enriched fecal broths were positive for *stx* and 61 % (78 out of 128)

of those were also positive for *eae* and 38 % (30 out of 78) of those were positive for at least one

of the 8 serogroups. Of the 30 samples that screened positive for *stx, eae* and a serogroup, 25 isolates of the target serogroups (at least one from every serogroup tested for except O145) were recovered, however only 4 of those isolates (1 O91 and 3 O157) harbored *stx* classifying them as STEC.

Fukushima and Seki, (2004) collected 605 fecal samples from cattle presented at an abattoir in Japan. One-tenth of a gram of feces was inoculated into 0.9 ml of 0.85% NaCl solution and ten-fold serial diluted; then 0.7 ml of the ten-fold dilution was inoculated into 10 ml of TSB and incubated for 6 h at 42°C. Immunomagnetic separation was performed with 1ml sub-sample of the fecal enrichment using commercial IMS beads (O26, O111 and O157). Both the fecal enrichment and recovered IMS beads were streaked onto sorbitol-MacConkey agar (O157 IMS beads) and Chromocult agar (fecal enrichment, O26 and O111 IMS beads) then incubated at 37°C for 18h. A colony sweep was screened with a PCR to detect *stx* genes. Fifty colonies from *stx* positive samples were individually sub-cultured and five isolates were then pooled together and screened a second time for *stx* before individual isolates were tested for *stx* and serotyped. The *stx* gene was detected in 37.5% of the colony sweeps and STEC isolates were recovered from 50% of the *stx* positive colony sweeps. While the most prevalent STEC was the O26 serogroup, 68 different STEC serogroups were isolated.

Hofer et al., (2012) collected fecal samples (n = 573) over an eleven month period from 7 abattoirs in Switzerland. Fecal samples were collected using swabs after evisceration. The swabs were placed in a bag with 20 ml of modified tryptic soy broth (16mg/l of novobiocin), stomached and incubated 16 h at 37°C. Fifty micro-liters of this enrichment was screened for *stx* with PCR and *stx* positive samples were further screened for serogroup specific genes for O26, O103, O111, O145 and O157. Strain isolation was attempted on samples that were positive for

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stx and O26, O145 or O157. For the strain isolation a secondary enrichment was performed by adding 1 ml of the original fecal enrichment to10 ml of brain heart infusion broth which was incubated overnight at 37°C. Then IMS was performed with O26, O145 and O157 beads. Beads were plated on sheep blood agar and incubated overnight at 42°C. Colony hybridization for the serogroup specific genes was performed. One to four positive colonies were picked from the sheep blood agar and serogroups were confirmed along with *stx* status by PCR. Of the 573 enriched fecal samples, 72.7% (417) tested positive for the *stx* genes and 23.9, 25.9, 0.8, 41.9 and 7.8% of the 417 samples were positive for O26, O103, O111, O145 and O157 serogroups, respectively. For O26, O145 and O157 positive samples, isolates were obtained from less than 18% of O26 and O145 and 40% for O157. However, most isolates were not STEC; only 9 out of 17 O26 isolates, 4 out of 28 O145 isolates, 5 out of 12 O157 isolates were *stx* positive.

Lynch et al., (2012) collected five rectal fecal swabs six dairy farms in Ireland over a seventeen month period. The rectal fecal swabs were submerged in the 225 ml of modified TSB (cefixime and vancomycin), vortexed and incubated overnight at 37°C. A DNA extraction was performed using 1.0 ml of enrichment broth and a commercial DNA extraction kit. The extracted DNA was screened for stx genes by real-time PCR. Samples positive for *stx* genes were subjected to a serogroup-specific real-time PCR (O157, O111, O26, O103, and O145). All samples that tested positive for *stx* and a targeted serogroup were further processed by IMS (O157, O26, O103, and O145). Samples positive for O157 were than plated on MacConkey agar with cefixime (0.05 mg/l) and potassium tellurite (0.25 mg/l). Non-O157 serogroups were plated on Chromocult supplemented with cefixime (0.05 mg/l), cefsulodin sodium salt (5 mg/l) and vancomycin hydrochloride hydrate (8 mg/l). All plates were incubated at 37°C for 18h and isolates were tested by PCR for *stx* and serogroups. Three hundred eight six of the 600 (63.3%)

rectal fecal swabs screened positive for *stx* genes of these positive samples 39% (O145), 19% (O103), 7% (O26), 3% (O157), and 0.001% (O111) were positive for the indicated serogroups. Isolates were recovered from 56% of the O157, 31% of the O26, 21% of the O103 and 8% of the O145 positive samples. However of the isolated *E. coli*, only 8.3% of the O26, 40.0% of O157, 3.8% of O103 and 0% of the 0145 were considered STEC.

Shinagawa et al., (2000) collected fecal samples from 510 cattle near Tohoku, Japan. One gram of feces was added to 20 ml of modified TSB (novobiocin at 25µg/ml) and incubated for 18 h at 37. A second enrichment was then performed by removing 1 ml of the primary enrichment and adding it to 10 ml TSB and incubating at 37°C for 18h. One milliliter of this secondary enrichment was used a PCR reaction to detected *stx*. For those enrichments that were *stx* positive the original fecal sample was inoculated into modified EC broth with novobiocin, however no incubation was performed. Immunomagnetic separation was performed on these un-incubated fecal broths with beads for O157, O26, and O111. The IMS beads were plated onto CT-SMAC agar and CHROMagar then incubated at 37°C overnight. Five to 6 colonies from each plate were test by agglutination for serogroup (O157, O26 and O111) and then tested by PCR for *stx*. Fifty percent (258 out of 510) of enriched fecal samples were positive for *stx*; however only 4 O26, 1 O111 and 5 O157 STEC isolates were recovered.

Thomas et al., (2011) collected 1.0 g rectal fecal samples over a 14 month period from 402 different animals. The 1.0 g fecal samples were added to 19 ml of mTSB (vancomycin at 6 mg/l and cefixime at 50 µg/l) and incubated at 37°C for 6h. DNA was then extracted from a sub-sample of the enriched fecal broth using a commercial kit and screened by PCR *stx* genes. Samples positive for *stx* were than screened for serogroup specific genes (O157, O26, O111, O103 and O145). Automated IMS was performed on samples positive for *stx* and a serogroup

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with beads specific for O26, O111, O103, O145 and O157. The beads were plated onto CT-SMAC agar (O157) and modified CHROMagar (supplemented with cefixime at 50 µg/l, cefsulodin at 50 mg/l and vancomycin at 8 mg/l; O26, O103, O111 and O145) and incubated at 37°C for 24h. Up to 5 colonies from these plates were sub-cultured and tested by PCR for serogroup then presences of *stx* genes. The majority of samples (301 out of 402) were found to be positive for *stx* and O157 or O111; interestingly all samples screened positive for *stx* and O26, O103 or O145. However only 8 (1.99%) O26, 34 (8.46%) O103, 0 O111, 3 (0.75%) O145 and 8 (2.66%) O157 isolates were recovered of which 6 of the O26, 4 of the O103, all 3 of the O145 and 7 of the O157 isolates possessing *stx* and therefore being considered STEC.

From these studies it can be concluded that a large majority cattle feces contains STEC; however due to deficiency's in isolation techniques, determining if these STEC are among serogroups commonly observed in human illness cannot be reliably determined. Furthermore when serogroups common to human illness were isolated, they did not necessarily harbor *stx* and therefore may not produce human illness. Better isolation techniques will be need to isolate STEC from bovine fecal samples in order to determine if the high rates of STEC observed in cattle feces are a threat to human health.

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Figure 1.1. Neighbor joining cluster analysis of *stx* sequences, high-lighting there serogroup and proposed classification. Adapted from Scheutz et al., (2012).
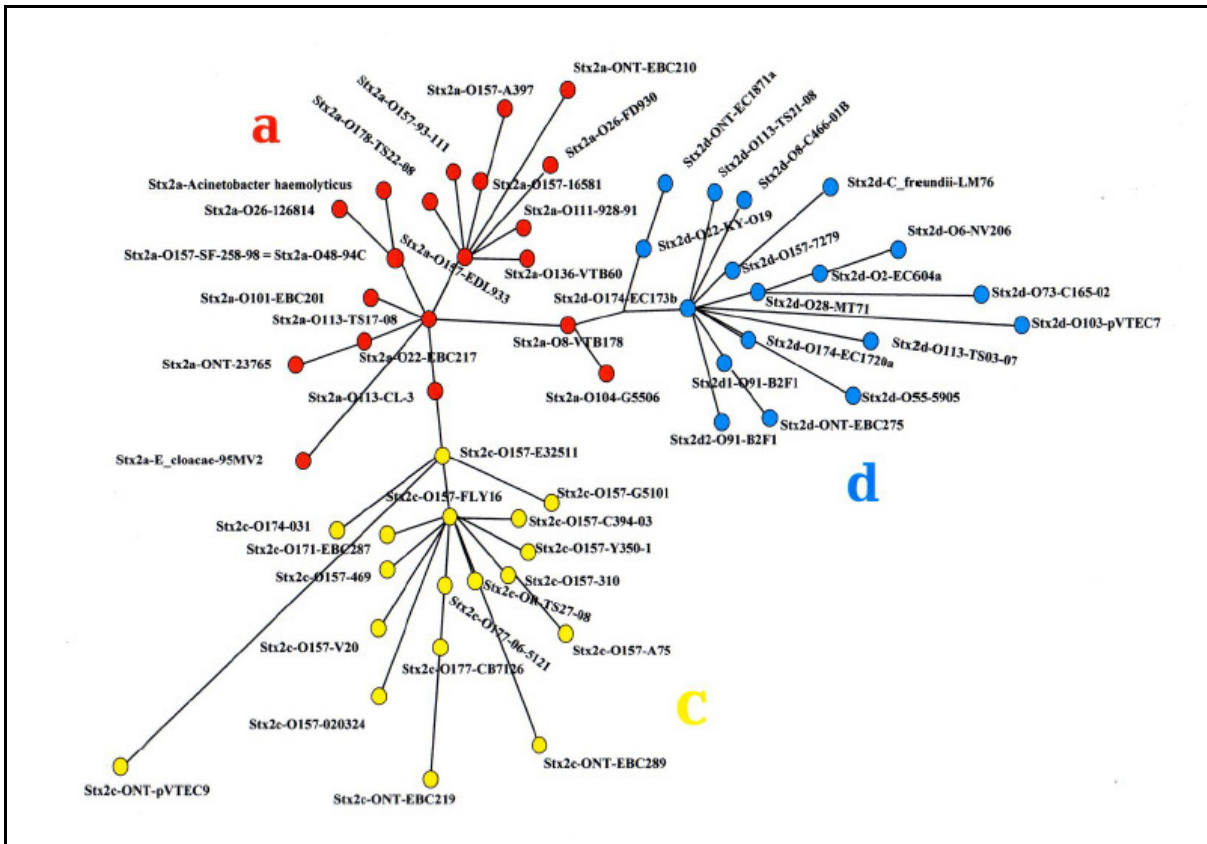
Table 1.1. Association between *eae* variant and serogroup in STEC from human clinical cases.[1]

E. coli serotype	N=	*eae* variant
O26:H11	6	β
O26:H-unknown	9	β
O26:H-unknown	1	Negative
O103:H2	9	θ
O103:H2	2	ε
O103:H-unknown	2	θ
O103:H-unknown	1	Negative
O111: H-unknown	12	γ
O111:H-unknown	3	ε
O145:H-unknown	13	γ
O157:H7	25	Γ
O157:H-unknown	1	Negative

[1]Adapted from Posse et al., (2007)

Table 1.2. STEC O-group and commonly associated H-group[1]

STEC O-group	Common H-group
O26	H11
O103	H2
O111	H8
O121	H19
O145	H28
O157	H7

[1]Adapted from Beutin et al., 2004

Chapter 2 - Dietary monensin level, supplemental urea, and ractopamine on fecal shedding of *Escherichia coli* O157:H7 in feedlot cattle

Abstract

Inclusion of distiller's grains (DG) in cattle diets has been shown to increase fecal shedding of *E. coli* O157:H7. It is hypothesized that altered gut fermentation by DG may be responsible for the positive association. Therefore, feed additives affecting ruminal or hindgut fermentation of DG also may impact fecal shedding of *E. coli* O157:H7. The objectives of the study were to evaluate effects of monensin level (33 or 44 mg/kg DM), supplemental urea (0, 0.35, or 0.70% of DM), and ractopamine (0 or 200 mg/steer daily administered during the last 42 d of finishing) in a steam-flaked corn grain-based diet containing 30% wet sorghum DG on fecal shedding of *E. coli* O157:H7. Seven-hundred and twenty crossbred beef steers, housed in 48 pens (15 steers/pen), were assigned to dietary treatments in a randomized complete block design with a 2 x 3 x 2 factorial treatment arrangement. Fresh pen floor fecal samples (10 per/pen) were collected every 2 wk for 14 wk (July through November) and cultured for *E. coli* O157:H7. Isolation of *E. coli* O157:H7 was by selective enrichment of fecal samples in an enrichment broth, immunomagnetic separation, followed by plating onto a selective medium. Samples that yielded sorbitol-negative colonies, which were positive for indole production, O157 antigen agglutination, and contained *rfbE, fliC,* and *stx2* were considered positive for *E. coli* O157:H7. Fecal prevalence data were analyzed as repeated measures using negative binomial regression to

examine effects and interactions of sampling day, urea, monensin, and ractopamine. Mean fecal prevalence of *E. coli* O157:H7 was 7.6%, and ranged from 1.6 to 23.6%. Cattle fed monensin at 44 mg/kg of feed had lower ($P = 0.05$) fecal *E. coli* O157:H7 prevalence than cattle fed 33 mg/kg (4.3 vs 6.8%). Although the reason for the reduction is not known, it is likely because of changes in the microbial ecosystem induced by the higher dose of monensin in the hindgut. Supplemental urea at 0.35 or 0.70% had no effect ($P = 0.87$) on fecal shedding of *E. coli* O157:H7. Fecal prevalence of *E. coli* O157:H7 were 5.3, 5.7, and 5.9 % for groups fed 0, 0.35 and 0.7% urea, respectively. The inclusion of ractopamine at 0 or 200 mg/head/d had no effect ($P = 0.89$) on fecal prevalence of *E. coli* O157:H7 (4.4 vs 4.0 %). Additional research is needed to confirm the reduction in fecal shedding of *E. coli* O157:H7 in cattle fed monensin at 44 mg/kg of feed compared cattle fed 33 mg/kg of feed.

Introduction

Escherichia coli O157:H7 is a food borne pathogen that inhabits the hindgut (Naylor et al., 2003; Walker et al., 2010) and is shed in the feces, which can subsequently contaminate food products (Rangel et al., 2005). Fecal shedding of *E. coli* O157 is influenced by a number of factors, including diets (Callaway et al., 2009). It is hypothesized that dietary ingredients that flow into the hindgut are likely to affect fecal shedding of *E. coli* O157 (Jacob et al., 2009). Distillers grains (**DG**), which contain little starch and approximately three times higher lipid, protein, and fiber than corn (Spiehs et al., 2002), increase fecal *E. coli* O157 prevalence in cattle (Jacob et al., 2008). Increased flow of lipid, protein and fiber to the hindgut are likely to alter ecology of the hindgut and have an impact on *E. coli* O157. Provision of ammonia as urea may enhance ruminal fermentation and decrease flow of fiber to the hindgut. Monensin at 33 mg/kg

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DM has been shown to have no effect on *E. coli* O157 in cattle (Jacob et al., 2008; McAllister et al., 2006). In 2006, 44 mg of monensin/kg of feed was approved as the upper limit in feedlot cattle diets (NADA 095-735, 2006). The impact of feeding 44 mg/kg of monensin on fecal shedding of *E. coli* O157 has not been determined. Ractopamine, a beta-agonist, (Schroeder et al., 2003; Laudert et al., 2004) has been shown to reduce (Edrington et al., 2006b) or have no effect (Edrington et al., 2009) on fecal *E. coli* O157. These conflicting data suggest that further studies are needed. Therefore, our objectives were to evaluate the effects of monensin level (33 or 44 mg/kg DM), supplemental urea (0, 0.35, or 0.70% of DM), and ractopamine (0 or 200 mg/steer) on fecal shedding of *E. coli* O157:H7 in cattle fed diets supplemented with wet sorghum DG.

Materials and Methods

All procedures in the care and management of cattle were approved by the Kansas State University Institutional Care and Use Committee.

Study Design

Seven hundred and twenty crossbred beef steers (initial BW = 433 ± 23.1 kg) were used in a randomized complete block design with a 2 x 3 x 2 factorial treatment arrangement. Factors were monensin level (33 or 44 mg/kg DM), level of supplemental urea (0, 0.35, or 0.70% of DM), and ractopamine (0 or 200 mg/steer daily, fed during the last 42 d before harvest; Figure 1). The basal diet consisted of steam-flaked corn with 30% wet sorghum DG (Table 1). Steers were allowed *ad libitum* access to alfalfa hay and water from arrival until processing at the start of the adaptation phase. On day 0 of the adaptation period, steers were weighed, vaccinated,

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stratified by weight into two blocks and then randomly assigned to pens. Forty-eight dirt-floor pens (10.4 x 26.8 m) containing 15 steers each were randomly assigned to 1 of 12 treatment combinations. Two transition diets and a final diet were fed over a 21 day step-up period. The amount of feed offered to the pen was determined at approximately 7:00 a.m., and the daily rations for each pen was delivered at approximately 8:00 a.m. and 2:00 p.m. each day. Cattle were fed amounts sufficient to result in traces of residual feed at the next feeding and fresh water was available at all times.

The three treatments were not started at the same time point of the study, and therefore, not all sample events occurred in steers fed diets with all treatments (Figure 1). Monensin was included in the transition and final diets while urea supplementation was initiated at the end of the adaptation phase and after the first sampling event. Ractopamine administration began after five sampling events, and therefore, the last four samples were from steers fed diets that included all three treatments.

Fecal samples (10/pen) were collected, July through November, from the surface of each pen approximately every 2 wk for 14 wk (n = 9). Samples (approximately 100 g) were collected from freshly defecated fecal pats using a plastic spoon, and care was taken to avoid ground contamination or repeat sampling from the same steer on the same sampling. The spoon with feces was placed into a Whirl-pack bag (Nasco, Ft. Atkinson, WI), and once samples were collected, they were placed in a cooler with ice packs and delivered to the Kansas State University College of Veterinary Medicine Pre-harvest Food Safety Laboratory for processing.

Isolation of E. coli O157:H7

Fecal samples were cultured for *E. coli* O157:H7 as previously described (Greenquist et al., 2005). Briefly, samples were kneaded for 30 sec and approximately 1 g of feces was placed in tubes containing 9 mL Gram-negative (**GN**) broth (Becton Dickinson, Franklin Lakes, NJ) with cefixime (0.05 mg/L), cefsoludin (10 mg/L), and vancomycin (8 mg/L; **GNccv**). The GNccv broth was then incubated for 6 h at 37°C. After enrichment, immunomagnetic bead separation (**IMS**) was performed, followed by plating onto sorbitol-MacConkey agar with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; **CT-SMAC**). Plates were incubated overnight at 37°C, and up to six sorbitol negative colonies were streaked onto blood agar plates. Colonies were tested for indole production and latex agglutination for the O157 antigen, and further characterized by multiplex PCR to identify *eae* (intimin)*, stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *hly* (hemolysin)*, rfbE* (O157 antigen) and *fliC* (flagella) genes (Bai et al., 2010). Personnel conducting sampling and laboratory analyses were blinded to the treatment assignments.

Statistical Analysis

Descriptive statistics on prevalence outcomes were assessed prior to multivariable analysis. A repeated measures negative binomial generalized linear model (PROC GENMOD, SAS v. 9.2, Cary, NC) with a log links was used to analyze data. The negative binomial function was chosen, as it best represented data having a high number of zero prevalence samples and few samples with very high prevalence. It was verified to be the correct function by examining the dispersion parameter point estimate (α). Because the 95% confidence interval did not include 0, the negative binomial model was deemed appropriate (Heeringa et al., 2010). Pen was considered the experimental unit with the count of positive samples as the outcome. This was

offset by the natural log of the number of samples processed for each pen. Pen over time was included as a repeated (random) effect to account for the lack of independence among samples from the same pen over sampling days. Score statistics for the generalized estimating equations were used to assess significance of sampling day, monensin, urea, ractopamine, and their interactions. Because urea and ractopamine were not fed throughout the trial, the data were analyzed in three phases. Results were considered significant at $P < 0.05$ and manual backward selection was used to remove variables that were not significant.

Results and Discussion

Overall fecal prevalence of *E. coli* O157:H7 was 7.6% (327 of 4,300 fecal samples), and ranged from 23.6% on the first collection (July) to 1.6 % on the eighth collection day (October; Figure 2). The multiplex PCR analyses revealed that all isolates (N=327) were positive for *eae, stx2, hly, rfbE* and *fliC,* and only 14% of the isolates (47/327) were positive for *stx1*. There were no two- or three-way interactions observed between monensin, urea and ractopamine treatment; however, there was a tendency for interaction between monensin and sampling week ($P = 0.06$), indicating that *E. coli* O157:H7 prevalence between monensin treatments tended to vary by sampling week. Fecal shedding of *E. coli* O157:H7 in cattle is influenced by a number of factors, including diets and dietary additives fed to the animal (Callaway et al., 2009; Jacob et al., 2009). It is believed that dietary ingredients that flow into the hindgut and alter the microbial population and fermentation products are likely to have an impact persistence and fecal shedding of *E. coli* O157:H7 (Jacob et al., 2009).

Cattle fed monensin at 44 mg/kg of feed had lower ($P = 0.05$) *E. coli* O157:H7 prevalence than cattle fed 33 mg/kg (4.3 vs 6.8%, Figure 3). Previous studies generally examined monensin

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at 33 mg/kg and compared it to a control group that lacked monensin in the diet. Van Baale et al., (2004) in a challenge study (n= 12) fed at a slightly higher dose of 39.6 mg/kg, found no difference in fecal *E. coli* O157 over a 63-d study period, but observed an initial decrease (first 5 d) in fecal concentration of *E. coli* O157 in monensin-fed cattle on a grain diet, but not in forage-fed animals. McAllister et al. (2006) observed no difference in fecal shedding of *E. coli* O157 (66 vs. 71%) when cattle (n = 32) were fed monensin at 0 or 33 mg/kg in the diet. Jacob et al., (2008) in a natural prevalence study found no differences ($P = 0.8$) in fecal *E. coli* O157:H7 between cattle fed diets with 0 and 33 mg/kg monensin. Although monensin is not generally inhibitory to gram negative bacteria, some are susceptible to high concentrations (Nagaraja and Taylor, 1987). *In vitro* studies have shown that monensin at concentrations equal to (Bach et al., 2002) or higher (Van Baale et al., 2004) than concentrations expected in the rumen when 33 mg/kg of monensin is fed did not affect the growth of *E. coli* O157:H7. Because a substantial proportion of monensin passes through the intestinal tract intact, it is likely that the reduction in fecal shedding of *E. coli* O157:H7 observed with the higher dose of monensin is because of changes in the microbial populations and fermentation products in the hindgut. The effects of monensin on hindgut fermentation, such as increased propionic acid, are similar to that described in the rumen (Yokoyama et al., 1985; Marounek et al., 1990).

Supplemental urea at 0.35 or 0.7 % had no effect ($P = 0.87$) on fecal shedding of *E. coli* O157:H7. Fecal prevalence of *E. coli* O157:H7 were 5.3, 5.7, and 5.9 % for groups fed 0, 0.35 and 0.7% urea, respectively (Figure 4). Distillers grains, which contain little starch (< 5% in DG vs. >70% in corn, Belyea et al., 2004) and approximately three times higher lipid, protein, and fiber contents than corn grain (Spiehs et al., 2002), have been shown to increase *E. coli* O157:H7 prevalence in cattle (Jacob et al., 2008; Wells et al., 2009). Increased flow of lipid, protein and

fiber to the hindgut are likely to alter ecology of the hindgut, which may have an impact on persistence of *E. coli* O157:H7. Increased flow of lipid into the hindgut may have a negative effect on *E. coli* O157:H7 because of the antibacterial effects of the released free fatty acids (Annamalai et al., 2004). It is possible that lipid may get digested and absorbed from the small intestine and not flow in sufficient amounts into the hindgut to have a negative effect. Dietary fiber is known to alter the physiology and stimulate bacterial growth in the human colon (Cummings and Stephan, 1980). Possibly, increased flow of fiber into the hindgut stimulates mucus production, which may be stimulatory to *E. coli* O157:H7 growth (Fox et al., 2009). Because DG contain less degradable protein (Klopfenstein et al., 2008), ruminal availability of nitrogen may be limiting, which may hinder fiber (Firkins et al., 1986) or starch (Fron et al., 1996) digestion in the rumen. Provision of supplemental ruminal ammonia as urea may enhance ruminal fermentation and decrease flow of fiber to the hindgut. Firkins et al. (1986) demonstrated that diets containing DG had less ruminal NDF digestion compared to a diet containing dry corn gluten feed, but the total tract NDF digestion was greater for DG diet suggesting greater hindgut fermentation. Based on *in vitro* and *in vivo* studies, Fron et al. (1996) demonstrated that inclusion of condensed distiller's byproducts altered microbial activities (increased ruminal populations of starch and lactate utilizing bacteria) and increased the rate of fermentation of lactic acid, however, ruminal pH was unaffected. Varying levels of urea, which could potentially improve ruminal fiber digestion, did not affect fecal prevalence of *E. coli* O157:H7 in this study. It is possible that ammonia was not a limiting factor, hence urea supplementation did not have an effect fecal shedding of *E. coli* O157:H7. Increased availability and degradation of protein in the hindgut and therefore, increased ammonia production and elevated pH may favor persistence of *E. coli* O157:H7.

The inclusion of ractopamine at 0 or 200 mg/head/d had no effect (P = 0.89) on fecal

prevalence of *E. coli* O157:H7 (4.4 vs 4.0 %; Figure 5). However, the power to detect an effect

was low due to low *E. coli* O157:H7 prevalence in the final phase of the study. The mean fecal

prevalence in the last four samples (collected in the month of October) was 4.2% compared to a

mean of 10.3% in the first five samples (collected in July, August, and September). The

reduction in prevalence in October is likely because of seasonal influence on fecal shedding of *E.

coli* O157:H7 (Edrington et al., 2006a; Fernández et al., 2009). Ractopamine, a beta-agonist, is

used to accelerate gain and improve efficiency in cattle during the final 4 to 6 wk of the finishing

phase (Schroeder et al., 2003; Laudert et al., 2004). Ractopamine may potentially interact with

the bacterial quorum-sensing systems (Sperandio et al., 2003) to affect *E. coli* O157:H7

prevalence. Studies have shown that the neuroendocrine environment of the gastrointestinal

tract, particularly norepinephrine released by the enteric nervous system, has influence on

bacterial growth, and in certain pathogens, including *E. coli* O157:H7, expression of virulence

factors (Lyte etal., 1997). Edrington et al. (2006b) conducted a study with a small number of

cattle (n=20) that were administered 0 or 20 mg of ractopamine daily for 28 d and observed a

reduction in fecal shedding (P = 0.006) with ractopamine (51.2 vs 41.8%, for 0 and 20 mg

ractopamine, respectively). In another study (Edrington et al., 2006b) with 1,800 feedlot heifers,

in three replicates, ractopamine fed at 200 mg/animal daily for 28 d lowered (P = 0.05) *E. coli*

O157:H7 prevalence (8.5 vs 13.8%) in feces compared to control heifers fed no ractopamine.

However, there were no significant differences in two of the three replicates. Recently,

Edrington et al., (2009) used 504 feedlot heifers in a 2 x 3 factorial design, with ractopamine

included at 0 or 200 mg/heifer daily for 14, 28, or 42 d. Fecal samples were obtained at day 0

and then at days 14, 28, or 42 corresponding to each treatment period. There were no significant differences in prevalence among treatments.

In conclusion, our results suggest inclusion of supplemental urea in the diet had no effect on fecal shedding of *E. coli* O157:H7 in cattle. Our premise in testing the effect of supplemental urea was the potential effects of altered ruminal and possibly hindgut fermentation on *E. coli* O157:H7. Because we did not monitor ruminal or hindgut fermentation changes, we have no evidence that fermentation was altered by feeding supplemental urea. Similarly, the low prevalence of *E. coli* O157:H7 during ractopamine feeding precluded us from assessing the impact of ractopamine. The reduction in fecal shedding of *E. coli* O157:H7 associated with feeding the high dose of monensin is interesting and somewhat surprising considering that prior studies (although with lower doses) had shown no effect. Therefore, additional research is needed to confirm the reduction in fecal shedding of *E. coli* O157:H7 in cattle fed 44 mg/kg monensin.

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Figure 2.1. Timeline for administration of dietary treatments and fecal sampling events (indicated by arrows).

Sampling week
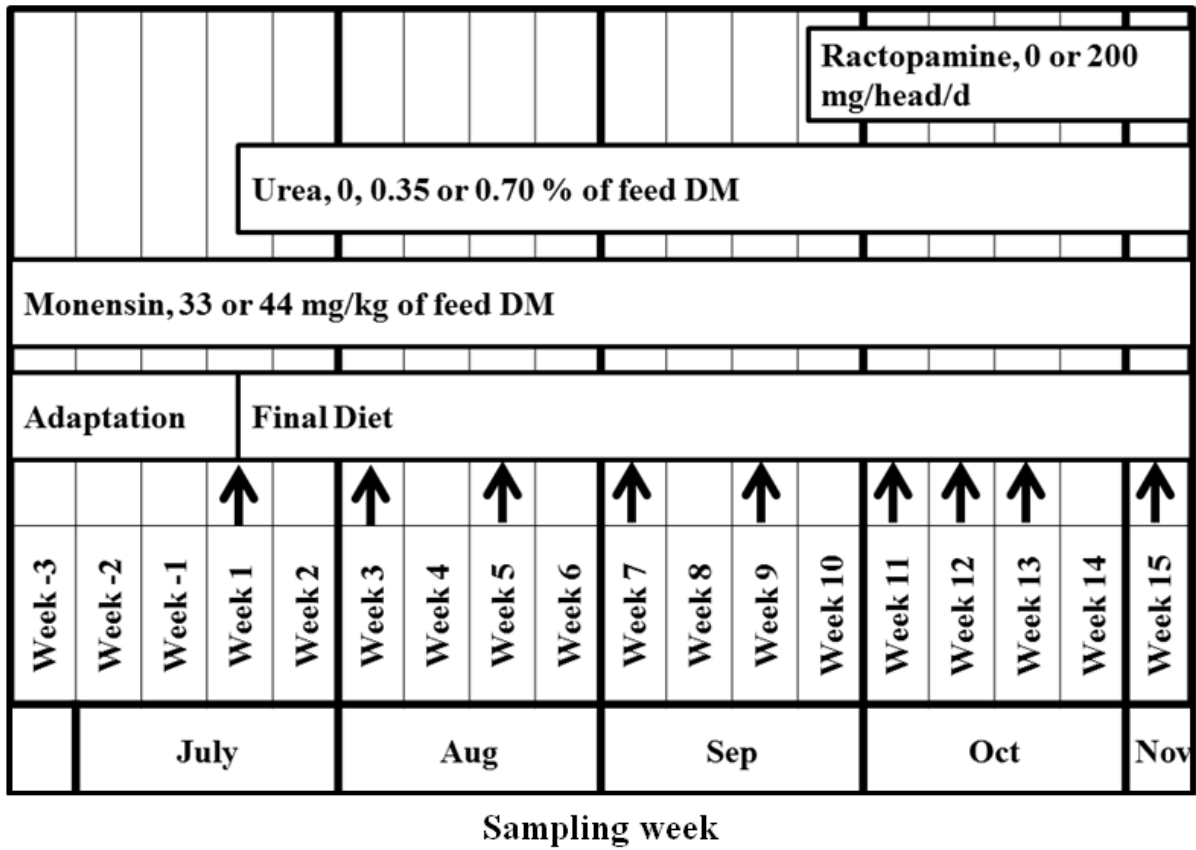
Figure 2.2. Overall fecal prevalence of *Escherichia coli* O157:H7 in cattle fed steam-flaked corn grain based diet supplemented with 30% wet distiller's grains (n = 48 pens; SEM = 0.7).
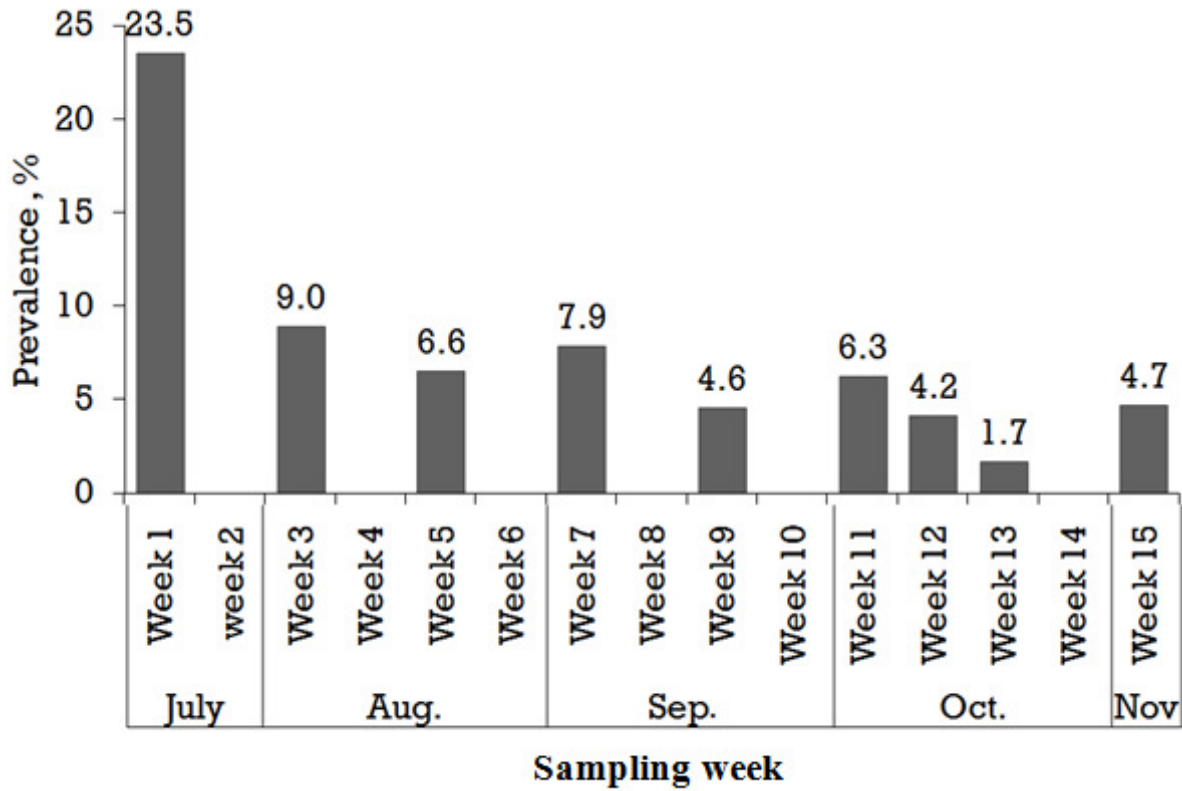
Figure 2.3. Fecal prevalence of *Escherichia coli* O157:H7 in steers fed steam-flaked corn grain-based diet with 30% wet sorghum distiller's grains and supplemented with monensin at 33 or 44 mg/kg of feed (n = 24 pens/treatment; SEM = 1.2).
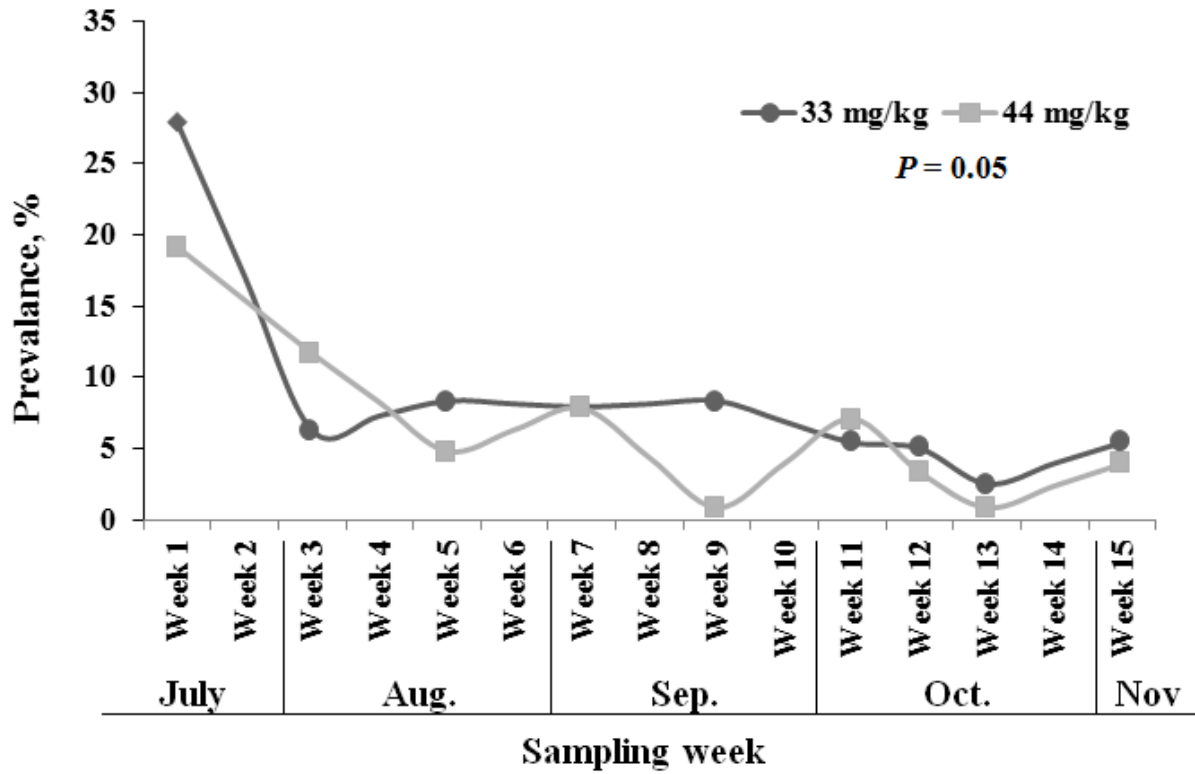
Figure 2.4. Fecal prevalence of *Escherichia coli* O157:H7 in steers fed steam-flaked corn grain-based diet with 30% wet sorghum distiller's grains and supplemented with urea at 0, 0.35, or 0.70 % of the diet (n = 16 pens/treatment SEM = 1.2).
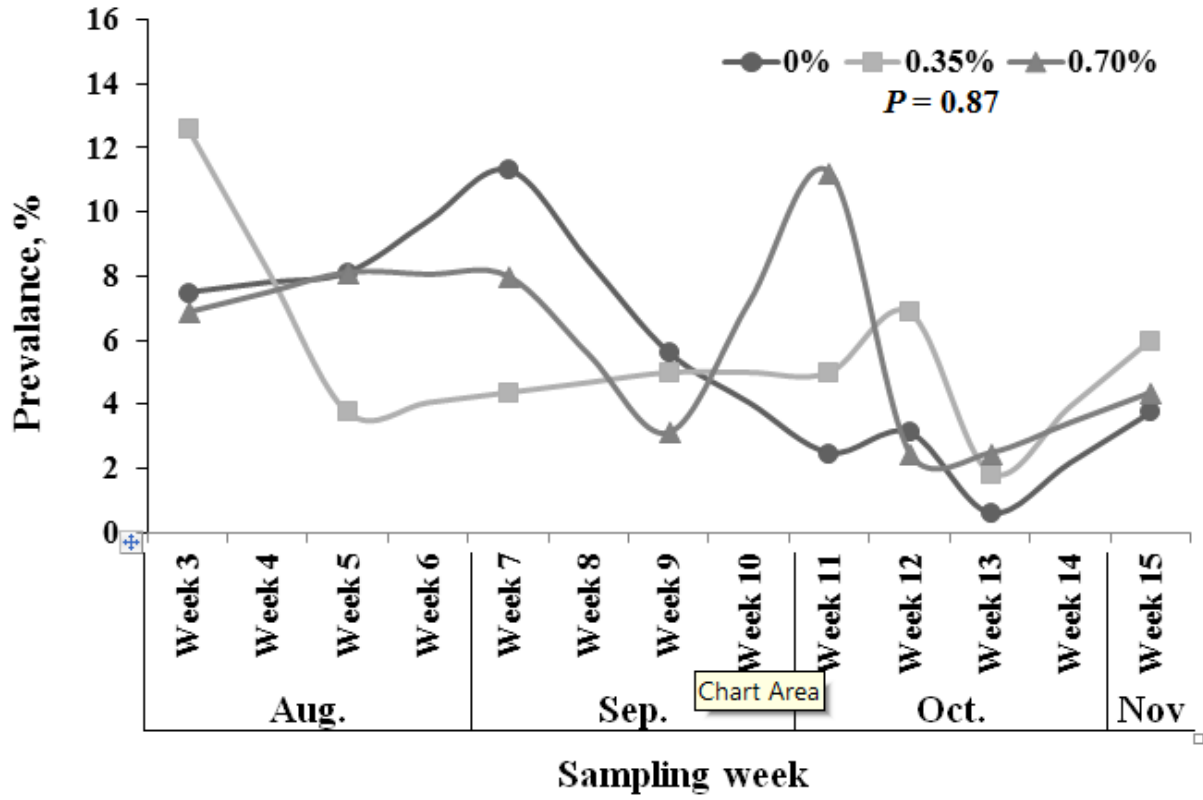
Figure 2.5. Fecal prevalence of *Escherichia coli* O157:H7 in steers fed steam-flaked corn grain-based diet with 30% wet sorghum distiller's grains and supplemented daily with ractopamine at 0 or 200 mg/steer (n = 24 pens/treatment; SEM = 1.2).
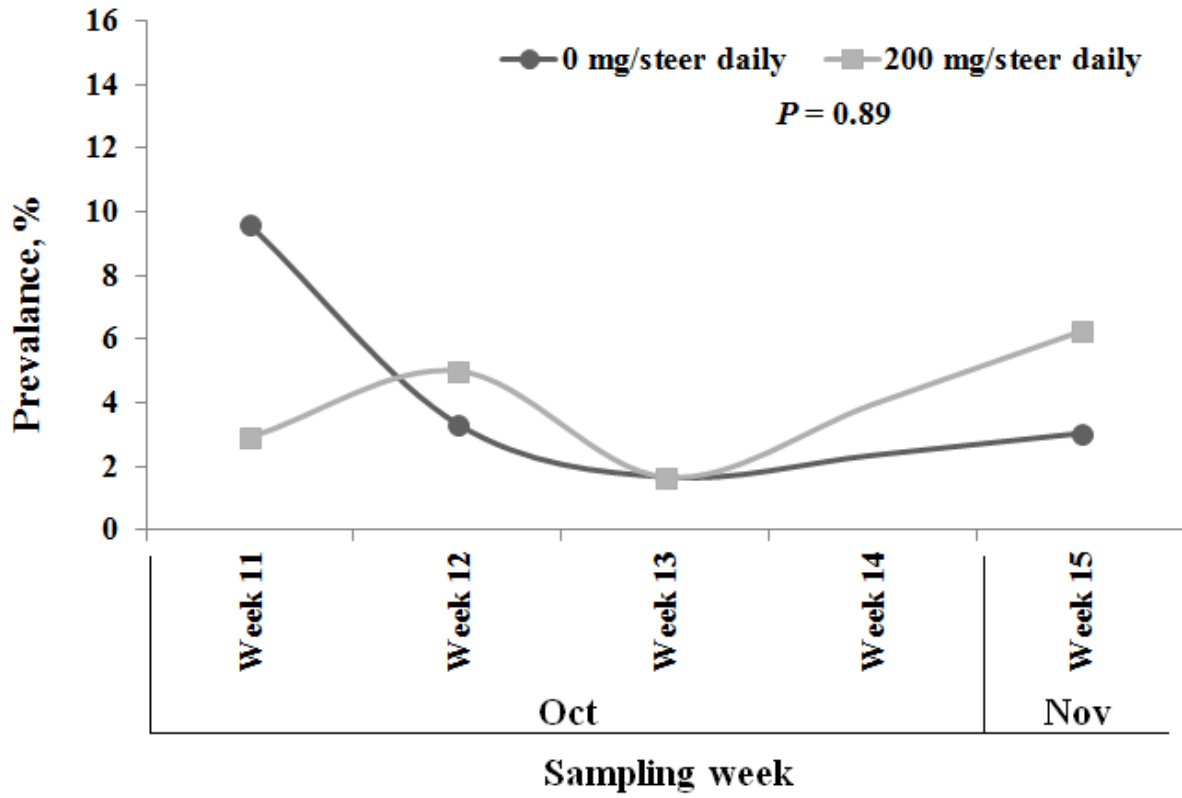
Table 2.1. Ingredient and chemical composition of the diet

Item	Percentage, DM basis
Ingredient	
Steam-flaked corn	58.1, 57.7, or 57.3
Wet sorghum distiller's grain	30.0
Wheat straw	7.0
Premix[1]	2.38
Limestone	1.80
Urea	0, 0.35, or 0.70
Supplement[2]	0.71
Nutrient Composition	
Dry matter, %	57.5
CP, % DM	14.2, 15.4, or 16.5
NDF, % DM	19.2

[1]Feed additive premix provided 33 or 44 mg of monensin sodium (Elanco Animal Health, Greenfield, IN) and 11 mg tylosin (Elanco Animal Health, Greenfield, IN) per kg of feed.

[2]Supplement contained 2,650 IU vitamin A; 10 mg Cu; 0.5 mg I; 0.15 mg Co; 0.25 mg Se; 50 mg Zn; and 50 mg Mn per kg of diet DM; Ractopamine hydrocholoride (Elanco Animal Health, Greenfield, IN) included in the diet at 0 or 200 mg per animal daily the last 42 d before harvest.

Chapter 3 - Effects of feeding dried distillers grains with supplemental starch on fecal shedding of *Escherichia coli* O157:H7 in experimentally inoculated steers

Abstract

Distillers grains (DG), a co-product of ethanol production used as protein and energy supplements in cattle diets, have been shown to increase fecal shedding of *Escherichia coli* O157:H7, a major foodborne pathogen. The reason for the positive association is not known. Because DG often replaces grain in the diet, decreased starch content and flow to the hindgut may create a favorable environment for *E. coli* O157:H7. Our objective was to determine whether the addition of starch to a DG-supplemented diet negates the effects of DG on fecal shedding of *E. coli* O157:H7. We conducted a study with 21 steers fed one of three diets: a corn grain-based basal diet (CON), basal diet supplemented with 25% dried DG (DDG), and basal diet supplemented with 25% DDG with corn starch (DDG+S) added at a level intended to raise starch concentration to that of the CON diet. Steers, housed individually in a biosafety level 2 animal facility, were randomly allocated to treatment diets and orally inoculated with a 5-strain mixture (10^9 cfu per steer) of nalidixic acid-resistant (Nal^R) *E. coli* O157:H7. Fecal samples were collected for 5 wk, and on d 35, steers were euthanized and necropsied to collect gut content samples. Fecal or gut samples were cultured to determine prevalence and concentrations of Nal^R *E. coli* O157:H7. Dietary starch concentrations, based on feed analysis, were 46.3% in

the CON compared with 43.3% and 41.3% in the DDG and DDG+S diets, respectively. Steers fed DDG or DDG+S diets shed Nal^R *E. coli* O157:H7 more often ($P < 0.01$) and at higher concentrations (1.9 or 2.0 cfu/g; $P < 0.01$) than those fed CON diet (1.4 cfu/g), but no difference was observed between DDG and DDG+S diets. Cumulative prevalence and concentrations of Nal^R *E. coli* O157:H7 were higher in gut samples collected at necropsy in steers fed DDG and DDG+S diets compared with those fed CON diet, but no difference was observed between DDG and DDG+S diets. The study confirms our previous finding of the positive association between feeding DG and fecal shedding of *E. coli* O157:H7. The lack of effect of starch addition to the DDG diet on fecal shedding of *E. coli* O157:H7 may be because either the decreased starch content in the DG-supplemented diet is not a factor in the increased shedding of *E. coli* O157:H7 or added starch was completely digested in the rumen and small intestine with no starch reaching the hindgut to have an impact.

Introduction

Escherichia coli O157:H7, a major food borne pathogen, inhabits the hindgut of cattle and is shed in the feces (Naylor et al., 2003; Walker et al., 2010). Cattle feces can be a source of human infection through direct or indirect contamination of food and water (Rangel et al., 2005). Fecal shedding of *E. coli* O157:H7 is influenced by a number of factors, including diet (Callaway et al., 2009). Dietary ingredients that flow into the hindgut and potentially alter the ecosystem are likely to affect fecal shedding of *E. coli* O157:H7 (Jacob et al., 2009). Distillers grains (**DG**), a co-product of ethanol production, are widely used as an energy or protein supplement in cattle diets. Feeding DG, which contain no or very little ($< 5\%$) starch (Spiehs et al., 2002; Belyea et al., 2004), has been shown to increase fecal shedding of *E. coli* O157:H7 in

cattle by more than 30% (Jacob et al., 2008b; Jacob et al., 2010; Wells et al., 2009). The reason

for the increase is not known. Distillers grains could contain components that directly or

indirectly stimulate growth of *E. coli* O157:H7 (Jacob et al., 2008a); indirect stimulation is likely

mediated by the impact of DG component(s) on hindgut microbial ecology. Replacing corn

grain with DG supplementation in the diet reduces starch content and results in decreased starch

flow to the hindgut, which likely alters ecology of the hindgut and creates an environment

favorable to the growth and persistence of *E. coli* O157:H7 (Jacob et al., 2009). Therefore, we

hypothesized that decreased starch content of the DG-supplemented diet may account for the

increased fecal shedding of *E. coli* O157:H7. Our objective was to determine whether addition

of starch to the DG-supplemented diet would negate the increased fecal shedding of *E. coli*

O157:H7. We formulated a DG-supplemented diet to equal the starch content of corn grain-

based diet and used an experimental inoculation model to test our hypothesis.

Materials and Methods

All procedures in the care and management of cattle in the study were approved by the

Kansas State University Institutional Care and Use Committee.

Animals and Treatment Groups

Twenty-one Holstein steers (mean BW = 166.4 ± 4.3 kg) were randomly assigned using a

spreadsheet algorithm to one of three dietary treatments (Table 1). Initially, animals within each

treatment group were housed together in outdoor concrete-surfaced pens. A high-grain diet

consisting of 70.1% dry-rolled corn grain constituted the control diet (**CON**). The second dietary

treatment had 25% dried DG (**DDG**) added, on a DM basis, to replace a portion of the dry-rolled

corn. The third dietary treatment (**DDG+S**) consisted of 25% DDG with 23.5% of the remaining

dry-rolled corn grain replaced with corn starch in order to have starch and nonfiber carbohydrate (**NFC**) concentrations similar to that of the CON diet. The diets were formulated to meet NRC requirements and adjusted to contain as similar concentrations of all other nutrients as possible (Table 1). Steers were stepped-up with 3 increments of their respective diets (50, 65, and 85% concentrate) approximately every 4 d over a 2 wk period. Steers were then moved to individual pens (1.52 × 3.05 m) within a biosafety level 2 facility and allowed to acclimate for 1 wk prior to oral inoculation with *E. coli* O157:H7. Steers were randomly (spreadsheet algorithm) assigned to the individual pens that have walls high enough (1.8 m) to prevent physical contact between adjacently penned animals. Each pen was equipped with a built-in head catch to facilitate stomach tubing, so the steers were not moved from their pens. Diets were fed twice daily at levels targeting 5% refusals, which were mixed into the next feeding; all steers had *ad-libitum* access to fresh water. Pens were surface washed with minimal splashing and cleaned once daily; pen floors and drains were designed to prevent wash water from contaminating adjacent pens. Body weights were recorded on the day steers were moved to the individual pens and on the necropsy day to determine ADG over the duration of the experiment.

Escherichia coli O157:H7 Inoculation

Steers were orally inoculated with a 5-strain mixture (KSU 01-2-8970, 01-2-10004, 01-2-10530, 01-2-7443, and 01-2-12329) of *E. coli* O157:H7, previously isolated from feedlot cattle feces (Sargeant et al., 2003) and made resistant to 50 µg/mL nalidixic acid (Nal^R *E. coli* O157:H7). All 5 strains were PCR positive for the *eae* (intimin), *stx*2 (Shiga toxin 2), *ehx*A (enterohemorrhagic hemolysin), and *fli*C (flagellar antigen) genes, and 1 strain (01-2-8970) was also positive for the *stx*1 (Shiga toxin 1) gene. Individual isolates from frozen storage beads

were streaked on blood agar plates (**BAP**; Remel, Lenexa, KS) and grown overnight at 37°C. A single colony was selected, inoculated into 10 mL of tryptic soy broth (**TSB**; Difco, BD, Sparks, MD) and incubated overnight at 37°C. One milliliter of TSB culture was then inoculated into 100 mL of TSB and incubated for 7 h at 37°C to reach an absorbance of 0.6 at 600 nm. Aliquots of 5 strains cultures were mixed together and 1 mL of the pooled culture was serially diluted 10-fold in buffered peptone water (Remel) and spread plated (4 plates per dilution) onto sorbitol-MacConkey agar (Difco) plates with cefixime (0.5 mg/L), potassium tellurite (2.5 mg/L), and nalidixic acid (50 µg/mL; **CTN-SMAC**) to determine the concentration of Nal^R *E. coli* O157:H7. Each steer was dosed orally, via stomach tube, with 5 mL of pooled culture mixed with 100 mL of 1% sterile skim milk (Oxoid, Basingstoke Hampshire, UK). The final inoculum (5 mL) contained 5.6×10^9 cfu of Nal^R *E. coli* O157:H7. Steers were monitored twice daily following inoculation for clinical signs of infection including general depression, respiratory distress, depressed feed intake, and loose stool.

Fecal Sampling and Detection of E. coli O157:H7

Fecal samples were collected from each animal before inoculation (d -7 and 0) and tested to for *E. coli* O157:H7 as described previously (Greenquist et al., 2005). Briefly, 1 g of fecal sample was enriched in 9 mL of Gram Negative broth (Difco) with cefixime (0.5 mg/L), cefsoludin (10 mg/L), and vancomycin (8 mg/L; **GNccv**). Immunomagnetic bead separation (Dynal Inc., New Hyde Park, NY) was performed and samples were plated onto sorbitol-MacConkey agar (Difco) with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; CT-SMAC). Six sorbitol-negative colonies were picked and tested for indole production and O157 antigen latex agglutination (Oxoid).

After oral inoculation with Nal^R E. coli O157:H7 (d 0), fecal samples were collected from each steer 3 times a wk (Monday, Wednesday, and Friday). Foot baths with 1% Virkon S/Trifectant (21.4% potassium peroxymonosulfate; 1.5% sodium chloride solution; Vètoquinol, Fort Worth, TX) were used and gloves were changed between each steer. Fecal samples were collected via rectal palpation or from pen floors if defecation was observed, placed in Whirl-pack bags (Nasco, Ft. Atkinson, WI), and transported to the laboratory. Approximately 10 g of feces was also placed in a 50 mL conical tube containing 25 mL of double-deionized water and vortexed for 1 min. The pH of the fecal suspension was immediately measured using an Accumet AB15 Basic pH meter (Fisher Scientific, Waltham, MA). Aliquots of fecal samples collected on d 5, 12, 21, 28, and 35 were frozen at -20° C for nutrient (ADF, NDF, CP and starch) analysis.

Detection and Enumeration of Nal^R E. coli O157:H7

Approximately 1 g of feces was added to a pre-weighed tube containing 9 mL of GNccv broth and the tube was weighed again to record the sample weight. One milliliter of the GNccv fecal suspension was serially diluted 10-fold in buffered peptone water. One hundred microliters of different dilutions were spread-plated, in triplicate, on CTN-SMAC. Plates were incubated overnight at 37˚C and the sorbitol-negative colonies were counted to determine the concentrations of Nal^R E. coli O157:H7. The remaining GNccv broth was enriched for 6 h at 37º C, after which 1 mL was removed and inoculated into another 9 mL tube of GNccv broth (secondary enrichment) and incubated for 16 to 18 h at 37ºC. If colonies were not recovered by direct plating, 100 µL of the secondary enrichment was plated onto CTN-SMAC. A colony from

each direct or secondary enrichment plate was picked, plated on BAP and tested for indole production and O157 antigen latex agglutination.

Necropsy and collection of gut contents

Thirty-five days after inoculation, steers were euthanized and necropsied. Steers were divided into 2 groups and euthanized on 2 consecutive days with treatment groups balanced across euthanasia days. The order of animals euthanized was random and steers were not fed on the day of euthanasia, which was performed in the morning. Steers were then immediately necropsied and contents from the rumen, cecum, colon, and rectum were collected, transported to the laboratory and concentrations of Nal^R *E. coli* O157:H7 were determined as above. The pH of rectal contents were measured after preparing a suspension in double-deionized water and that of rumen, cecum, and colon samples were measured without the addition of double-deionized water. The rectum was removed and cut open, the mucosa was rinsed with water to remove visible fecal material and the 3- to 5- cm area proximal to the rectoanal junction, was swabbed with a foam-tipped applicator (RAMS; VWR International, Buffalo Grove, IL; Fox et al., 2008). Swabs were placed in 3 mL of GNccv broth, transported to the laboratory, vortexed and processed for detection of Nal^R *E. coli* O157:H7 as described above.

Feed and Fecal Nutrient Analyses

Approximately, 0.5 kg of each diet was collected (n = 15 per treatment) on each day of fecal sampling, and composited within treatment. The composite samples were sent to Cumberland Valley Analytical Services (Hagerstown, MD) for DM, NFC, starch (according to Hall, 2009), CP and NDF analyses. Frozen fecal samples from d 5, 12, 21, 28, and 35 were submitted to the

Animal Science Analytical Lab (Kansas State University, Manhattan, KS) for DM, ADF, NDF, CP, and starch analyses.

Statistical Analysis

Dietary treatment effects were assessed in linear or generalized linear mixed models (LMM and GLMM, respectively; Proc Glimmix; SAS Version 9.2, SAS Institute inc., Cary, NC) depending on whether the outcome variable was continuous or categorical, respectively. When the outcome variable was continuous, as was the case for concentrations of fecal Nal^R *E. coli* O157:H7 and nutrient components (DM, starch, CP, NDF and ADF) and fecal pH, a LMM was used to access the effects of dietary treatment. When a categorical outcome variable, such as whether an animal's sample from feces or necropsy was positive or negative for Nal^R *E. coli* O157:H7, was being analyzed, a GLMM was used assuming a binomial distribution and utilizing a logit link function. Concentrations of Nal^R *E. coli* O157:H7 were $\log_{10}$ transformed before data analyses. If Nal^R *E. coli* O157:H7 was not detected by direct plating, but the enriched sample was positive, the lowest enumerable concentration was assigned to account for sample weight (100 cfu/g). The concentration in RAMS samples was not determined because the size of the inoculum was unknown; therefore, RAMS were considered positive (direct plating or enrichment) or negative and included only in analysis of prevalence data.

Initially in both LMM and GLMM for data from individual fecal samples collected throughout the trial, the linear predictors were dietary treatment, sampling day and dietary treatment × sampling day interaction. For data from samples taken at necropsy, dietary treatment, sample site (rumen, cecum, colon, rectum or RAMS) and dietary treatment × sampling site interaction were initially included in the model as linear predictors, for both LMM and

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GLMM. In addition, animal was included as a repeated effect in all models of data from repeated sampling in order to account for the lack of independence between samples. When GLMM with repeated measures failed to converge properly, additional GLMM specified in an events/trials format were used to model cumulative prevalence (total sampling days positive or total gut sampling sites positive for each animal (events) over total number of samples collected (trials) for each animal) with dietary treatment as the linear predictor. For animal performance outcomes (ADG and DMI) that were averaged over the trial, a LMM with dietary treatment included as a single linear predictor was used. Model-adjust means (LSmeans; back transformed to the original scale when appropriate) and corresponding standard error of the means are reported. For all models, P values < 0.05 were considered statistically significant and P values ranging from 0.05 to 0.10 were considered statistical trends.

Results

All steers tested negative for fecal prevalence of *E. coli* O157:H7 on the day of and the 7 d prior to oral inoculation. None of the steers exhibited any adverse clinical signs following oral inoculation of *E. coli* O157:H7. We detected no differences between dietary treatment groups in ADG and DMI of steers during the 35 d of the experiment. The 3 treatment diets were formulated to be as similar as possible in all nutrient composition except starch and NFC. The starch concentrations, based on feed analysis, were 46.3% in the CON diet compared with 43.3% and 41.3% in the DDG and DDG+S diets, respectively. The NFC concentrations were 61.7 % in the CON, 58.3% in the DDG, and 61.9% in the DDG+S diets, respectively.

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Fecal Shedding of Nal^R E. coli O157:H7

Two days after oral inoculation with *Nal^R E. coli* O157:H7, all steers were shedding *Nal^R E. coli* O157:H7, which was detected only after samples were enriched and plated on CTN-SMAC. The level of shedding could not be quantified because the dilutions chosen (10^{-3} to 10^{-6}) to inoculate the plates showed no growth, indicating that fecal concentration was below 10^4 cfu/g of feces. However, in the samples collected on d 5, concentrations of *Nal^R E. coli* O157:H7 in feces from all three groups of steers ranged from 10^4 to 10^5 cfu/g. Data analyses of the fecal concentrations of *Nal^R E. coli* O157:H7 showed that the sampling day × dietary treatment interaction was not significant ($P = 0.21$). Sampling day had a significant effect ($P < 0.001$) on the fecal concentration of *Nal^R E. coli* O157:H7. After d 5 in the CON group, fecal concentrations of *Nal^R E. coli* O157:H7 declined gradually, and positive samples were only detectable by direct plating of enriched samples by d 19 (≤ 100 cfu/g; Figure 1). The fecal concentrations of *Nal^R E. coli* O157:H7 were different ($P < 0.001$) between dietary treatments. Feces from steers in the DDG (1.94±0.10 log10 cfu/g) and DDG+S (2.03±0.10 log10 cfu/g) groups had higher ($P < 0.01$ and $P < 0.001$, respectively) *Nal^R E. coli* O157:H7 concentrations than the CON group (1.44±0.10 log10 cfu/g), but no difference ($P = 0.52$) was observed between the DDG and DDG+S groups (Figure 1).

Analysis of cumulative fecal prevalence of *Nal^R E. coli* O157:H7 (total number of sampling days a steer was positive from d 2 to 35) demonstrated a significant dietary treatment effect where more steers were positive for fecal shedding of *Nal^R E. coli* O157:H7 in DDG ($P < 0.01$) and DDG+S ($P < 0.001$) compared with the CON group, and again there was no difference ($P = 0.26$) between the DDG and DDG+S groups (Figure 2).

Concentrations and Prevalence of Nal^R E. coli O157:H7 in Necropsy Samples

At the time of necropsy, the concentrations of Nal^R *E. coli* O157:H7 in gut contents was not associated with the gut sample site (rumen, cecum, colon, or rectum) × dietary treatment interaction ($P = 0.85$), but was significantly affected by gut sample site ($P = 0.03$) and tended to be affected by dietary treatment ($P = 0.06$). The rectal contents contained significantly higher ($P < 0.01$) concentrations of Nal^R *E. coli* O157:H7 compared with the contents of the rumen, cecum or colon, which did not differ. Overall across all sites, the DDG+S group contained higher concentrations of Nal^R *E. coli* O157:H7 than the CON ($P = 0.02$), and tended to have higher concentrations compared to DDG ($P = 0.09$) group (Table 2); CON and DDG groups did not differ ($P = 0.48$). Recto-anal mucosal swabs were not included in the gut sampling site concentration analysis because the RAMS method does not use a measured amount of sample.

The cumulative prevalence of Nal^R *E. coli* O157:H7 (total sampling sites positive per animal) at necropsy was significantly affected by dietary treatments ($P = 0.05$). Across all gut sample sites, dietary effects showed that steers in the DDG+S group had higher prevalence of Nal^R *E. coli* O157:H7 compared to CON ($P = 0.05$) or DDG ($P = 0.03$) group (Table 2). No difference ($P = 0.80$) in prevalence was detected between steers in the DDG and CON groups.

Fecal pH

Overall, fecal pH was significantly different over sampling days ($P < 0.01$) and between dietary treatment groups ($P < 0.001$; Figure 3), but sampling day × dietary treatment interaction was not significant ($P = 0.18$). The average fecal pH of steers in the DDG group (pH = 6.18) was lower ($P < 0.01$) than that of the CON and DDG +S groups (pH = 6.34 and 6.26,

respectively). The average fecal pH of steers of the DDG+S group tended to be lower ($P = 0.07$) than the CON group.

Gut contents pH at necropsy

A tendency ($P = 0.07$) was observed for gut pH to be associated with sample sites (rumen, cecum, colon, and rectum) × dietary treatment interaction; whereas pH was significantly different across gut sample sites ($P = 0.05$) and between dietary treatment groups ($P < 0.001$; Table 3). Ruminal pH was lower than the cecum, colon, and rectum, which were not different from each other. The pH of cecal and colonic contents were higher in the control group compared to DDG or DDG+S groups. The pH of gut contents did not differ ($P = 0.35$) between DDG and DDG+S treatment groups.

Fecal Composition

Overall, fecal protein and starch concentrations were significantly different ($P < 0.001$ and $P = 0.04$, respectively) between dietary treatment groups (Table 4), but did not change significantly over time, and no dietary treatment x sampling day interactions occurred. The fecal starch concentration of steers in the DDG+S group (11.5%) was lower than those of the CON group (16.0%; $P = 0.01$) and tended to be lower than DDG group (13.1%; $P = 0.09$); the CON and DDG groups were not different from each other. The fecal protein concentration of steers in the DDG+S group was lower (13.5%) than that of the CON (16.2%; $P < 0.001$) and DDG groups (15.8%; $P < 0.01$), which were not different ($P = 0.50$) from each other.

Discussion

Cattle fed high grain diets supplemented with DDG had increased fecal shedding of Nal^R *E. coli* O157:H7, and more animals were fecal positive for Nal^R *E. coli* O157:H7 for a longer duration compared with the control that had no DDG supplement. These findings are in agreement with previous research on DG effects on fecal shedding of *E. coli* O157:H7 (Jacob et al., 2008a,b, 2010; Wells et al., 2009, 2011). The reason(s) behind the positive association are unknown. *Escherichia coli* O157:H7 colonizes within the hindgut of cattle (Naylor et al., 2003; Walker et al., 2010), therefore, changes in hindgut microflora, pH and VFA concentrations associated with altered nutrient flow and availability may directly or indirectly affect the ability of *E. coli* O157:H7 to proliferate, colonize and persist in the hindgut. Our hypothesis was that lower starch concentrations in DG-supplemented diets, due to corn grain replacement, may contribute to higher fecal prevalence of *E. coli* O157:H7. We added corn starch to the DDG diet to achieve estimated NFC and starch concentrations similar to that of the control diet (60.0% estimated NFC; 49.0% estimated starch). We expected that some of the starch would flow into the hindgut and alter the conditions to have an effect on *E. coli* O157:H7; however, the fecal concentration and number of steers shedding *E. coli* O157:H7 in DDG+S were similar to that of DDG group, suggesting that inclusion of additional starch to the DDG diet had no effect on fecal shedding of Nal^R *E. coli* O157:H7. Because the starch added to the diet was pure, the starch could have been digested completely in the rumen and small intestine, with no starch reaching the hindgut to have an impact on hindgut fermentation. This possibility is supported by fecal starch concentrations, which were lowest in steers fed the DDG+S diet. The amount of corn grain that was replaced with corn starch (23.5% of DM) in the DDG+S diet was estimated from NRC (2000) values for corn grain (75.5% NFC; 68.0% starch) and Hall, (2009) for corn starch

(≥ 95% NFC; 95% starch). The added corn starch in the DDG+S diet did not achieve a similar concentration of starch compared with the CON diet (46.3% in CON vs. 41.3% in DDG+S). Furthermore, the DDG diet had a slightly greater concentration of starch compared to the DDG+S diet (43.3% and 41.3%, respectively); however, the amount of corn starch added to the DDG diet achieved a concentration of NFC similar to the CON diet (61.7 % in CON vs. 61.9 % in DDG+S), whereas the DDG diet had slightly lower than expected NFC concentration (58.3%). The discrepancy between NFC and starch concentrations in the DDG+S diet could not be explained and individual ingredient samples were not collected, so determining the exact cause of the discrepancy was not possible. Even with these differences in NFC and starch concentrations among dietary treatments, we observed a significant increase in the fecal shedding of *E. coli* O157:H7 by steers on DDG-supplemented diets. This result suggests that other nutrients or substances in DDG supplemented diets may be responsible for fecal shedding increase of *E. coli* O157:H7. We were not able to balance the diets for NDF due to the high concentration of NDF in DDG-supplemented diets, which resulted in DDG and DDG+S diets having 30.2% and 16.3% increase in NDF, respectively, over the CON diet, suggesting that NDF may influence the fecal shedding of *E. coli* O157:H7.

We had previously hypothesized (Jacob et al., 2009) that higher hindgut starch concentrations provide more substrate for the microbes and lead to low pH and high VFA concentration, thereby decreasing *E. coli* O157:H7. We observed a significantly lower fecal pH in the DDG and DDG+S groups compared with the CON diet, suggesting higher hind gut microbial activity, although we did not measure VFA concentrations. The higher concentration of fecal protein in the CON and DDG diets compared to the DDG+S diet suggests greater microbial growth in the hindgut (Ørskov et al., 1970). Kudva et al. (1997) showed that sheep,

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experimentally inoculated with *E. coli* O157:H7 and fed a grass hay diet, shed the organism for a

longer duration and at higher concentrations than sheep fed a corn and alfalfa pellet diet. These

researchers also observed fewer positive animals when the grass hay diet was abruptly changed

to a corn and alfalfa pellet diet, suggesting feeding of a starch-based diet hinders *E. coli*

O157:H7's ability to persist in the hindgut. Fox et al. (2007) and Jacob et al. (2008a) showed

that increased ruminal degradability of grain, associated with processed grains (steam-flaked vs.

dry-rolled), increased the prevalence of *E. coli* O157:H7, probably due to less starch reaching the

hindgut.

Distillers grains have increased concentrations of protein, fiber, and lipids (Spiehs et al.,

2002), which alter the flow and availability of nutrients in the hindgut. Increased protein and

fiber contents may allow for greater protein and fiber fermentation within the hindgut, thereby

altering pH and concentrations of fermentation products. Increased flow of fiber may stimulate

mucus production, and constituents of mucus, particularly gluconic acid, have been shown to

stimulate growth of *E. coli* O157:H7 (Fox et al., 2009). In our study, dietary protein was held

constant across all diets by altering the amount of soybean meal in the diet (10.8% in the CON

diet and 1.5% in the DDG+S diet). Dietary NDF was highest in the DDG diet (16.1%), lowest in

the CON diet (11.8%) and the amount in the DDG+S diet (14.1%) was between DDG and CON

diets. However, fecal concentration and prevalence of Nal^R *E. coli* O157:H7 did not differ

between DDG and DDG+S groups, and DDG+S tended to have a higher concentration and

significantly higher prevalence of Nal^R *E. coli* O157:H7 in necropsy samples. Increased dietary

lipid concentrations will increase the flow of free fatty acids, which could exert antimicrobial

effects (Annamalai et al., 2004), but free fatty acids are likely to be absorbed and not reach the

hindgut; also, the lipid concentrations across all 3 diets were held constant by adding fat (white

grease) to the CON diet. Another possibility (Jacob et al., 2008a, 2009) is that DG may contain unknown substances that have a stimulatory effect on *E. coli* O157:H7, and this idea is supported by an apparent stimulatory effect of DG when added to *in vitro* rumen fluid cultures inoculated with *E. coli* O157:H7; however, the same effect was not seen in fermentations with fecal bacterial cultures (Jacob et al., 2008a).

Regardless of the nutritional mechanism, feeding DG to cattle results in higher levels of *E. coli* O157:H7 in the feces which leads to higher levels of environmental *E. coli* O157:H7. Varel et al. (2008) demonstrated that *E. coli* O157:H7 inoculated into manure slurries from cattle fed DG (20 or 40% of DM) persisted longer and at higher concentrations over a 14 d- room temperature incubation compared with control manure slurries from cattle fed 0% DG. Higher concentrations of *E. coli* O157:H7 in a feedlot pen would create an environment more likely to reinoculate the bacteria into the gastrointestinal tract of cattle, furthering its persistence within a cohort of animals.

In conclusion, our experimental inoculation study confirms the positive association between feeding DG and fecal shedding of *E. coli* O157:H7. Furthermore, addition of starch to the DDG diet to achieve concentration of estimated starch similar to that of the corn grain-based diet without DG supplement diet had no effect on fecal shedding of *E. coli* O157:H7. However, inclusion of pure starch in the diet may not have achieved our intended goal to have starch flow into the hindgut similar to that of corn grain. Perhaps use of ruminally- protected starch or infusion of starch post ruminally would have been a better approach to test our hypothesis. Also, research is needed to understand whether other components of DG, protein or fiber, may be responsible for the positive association with *E. coli* O157:H7 fecal shedding.

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Figure 3.1. Fecal concentration of nalidixic acid-resistant (Nal^R) *Escherichia coli* O157:H7

following oral inoculation with a 5-strain mixture of Nal^R *E. coli* O157:H7 in steers fed a corn

grain-based, high grain diet supplemented with no dried distillers grains (CON; n = 7;

mean=1.44 ± 0.10 log10 cfu/g), 25% dried distillers grains (DDG; n = 7; mean=1.94 ± 0.10

log10 cfu/g) or 25% DDG plus corn starch (DDG+S; n = 7; mean=2.03 ± 0.10 log10 cfu/g).

Steers in the CON group had significantly lower ($P < 0.001$) fecal concentrations of Nal^R *E. coli*

O157:H7 compared with DDG or DDG+S; all other treatment comparisons, the day x treatment

interaction, and the main effect of day were not statistically significant.
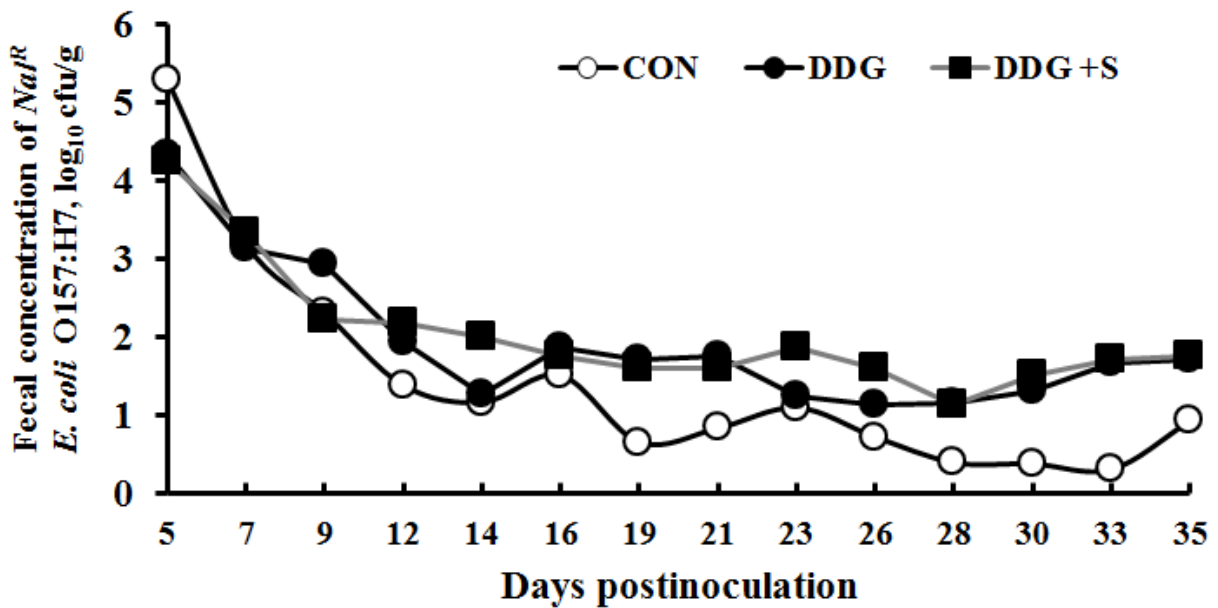
Figure 3.2. The number of cattle that tested fecal positive for nalidixic acid-resistant (Nal^R)

Escherichia coli O157:H7 by sampling day following oral inoculation with a 5-strain mixture of

Nal^R *E. coli* O157:H7. Cattle were fed corn grain based high-grain diets supplemented with no

dried distillers grains (CON; n = 7), 25% dried distillers grains (DDG; n = 7) or 25% DDG plus

corn starch (DDG+S; n = 7). The cumulative prevalence of Nal^R *E. coli* O157:H7 between days

2 and 35 was higher in steers fed DDG ($P < 0.01$; mean=80.6 ± 4 %) and DDG+S ($P < 0.001$;

mean 86.7 ± 3.4%) compared with CON (mean=57.1 ± 5.0 %) steers.
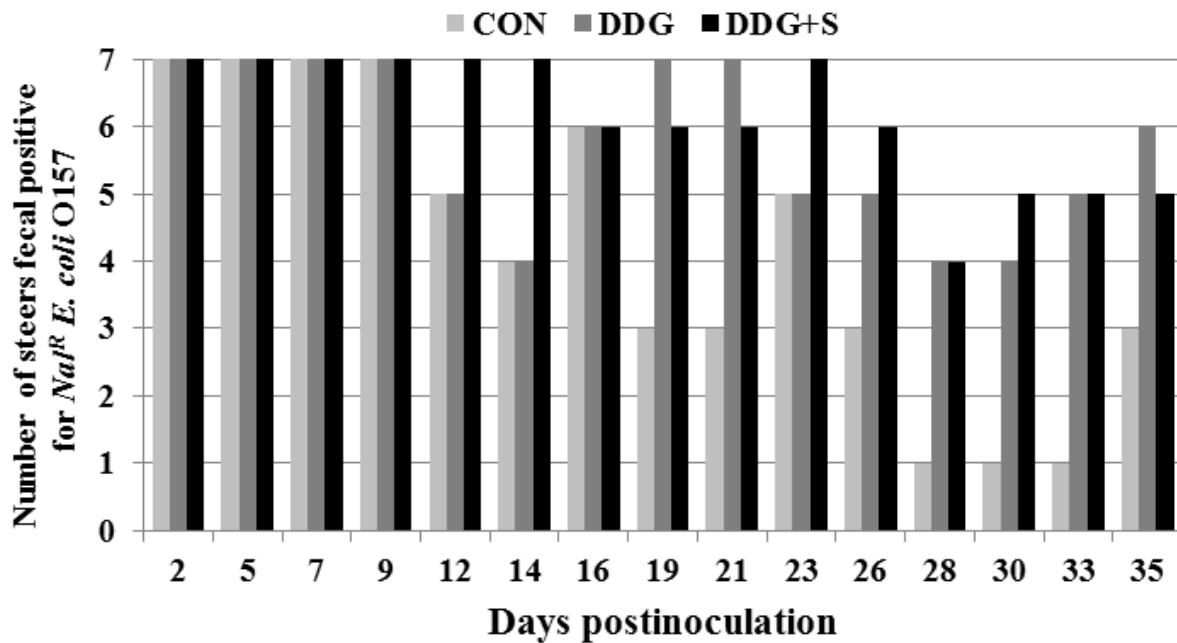
Figure 3.3. Fecal pH of steers fed corn grain-based high-grain diets supplemented with no dried distillers grains (CON; n = 7; mean=6.34 ± 0.03), 25% dried distillers grains (DDG; n = 7; mean=6.18 ± 0.03) or 25% DDG plus corn starch (DDG+S; n = 7; mean=6.26 ± 0.03) by sampling day following oral inoculation with nalidixic acid-resistant *Escherichia coli* O157:H7. Fecal pH was significantly different between dietary groups ($P < 0.001$), and between sampling days ($P < 0.01$), but the dietary group x sampling day interaction was not significant ($P = 0.18$).
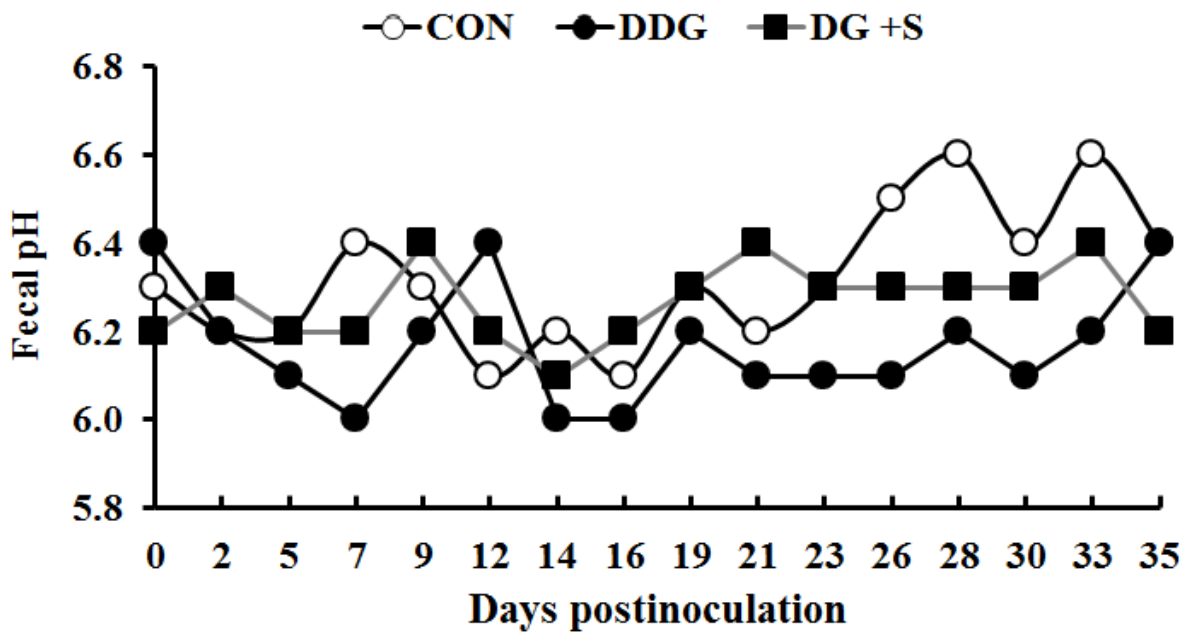
Table 3.1. Ingredient and chemical composition of the treatment diets

Item	Corn grain-based high grain diet supplemented with (% DM):		
	0% dried distillers grain (DDG; Control)	25% DDG	25% DDG plus corn starch
Ingredients			
Dry rolled corn	70.1	60.6	36.1
Corn DDG	-	25.0	25.0
Chopped alfalfa hay	10.0	7.0	7.0
Soybean meal	10.8	-	1.5
Choice white grease[1]	2.3	-	-
Molasses	2.5	3.0	2.5
Corn starch	-	-	23.5
Supplement[2]	2.0	2.0	2.0
Medicated premix[3]	2.4	2.3	2.4
Nutrient composition			
DM, %	91.1	90.9	90.5
Nonfiber carbohydrates, % DM	61.7	58.3	61.9
CP, % DM	14.6	14.1	13.8
Starch, % DM	46.3	43.3	41.3
NDF, % DM	11.8	16.9	14.1

[1]Choice white grease was from Key Feeds, Clay Center, KS.

[2]Supplement contained 15.5 g of limestone, 3.2 g of CaCl, 1,749 IU vitamin A, 0.1 mg Co, 6.6 mg Cu, 0.33 mg I, 0.17 mg Se, 33 mg Mn, and 33 mg Zn per kg of diet DM.

[3]Medicated premix provided 33 mg of monensin sodium (Elanco Animal Health, Greenfield, IN) and 11 mg tylosin (Elanco Animal Health) per kg of feed in a ground corn carrier.

Table 3.2. The prevalence and concentration of nalidixic acid-resistant (Nal^R) *Escherichia coli* O157:H7 in gut contents at necropsy from cattle fed corn grain-based, high-grain diets supplemented with no dried distillers grains (0% DDG [Control]; n = 7), 25% DDG (n = 7), or 25% DDG and corn starch (DDG+S; n = 7)[1].

	Corn grain-based, high grain diet supplemented with:						
	0% DDG (Control)	25% DDG	25% DDG+S	0% DDG (Control)	25% DDG	25% DDG+S	SEM
Gut site	Prevalence (Positive/Total)			Concentration ($\log_{10}$ cfu/g)			
Rumen	0/7	0/7	2/7	0.00	0.00	0.43	0.09
Cecum	2/7	1/7	4/7	0.44	0.19	0.94	0.16
Colon	1/7	2/7	4/7	0.30	0.43	1.10	0.19
Rectum	3/7	3/7	5/7	0.74	1.70	1.95	0.39
Rectoanal mucosal swab	6/7	5/7	6/7	-	-	-	-
Rumen+Cecum+Colon+Rectum	6/28[b]	6/28[b]	15/28[a]	0.37[b]	0.58[ab]	1.10[a]	0.21

[1] The concentrations of Nal^R *E. coli* O157:H7 in gut contents was not associated with the gut sample site (rumen, cecum, colon, or rectum) × dietary treatment interaction ($P = 0.85$), but was significantly affected by gut sample site ($P = 0.03$) and tended to be affected by dietary treatment ($P = 0.06$). The cumulative prevalence of Nal^R *E. coli* O157:H7 (total sampling sites positive per animal) at necropsy was significantly affected by dietary treatments ($P = 0.05$).

[a, b] Within cumulative prevalence values, treatments without a common superscript letter differ at $P < 0.05$; within concentration values, treatments without a common superscript letter differ at $P < 0.05$

Table 3.3. Gut content pH at necropsy of cattle fed corn grain-based, high-grain diets supplemented with no dried distillers grains (0% DDG [Control]; n = 7), 25% DDG (n = 7), or 25% DDG and corn starch (DDG+S; n = 7) following oral inoculation with nalidixic acid-resistant *Escherichia coli* O157:H7.[1]

| Source | Corn grain-based, high grain diet supplemented with: | | | SEM |
	0% DDG (Control)	25% DDG	25% DDG+S	
Rumen	5.62	5.78	5.77	0.097
Cecum	6.51[a]	6.13[b]	6.09[b]	0.057
Colon	6.57[a]	6.17[b]	6.08[b]	0.055
Rectum	6.42	6.47	6.28	0.055

[1] Fecal pH was significantly different between gut sampling site ($P < 0.05$), and between dietary treatment group ($P < 0.001$), but the gut sampling site x dietary group interaction was not significant ($P = 0.07$)

[a, b] Within a row, means without a common superscript letter differ at $P < 0.05$.

Table 3.4. Dry matter content and nutrient composition of feces from of cattle fed corn grain-based high-grain diets supplemented with no dried distillers grains (0 % DDG [Control]; n = 7), 25% DDG (n = 7), or 25% DDG and corn starch (DDG+S: n = 7) following oral inoculation with nalidixic acid-resistant *Escherichia coli* O157:H7

| Nutrient | Corn grain-based high grain diet supplemented with: | | | SEM | *P*-value |
	0% DDG (Control)	25% DDG	25% DDG+S		
DM, %	23.0[b]	23.3[b]	25.0[a]	0.54	0.04
Starch, % of DM	16.0[a]	13.1[ab]	11.5[b]	1.30	0.04
CP, % of DM	16.2[a]	15.8[a]	13.5[b]	0.46	< 0.001
NDF, % of DM	30.2[c]	39.3[a]	32.8[b]	0.90	< 0.001
ADF, % of DM	14.8[a]	15.0[a]	12.9[b]	0.49	< 0.001

[a,b,c] Within a row, means without a common superscript letter differ at *P* < 0.05.

Chapter 4 - Detection of *Escherichia coli* O104 in the Feces of O104 in the Feces of Feedlot Cattle by a Multiplex PCR Assay Designed to Target Major Genetic Traits of the Virulent Hybrid Strain Responsible for the 2011 German Outbreak

Abstract

We have developed an 11-gene multiplex PCR (mPCR), based on genes that code for serogroup-specific O-antigens and four major virulence factors (intimin, hemolysin and Shiga toxins [Stx] 1 and 2), to detect O157 and the 'top six' non-O157 (O26, O45, O103, O111, O121, and O145) Shiga toxin-producing *Escherichia coli* (STEC). The assay specificity was validated with pure cultures of the seven STEC (185 strains), 26 other STEC (65 strains), non-STEC (5 strains) and 33 strains of other species. Sensitivity of the assay with fecal sample spiked with pooled cultures of seven STEC was 10^5 CFU/g before enrichment and 10^2 CFU/g after enrichment. The applicability of the assay to detect STEC in fecal samples (n=50), before and after enrichment, was evaluated by comparing with culture-based methods for O26, O111, and O157. The mPCR assay of 50 fecal samples showed seven (14%) positive for a total of nine O-groups compared to 23 (46%) positive for 34 O-groups after an enrichment step. Overall, 17 and 27 of 50 fecal samples were positive, by culture-based methods, for O157 and non-O157 (4 for

O26, 3 for O45 and 20 for O103) serogroups, respectively. None of the 27 non-O157 isolates possessed the *stx* genes, suggesting that cattle harbor Shiga toxin negative *E. coli* belonging to the 'top six' non-O157 serogroups. Our data, although based on a limited number of samples, suggest that the sensitivities of the mPCR and culture-based methods in detecting the seven serogroups of STEC in feces differed between O-groups. An obvious limitation of our mPCR is that the concurrent detection of virulence genes and the serogroups in a sample does not necessarily associate the virulence genes with the prevalent serogroups in the same sample. The major application of our 11-gene mPCR assay may be in identifying putative colonies of STEC obtained by culture-based methods.

Introduction

Cattle harbor Shiga toxin-producing *E. coli* (STEC) in the gastrointestinal tract, particularly in the hindgut (Hussein and Bollinger, 2005), and serve as a primary reservoir. The STEC is shed in the feces, which serves as a major source of food and water contaminations for food borne infections (Ferens and Hovde, 2011; Rangel et al., 2005). Among STEC, the O157 serogroup has long been recognized as a major foodborne pathogen (Paton and Paton, 2003; Pennington, 2010). Recently, non-O157 serotypes, belonging to six O groups, O26, O111, O103, O121, O45, and O145, have been recognized as a growing public health concern (Brooks et al., 2005). According to the Centers for Disease Control and Prevention, STEC account for approximately 175,000 illnesses annually, and O157 and non-O157 are responsible for 36 and 64% of the total STEC infections, respectively (Scallan et al., 2011). The traditional method to detect and isolate *E. coli* O157 from cattle feces involves an enrichment step, followed by

immunomagnetic bead separation (IMS) and plating on a selective medium that can phenotypically and presumptively distinguish O157 from other *E. coli*. The confirmation is made with O157-specific agglutination, followed by PCR for genes that code for O-group antigen (rfb_{O157}), H7 (*fli*C), Shiga toxins and other virulence factors (Bai et al., 2010). Cultural procedures for detection and isolation of non-O157 *E. coli*, however, are not yet established. Commercial IMS beads are available for four of the 'top six' non-O157 serogroups and selective media that can phenotypically distinguish non-O157 STEC serogroups have not been developed.

Multiplex PCR (mPCR)-based detections have been developed to identify non-O157 STEC in food and carcass samples. Genes in the O-antigen cluster, especially *wzx*, which encodes a flippase required for O-polysaccharide export, has been used in various PCR-based detections (DebRoy et al., 2005; DebRoy et al., 2011; Fratamico et al., 2009; Monday et al., 2007; Perelle et al., 2004; Perelle et al., 2007; Valadez et al., 2011). However, none of them has evaluated applicability of the mPCR to detect O157 and the 'top six' non-O157 serogroups in fecal samples of cattle. A PCR assay that detects the major virulence genes concurrently with the identification of the major serogroups will have an obvious advantage. Therefore, we designed an 11-gene multiplex PCR assay to detect the seven major STEC serogroups (O26, O45, O103, O111, O121, O145, and O157) concurrently with the four major virulence genes (*stx*1, *stx*2, *hlyA*, and *eae*). The assay was validated with pure cultures of STEC and applicability of the assay to detect the seven STEC in fecal samples was evaluated.

Materials and Methods

Primer design

The *wzx* gene, which encodes for a flippase required for O-polysaccharide export, was used to design primers for serogroups O26, O45, O103, O111, and O145. The *wbqE* gene, which encodes for a putative glycosyl transferase, and *wbqF*, which encodes for a putative acetyl transferase (Fratamico et al., 2003), were used to design primers for O121. Primers used for *stx*1, *stx*2, *hlyA, eae,* and *rfb*$_{O157}$ genes were validated in our previous study (Bai et al., 2010). Primers were designed to amplify the targets with distinct amplicon sizes, and were designed to match all available sequences for the respective O-serogroups at the time of designing. The *wzx* gene in each serogroup was found specific, except for O121 in which the *wzx* gene was nearly identical to that from a *Shigella* strain (AY380835.1), thus *wbqE-F* were used to specifically amplify *E. coli* O121. All primer sequences are given in Table 1.

PCR reactions and visualization

All primer stocks were prepared in 1X TE buffer (Integrated DNA Technologies, Inc., Coralville, Iowa) at concentrations of 100 pM/μl. Equal volumes of the 11-primer pairs (22 primers) except for O111, which was doubled, were mixed together. One microliter of the primer mix was used in a 20 μl PCR reaction resulting in final primer concentrations of 0.42 μM for O111 and 0.21 μM for all other primers in the reaction. Each reaction also contained 10 μl of BioRad iQ Multiplex Powermix (without additional supplement), 1 μl of boiled bacterial cells, or DNA extracted from fecal, spiked fecal or enriched fecal samples, and 8 μl nuclease-free water.

The PCR amplification program included a 5 min denaturation at 94°C, followed by 25 (for pure culture) or 35 cycles (for all others) of 94°C for 30 sec, and 67°C for 80 sec. The PCR products were run on 1.2% agarose gel and visualized with a GelDoc 2000 Fluorescent Imaging System (BioRad, Hercules, CA).

Template DNA preparation

Bacterial cultures, stored in protect beads at $-80°C$, were streaked on blood agar plates (Remel, Lenexa, KS) and incubated overnight at 37°C. One or two colonies of each strain were suspended in 1 ml of distilled water and boiled for 10 min. After a quick centrifugation, 1 μl of the supernatant was used as DNA template. GeneClean DNA extraction kit (MP Biomedicals, Solon, OH) was used for all *Escherichia coli* (EC)-broth (Oxoid Ltd., Hampshire, England; (Vimont et al., 2007) enriched fecal samples, and QIAamp DNA stool mini kit (Qiagen, Valencia, CA) was used for all fecal and fecal samples spiked with pure cultures.

STEC strains used in the initial assay development

Strains representing the seven O-serogroups were used for the initial assay development and to determine detection sensitivity. Strains TW 1597 (O26), 2566:58 (O45) were kindly provided by The Thomas S. Whittam Microbial Evolution Laboratory, Michigan State University; Strains 15612-1 (O103), 4190 (O121), 7726-1 (O111), 1234-1 (O145) were from our collection (Renter et al., 2005); and strain 43894 (O157) was from the American Type Culture Collection (Manassas, VA). For assay development, single colonies of the seven strains from BAP were cultured overnight in Luria Bertani (LB) broth individually, and 100 μl was inoculated into 10

ml LB broth and incubated for 4-5 h until they reached an absorbance of 0.5 at 600 nm (~10^8 CFU/ml). The seven strain-cultures were mixed in equal amounts, and 1 ml of the pooled mixture was boiled for 10 min, and 1 μl of the supernatant after a quick spin was used in the mPCR reactions. For the pure culture sensitivity test, six 10-fold dilutions were made from the seven strain-culture mixture, and 1 ml of each dilution was boiled and centrifuged as before for use as template in mPCR. Aliquots of the same dilutions were spread-plated on MacConkey agar to determine bacterial cell concentrations.

E. coli and non-E. coli strains used for assay specificity validation

A collection of 185 strains belonging to the seven major STEC (Table 2), 65 strains belonging to 26 other STEC serogroups (O6 [2], O8 [3], O15 [2], O22 [1], O38 [3], O39 [3], O49 [1], O74 [3], O84 [3], O88 [3], O91 [2], O96 [3], O109 [3], O113 [3], O116 [3], O117 [3], O118 [2], O130 [4], O136 [3], O141 [3], O146 [1], O153 [2], O159 [1], O163 [3], O171 [3], O172 [2]), five strains of non-STEC (ATCC 43886, 12014, 35401, 43896, and 25922), and 33 strains of other Gram negative and Gram positive bacteria (*Enterobacter aerogenes* [1]*, Enterococcus casseliflavus* [1]*, Enterococcus faecalis* [3]*, Enterococcus faecium* [2]*, Enterococcus gallinarum* [1], *Klebsiella pneumoniae* [1], *Listeria monocytogenes* [1], *Morganella morganii* [1], *Proteus mirabilis* [1], *Salmonella enterica* serotypes [19 strains from 16 serotypes: *S. Agona, S. Anatum. S. Bareilly, S. Braenderup, S. Derby, S. Enteritidis, S. Infantis, S. Kentucky, S. Mbandaka, S. Montevideo, S. Muenchen, S. Oranienberg, S. Orion, S. Reading, S. Senftenberg,* and *S. Typhimurium*]*, Serratia marcescens* [1], and *Streptococcus pyogenes* [1]. The bacterial cultures

stored at -80°C were grown on BAP to obtain single colonies. One or two colonies were suspended in 1 ml of water, boiled and centrifuged as before for use in mPCR reactions.

PCR Assay sensitivity with pure cultures and cattle fecal sample spiked with pure cultures

The seven STEC strains were grown individually to an absorbance of 0.5 at 600 nm (~10^{8} CFU/ml), and equal volumes of the cultures were pooled together. A serial of 10-fold dilutions were made, and 1 ml of each dilution was boiled and used for the mPCR assay. For fecal samples spiked with the seven STEC strains, the pooled cultures were prepared as before, and 10-fold serially diluted mixtures were inoculated into aliquots of a fecal sample collected from a feedlot steer. DNA extractions were performed with QIAamp DNA stool mini kit on fecal samples spiked with different culture dilutions. One gram of each fecal sample inoculated with different dilutions of the culture mixture was also enriched in 9 ml EC broth for 6 h at 40°C, and DNA was extracted by GeneClean kit. The dilutions of the seven strain mixture were also spread-plated on MacConkey agar to determine bacterial cell concentrations. The experiment to determine assay sensitivity was repeated with a different fecal sample.

PCR assay vs. culture detection of STEC in cattle fecal samples

A total of 50 fecal samples were collected from feedlot cattle and ~1 g of each sample was mixed in 9 ml EC broth, and incubated at 40°C for 6 h. DNA was extracted from fecal suspensions in EC broth, before and after enrichment, with the GeneClean kit and used in mPCR assay. The enriched samples were also subjected to immunomagnetic separation (IMS) with

Dynabeads (Invitrogen, Carlsbad, CA) for O26, O111, and O157. The O157 beads were plated

on sorbitol MacConkey agar (SMAC) with cefixime (0.5 mg/L) and potassium tellurite (2.5

mg/L), and six sorbitol-negative colonies were picked for O157 antigen latex agglutination test

and colonies positive for agglutination were subjected to mPCR assay. The O26 and O111 beads

were streaked on MacConkey agar plates and ten colonies, randomly picked from each sample,

were streaked individually on BAP and incubated overnight. A single colony from each of the

ten cultures was suspended individually in 1 ml of distilled water and 100 µl of suspensions from

each of the ten colonies from each sample were pooled together and subjected to the mPCR

assay. For samples that were positive for any of the STEC based on the mPCR assay of the

pooled cultures, the ten individual colonies were subjected to mPCR to identify the positive pure

culture. The isolates positive for STEC were stored at -80°C.

Results

mPCR assay with pure cultures

The specificity of each primer pairs was tested individually using the mixture of the seven *E.

coli* serogroups, O26, O45, O103, O111, O121, O145, and O157. The primers only amplified

the corresponding STEC serogroup (data not shown). The serogroup-specific primers were then

mixed with the primers for the four virulence genes, *stx*1, *stx*2, *eae* and *hlyA*. After optimizing

PCR conditions (stated in the materials and methods section), 11 distinct bands were obtained

(Fig. 1). The sizes of the amplicons were: 890 (*wzx*$_{O45}$), 740 (*wzx*$_{O103}$), 655 (*stx*1), 587 (*wbq*$_{O121}$),

523 (wzx_{O145}), 477 (*stx*2), 417 (wzx_{O26}), 375 (*eae*), 296 (rfb_{O157}), 230 (wzx_{O111}), and 199 bp

(*hlyA*).

A total of 185 strains belonging to the seven major serogroups (30 [O26], 4 [O45], 41

[O103], 39 [O111], 12 [O121], 15 [O145], and 44 [O157]) were tested. The 11-gene mPCR

assay confirmed the serogroup of each of the 185 strains and their virulence gene profiles. The

prevalence of the four virulence genes varied between the serogroups. In case of O157, all

strains contained *eae* and *hlyA,* and 42 and 21 of 44 strains were positive for *stx*2 and *stx*1,

respectively. Of the 141 non-O157 STEC strains, 114 had *stx*1, 39 had *stx*2, 82 had *eae*, and 119

strains had *hlyA* (Table 2). Next to O157, O111 strains (n=39) possessed more virulence genes;

38 *stx*1, 22 *stx*2, 38 *eae*, and 37 *hlyA* genes (Table 2). None of the O-serogroup primer pairs

amplified any of the 65 strains in the other STEC O-serogroups or from the five non-STEC

ATCC strains; however, the primer pairs of the virulence genes amplified the virulence genes in

other STEC O-serogroups. The virulence gene profiles the other STEC-O groups matched the

original descriptions (data not shown). None of the 33 non-*E. coli* strains, representing both

gram positive and gram negative bacterial species, showed any amplification (data not shown).

Assay sensitivity with pure cultures and spiked fecal samples

The initial concentration of pooled cultures of the seven O-serogroups was 6.5 x 10^7 CFU/ml

(mean of two replications), which was subjected to four additional ten-fold dilutions to obtain 6.5

x 10^6, 6.5 x 10^5, 6.5 x 10^4, and 6.5 x 10^3 CFU/ml. The minimum concentration of the pooled

culture that amplified all 11 genes was 6.5 x 10^4 CFU/ml (Fig. 2). In fecal samples spiked with

different concentrations of the pooled cultures of the seven O groups, the sensitivity of detection

was 6.5 x 10^5 CFU/g before enrichment (Fig. 3) and 6.5 x 10^2 CFU/g after enrichment for 6 h in EC broth (Fig. 4).

PCR assay vs. IMS-based detections of O26, O111 and O157 in cattle fecal samples

The mPCR assay of the 50 fecal samples suspended in EC broth before enrichment showed seven samples (14%) positive for a total of nine O groups (O26=1, O45=4, O103=1, O121=2, and O157=1) compared to 23 samples (46%) positive for 34 O groups (O26=9, O45=6, O103=8, O121=3, and O157=8) after 6 h enrichment (Table 3). Similarly, more samples were positive for the four virulence genes after enrichment compared to samples before enrichment. None of the samples was positive for O111 and O145 serogroups. Of the 50 fecal samples, 27 (54%) samples were negative for any of the seven major O-serogroups, and 14 (28%) were positive for one, 7 (14%) were positive for two, two (4%) were positive for three of the seven serogroups.

We plated a subset of 20 of the 50 samples that were enriched in EC broth directly on MacConkey agar without the IMS procedure, and testing of the pooled colonies (10 randomly picked colonies per sample) yielded only three samples positive for O103. After O157 IMS separation and agglutination confirmation of the sorbitol-negative colonies on CT-SMAC plates, 17 of the 50 samples (34%) were positive for O157 serogroup. All 17 O157 isolates possessed *eae* and *hlyA*, 15 had *stx*1 and *stx*2, and two isolates had *stx*2 only. The use of O26 IMS beads and testing of the pooled 10 colonies from MacConkey agar plates yielded four samples positive for O26, two samples positive for O103, and one sample positive for O45 serogroups (Table 3). Samples after O111 IMS procedure had no positive O111, but 18 of 50 (36%) fecal samples were positive for O103 and two samples were positive for O45. The 18 samples that were

positive for O103 included the three samples that were identified as positive by direct plating of the enriched samples (without IMS) on MacConkey agar. Table 4 shows the comparison between mPCR (pre- or post-enriched samples) and culture-based method with IMS in detecting O26, O103 (with O111 IMS beads), and O157 serogroups in feces. Fewer fecal samples were positive for any of the three serogroups by either mPCR or IMS than the number of samples positive regardless of the method used. In contrast, only few samples were positive for any of the three serogroups by both mPCR and IMS. For example, a total of 21 of 50 samples were positive for O157 based on either mPCR or IMS and only 4 of 21 samples were positive by both mPCR and IMS (Table 4).

Overall, 50 fecal samples were positive for 27 non-O157 serogroups (4 for O26, 3 for O45 and 20 for O103) based on testing of pooled colonies on MacConkey agar after the IMS procedure. Because all samples were subjected to O26, O111 and O157 IMS procedures, multiple serogroups were identified from the same samples (nine samples had two, and four samples had three serogroups). We tested the original single colonies that were stored in -80°C of each positive sample by mPCR for O-groups and virulence genes. We identified the 27 non-O157 serogroups in pure cultures, however, none of the 27 strains had *stx* genes. One O103 strain had *eae* and *hlyA*, five O103 and one O26 strains had only *hlyA*. The serogrouping of the 27 non-O157 *E. coli* positives were confirmed by the *E. coli* Reference Center at Pennsylvania State University. When ten individual colonies from each positive samples were tested, 13 isolates were identified that did not belong to the seven O-groups, but carried the virulence genes (4 had *eae* and *hlyA*, 2 had *stx*1 and *hlyA*, 4 had *stx*2 only, 2 had *stx*1 only, and 1 had *hlyA* only). All 13 isolates were confirmed as *E. coli* based on the API Rapid 20E kit (bioMérieux Inc.,

Durham, NC). The serogroups of the 13 strains were identified, by the Pennsylvania State University *E. coli* Reference Center, as O171 (n=4), O104 (n=2), O71 (n=2), and one each of O8, O108, O116, O153 and O109/O119 strain (Table 5).

Discussion

We developed the 11-gene mPCR to concurrently detect O157 and the 'top six' non-O157 serogroups with the four major virulence genes. According to the CDC, the 'top six' O-groups are responsible for 74% of non-O157 STEC infections in the US (Scallan et al., 2011). The four virulence genes chosen, *stx*1, *stx*2, *eae*, and *hlyA,* code for key virulence factors in human infections with O157 and non-O157 STEC (Brooks et al., 2005; Karmali et al., 2010), and are routinely included in characterizations of STEC (Bai et al., 2010; Hu et al., 1999; Lefebvre et al., 2005; Paton and Paton, 1998). Few mPCR-based detections for identifications of O157 and the major non-O157 STEC have been published (DebRoy et al., 2011; Monday et al., 2007; Valadez et al., 2011). Monday et al. (2007) developed a mPCR to detect O157 and 5 of the top six (O26, O103, O111, O121, and O145; no O45) STEC. The assay also included primers for *eae* and Shiga toxins (a single primer pair to detect both *stx*1 and *stx*2). The assay was validated with pure cultures of STEC only. DebRoy et al. (2011) have reported on a mPCR to detect 8 STEC serogroups (the seven major STEC and O113) by targeting the *wzx* genes of the O-antigen gene clusters and specificity and sensitivity of the assay were evaluated with pure cultures and with artificially inoculated apple juice. The assay did not target virulence genes and the applicability of the assay to detect STEC in cattle feces was not evaluated.

The primers in our study were designed to obtain 11 amplicons that formed distinct bands on agarose gels. The designed primers were specific for the seven serogroups, based on specificity assays determined with pure cultures of a number of STEC, non-STEC, and other related or unrelated gram negative and gram positive bacteria. The specificity, sensitivity and applicability of the assay to detect the seven serogroups were also evaluated with fecal samples inoculated with the pooled mixture of the seven STEC. A minimum concentration of 6.5×10^5 CFU/g (650 CFU/µl of sample volume in PCR reaction) was needed to detect all 11 genes. The sensitivity of the assay was improved to 6.5×10^2 CFU/g with the inclusion of a 6-h enrichment step in EC broth. The sensitivity of the 11-gene assay was a half to one log lower than the sensitivity reported by DebRoy et al. (2011) for the mPCR of 8 O-groups with no virulence genes, and that of the six-genes mPCR (rfb_{O157}, $fliC_{H7}$, stx1, sxt2, eae and $hlyA$) without O-groups of non-O157 reported by Bai et al. (2010). We tested the 11-gene PCR primers on a single O157 strain that was positive to the four virulence genes (rfb_{O157}, stx1, sxt2, eae and $hlyA$), and the sensitivity was similar (10^4 before enrichment and 10^1 after enrichment) to that of 8 O-groups (DebRoy et al., 2011) and 6 virulence genes mPCR (Bai et al., 2010). The difference in sensitivity may be reflective of the number of genes involved in the amplification (11 vs. 8 or 6).

We evaluated the applicability of mPCR to detect the seven major serogroups of *E. coli* in cattle feces by comparing to that of culture-based detection methods (IMS followed by plating on CT-SMAC for O157 or MacConkey agar for the six non-O157) with a limited number of fecal samples (n=50). The culture method was used only for three (O26, O111, and O157) of the seven STEC because of our inability to procure IMS beads for O103 and O145. Beads for O45 and O121 are not yet commercially available. However, mPCR of the post-enrichment sample

identified 6 samples positive for O45 and 3 samples positive for O121. The mPCR assay of the

fecal samples suspended in EC broth before incubation for enrichment identified O-groups in

seven of the 50 fecal samples (O26=1, O45=4, O103=1, O121=2, and O157=1), suggesting that

the samples possibly contained high concentrations ($> 10^4$ per g) of the O-groups of *E. coli*.

When we plated a subset of 20 samples after enrichment, without IMS, on to MacConkey agar,

only three samples yielded O-groups and all three were O103. The same 20 samples, after IMS

with O26, O111, or O157 beads, yielded 12 O157 and 18 non-O157 strains (O26=4, O45=2,

O103=12), which illustrates the advantage of using IMS beads. The sensitivity of the mPCR and

culture-based methods in detecting the major serogroups in feces differed between O-groups. In

case of O157, the IMS procedure with O157 beads identified 17 (34%) positive samples

compared to eight (16%) of 50 samples by mPCR, which included nine of the 17 samples

positive by IMS that were not detected by mPCR and four of the eight samples positive by

mPCR that were not detected by IMS. Interestingly, only four samples were positive by both

methods. In case of O111 beads, which identified O103, more samples were positive by IMS

(18 of 50) compared to mPCR (8 of 50). The difference in sensitivity is not surprising because

for a PCR assay to be positive, the sample should contain at least 1,000 cells per g or ml of

sample to assure that a sample volume of 1 µl in the PCR reaction contains DNA from at least

one cell. However, in case of O26, mPCR identified more positives (9 of 50) compared to IMS

(4 of 50). Additional samples need to be evaluated before suggesting that sensitivity of

commercial O26 beads need improvement. Interestingly, the commercially available O111

beads did not yield any O111, but yielded several O103, suggesting that the beads were either

nonspecific or perhaps mislabeled by the manufacturer. Our data, although based on a limited

number of fecal samples, suggest that neither PCR nor culture based method was better in detecting the prevalence of the seven serogroups in cattle feces. The PCR has the advantage in that it is amenable to rapid through-put and automation. On the other hand, the culture-based method, which is labor-intensive and expensive, yields isolates that could then be tested for the presence of virulence genes.

A total of 27 non-O157 strains (O26=4, O45=3, and O103=20) were isolated from 50 fecal samples. Interestingly, none of the 27 strains carried *stx* genes, suggesting that cattle harbor Shiga toxin-negative *E. coli* belonging to the 'top six' non-O157 serogroups. The other possibility is that the primers used in the PCR were not amplifying the *stx* from the non-O157 serogroups. The latter is unlikely because the primers amplified *stx* genes of all the known strains (n=250) that were tested in this and in a previous study (Bai et al., 2010). An analysis of the *stx*2 gene in the current database indicated that the primers matched majority of non-O157 STEC strains, except the *stx*2f subtype, which appear to be distantly related to all other *stx* subtypes (~40-60% identical to other subtypes; data not shown). The *stx*2f subtype was mainly found in STEC isolated from pigeons (Schmidt et al., 2000; Unkmeir and Schmidt, 2000), and occasionally in isolates from humans with mild symptoms of enteritis (van Duynhoven et al., 2008). In addition to *stx*2f subtype, there is a single base mismatch in our stx2 reverse primer against the subtype *stx*2g. However, the modified reverse primer (*stx*2R2: TGTCGCCA**S**TTATCTGACATTC) did not detect *stx*2 from the 27 non-O157 strains (data not shown). Studies on the prevalence of non-O157 STEC in cattle feces, generally from countries outside the US, have shown that many non-O157 strains do carry the *stx* genes (Jenkins et al., 2003; Pearce et al., 2004; Pradel et al., 2000; Renter et al., 2005). There are also reports of

prevalence of non-O157 STEC in cattle that lacked *stx* and other major virulence genes (Barlow and Mellor, 2010; Bosilevac and Koohmaraie, 2011; Bugarel et al., 2011). A recent study of *E. coli* O26 strains in Germany indicated that 133 of 250 strains did not possess either *stx* genes, and the authors speculated that the virulent O26 strains were most likely the less virulent O26 that acquired one or both *stx* genes (Bugarel et al., 2011). In a study (Bosilevac and Koohmaraie, 2011) on the prevalence of non-O157 STEC in commercial ground beef in the US, only a small proportion of STEC isolates (10 of 338) recovered possessed major virulence genes to be considered of significant food safety threat.

An obvious limitation of our mPCR is that the concurrent detection of virulence genes and the seven serogroups in a sample does not necessarily mean that the virulence genes are associated with the seven serogroups in the same sample. Therefore, when more than one O-serogroup is present in a sample, which is often the case (Barlow and Mellor, 2010; Renter et al., 2007), the assay will not be able to associate the virulence gene(s) to any particular serogroup. Also, the virulence genes in the same sample could be from a serogroup other than the seven major STEC that have been shown to carry some of the virulence genes, including *stx* genes and more often the *eae* and the *hly*A. In our study, there were eight *E. coli* isolates from 50 fecal samples that did not belong to the top seven serogroups, but carried *stx*1 and/or *stx*2 genes. Blanco et al. (2005) also demonstrated that the *eae* gene is present in cattle *E. coli* serogroups O2, O8, O10, O15, O34, O64, O77, O113, O119, O128, O156, O177 and several ONT (O-antigen nontypeable) strains. The *eae* gene was also found in both *stx*-positive, and *stx*-negative *E. coli* serogroups (Ito et al., 2007). Similarly, *hlyA* is found in *E. coli* O5, O69, O76, O84, O98, and O156 of both cattle and human origins (Boerlin et al., 1998). Using our *eae* and *hlyA*

primers as queries to search the GenBank, we identified perfect match to our primers from multiple *E. coli* O-groups, including those that were not within the seven major STEC serogroups (data not shown). The confirmation of the association of virulence gene(s) with a particular serogroup would require that the isolate be obtained in pure culture and then tested again by mPCR. The major application of our11-gene mPCR assay may be in identifying serogroup and virulence genes of putative colonies of STEC that have been obtained by culture-based method.

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Figure 4.1. Gel image of amplicons of 11genes amplified by multiplex PCR of a pooled mixture of pure cultures of *Escherichia coli* O26, O45, O103, O111, O121, O145 and O157 strains. Lane 1: molecular size markers; Lane 2: Amplicons of 11 gene segments.

Gene	Size
wzx_{O45}	890 bp
wzx_{O103}	740 bp
stx1	655 bp
wbq_{O121}	587 bp
wzx_{O145}	523 bp
stx2	477 bp
wzx_{O26}	417 bp
eae	375 bp
rfb_{O157}	296 bp
wzx_{O111}	230 bp
hlyA	199 bp
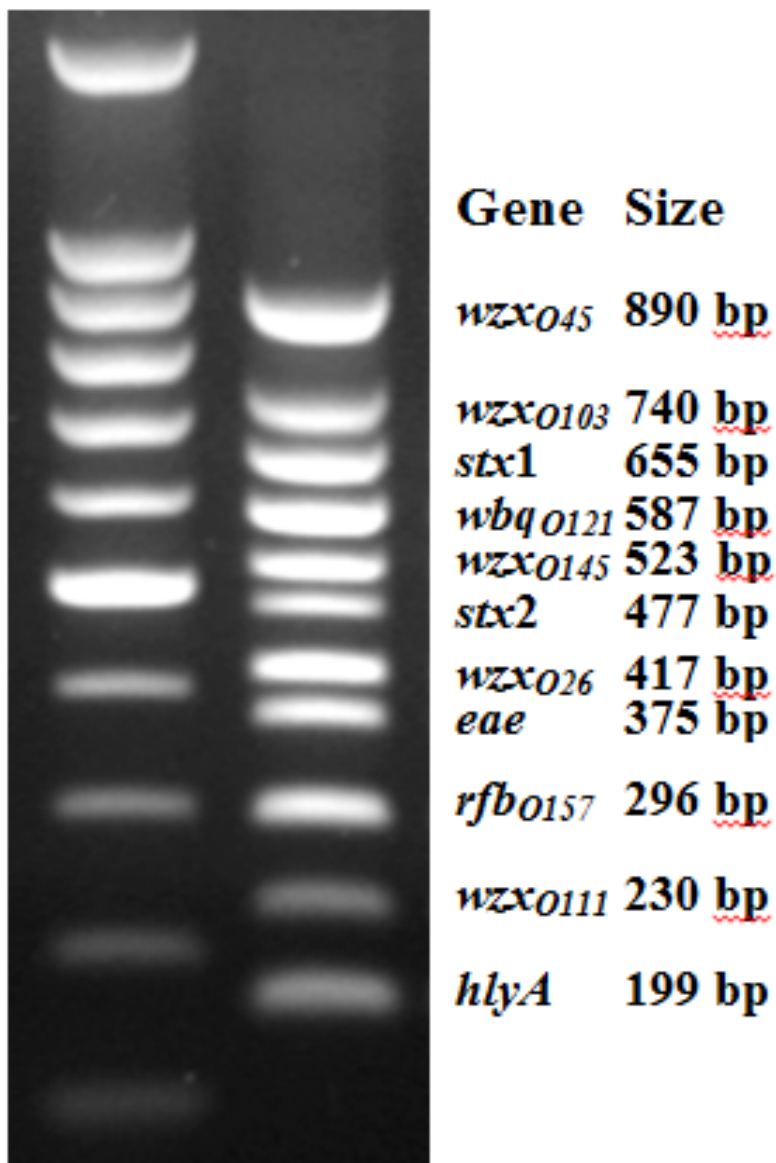
Figure 4.2. Sensitivity of the 11-gene multiplex PCR tested with ten-fold, serially diluted pooled cultures of *Escherichia coli* O26, O45, O103, O111, O121, O145 and O157 strains. Lanes 1 and 7: Molecular size markers; Lanes 2-6: Amplicons from pooled cultures with concentrations of 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/ml.
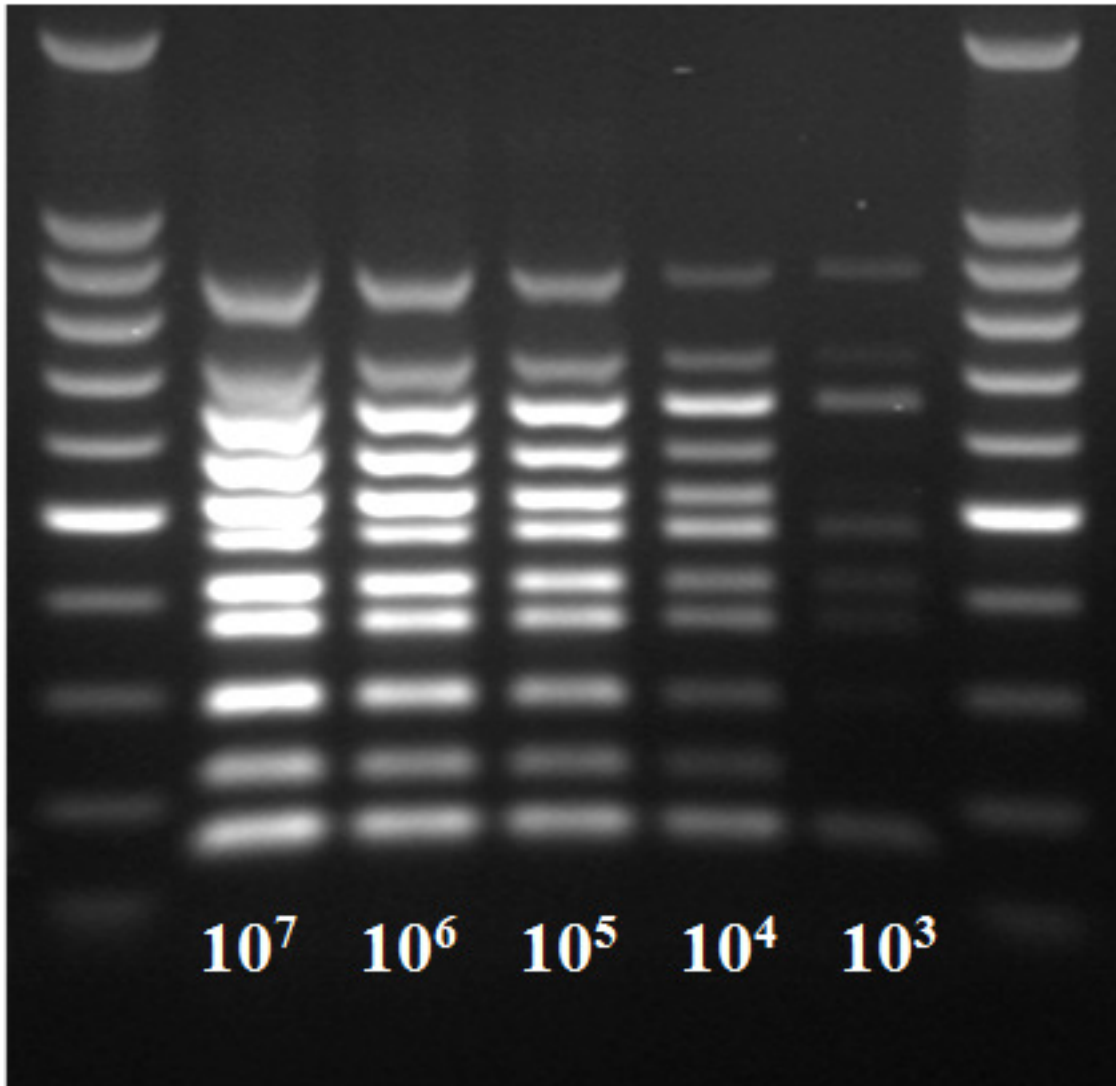
Figure 4.3. Sensitivity of the multiplex PCR assay determined with cattle fecal sample spiked with ten-fold, serially diluted pooled cultures of *Escherichia coli* O26, O45, O103, O111, O121, O145 and O157 strains. Lanes 1 and 6: Molecular size maker; Lanes 2-4: Amplicons obtained from cattle fecal sample spiked with pooled cultures of 10^6, 10^5, and 10^4 CFU/g of feces; Lane 5 (CK): Cattle feces before spiking with *E. coli* cultures.
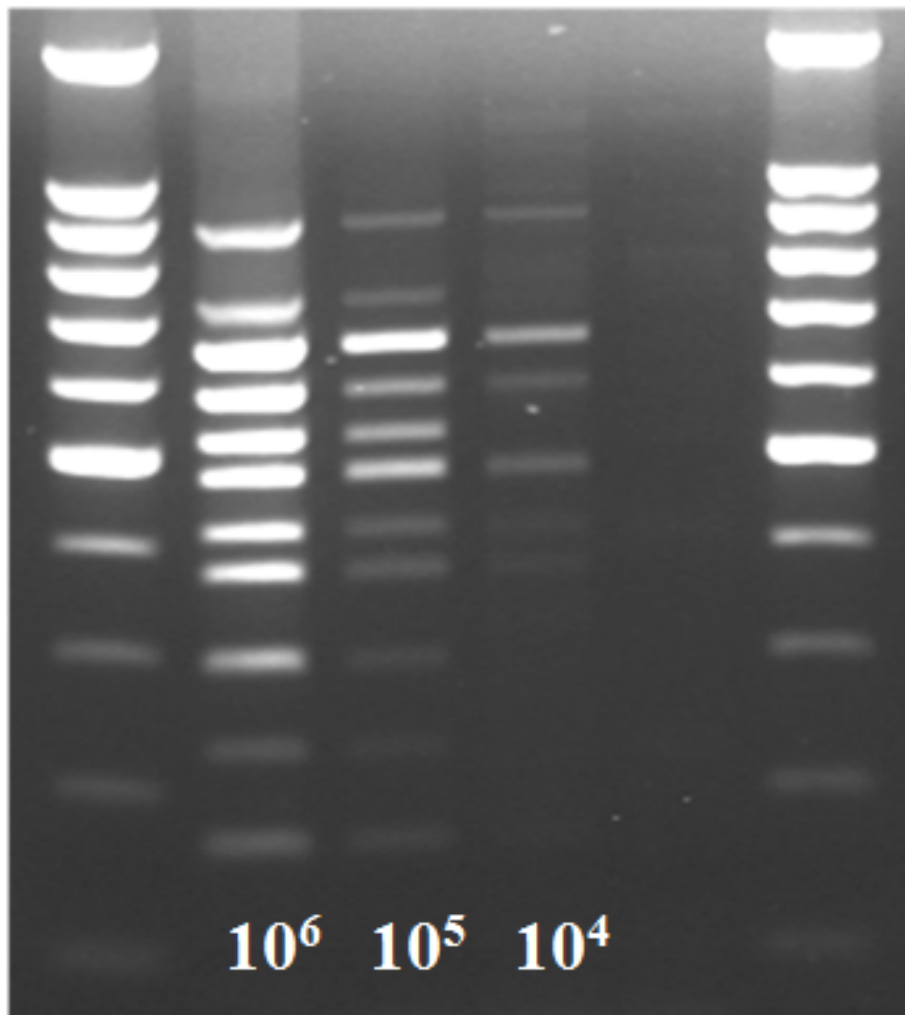
Figure 4.4. Sensitivity of the multiplex PCR assay determined with cattle fecal sample spiked with ten-fold, serially diluted pooled cultures of *Escherichia coli* O26, O45, O103, O111, O121, O145 and O157 strains after enrichment in *Escherichia coli* broth at 40 C for 6 h. Lanes 1 and 10: Molecular size makers; Lanes 2-8: Amplicons from enriched cattle fecal sample spiked with pooled cultures of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/g of feces; Lane 9 (CK): Enriched cattle feces without added *E. coli* cultures.
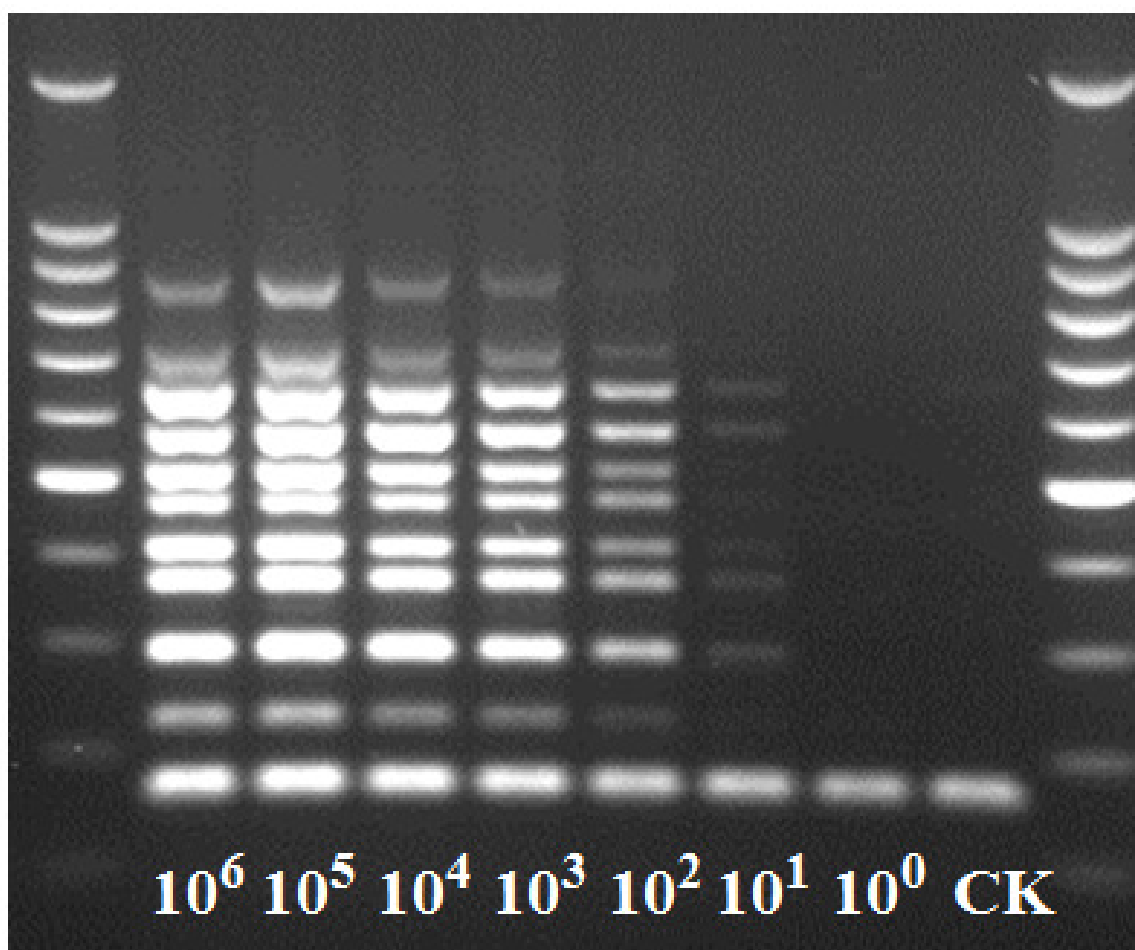
Table 4.1. Target genes, primer sequences and amplicon sizes

Target genes	Primer	Primer sequence (5'—3')	Amplicon size (bp)	Reference
wzx_{O45}	O45-F	GGGCTGTCCAGACAGTTCAT	890	This study
	O45-R	TGTACTGCACCAATGCACCT		
wzx_{O103}	O103F2	GCAGAAAATCAAGGTGATTACG	740	This study
	O103R2	GGTTAAAGCCATGCTCAACG		
$stx1$	stx1-F	TGTCGCATAGTGGAACCTCA	655	Bai et al., 2010
	stx1-R	TGCGCACTGAGAAGAAGAGA		
$wbqE_{O121}$	O121-F2	TCAGCAGAGTGGAACTAATTTTGT	587	This study
$wbqF_{O121}$	O121-R2	TGAGCACTAGATGAAAAGTATGGCT		
wzx_{O145}	O145F5	TCAAGTGTTGGATTAAGAGGGATT	523	This study
	O145R5	CACTCGCGGACACAGTACC		
$stx2$	stx2-F	CCATGACAACGGACAGCAGTT	477	Bai et al., 2010
	stx2-R	TGTCGCCAGTTATCTGACATTC		
wzx_{O26}	O26F4	AGGGTGCGAATGCCATATT	417	This study
	O26R4	GACATAATGACATACCACGAGCA		
eae	eae-F	CATTATGGAACGGCAGAGGT	375	Bai et al., 2010
	eae-R	ACGGATATCGAAGCCATTTG		
rfb_{O157}	rfbE-F	CAGGTGAAGGTGGAATGGTTGTC	296	Bertrand and Roig 2007; Bai et al., 2010
	rfbE-R	TTAGAATTGAGACCATCCAATAAG		
wzx_{O111}	O111F2	TGCATCTTCATTATCACACCAC	230	This study
	O111R2	ACCGCAAATGCGATAATAACA		
$hlyA$	hlyA-F	GCGAGCTAAGCAGCTTGAAT	199	Bai et al., 2010
	hlyA-R	CTGGAGGCTGCACTAACTCC		

Table 4.2. Multiplex PCR confirmation of O-serogroups and virulence genes of

seven major shiga toxin-producing *E. coli* strains

Serogroups	Number of strains	Virulence genes			
		*stx*1	*stx*2	*eae*	*hly*A
O26	30	24	5	17	24
O45	4	2	0	0	1
O103	41	41	2	12	41
O111	39	38	22	38	37
O121	12	0	3	0	3
O145	15	9	7	15	13
O157	44	21	42	44	44
TOTAL	185	135	81	126	163

1 **Table 4.3.** Detection of the seven serogroups of *Escherichia coli* in cattle feces by multiplex PCR or culture-based methods

Enrichment in *Escherichia coli* broth	Immunomagnetic bead separation	Number of fecal samples (n=50) positive for O-specific and virulence genes										
		O26	O45	O103	O111	O121	O145	O157	*stx*1	*stx*2	*eae*	*hlyA*
Pre-enrichment	None	1	4	1	0	2	0	1	3	8	22	31
Post-enrichment	None	9	6	8	0	3	0	8	17	22	35	44
	O26	4	1	2	0	0	0	0	4	4	2	5
	O111	0	2	18	0	0	0	0	1	0	4	10
	O157	0	0	0	0	0	0	17	15	17	17	17

2

Table 4.4. Prevalence of *Escherichia coli* serogroups in fecal samples (n=50) by multiplex PCR (mPCR) or culture-based method with immunomagnetic separation (IMS):

Method of detection	Serogroups		
	O26	O103	O157
mPCR only	9	8	8
IMS only	4	18	17
mPCR and IMS	2	6	4
mPCR or IMS	11	20	21

Table 4.5. Prevalence of virulence genes in serogroups, other than the seven major serogroups, of *Escherichia coli* in Cattle Fecal Samples (n=50)

Serogroups	Number of strains	Number of strains positive for virulence genes			
		*stx*1	*stx*2	*eae*	*hlyA*
O171	4	0	4	0	0
O104	2	2	0	0	2
O71	2	0	0	2	2
O8	1	1	0	0	0
O108	1	0	0	1	1
O116	1	0	0	0	1
O153	1	0	0	1	1
109/119	1	1	0	0	0
Total	13	4	4	4	7

Chapter 5 - *Escherichia coli* O104 in the feces of feedlot cattle do not appear to carry traits of the virulent hybrid strain responsible for the 2011 German outbreak

(*Accepted in Applied and Environmental Microbiology*)

Abstract

Shiga toxin-producing *E. coli* (STEC) O104:H4 was the cause of a large food-borne outbreak of hemorrhagic colitis and hemolytic uremic syndrome in Germany in 2011. The serotype was a hybrid of STEC and enteroaggregative *E. coli* (EAEC) pathotypes that carried genes for Shiga toxin 2 (*stx*2), and enteroaggregative fimbrial adhesins, but lacked intimin (*eae*) and enterohemolysin (*ehx*A). Because cattle are known reservoirs of STEC, we investigated fecal carriage of *E. coli* O104 in feedlot cattle. A multiplex PCR (mPCR) was designed and validated to detect the following 8 genes: *stx*1, *stx*2, *ter*D (tellurite resistance), *eae*, wzx_{O104} (O104 specific O-antigen flippase), $fliC_{H4}$ (H4 flagella), *ehx*A and *agg*A (aggregative adherence fimbria 1). A total of 248 fecal samples from 8 feedlots were collected, enriched in *E. coli* broth, extracted DNA and subjected to mPCR. Overall, 20.6% (51/248) of fecal samples were positive for wzx_{O104}. None of the fecal samples was positive for *agg*A gene. Enriched fecal samples positive for wzx_{O104} (n=51) were plated on several selective and differential media and presumptive *E. coli* colonies were tested by the mPCR. Only 7 (13.7%) samples yielded pure cultures of serogroup O104 and all 7 isolates were negative for genes characteristics of STEC and EAEC. The lack of selectivity in culture methods may have limited our ability to identify more positive

samples. Our results suggest that *E. coli* O104 is present in cattle feces, but the strains do not appear to carry genes characteristic of the virulent hybrid strain responsible for the 2011German outbreak.

Introduction

In the summer of 2011, a large outbreak of food-borne illness caused by an unusual serotype of Shiga toxin-producing *Escherichia coli* (STEC) was reported in Europe (Frank et al., 2011). The striking feature of the illness was a surprisingly high incidence of hemolytic uremic syndrome (24%) in adults (Frank et al., 2011). Epidemiological investigation implicated organic fenugreek sprouts as the point source of the outbreak (King et al., 2012). The *E. coli* serotype was O104:H4, characterized as a hybrid strain of enteroaggregative *E. coli* (EAEC) and STEC (Bielaszewska et al., 2011). The hybrid strain carried *stx*2 gene, lacked intimin (*eae*) and enterohemolysin (*ehx*A), and possessed an operon with genes (*aggA, aafA, agg3A,* and *agg4A*) coding for aggregative adherence fimbrial adhesins I, II, and III (AAF/I-III)*,* typical of EAEC (Bielaszewska et al., 2011; Frank et al., 2011). Such a hybrid pathotype with enteroaggregative and Shiga toxigenic features have been reported with two other serogroups, O111:H2 (Morabito et al., 1998) and O86:NM (Iyoda et al., 2000).

Cattle are a primary reservoir of STEC (Karmali et al., 2010) in which the organisms reside in the hindgut and are shed in the feces. Cattle feces can be a source of direct or indirect contamination of food and water leading to human STEC illnesses (Ferens and Hovde, 2011; Rangel et al., 2005). Therefore, it is of interest to determine whether cattle harbor serogroup

O104 and whether harbored strains of O104 carry virulence genes characteristic of STEC and or EAEC. In Germany, no O104:H4 strain was detected in cattle feces in the outbreak area (Wieler et al., 2011). In a report from France, based on a real-time PCR assays that targeted *stx*2, *wzx*~O104~, *fliC*~H4~, and *aggA*, none of the cattle fecal samples examined carried the four genes simultaneously, and the authors suggested that cattle are not likely a reservoir of *E. coli* O104:H4 (Auvray et al., 2012). However, similar studies to assess presence of O104 in feces of cattle in the US have not been conducted.

There are reports of prevalence of STEC O104 in cattle and sheep feces with flagellar types other than H4 (Blanco et al., 2003, 2004). The O104 serogroup with H21 flagellar type, which was also negative for intimin (similar to O104:H4 outbreak strains)*,* was implicated in a US outbreak of hemorrhagic colitis associated with consumption of raw milk in Montana in 1994 (CDC, 1995; Feng et al., 2001). In order to detect serogroup O104 with STEC and or EAEC traits, we designed and validated a multiplex PCR (mPCR) to detect the following 8 genes: *stx*1 (Shiga toxin 1), *stx*2 (Shiga toxin 2), *ter*D (tellurite-resistance), *eae* (intimin), *wzx*~O104~ (O104 specific O-antigen flippase), *fliC*~H4~ (H4 specific flagella), *ehx*A (enterohemolysin) and *agg*A (pilin subunit of aggregative adherence fimbria 1[AAF/1; Bielaszewska et al., 2011). Our objectives were to screen feedlot cattle feces to detect the presence of the serogroup O104, and isolate and characterize *E. coli* O104 from PCR-positive fecal samples.

Materials and Methods

Bacterial strains

The following strains of *E. coli* were used in the development of the mPCR: a strain of O104:H4 (ATCC BAA-2326; Rohde et al., 2011) involved in the 2011 German outbreak, a strain of O104:H21 (ATCC 172801; CDC, 1995) involved in the outbreak of hemorrhagic colitis in Montana in 1994 (CDC, 1995) and *E. coli* O157:H7 (ATCC 43894). All strains were stored on CryoCare® beads (Key Scientific Products, Stamford, TX) at -80°C and to revive the frozen strains, a single bead was streaked on to blood agar (BAP; Remel, Lenexa, KS) and incubated overnight at 37°C.

Multiplex PCR assay

A multiplex PCR was designed to target the following 8 genes: wzx_{O104}, (O104 specific O-antigen flippase), $fliC_{H4}$ (H4 specific flagella), $stx1$ (Shiga toxin 1), $stx2$ (Shiga toxin 2), $terD$ (tellurite resistance), eae (intimin), $ehxA$ (enterohemolysin) and $aggA$ (pilin subunit of aggregative adherence fimbria 1[AAF/1]). Primers for wzx_{O104}, $fliC_{H4}$ and $aggA$ genes were designed with Primer3 software (Version 0.4.0; Rozen and Skaletsky, 2000). Target genes, primer sequences, and amplicons size are listed in Table 1.   Primers were individually reconstituted in 1 x TE buffer (pH 7.5) to concentrations of 100 pM/µl and equal amounts of each primer were mixed together. Twenty microliter volume PCR reactions consisting of 10 µl iQ Multiplex Powermix (Bio-Rad, Hercules, CA), 8 µl of PCR grade H_20, 1 µl of DNA template and 1 µl of the primer mixture (final individual primer concentration in PCR reaction of 0.31 µM) were used. The PCR program was: 94°C denaturation for 5 min, 25 cycles for pure culture

DNA or 35 cycles for fecal DNA, 94°C denaturation for 30 sec, and 65°C annealing for 30 sec and 68°C extension for 75 sec. The final step was a 68°C extension for 7 min. Amplified DNA was separated on 2.0 % agarose gel, stained with 0.5 µg/ml of ethidium bromide and visualized with a Bio-Rad GelDoc 2000 Fluorescent Imaging System.

PCR assay specificity validation

The specificity of the mPCR assay was tested with 185 strains belonging to the seven STEC serogroups most common in the US (O26, O45, O103, O111, O121, O145 and O157), 81 strains belonging to other STEC serogroups (O6, O8, O15, O22, O38, O39, O49, O74, O84, O88, O91, O96, O109, O113, O116, O117, O118, O130, O136, O141, O146, O153, O159, O163, O171 and O172), five strains of non-Shiga toxin producing *E. coli* (ATCC 43886, 12014, 35401, 43896, and 25922) and three species of related Gram negative bacteria (*Salmonella enterica, Klebsiella pneumonia* and *Proteus mirabilis*). Strains were revived from frozen beads by streaking onto BAP and incubating overnight at 37°C. Template DNA for the mPCR reaction was obtained by mixing 1 or 2 colonies in a microcentrifuge tube in 1.0 ml of double deionized H_2O, boiling for 10 min and centrifuging at 9,300 x g for 5 min.

PCR assay sensitivity with pure culture and cattle fecal sample spiked with pure culture

Escherichia coli O104:H4 (ATCC BAA-2326) was grown in Luria-Bertani (LB; Becton Dickinson Co., Sparks, MD) broth to an absorbance of 0.5 at 600 nm (approx. 10^8 CFU/mL) and serial 10-fold dilutions were made. A 1.0 ml aliquot of each dilution was removed, boiled for 10 min, centrifuged at 9,300 x g for 5 min and tested by the mPCR to determine the detection limit

in pure culture. Another 1.0 ml of each dilution was mixed with aliquots of fresh fecal sample (1

ml per 10 g of fecal sample) collected from a feedlot steer. An aliquot (10 g) of feces mixed

with 1 mL of sterile LB broth served as the uninoculated control. One gram of spiked feces of

each dilution was suspended in 9.0 ml of *Escherichia coli* broth (EC; Oxoid Ltd., Hampshire,

UK), vortexed and incubated at 40°C for 6 h. Aliquots (1.0 ml) of fecal suspensions before and

after enrichment were placed in 1.5 ml microcentrifuge tube, boiled for 10 min and centrifuged

at 9,300 x *g* for 5 min. The dilutions of the O104:H4 culture used for inoculating feces were

spread-plated on MacConkey agar (3 plates per dilution; Difco; Becton Dickinson, Sparks, MD)

to determine colony counts. DNA from both pre- and post-enrichment sub-samples were

extracted and purified with a GeneClean DNA extraction kit (MP Biomedicals, Solon, OH), then

subjected to the mPCR. The detection limit of the mPCR was the lowest concentration of the

serially diluted pure culture, pre- and post-enrichment spiked fecal samples in which all 5 bands

were visualized.

 PCR assay and culture detection of E. coli O104 in cattle fecal samples

In the summer of 2012, a total of 248 fecal samples were collected based on a convenience

sample of eight feedlots. In 7 feedlots, 24 fresh pen floor fecal samples were collected from 10

different pens (2-3 samples per pen). From the eighth feedlot, a total of 80 fecal samples were

collected from 18 different pens (4 or 5 samples per pen). Fecal samples were placed in Whirl-

Pak bags, transported in coolers with ice packs to the laboratory, and processed within 24 h of

sampling. One gram of feces was placed in 9.0 ml of *EC* broth (Oxoid Ltd.), vortexed, and

incubated at 40°C for 6 h. Aliquots (1.0 ml) of fecal suspensions, before and after enrichment,

were placed in 1.5 ml microcentrifuge tube, boiled for 10 min and centrifuged at 9,300 x g for 5 min. DNA was extracted from the pre- and post-enrichment samples with a GeneClean DNA extraction kit and subjected to the mPCR. The remaining post-enrichment fecal suspension was stored at 4°C and used for isolation.

Fecal samples (n=51) that were positive for *E. coli* O104 by mPCR were streaked on to MacConkey agar (BD), Rainbow agar (Biolog Inc., Hayward, CA), non-O157 STEC differential agar (Possé et al., 2008), CHROMagar™ STEC (CHROMagar Microbiology, Paris, France, distributed by DRG International, Mountainside, NJ) and CHROMagar™ STEC with O104 supplement (CHROMagar Microbiology) plates. All plates were incubated at 37°C for 24 h, and 10 presumptive colonies per plate (based on colony appearance and color of pure culture O104:H4 on the same media; Table 3) were picked and streaked on to BAP. A single colony from each of the ten isolates of each sample was suspended individually in 1 ml of distilled water and 100 µl of suspensions of each of the ten colonies were pooled together and subjected to the mPCR assay for the 8 genes. If the DNA from the pooled colonies amplified the wzx_{104} gene, then each of the ten colonies was tested individually by the mPCR to identify the pure culture of the serogroup O104. Isolates that were positive for the wzx_{104} gene were submitted to the *E. coli* Reference Laboratory at Pennsylvania State University for serotype confirmation.

Statistical analysis

Sample-level crude prevalence estimates were calculated based on the overall proportions of samples that tested positive for each gene. Associations between presence of the serogroup

O104-specific gene (wzy_{O104}) and stx1, stx2, eae or $fliC_{H4}$ genes within enriched fecal samples were analyzed in generalized linear mixed models specified with a binomial distribution and logit link function. Random effects were used to account for the hierarchical structure of to the data (samples within pens and pens within feedlots). Odds ratios and confidence intervals (back transformed original scale) are reported. Due to the small numbers of samples positive by culture-based methods, only descriptive statistics are provided for these data.

Results

Multiplex PCR assay with pure cultures and spiked fecal samples

The specificity of each primer pair was validated individually with the DNA of O104:H4 ATCC strain, O104:H21 ATCC strain, O157:H7 ATCC strain or pooled DNA of O104:H4 and O157:H7 serotypes for amplifications of wzx_{O104}, $fliC_{H4}$, stx1, stx2, eae, $terD$, $ehxA$ and $aggA$ genes. When tested individually, primers amplified only single bands corresponding to the expected sizes of each amplicon: 655 bp for stx1, 477 bp for stx2, 434 bp for $terD$, 375 bp for eae, 337 bp for wzx_{O104}, 244 bp for $fliC_{H4}$, 199 bp for $ehxA$ and 151 bp for $aggA$. When primers were combined into a single reaction and assay conditions were optimized, 8 distinct bands of the expected amplicons were detected with the pooled DNA of serotypes O104:H4 and O157:H7 (Fig. 1). The O104:H4 German strain was negative for all except stx1, $eae,$ and $ehxA,$ the Montana strain was positive for wzx_{O104}, stx2, and $ehxA$ and negative for stx1, $fliC_{H4}$, $eae,$ and aggA, and the O157:H7 strain was positive for stx1, stx2, $eae,$ and $ehxA,$ but negative for wzx_{O104}, $fliC_{H4}$ and aggA (Fig. 1). The mPCR assay did not show amplifications of wzx_{O104},

*fliC*_{H4} or *agg*A genes in any of the 274 strains of STEC, non-STEC, and other related bacteria
(data not shown). All STEC strains were positive for at least one of the *stx* genes.

The initial concentration of the O104:H4 ATCC strain, which was serially diluted to
determine assay sensitivity, was 1.5×10^8 CFU/ml. The minimum concentration of the pure
culture that amplified the five expected genes (*wzx*$_{O104}$, *fliC*$_{H4}$, *stx*2, *terD*, and *aggA*) was 1.5 x
10^4 CFU/ml. In fecal samples spiked with serially diluted (10-fold) concentrations of O104:H4,
the sensitivity of detection was 1.5×10^5 CFU/g before enrichment and 1.5×10^2 CFU/g after
enrichment (Fig. 2). Although lower concentrations showed amplifications of some genes (*fliC*$_4$,
terD, and *ehxA*), only 10^2 or higher concentrations of cells, after enrichment, showed all 5 bands
of O104. The fecal sample inoculated with sterile LB broth showed distinct bands of *terD* and
ehxA, and a faint band of *fliC*$_4$.

Multiplex PCR detection of O104 from cattle fecal samples

Results from the mPCR assay of the 248 fecal samples from feedlots showed only 3 samples
(1.1%) positive for the O104 serogroup before enrichment and 51 samples (20.6%) positive after
enrichment in EC broth (Table 2). The crude within-feedlot sample prevalence of *wzx*$_{O104}$ gene
in cattle feces ranged from none (0/24 fecal samples) to 11 of 24 fecal samples (45.8%).
However, none of the 248 fecal samples was positive for the *aggA* gene. More fecal samples
were positive for the other six genes (*fliC*$_{H4}$, *stx*1, *stx*2, *eae, ehxA*, and *terD*) after enrichment (58
to 97.8%) compared to samples before enrichment (14.9 to 84.5%; Table 2). Samples were more
commonly positive for the *stx*2 gene (75.8%) the *stx*1 gene (58.1%). A high percentage of fecal
samples (82 to 98%) were positive for *fliC*$_{H4}$, *eae, ehxA*, and *terD* genes. Forty-one fecal

samples (16.5%) contained the three genes, wzx_{O104}, $fliC_{H4}$, and $stx1$ or $stx2$ (Table 2). The

presence of wzx_{O104} in enriched fecal samples was positively associated with the presence of

$fliC_{H4}$ (odds ratio [OR] of 11.8; confidence interval 1.89 to 71.43; $P < 0.01$) and eae (OR of 5.0;

confidence interval 1.29 to 19.23; $P = 0.02$). However, there was no significant association

between the presence of wzx_{O104} and either of the Shiga toxin genes

Isolation of E. coli O104 from cattle fecal samples

Fecal samples that were positive for wzx_{O104} gene (n=51) were plated on several media to

isolate the serogroup O104 in pure culture. Initially, MacConkey and Rainbow agar were used.

Subsequently, three additional media, a selective and differential non-O157 STEC medium

described by Posse et al (2008) and two commercial chromogenic media, CHROMagar STEC

and CHROMagar STEC with O104 supplement were included. The non-O157 STEC

differential medium described by Posse et al (2008) consisted of lactose- free MacConkey agar

base supplemented with sugars (sucrose and sorbose), additional bile salt, isopropyl β-D-1-

thiogalactopyranoside (IPTG), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal), sodium

tellurite, and novobiocin. The plating of O104:H4 ATCC strain on all four media yielded

lactose- fermenting pink colonies on MacConkey agar, and shades of light to dark blue to purple

colored colonies on non-O157 STEC differential agar, Rainbow agar, CHROMagar STEC and

CHROMagar STEC O104 (Table 3). After plating enriched fecal samples that were positive for

the serogroup O104 (n=51) by mPCR, 10 presumptive colonies (based on color) were randomly

picked from each medium and streaked individually on BAP and incubated overnight. If the

DNA from the pooled colonies of 10 amplified the wzx_{104} gene, then each of the ten colonies was

tested by mPCR to identify the serogroup O104. Of the 51 fecal samples, only 10 isolates (2 from 51 samples plated on MacConkey, 3 from 51 samples plated on Rainbow agar, 0 from 42 samples plated on non-O157 STEC differential agar, 1 from 42 samples plated on CHROMagar STEC and 4 from 16 samples plated on CHROMagar STEC O104) were positive for the O104 serogroup-specific gene (wzx_{104}). None of the 10 isolates in pure culture was positive for Shiga toxin genes, *eae,* or *aggA*. The four O104 isolates from CHROMagar STEC-O104 and 1 isolate from the Rainbow agar possessed *ter*D and *ehxA*, while the other 6 isolates did not possess any of the other 4 genes (*stx*1, *stx*2, *eae,* and *ehx*A) tested by the mPCR. Of the 10 isolates, 2 isolates on three occasions were from the same fecal sample on two different agars (MacConkey and Rainbow, MacConkey and CHROMagar STEC, Rainbow and CHROMagar STEC O104 agars), therefore, only seven samples were considered to have distinct isolates from different fecal samples (Table 4). Of the seven isolates, five were confirmed as O104 with H7 flagellar type by the *E. coli* Reference Center at Pennsylvania State University. Two of the seven isolates were identified as O8:H11 and O8:H21 by the *E. coli* Reference Center. The H7 flagellar type of the five O104 isolates was confirmed with the primers designed to identify *fliC*$_{H7}$ of *E. coli* O157 (Bai et al., 2010). A PCR described by Wang et al., (2001), designed for O8/O9 (F-GGCATCGGTCGGTATTCC and R-TGCGCTAATCGCGTCTAC), was performed on the seven isolates. The two isolates identified as O8 by the *E. coli* Reference Center yielded positive bands and the other 5 isolates were negative (Fig. 3). We then retested *E. coli* O8 strains (n=19) that were in our culture collection with the primers designed for wzx_{O104} gene, and none of the strains yielded a positive band (Fig. 4). The flagellar types (H11 and H21) of the two O104/O8 isolates were confirmed by PCR assays (Durso et al., 2005; Sekse et al., 2011).

Discussion

An 8-gene mPCR was designed to screen fecal samples of cattle for the presence of the serogroup O104 in association with the genes characteristic of the hybrid strain of STEC (*stx*2) and EAEC (*aggA*) with H4 flagellar antigen (*fliC*$_{H4}$). The *aggA* gene is one of four genes (*aggA* to *aggD*) in a cluster that encode for a type 1 aggregative adherence fimbrial (AAF) subunit required for the phenotypic expression of the aggregative adhesion pattern (Suzert et al., 2001). The *aggA* was chosen because it is more conserved than the other genes in the cluster, including the master regulator gene *aggR* of the AAF operon, typical of EAEC. The AAF adhesins are responsible for the characteristic "stacked brick" aggregative adherence of EAEC demonstrated on Hep-II cells, a human cell line (Nataro et al., 1987). Although 20.6% of the 248 fecal samples tested contained the serogroup O104, none of the fecal samples was positive for the *aggA,* which was not surprising because EAEC pathotype is generally considered as a human diarrheal pathogen, particularly of children in developing countries (Giammanco et al., 1996). However, *E. coli* strains displaying aggregative adherence pattern have been isolated from different animal species, including calves with diarrhea (Aidar et al., 2000; Uber et al., 2006). The EAEC strains of animal origin were classified as atypical EAEC because they lacked *aggR* and aggregative adherence fimbrial genes (Uber et al., 2006). The pathotype with a combination of enteroaggregative and Shiga toxigenic traits is rare and prior to the German outbreak strain, it was only previously described in strains of O111:H2 and O86:NM involved in outbreaks of bloody diarrhea or hemolytic uremic syndrome in children in France and Japan, respectively (Morabito et al., 1998; Iyoda et al., 2001). Similar to the German outbreak strain, the O111:H2

strain was negative for *eae,* but contained *stx*2 and genes that code for the aggregative fimbriae, but was positive, unlike the German strain, for the enteroaggregative heat stable enterotoxin.

The specificity of the assay to detect the serogroup O104-specific gene was confirmed with a number of STEC (O157 and non-O157) strains and other related gram negative bacteria. The sensitivity and applicability of the assay to detect the serogroup O104-specific gene were evaluated with a fecal sample inoculated with pure culture of O104:H4 strain. A minimum concentration of 1.5 x 10^5 CFU/g (150 CFU per PCR reaction) was needed without an enrichment step to detect the five targeted genes of *E. coli* O104:H4 in cattle feces. However, inclusion of an enrichment step in EC broth increased the sensitivity to 1.5 x 10^2 CFU/g of feces, which was similar to the sensitivity of detection by mPCR of other STEC (Paddock et al., 2012; Bai et al., 2012; DebRoy et al., 2011; Zhang et al., 2012). The real-time PCR described by Zhang et al. (2012) targeting wzx_{O104}, *stx*2, and $fliC_{H4}$ was shown to have a detection limit of 7 x 10^3 CFU/ml in human stool samples spiked with three different strains of *E. coli* O104:H4 and enriched for 4 h in Gram negative broth.

Overall, the O104 serogroup-specific gene was present in 20.6% of fecal samples obtained from feedlot cattle. The $fliC_{H4}$ gene was detected more frequently (x%), suggesting that the flagellar type is present in other serotypes or species of bacteria. The high overall prevalence of some of the virulence genes (*stx*1, *stx*2, *eae,* and *ehx*A) in fecal samples subjected selective enrichment has been previously reported (Bai et al., 2012; Paddock et al., 2012; Pradel et al., 2000; Auvray et al., 2012). A major limitation of a mPCR that detects serogroup, flagellar type and virulence genes in a fecal sample is that it does not indicate that the flagellar gene or virulence genes are associated with any particular serogroup. Therefore, our estimation of the

crude prevalence of O104 in feces of cattle is based entirely on the detection of the gene that

codes for the O antigen of O104. Serogroup O104 with H4 flagellar type, which may be an

STEC (positive for Shiga toxins and negative for enteroaggregative adhesins) or EAEC (positive

for enteroaggregative adhesins, heat stable enterotoxin and negative for Shiga toxins) has been

reported rarely to cause human infections (Bielazewaska et al., 2011; Germani et al., 1998; Kim

et al. 2011; Mellman et al., 2008; Scavia et al., 2011). Interestingly, the serotype of O104:H4,

either Shiga toxigenic or enteroaggregative, has never been reported in animals or food (Mora et

al., 2011). However, strains of O104 with no H antigen (non motile) or different from H4 (e.g.,

H7, H11, H12, H21, etc.) have been reported in cattle feces (Blanco et al., 2003, 2004; Mora et

al., 2011). Wieler et al. (2011) tested 2,000 *E. coli* strains isolated from 100 fecal samples from

cattle housed in farms located in the outbreak region of Germany with a multiplex PCR designed

to detect rfb_{104}, $stx2$, $terD$ and $fliC_{H4}$ (Bielaszewaska et al., 2011). None of the strains showed

the combination of four genes characteristic of the outbreak strain, which led the authors to

conclude that cattle, in contrast to the other STEC, were not a reservoir for the O104:H4

serotype. A similar conclusion was reached by Auvray et al. (2012) after testing cattle feces in

France. A total of 1,468 French cattle were tested for fecal carriage of O104:H4 by PCR assay

targeting wzx_{104}, $stx2$, $fliC_{H4}$, and $aggR$ genetic markers and none of the fecal samples contained

the four markers together. Because the full combination of four genes typical of the German

outbreak strain was not detected in any cattle feces, the authors concluded that French cattle are

not a reservoir of the hybrid pathotype. However, a small proportion (6.1%) of fecal samples

contained the three genes, wzx_{104}, $stx2$, and $fliC_{H4}$. In our study, we found 15.3% of fecal

samples harbored the combination of wzx_{104}, $fliC_{H4}$ and $stx2$ genes, which does not necessarily

mean that *stx*2, and *fliC~H4~* were carried by O104 as the genes could have been carried separately by distinct serotypes.

Of the seven O104 strains recovered in this study, five had H7, and the other two had H11 and H21. None of the seven strains carried genes for Shiga toxin or enteroaggregative fimbrial subunit. Two of the 7 strains carried the enterohemolysin gene. Interestingly, 2 of the 7 strains were identified as O8 serogroup based on the serology performed by the *E. coli* Reference center. The two strains were positive for the *wzx~104~* gene by our mPCR and also gave positive bands with primers targeting O8 serogroup (Wang et al., 2001). The oligosaccharide unit of the serogroup O104 has the identical structure as the *E. coli* K9 capsular antigen and the gene cluster that codes for O104 has the same genes in the same order as K9 gene cluster (Wang et al., 2001). The K9 antigen is generally present in strains of *E. coli* serogroups O8, O9, and O9a (Whitfield and Roberts, 1999). Published PCR assays designed to detect O104 also were shown to detect the K9 positive O8/O9 *E. coli* (Wang et al., 2001; Delannoy et al., 2012; Zhang et al., 2012). Two of our isolated O104 strains were serotyped by the *E. coli* Reference lab as O8 and these isolates showed amplicons when tested with O8/O9-specific primers (Wang et al., 2001). However, the remaining five strains of O104 did not show amplification with the O8/O9 primers. In our validation assay, we tested 19 strains of O8 and none of them showed amplification with our designed O104 primers, which suggests the specificity of the O-gene region of O104 targeted for amplification.

Because O104:H4 lacks unique biochemical characteristic no selective and differential medium has been developed for presumptive phenotypic identification. Also, monoclonal antibodies have not been developed for use in immunomagnetic bead separation, a procedure

commonly used for selective isolation of O157 and some serogroups of non-O157 STEC. In an attempt to isolate the serogroup O104 in pure culture from fecal samples that were PCR positive for O104 (n=51), we streaked enriched fecal suspension on to five different selective and differential media that allow color-based identification of non-O157 STEC. The commercial chromogenic media (Rainbow agar and CHROMagar) have been used to isolate a variety of non-O157 STEC serogroups (Hirvonen et al., 2012; Monaghan et al., 2012; Tillman et al., 2012; Tzschoppe et al., 2012). Of the 5 media that we used, CHROMagar STEC with O104 supplement, a proprietary preparation of undisclosed composition, identified O104 serogroup in 4 of 16 PCR-positive (25%) fecal samples tested. The commercial product was designed specifically to allow the growth of extended spectrum beta lactamase phenotype, a characteristic feature of the German outbreak strain. Overall, culture-based methods identified the O104 serogroup in only 7 of 51 (13.7%) PCR-positive fecal samples. Obviously, lack of immunomagnetic separation step and suitable selective and differential medium may have limited our ability to isolate O104 from fecal samples that were PCR positive for the serogroup-specific gene.

In conclusion, the 8-gene PCR assay will be useful to confirm putative isolates of serogroup O104 and determine the presence of major traits that are characteristics of STEC and EAEC pathotypes. Additionally, the assay could be used to screen fecal samples for the prevalence of the serogroup O104 before subjecting the samples for culture-based detection and isolation. Although mPCR detected 21% of fecal samples as positive for serogroup O104, O104 isolates were only recovered by culture-based procedures from few (13.7%) of the PCR-positive samples. Culture-based detection may have been limited by lack of selectivity in the isolation

procedure and further research to refine culture methods is needed. Because none of the fecal

samples contained the *aggA,* the enteroaggregative gene, and none of the isolated strains carried

*fli*C_{H4}, *aggA, or stx* genes, cattle do not appear to be a likely a reservoir for *E. coli* O104:H4 with

characteristics of STEC and EAEC. Further research is needed to determine the predominant

pathotype (STEC, EAEC or non-pathogenic) of serogroup O104 that are shed in cattle feces.

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Figure 5.1. Agarose gel images of amplicons obtained from multiplex PCR performed with two strains of *Escherichia coli* O104 (German [Lane 2] and Montana [Lane 3] strains), a strain of *E. coli* O157:H7 (ATCC strain 43894; Lane 4) and pooled DNA mixtures of O104 and O157 strains (Lane 5). Lane 1 is the molecular size markers (100 bp).
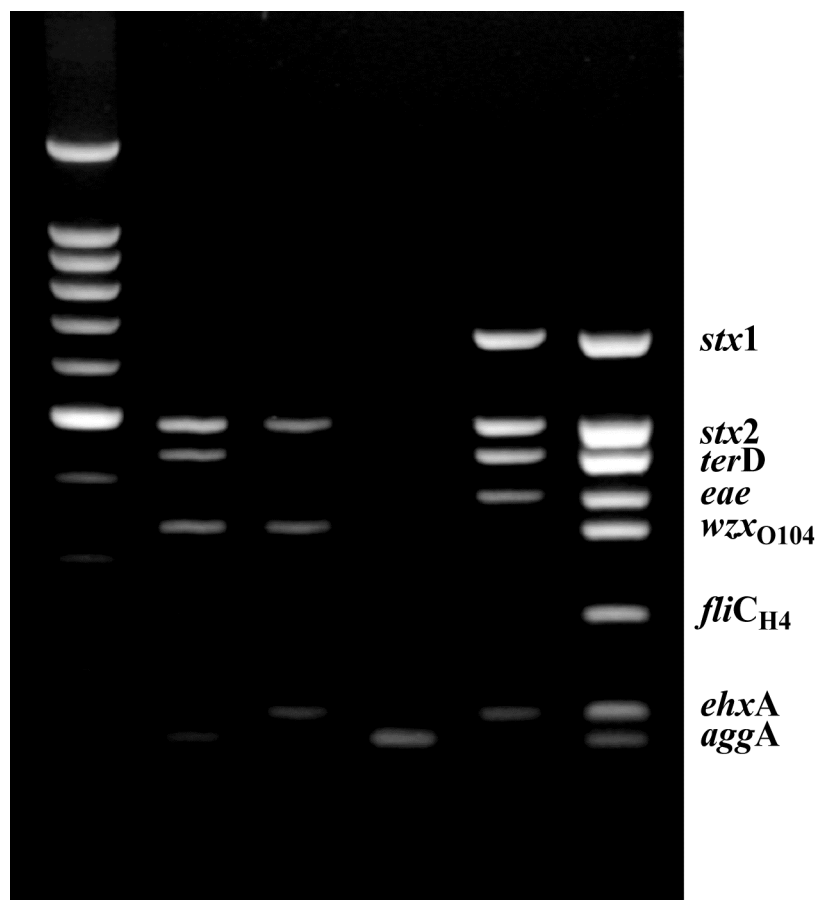
Figure 5.2. Agarose gel images of amplicons obtained from multiplex PCR performed on fecal sample inoculated with ten-fold serial dilutions of *Escherichia coli* O104:H4 (1.5×10^7–1.5×10^1 CFU/g) and enriched in *Escherichia coli* broth. Lanes 1 and 10 are molecular size markers (100 bp). The positive control (POS CON; Lane 11) was pooled DNA of ATCC strains of O104:h4 and O157:H7. The negative control (NEG CON; Lane 9) was fecal sample mock-inoculated with sterile broth.
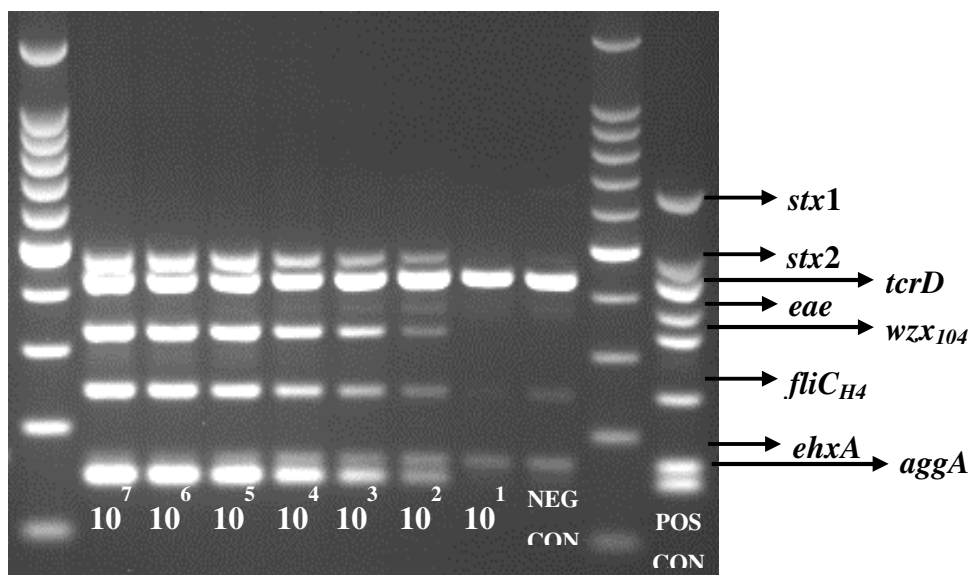
Figure 5.3. Agarose gel images of amplicons obtained from PCR with primers designed for *Escherichia coli* serogroups O104 (A) and O8 (B). Lanes 1 and 12 are molecular size markers Lanes 3 to 9 are the seven *E. coli* O104 strains isolated in this study of cattle feces. Lane 10 is *E. coli* O8 and lane 11 is negative control.
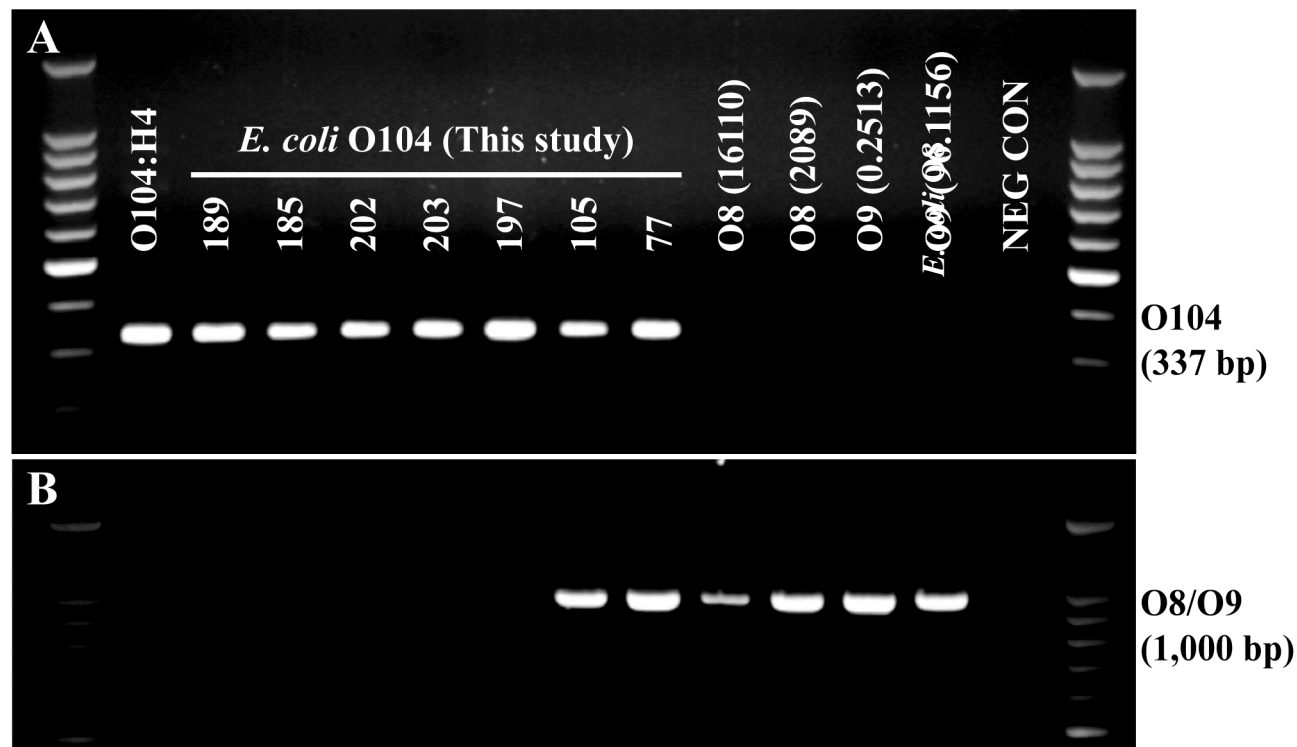
Figure 5.4. Agarose gel images of amplicons obtained from PCR with primers designed for *Escherichia coli* O104. Lane 2 is O104:H4 (German), lane 3 is O104:H21 (Montana), Lane 4 is serogroup O8, lanes 5 and 6 are strains of O104 and O8 isolated in this study, lane 7 is O157:H7, lane 8 is positive control (pooled DNA of O104 and O157 strains), lane 9 is negative control, and lanes 1 and 10 are molecular size markers (100 bp).

	O104:H4 (Ger.)	O104:H21 (MT)	O8 (KSU 16110)	O104/O8 (F77)	O104/O8 (F105)	O157:H7 (ATCC 43894)	POS CON	NEG CON	

*stx*1   655 bp

*stx*2   477 bp
terD   434 bp
eae   375 bp
wzx_{104} 337 bp

fliC H4   244 bp

exhA   199 bp
aggA   151 bp
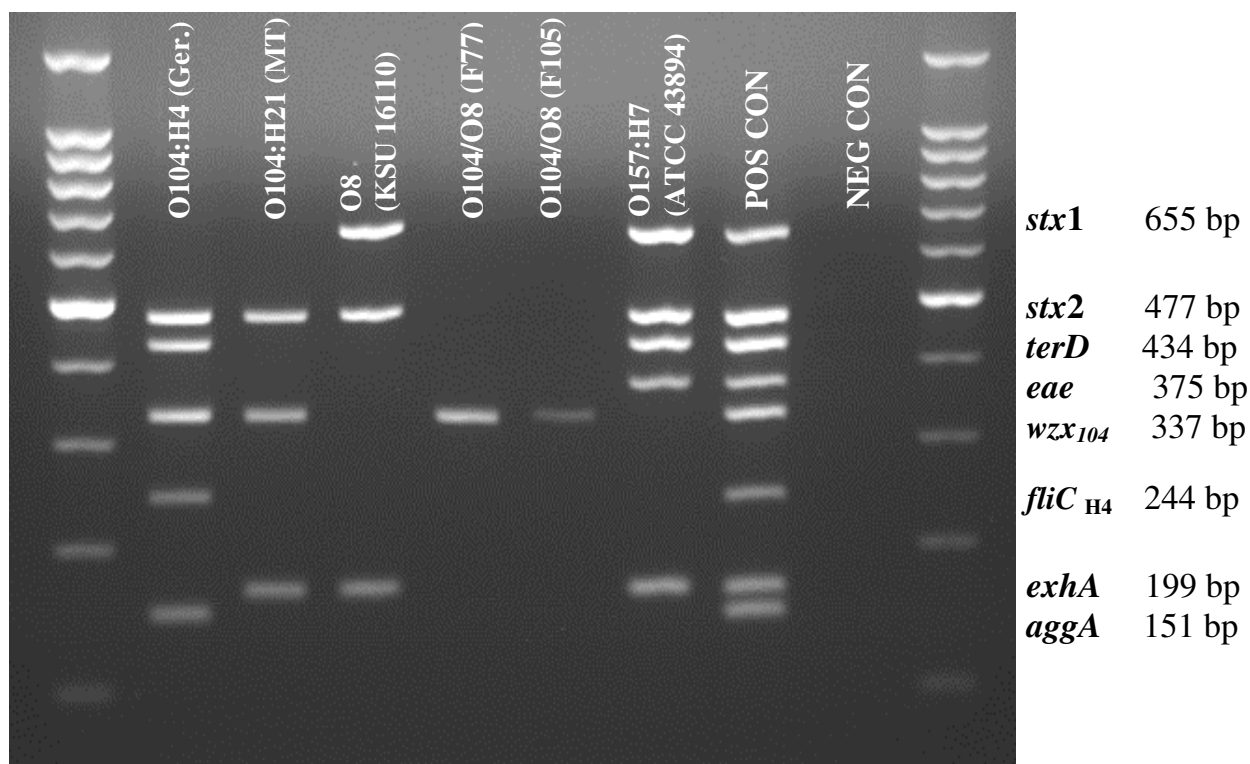
Table 5.1. Target genes, primers and amplicon sizes used for the multiplex PCR procedure

Target genes	Primer	Primer sequence (5'—3')	Amplicon size (bp)	Reference
wzx_{O104}	$wzx_{O104\text{-}F}$	GGTTTTATTGTCGCGCAAAG	337	This study
	$wzx_{O104\text{-}R}$	TATGCTCTTTTTCCCCATCG		
$fliC_{H4}$	$fliC_{H4}$-F	ACGGCTGCTGATGGTACAG	244	This study
	$fliC_{H4}$-R	CGGCATCCAGTGCTTTTAAC		
stx1	stx1-F	TGTCGCATAGTGGAACCTCA	655	Bai *et al.*, 2012
	stx1-R	TGCGCACTGAGAAGAAGAGA		
stx2	stx2-F	CCATGACAACGGACAGCAGTT	477	Bai *et al.*, 2012
	stx2-R	TGTCGCCAGTTATCTGACATTC		
eae	eae-F	CATTATGGAACGGCAGAGGT	375	Bai *et al.*, 2012
	eae-R	ACGGATATCGAAGCCATTTG		
ehxA	ehxA-F	GCGAGCTAAGCAGCTTGAAT	199	Bai *et al.*, 2012
	ehxA-R	CTGGAGGCTGCACTAACTCC		
terD	terD-F	AGTAAAGCAGCTCCGTCAAT	434	Bielaszewska *et al.*, 2011
	terD-R	CCGAACAGCATGGCAGTCT		
aggA	aggA-F	CGTTACAAATGATTGTCCTGTTACTAT	151	This study
	aggA-R	ACCTGTTCCCCATAACCAGAC		

Table 5.2. Number (and percentage) of cattle fecal samples positive for genes that encode for *Escherichia coli* O104 serogroup-specific traits before and after enrichment in *Escherichia coli* broth

Genes (encoded protein or function)	No. of samples (n=248) positive for (%):	
	Before enrichment[1]	After enrichment
*wzx*_{O104} (O104-antigen flippase)	3 (1.2)	51 (20.6)
*fli*C$_{H4}$ (H4 flagellar antigen)	103 (41.5)	214 (86.3)
*stx*1 (Shiga toxin 1)	37 (14.)	144 (58.1)
*stx*2 (Shiga toxin 2)	100 (40.3)	188 (75.8)
eae (Intimin)	92 (37.1)	204 (82.3)
*ehx*A (enterohemolysin)	210 (84.5)	243 (97.8)
terD (tellurite resistance)	119 (48.0)	233 (94.0)
aggA (aggregative adherence fimbriae 1)	0	0
wzx$_{O104 +}$*fli*C$_H$	2 (0.8)	47 (20.0)
wzx$_{O104 +}$*fli*C$_H$ + *stx*1	2 (0.8)	30 (12.1)
wzx$_{O104 +}$*fli*C$_H$ + *stx*2	2 (0.8)	38 (15.3)
wzx$_{O104 +}$*fli*C$_H$ + *stx*1 or *stx*2	2 (0.8)	41 (16.5)

[1]Fecal samples were enriched by incubating 1 g of feces in 9 ml of *Escherichia coli* broth at 40°C for 6 h

Table 5.3. Occurrence of serogroup 104 (wzx_{104}), H$_4$ flagellar antigen ($fliC_{H4}$), Shiga toxins (stx1 and stx2), intimin (eae), enterohemolysin (ehxA), tellurite resistance (terD), and enteroaggregative (aggA) genes in pooled colonies from cattle fecal samples that were positive for $wzx_{104.}$

Culture medium for isolation	No. of fecal samples[1]	Colony morphology[2]	Number of pooled colonies positive for:							
			wzx_{O104}	$fliC_{H4}$	stx1	stx2	eae	ehxA	terD	aggA
MacConkey agar	51	Pink colored	2	13	1	2	2	7	15	0
Rainbow agar	51	Blue-purple centered colonies with purple edges	3	8	2	2	4	10	12	0
Non-O157 STEC Differential agar (Possé agar)	42	Dark purple colonies	0	10	1	5	7	8	29	0
CHROMagar™ STEC	42	Light purple colonies	1	19	2	13	11	16	31	0
CHROMagar™ STEC-O104	16	Light purple colonies	4	1	3	4	10	11	15	0

[1] Number of fecal samples that contained the wzx_{104} when extracted DNA was screened by the multiplex PCR
[2] Ten colonies from each plate exhibiting the indicated morphology were pooled together and tested by multiplex PCR for the indicated genes

Table 5.4. Virulence genes profiles of *Escherichia coli* O104 strains isolated from cattle feces.

Strain origin	Strain	No. of strains	O type	H type	Virulence genes					
					*stx*1	*stx*2	*eae*	*ehxA*	*terD*	*agg*A
German Outbreak	ATCC # BAA-2326	1	O104	H4	-	+	-	-	+	+
Montana Outbreak	ATCC # BAA-178	1	O104	H21	-	+	-	+	-	-
Cattle feces	This study	5	O104	H7	-	-	-	+	+	-
Cattle feces	This study	1	O104/O8	H11	-	-	-	-	-	-
Cattle feces	This study	1	O104/O8	H21	-	-	-	-	-	-

Chapter 6 - Chapter 6 *Escherichia coli* O26 in feedlot cattle: Fecal prevalence, isolation, characteristics and effects of an *E. coli* O157 vaccine and a direct-fed microbial intended

Abstract

Escherichia coli serogroup O26 is second only to O157 as a cause of food borne, Shiga toxin-producing *E. coli* infections. Our objectives were to determine fecal prevalence *E. coli* O26 and isolate and characterize virulence genes of *E. coli* O26 in cattle in a commercial feedlot that were enrolled in a study to evaluate an *E. coli* O157:H7 siderophore receptor and porin (SRP[®]) proteins-based vaccine (VAC) and direct-fed microbial (DFM; Bovamine[®]). Cattle were randomly allocated to 40 pens within 10 complete blocks; pens were randomly assigned to control, VAC, DFM, or VAC+DFM treatments. Vaccine was administered on days 0 and 21, and DFM was fed (10^6 CFU/animal/day) throughout the study period. Pen floor fecal samples (30/pen) were collected weekly for four consecutive weeks at the end of the study period. Samples were enriched in *E. coli* broth and subjected to a multiplex PCR designed to detect O26-specific *wzx* gene and four major virulence genes (*stx*1, *stx*2, *eae*, and *ehx*A) and to a culture-based procedure that involved immunomagnetic separation and plating on MacConkey agar. Ten presumptive *E. coli* colonies were randomly picked from the plate, pooled and tested by the multiplex PCR for O26 and four virulence genes. Pooled colonies that were positive for O26 serogroup were streaked on sorbose MacConkey agar and 10 colonies, randomly picked per sample, were tested individually again by the multiplex PCR. The overall prevalence was higher

by the culture-based detection method (1,089/4,800; 22.7%) compared to the detection by mPCR assay (502/4,800; 10.5%). No significant effects of treatment, or treatment by sampling week interaction, on O26 prevalence were detected by either PCR- or culture-based method. *Escherichia coli* O26 was recovered in pure culture from 23.9% (260 out of 1,089) of O26 PCR-positive pooled colonies. Only seven of the 260 strains of O26 were STEC and 90.1% of the strains possessed *eaeβ* gene that codes for intimin subtype *β,* but not the *bfpA* gene that codes for bundle-forming pilus, indicating that majority of the O26 strains in cattle feces belonged to atypical enteropathogenic *E. coli*.

Introduction

Shiga toxin-producing *E. coli* (STEC) are major food borne pathogens that cause illnesses in humans with symptoms ranging from diarrhea, with or without blood, to hemolytic uremic syndrome, and even death (Tarr et al., 2005). Among STEC, the serotype O157:H7 has caused a greater number of food borne outbreaks than any other serotypes (Rangel et al., 2005). Recent epidemiological data have shown that non-O157 STEC represent a larger portion of foodborne STEC infections compared to O157 (Scallan et al., 2011). Of the non-O157 STEC, the serogroup O26 accounts for the largest proportion of infections (Brooks et al., 2005; Stigi et al., 2012).

Ruminants, particularly cattle, are considered to be major reservoirs of non-O157 STEC (Karmali et al., 2010). However, not much is known about the fecal prevalence and ecology of serogroup O26 because isolation and detection methods have not been optimized (Jenkins et al., 2008; Ferens and Hovde, 2011). A fecal prevalence of 6.5 % (Lynch et al., 2011), 23.0% (Hofer

et al., 2012), 80.0 % (Renter et al., 2004) and 82.5 % (Paddock et al., 2012), based on PCR assay targeting the O26 serogroup-specific gene (wzx_{O26}), have been reported. However, because of lack of selective isolation procedures, the subsequent recovery rate of *E. coli* O26 isolates from positive samples has been low (4.3 to 35.7% of PCR-positive fecal samples [Hofer et al., 2012; Lynch et al., 2011; Paddock et al., 2012]).

Preharvest intervention strategies that include the use of direct-fed microbials and vaccines have been shown to reduce the fecal shedding of *E. coli* O157:H7 in cattle. An *E. coli* O157:H7 siderophore receptor and porin protein- based (SRP[®]) vaccine was shown to reduce the overall fecal shedding of *E. coli* O157:H7 (Fox et al., 2009; Thomson et al., 2009; Thornton et al., 2009; Cull et al., 2012). Direct-fed microbials (DFM), particularly *Lactobacillus acidophilus*-based products, have been shown to reduce fecal shedding of *E. coli* O157:H7 (Loneragan and Brashears, 2005). The effects of SRP vaccine or DFM on fecal shedding of non-O157 STEC in feedlot cattle have not been evaluated; but it is conceivable that the mechanisms responsible for the possible *E. coli* O157:H7 reduction may also reduce other serogroups. Our objective was to determine fecal prevalence of serogroup O26 in cattle feces, evaluate selective isolation procedures, characterize virulence genes of the isolates, and determine if vaccine (SRP) and direct-fed microbial (Bovamine[®]) intended to reduce the prevalence of *E. coli* O157:H7 in feedlot cattle have effects.

Materials and Methods

All procedures in the care and management of cattle were approved by the Kansas State University Institutional Care and Use Committee.

Animals and study design

The animals, study location and study design have been previously described by Cull et al. (2012). The study was a randomized complete block design with a 2 x 2 factorial treatment structure of control, vaccine (VAC), DFM, and VAC plus DFM. The DFM The control group (CON) received neither DFM nor VAC. The DFM was Bovamine® (Nutrition Physiology Corp., Guymon, OK), which was mixed into the cattle's diet to provide 10^6 CFU/animal/day of *Lactobacillus acidophilus* and 10^9 CFU/animal/day of *Propionibacterium freudenreichii* throughout the study for DFM and VAC + DFM groups. Cattle in the VAC and VAC + DFM groups received a 2 ml subcutaneous dose of the vaccine (*E. coli* SRP® vaccine, Pfizer Animal Health, New York, NY) on the day of study allocation and 21 days later. Cattle in all treatment groups were fed a high moisture corn (46.4%)-based finishing diet with wet distiller's grains (25%), silage (7.1%), corn steep (2.5%) and a mineral supplement mix (2.0%) with 280 mg of monensin and 90 mg of tylosin per animal per day (Elanco Animal Health, Greenfield, IN) on a dry-matter basis. Cattle were randomly assigned to 1 of 4 treatment groups within blocks (10 time-based blocks) based on the the time of allocation during an 7 week enrollment period. Treatment groups within a block were subsequently randomized to a pen within a pre-determined set of 4 consecutive pens (40 pens). During the 4 weeks prior to the end of study date for each respective block, fresh pen floor fecal samples (n = 30/pen/week) were collected. Samples were transported on ice to the Kansas State Universities pre-harvest food safety laboratory. Samples were assigned sequential numbers, thus blinding laboratory personal to treatment assignments.

PCR detection of E. coli O26

Fecal samples were processed within 24 h after collection. Samples were enriched by preparing a suspension of 1.0 g of feces in 9.0 ml of *E. coli* broth (EC; Oxoid Ltd., Hampshire, England) and incubating at 40° C for 6 h (Paddock et al., 2012). A 1.0 ml sample of the enriched fecal suspension was placed in 1.5 ml microcentrifuge tube, boiled for 10 min and centrifuged at 10,000 x *g* for 5 min. DNA was extracted and purified with a GeneClean DNA extraction kit (MP Biomedicals, Solon, OH) and subjected to an 11-plex PCR (mPCR; Bai et al., 2012) designed to detect the seven STEC serogroups (O26, O45, O103, O111, O121, O145, O157) and four major STEC virulence genes (*stx*1, *stx*2, *eae* and *ehx*A).

Culture-based detection of E. coli O26

Another 1.0 ml sample of the enriched fecal suspension was subjected to immunomagnetic separation (IMS) with Dynabeads® VTEC/STEC O26 (Invitrogen, Carlsbad, CA). Twenty microliters of the bead suspension were pipetted onto MacConkey agar (BD Biosciences, Franklin Lakes, NJ) and streaked with an inoculating loop to obtain isolated colonies. Plates were incubated at 37 C for 24 h and up to ten lactose-fermenting colonies were randomly picked and suspended in 1 ml of peptone broth. A 100 µl aliquot of the pooled colony mixture was boiled for 10 min and subjected to the11-plex PCR. The remaining colony mixture was preserved with glycerol (15% final concentration) and stored at -80°C. Pooled colony mixtures that were positive for serogroup O26 by the mPCR were then used to isolate *E. coli* O26 in pure culture.

Evaluation of culture media for isolation of E. coli O26

A lactose-free MacConkey agar base (BD Biosciences, Sparks, MD) with sorbose (10 g/l; SorboseMAC) or rhamonse (10 g/l; RMAC) as the sole sugar source (Posse et al., 2008; Hiramatsu et al., 2002) was evaluated. The sorboseMAC medium was evaluated with supplementation of no additives, novobiocin (8.0 mg/l; Sigma-Aldrich, St. Louis, MO; Hiramatsu et al., 2002), potassium tellurite (2.5 mg/l; Sigma-Aldrich), or novobiocin and potassium tellurite. The evaluation of the selectivity and ability to differentiate O26 serogroup from others were done with pure cultures of O26 (n = 36) and non-O26 (n = 116) STEC. The strains were streaked onto each medium to determine growth and fermentability of the sugar after a 24 h incubation at 37°C. Media on which all *E. coli* O26 strains grew and fermented the sugar to form colored colony were further evaluated for their selectivity in isolating and differentiating *E. coli* O26 from a subset (n = 34) of the O26 positive pooled colony mixtures.

Isolation of E. coli O26 from PCR-positive pooled colony mixtures

The colony mixtures were thawed and 10 µl was streaked onto each medium. Following 24 h incubation at 37°C, colonies (up to 10) with presumptive *E. coli* O26 fermenting characteristics were selected and individually tested by the mPCR. The medium with the highest recovery rate was then used to isolate *E. coli* O26 from the remaining O26 positive pooled colony mixtures. Isolates that were confirmed as O26 were stored on CryoCare beads at -80°C.

Characterization of E. coli O26 isolates

Isolated *E. coli* O26 were further characterized with individual PCR assays for additional genes; *bfp*A (bundle forming pilus; Gunzburg et al., 1995), *eae*β1 (intimin; Blanco et al., 2003), *esp*P (secreted effector protein; Cookson et al., 2002), *fli*C$_{H11}$ (flagellar gene for H11; Durso et al., 2005), and *tir* (translocated intimin receptor; Bardiau et al., 2011). Two strains, TW01597, a STEC O26 (Thomas Whittam *E. coli* Reference Laboratory at Michigan State University) and EDL933 (ATCC 700927; American Tissue and Culture Collection, Manassas, VA), a STEC O157:H7 were used as positive controls. Strains and isolates were grown on blood agar, DNA was extracted from 1 or 2 colonies by mixing with 1.0 ml of ddH$_2$0, boiling for 10 min and centrifugation at 10,000 x *g* for 5 min

Statistical analysis

Dichotomous outcome variables, whether an animal's sample was positive or negative for each serogroup or virulence gene, were analyzed as pen-level proportions for treatment effects in generalized linear mixed models (GLMM; Proc Glimmix; SAS Version 9.2, SAS Institute Inc., Cary, NC) assuming a binomial distribution and utilizing a logit link function as described by Cull et al. (2012). Treatment, sampling week and treatment × sampling week interaction were included in the model as linear predictors. Block was included as a random effect. In addition, pen over time was included as a repeated effect in all models accounting the lack of independence between the four samples from each pen. Model-adjust means (LSmeans; back transformed to the original scale) and standard error of the means are reported. For all models, *P* values < 0.05 were considered statistically significant and *P* values ranging from 0.05 to 0.10

were considered statistical trends. To determine if an individual fecal sample was more likely to be O26 positive by PCR on DNA extracted from enriched feces compared to culture method (IMS separation, plating on MacConkey and testing of pooled colonies),   data were analyzed by GLMM was used where the outcome was considered binary for each sample for each detection method. Block was included as a random effect. As before pen over time was included as a repeated effect in all models accounting the lack of independence between the four samples from each pen. The odds ratio and 95% confidence intervals were reported.

Results

Overall, 10.5% (502/4,800) of the fecal samples were positive for the O26 serogroup, based on the mPCR assay, and the prevalence of the four virulence genes, stx1, stx2, $eae,$ and $ehxA,$ were 12.4% (597/4,800), 24.0% (1,151/4,800), 45.4% (2,179/4,800), and 84.7% (4,066/4,800), respectively (Table 1). The prevalence of serogroup O26 in feces collected weekly, based on the mPCR assay, ranged from 0.4 to 41.5% (Fig 1A). Based on culture method (IMS followed by plating on MacConkey agar), the O26-positive samples ranged from 2.5 to 36.7% of the samples tested (Fig 1B). Neither vaccine or DFM, nor both had significant effects on the fecal prevalence of the serogroup O26 or the four virulence genes in cattle feces (Table 1). When a mixture of 10 randomly picked colonies, obtained from plating O26 IMS beads on MacConkey agar, were tested by the mPCR, the O26 serogroup was detected in 22.7 % (1,089 out of 4,800) of the fecal samples (Table 2). Although O26 IMS beads were intended to retrieve O26 from the enriched sample, the pooled colonies also contained six other STEC serogroups that were part of the mPCR assay. The other serogroups, in the order of prevalence, were 9.8 % O103 (470/4,800), 5.7 % O111 (273/4,800), 0.8 % O145 (37/4,800), 0.6% O157 (38/4,800), 0.4 % O121 (21/4,800)

and 0.4 % O45 (18/4,800). The prevalence of virulence genes in the pooled colonies were 2.4% of *stx*1 (117/4,800), 10.8% of *stx*2 (519/4,800), 7.9% of *eae* (378/4,800) and 16.6% of *ehx*A (776/4,800). The prevalence estimates of O26 based on IMS, plating on MacConkey agar and testing 10 pooled colonies picked randomly were not affected by the vaccine, DFM or both. There was no treatment group by sampling week interaction. However, more pooled colonies (*P* < 0.01) were O26 positive in weeks 3 and 4 compared to weeks 1 and 2 (data not shown). Of the 1,089 samples that were O26 positive based on testing of the pooled colonies, 561 (51.5%) were negative for all four virulence genes and the remaining (528/1,089; 48.5%) contained at least one of the four virulence genes. Among the O26-postive pooled colonies, 45 (8.5%), 216 (40.9%), 179 (33.9%) and 311 (58.9%) were positive for *stx*1, *stx*2, *eae* and *ehx*A, respectively (Table 3). Only a small proportion of O26 positive pooled colonies contained a combination of *stx*1 or *stx*2 and *eae* (10.4%). Among the other six serogroups detected in the pooled colonies, only O103 and O111 were in high numbers (168/1,089 [15.4%] and 259/1,089 [23.8%] for O103 and O111, respectively; Table 3). The proportions of pooled colonies positive for O103 and O111 that contained *stx*1 or *stx*2 and *eae* were 6.6 and 10.0%, respectively. The number of O26-positive pooled colonies that contained O45, O121, O145 or O157 ranged from 4 to 13 (Table 3).

In order to obtain pure culture of O26 from pooled colonies from samples that were PCR positive for O26 (n=1,089), use of lactose-free MacConkey agar containing sorbose as the sole sugar and without or with potassium tellurite (2.5 µg/ml) and or novobiocin (8 µg/ml) was evaluated. Initially, growth and fermentation of sorbose by pure cultures of O26 strains (n=36) and non-O26 STEC strains (O45, O103, O111, O121, O145, and O157; n=116) on media without or with potassium tellurite and or novobiocin were tested (Table 5). All O26 strains tested fermented sorbose sugar (pink-colored colonies), while sorbose fermentation was highly

variable among the other six serogroups. Only few strains of O45, O103, O111, O121, and O145 fermented sorbose, however, none of the O157 strains (n=20) fermented sorbose. Inclusion of potassium tellurite (2.5 µg/ml) inhibited the growth of 4 of 36 strains of O26, 2 of 2 O45, 9 of 40 O103, 0 of 29 O111, 6 of 9 O121, 2 of 16 O145, and none of the 20 O157 strains. Novobiocin (8 µg/ml) inclusion in the medium had no effect on the growth of O26 or other STEC strains (Table 5). A subset (n=34) of O26 PCR-positive pooled colonies were plated on sorbose-MacConkey agar without or with potassium tellurite and or novobiocin to evaluate the selectivity of the media to detect O26 serogroup (Table 5). Of the 34 samples of pooled colonies, O26 was recovered from 14 (41.2%) samples on the medium with sorbose alone or sorbose with potassium tellurite and novobiocin. Twelve of the 14 isolates from the two media were from the same samples and two isolates were from different samples. Fewer than 14 were detected on media containing sorbose with potassium tellurite or novobiocin (Table 5). Based on this data, we used sorbose MacConkey agar without potassium tellurite or novobiocin to isolate O26 from all the PCR positive O26 pooled colonies. Of the 1,089 samples of pooled colonies, O26 was obtained in pure cultures from 260 samples, representing a recovery rate of 23.9%. Because we used mPCR to test individual colonies, we also obtained 1 each of O121 and O145, 2 of O45 and 26 of O103 serogroups. However, no O157 strain was obtained. In order to enhance recovery rate of O26 from PCR-positive samples, we tested 3 additional media, sorbose MacConkey agar with novobiocin, rhamnose MacConkey agar with novobiocin and a selective and differential medium described by Posse et al., (2007; without potassium tellurite), with a subset (n=47) of O26 positive poled colonies that did not yield O26 in pure culture on sorbose MacConkey agar (Table 6). Rhamnose sugar is not fermented by O26 (Posse et al., 2007), therefore, gray-colored colonies (nonfermenters) were picked from the Rhamnose MacConkey

agar plates. The three different media yielded O26 in pure culture from six of 47 pooled colonies

that were tested.

Of the 260 isolates of O26 obtained, only seven isolates carried Shiga toxin genes, with six

carrying *stx*1 and one carrying *stx*2 (Table 7). From the other serogroups detected among the

individual isolates, only one O145 isolate carried a Shiga toxin gene (*stx*1). Interestingly, 59

additional isolates were recovered that contained one or both Shiga toxin genes but did not

belong to the seven serogroups detectable by the mPCR assay. In order to further characterize

the O26 isolates, PCR assay was conducted for five additional genes, *eae*β1, *bfp*A, *tir*, *fli*C$_{H11}$

and *esp*P that code for intimin subtype β1, bundle forming pilus, translocated intimin receptor,

flagellar H11 protein and secreted effector protein, respectively (Table 8). Seventeen of the 260

O26 isolates carried none of the virulence genes tested. One of the 17 isolates was positive for

the H11 gene. None of the isolates was positive for the *bfp*A, a gene characteristic of the typical

enteropathogenic *E. coli* (EPEC). Majority of the O26 isolates (236/260) carried the *eae*β1 gene

with no *stx*. These O26 atypical EPEC (aEPEC) possessed a variety of virulence gene profiles.

The majority of O26 aEPEC (234 out of 236) was positive for *tir,* which is commonly found on

the LEE with *eae*β1, and the presence of *fli*C$_{H11}$ identifies that these isolates were O26:H11

aEPEC. Fewer of the aEPEC isolates (37 out of 236) carried the *tir* and *eae*β1 but not the

*fli*C$_{H11}$. A small number of isolates of aEPEC O26 (7 out of 236) was positive for *tir*, *fli*C$_{H11}$ and

*ehx*A. Only three O26 aEPEC isolates were positive for *tir* and *fli*C$_{H11}$, as well as *esp*P which are

commonly observed in STEC. Of the seven O26 STEC, six different virulence gene profiles

were observed. All seven O26 STEC were positive for the *eae*β1 and *tir*. Six of the 7 O26

STEC were O26:H11. Only two isolates were *ehx*A positive and three isolates were *esp*P

positive. Samples were 2.64 times (CI: 2.35 to 2.98) more likely to be found O26 positive by

mPCR from DNA extracted from colony pools compared to DNA extracted from enriched feces.

Discussion

The study focused on the serogroup O26 because, according to the CDC, it is the most

frequent serogroup involved in human non-O157 STEC infections in the US (Brooks et al.,

2005). The O26 serogroup accounted for 22% of non-O157 STEC infections reported between

1983 and 2002. The fecal samples used in the study to test for O26 was part of a study that was

designed to study the effects of a *E. coli* O157 vaccine and a *Lactobacillus acidophilus*-based

DFM on fecal shedding of *E. coli* O157:H7 (Cull et al., 2012). The study showed that that a

two-dose vaccination significantly reduced fecal shedding of *E. coli* O157:H7. However, the

DFM had no effect on fecal shedding of *E. coli* O157:H7 and the lack of effect may be because

of the low dose used in the study compared to studies that have shown reduction in shedding

(Cull et al., 2012; Loneragan and Brashears, 2005). The vaccine is based on siderophore

receptor and porin proteins of *E. coli* O157:H7 and the efficacy in reducing fecal shedding is

attributed to induced immunity that blocks bacterial uptake of iron (Thomson et al., 2009).

Because O26 may have surface proteins similar to that of *E. coli* O157:H7, it is logical to expect

for the vaccine to have an effect on fecal shedding of *E. coli* O26. Direct fed microbials,

particularly products containing *L. acidophilus*, have been shown to reduce fecal shedding of *E.*

coli O157:H7 (Jacob and Nagaraja, 2012). Although the mode of action is not well accepted, it

is generally believed that the reduction of *E. coli* O157:H7 is because of alteration in the hindgut

ecosystem resulting in inhospitable environment for *E. coli* O157:H7. The effects of *E. coli*

O157:H7 vaccine and the DFM on fecal shedding of O26 or any other non-O157 STEC have not been investigated. Neither vaccine nor DFM had any effect on fecal O26 estimated by either mPCR assay or culture-based method. The lack of DFM effect on O26 was not unexpected because the product also had no effect on fecal shedding of *E. coli* O157:H7 (Cull et al., 2012). The lack of vaccine effect indicates that the surface proteins of O26 involved in iron uptake are antigenically different from those of *E. coli* O157:H7.

Cattle are considered to be a major reservoir of STEC (Karmali et al., 2010) and serogroup O26 was isolated from cattle feces almost 50 years ago (Bokhari and Orskov, 1952). However, not much is known about fecal prevalence and factors affecting fecal shedding of O26 in cattle, mainly because a selective medium that can phenotypically distinguish O26 from other serogroups has not been developed. In this study, two methods were used to obtain fecal prevalence estimates and weekly estimates of fecal prevalence of the serogroup O26, regardless of the treatment group, differed between the two methods of detection. The overall prevalence was higher by the culture-based detection method compared to the detection by mPCR assay (22.7 vs. 10.5%), in spite of plating the O26 specific-IMS beads on MacConkey agar, a relatively nondifferential medium, for detection and isolation. A similar difference in sensitivity of detection between culture- and PCR-based methods has been shown with O157 in fecal samples (Bai et al., 2012). Jenkins et al. (2003) have reported that IMS beads were 2.5 times more sensitive than the procedure that used PCR to detect Shiga toxins and then plating the positive samples on MacConkey to detect by colony hybridization with Shiga toxin probes. The difference in sensitivity may be because of the volume of sample used in the detection procedure. In the PCR assay, the volume of sample was 1 µl, which requires the sample to contain a minimum of 1,000 cells per g or ml to assure that the sample volume in the PCR reaction

contains DNA from at least one cell. However, in the same study (Bai et al., 2012) with a limited number of fecal samples (n=50), the mPCR identified more O26 (9 of 50) compared to IMS (4 of 50) method. The detection limits for enrichment, IMS, and plating for O157 or non-O157 serotypes have been reported to be 20-90 CFU/25 g of feces (Verstraete et al., 2010). Hall et al. (2006) have reported that the recovery of inoculated O26 from bovine fecal pats by IMS and plating on a chromogenic agar differed between different strains and bovine feces. The suggested reasons for the difference included variation in test sensitivity and interactions between inoculated strains and growth inhibitors in feces (Hall et al., 2006). In a survey conducted to determine the prevalence of O26 and other non-O157 STEC in feces of cattle in Scotland by culture-based method, O26 was detected in 4.6% of fecal pats (Pearce et al., 2006).

Although commercial IMS beads were coated with O26-specific antibodies, and therefore, intended to selectively retrieve O26 from cattle feces, the use of relatively non-differential MacConkey agar and mPCR assay allowed us to identify the other six serogroups contained in the pooled colonies. Of the six serogroups, O103 and O111 were identified more frequently (9.8% of O103 and 5.7% of O111) than the other four serogroups (0.4 to 0.8% of O45, O121, O145, and O157). The higher frequency of detection of O103 and O111 suggests some cross reactivity of the O26 antibodies with O-antigens of O103 and O111.

Relative to serogroup O157, only a few studies have been reported on the detection and or prevalence of O26 serogroup in cattle or sheep feces (Brandal et al., 2012; Cobbold et al., 2004; Renter et al., 2005, 2007; Barlow and Mellor, 2010; Hall et al., 2006; Pradel et al., 2000; Jenkins et al., 2003; Pearce et al., 2004; Sasaki et al., 2011; Wells et al., 1991). The majority of cattle or sheep shedding O26 are healthy, although the serogroup has been isolated from calves and lambs with diarrhea (Blanko et al., 1994; Caprioli et al., 1993, Cid et al., 2001; De et al., 2002).

Although O26-specific IMS beads are available, the limitation of culture-based methodology to detect or isolate O26 serogroup is lack of a selective medium, similar to sorbitol MacCOnkey agar for O157, for presumptive identification based on phenotypic characteristic (colony color),. Possé et al. (2008a) have described a selective (enteric Gram negative bacteria) and differential MacConkey agar base medium supplemented with a mixture of sugars (sucrose, sorbose, rhamnose) with inhibitory components (potassium tellurite and novobiocin) and a chromogenic compound to signal β-galactosidase activity that allowed color-based identification of O26 and other non-O157 *E. coli* (O103, O111, and O145). Based on sugar fermentation characteristics of non-O157 STEC described by Possé et al. (2008a), sorbose fermentation was used for phenotypic identification of O26 cultured on a lactose-free MacConkey agar. All strains of O26 serogroup of STEC in our culture collection (n=36) fermented sorbose, however it was not a unique characteristic of O26 because some of the strains of other STEC also fermented, although none of the O157 strains fermented sorbose. Inclusion of potassium tellurite or novobiocin in the sorbose MacConkey agar was inhibitory to certain strains of O26 and othernon-O157 STEC. The use of sorbose MacConkey agar allowed isolation of O26 in pure culture from 23.9% (260/1,089) samples of pooled colonies that were PCR positive for the serogroup O26. The poor recovery is reflective of lack of selectivity of the medium and the logistical limitation of finding a positive O26 from a mixture of 10 colonies that were pooled together. Fukushima and Seki, (2004) enriched and performed IMS on 605 fecal samples and streaked the resulting beads to Chromocult agar, a commercial chromogenic medium. A colony sweep from each plate was screened with a PCR to detect *stx* genes and subsequently recovered *stx*-positive pure cultures from 50% of the *stx* positive colony sweeps. While the most prevalent STEC was the O26 serogroup (20 out of 114 STEC isolates), 68 different STEC serogroups were isolated. Barlow

and Mellor, (2010) found that 78 of 300 enriched fecal samples were positive for *stx* and *eae,* and only three of the 78 were positive for the O26 serogroup. Attempt to isolate O26 from three enriched fecal samples that were positive for O26, *stx* and *eae* yielded only one O26 isolate.

   Escherichia coli O26 serogroup is broadly classified into Shiga toxigenic and enteropathogenic (EPEC) based on virulence factors, although both are capable of causing attaching and effacing lesions (Kaper et al., 2004). The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island that contains the genes (*eae*) encoding the intimin, a type III secretion system, a number of secreted (Esp) proteins and the translocated intimin receptor (Tir) (McDaniel et al., 1995). Intimin is the outer membrane protein responsible for the intimate attachment between *E. coli* and enterocytes and the 280-amino acid residues at the C-terminus has antigenic variation that allows classification of distinct subtypes among STEC and EPEC strains (Adu-Bobie et al., 1998). The Esp proteins (EspA, EspB, and EspD) are involved in the formation of a translocon to deliver effector proteins to the host cell to induce cytoskeleton rearrangement and subversion of the host cell function (Frankel et al., 1998). The translocated intimin receptor (Tir) is one of the proteins inserted in the host cell membrane to serve as a receptor for intimin (Kenney etal., 1997). Strains of EPEC produce a characteristic adherence, called local adherence, in which bacterial cells form microcolonies or clusters. This type of adherence is associated with the presence of a plasmid, called EAF (EPEC adherence factor) plasmid, which also has a cluster of genes that encode bundle-forming pili (BFP; Nataro and Kaper, 1998). Strains of EPEC carrying *bfpA* gene are called typical EPEC. In contrast to typical EPEC, certain strains that carry *eae* gene of the β subtype, but do not have the EAF plasmid encoding *bfpA,* are called atypical EPEC (aEPEC; Chen and Frankel, 2005). A total of 260 strains of O26 were obtained in the study and

seven were STEC, with 6 possessing *stx*1and one strain had *stx*2. Generally, STEC strains of

bovine origin, including O26, are more likely to carry *stx*1 than *stx*2 (Pearce et al., 2004, 2006;

Aktan et al., 2007). The majority of the O26 isolates (236/260; 90.1%) obtained in the study

contained *eae*β1, but none was positive for *bfpA,* suggesting they belonged to atypical EPEC.

Sekse et al., (2011) and Kobayashi et al., (2001) screened O26 isolated from ruminant feces (n =

142 and 9, respectively) and also reported that none of them carried the *bfpA*.

In conclusion, serogroup 026 was detected in in 10.5% (502/4,800) and 22.7 (1,089/4,800) of

fecal samples collected from feedlot cattle based on PCR- and culture-based methods,

respectively. The interventions (vaccine and or DFM) had no impact on fecal shedding of O26.

Only 260 of the 1,089 culture positive samples yielded pure cultures of O26. Only seven of the

260 strains of O26 were STEC and majority of the strains was aEPEC

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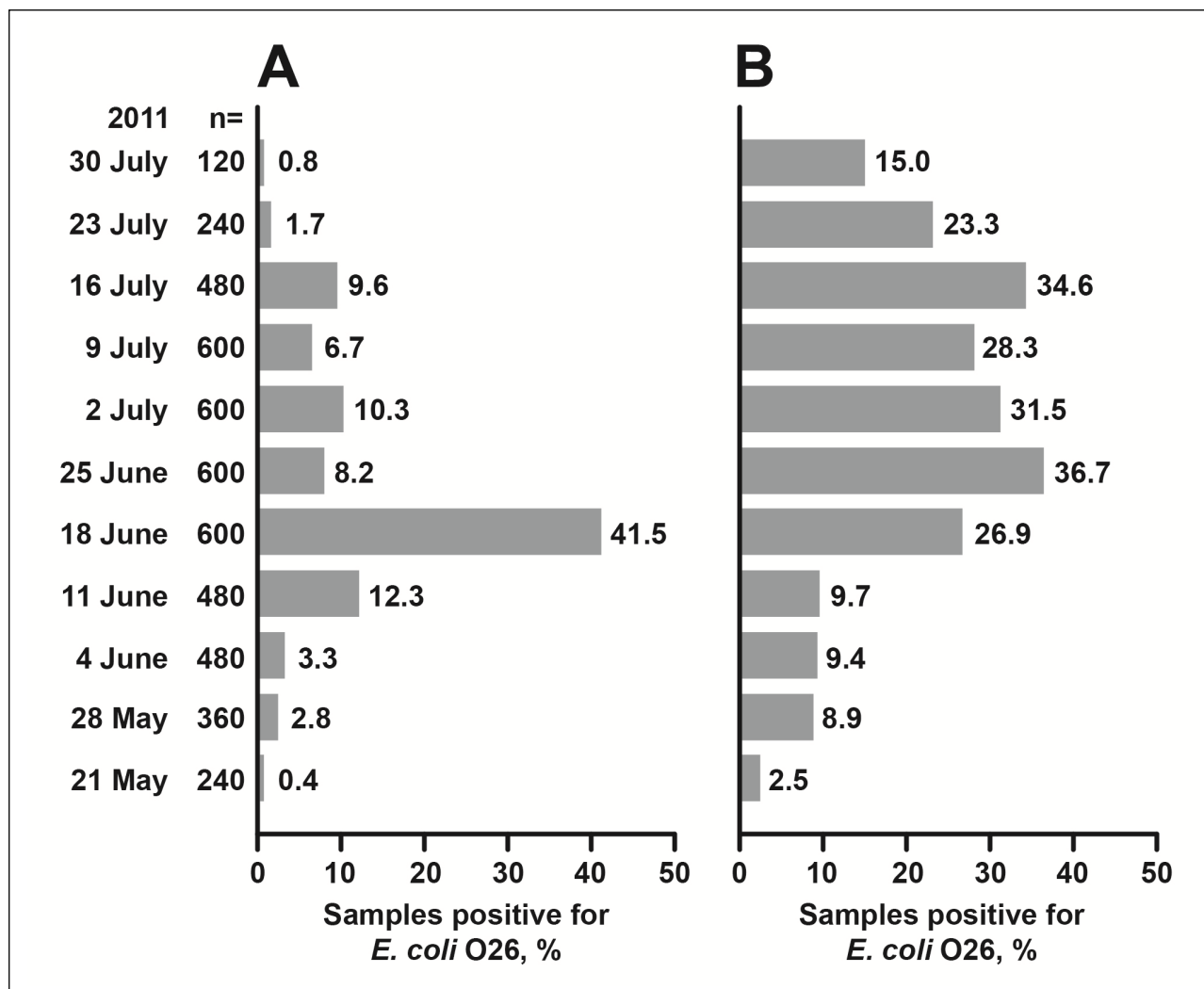
Figure 6.1. Prevalence of serogroup O26, based on multiplex PCR assay (A) and culture method (Immunomagnetic separation and plating on MacConkey agar; B), in feces of feedlot cattle (n=4,800).

Table 6.1. Prevalence of the O26 serogroup and virulence genes, based on multiplex PCR, in *Escherichia coli* broth-enriched fecal samples (n=4,800) of feedlot cattle that received no treatment (control), vaccine (siderophore receptor and proteins-based), direct-fed microbials (*Lactobacillus acidophilus* and *Propionibacterium freudenreichii*) or both.

Serogroup and virulence genes	Total	Number of samples (n=1,200) positive for O26 (%)			
		Control	Vaccine	Direct-fed microbials	Vaccine + Direct-fed microbials
O26	502 (10.5)	110 (2.3)	87 (1.8)	179 (3.7)	126 (2.6)
*stx*1	597 (12.4)	118 (2.5)	130 (2.7)	226 (4.7)	123 (2.6)
*stx*2	1,151 (24.0)	263 (5.5)	251 (5.2)	341 (7.1)	296 (6.2)
eae	2,179 (45.4)	537 (11.2)	546 (11.4)	629 (13.1)	467 (9.7)
*ehx*A	4,066 (84.7)	1,047 (21.8)	967 (20.2)	1,068 (22.3)	984 (20.5)

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Table 6.2. Prevalence of the seven serogroups of Shiga toxin-producing *Escherichia coli* and major virulence genes in pooled, randomly selected (n=10) colonies from MacConkey agar plated with fecal samples (n=4,800) that were enriched in *Escherichia coli* broth and subjected to O26 immunomagnetic beads separation.

Serogroup and virulence genes	No. of samples positive for serogroup and virulence genes (%)				
	Total (n=4,800)	Control (n=1,200)	Vaccine (n=1,200)	Direct-fed microbial (n=1,200)	Vaccine + Direct-fed microbial (n=1,200)
O26	1,089 (22.7)	263 (24.2)	275 (25.3)	270 (24.8)	281 (25.8)
O45	18 (0.4)	6 (33.3)	7 (38.9)	3 (16.7)	2 (11.1)
O103	470 (9.8)	140 (29.8)	99 (21.1)	95 (20.2)	136 (28.9)
O111	273 (5.7)	68 (24.9)	91 (33.3)	57 (20.9)	57 (20.9)
O121	21 (0.4)	10 (47.6)	5 (23.8)	4 (19.1)	2 (9.5)
O145	37 (0.8)	5 (13.5)	11 (29.7)	10 (27.0)	11 (29.7)
O157	28 (0.6)	6 (21.4)	8 (28.6)	5 (17.9)	9 (32.1)
*stx*1	117 (2.4)	37 (31.6)	25 (21.4)	30 (25.6)	25 (21.4)
*stx*2	519 (10.8)	137 (26.4)	125 (24.1)	102 (19.7)	155 (29.8)
eae	378 (7.9)	105 (27.8)	112 (29.6)	76 (20.1)	85 (22.5)
*ehx*A	776 (16.2)	186 (24.0)	184 (23.7)	197 (25.4)	209 (26.9)

[1] Ten colonies were randomly picked and pooled together for the DNA extraction and 11-plex PCR assay.
[2] Samples were collected from feedlot pen floor fecal pats weekly for four consecutive weeks before the end of the study

Table 6.3. Prevalence of the six serogroups and four major virulence genes in pooled colonies (n=10) from MacConkey agar plated with fecal samples (n=4,800) that were enriched in *Escherichia coli* broth and subjected to O26 immunomagnetic beads separation and were PCR-positive (n=1,089) for the serogroup O26

Serogroups	Number detected	None	stx1	stx2	eae	ehxA	stx1 + eae	stx2 + eae	stx1 or stx2 + eae
O26	1089	561 (51.5)	45 (8.5)	216 (40.9)	179 (33.9)	311 (58.9)	7 (1.3)	52 (9.9)	55 (10.4)
O45	4	2	1	2	0	1			
O103	168	75 (44.6)	12 (7.1)	44 (26.2)	29 (17.3)	54 (32.1)	1 (0.6)	8 (4.8)	11 (6.6)
O111	259	112 (43.2)	6 (2.32)	50 (19.3)	104 (40.2)	57 (22.7)	1 (0.4)	23 (8.9)	26 (10.0)
O121	13	4	0	4	6	3			
O145	12	2	1	2	6	7			
O157	10	10	2	5	7	7			

Table 6.4. Growth and fermentation of sorbose in lactose-free MacConkey agar with or without potassium tellurite and or novobiocin of pure cultures of the seven Shiga toxin-producing *Escherichia coli*

Culture medium	Number of strains fermenting sorbose/Number of strains that grew on the medium						
	O26 (n=36)	O45 (n=2)	O103 (n=40)	O111 (n=29)	O121 (n=9)	O145 (n=16)	O157 (n=20)
Lactose-free MacConkey agar base with:							
Sorbose (10 g/l)	36/36	1/2	14/40	2/29	3/9	4/16	0/20
Sorbose (10 g/l) and potassium tellurite (2.5 mg/l)	32/32	0/0	13/31	2/29	3/3	3/14	0/20
Sorbose (10 g/l) and novobiocin (8 mg/l)	36/36	0/0	14/40	2/28	3/9	3/16	0/20
Sorbose (10 g/l) + potassium tellurite (2.5 mg/l) + novobiocin (8 mg/l)	32/32	0/0	13/31	2/28	3/3	3/14	0/20

Table 6.5. Isolation of *Escherichia coli* O26 in pure culture from pooled colonies that were PCR-positive for serogroup O26.

Item[1]	Samples tested (n = 34)	PCR positive for O26	Virulence genes[5]			
			*stx*1	*stx*2	*eae*	*ehx*A
Pooled colonies (n=10)	34	34	3	3	3	4
Sorbose MacConkey	34	14	1	0	0	1
Sorbose MacConkey + Novobiocin (8 mg/l)	34	10	0	0	0	1
Sorbose MacConkey +Potassium tellurite (2.5 mg/l)	34	8	0	0	0	2
Sorbose MacConkey + Novobiocin (8 mg/l) and Potassium tellurite (2.5 mg/l)	34	14	2	0	0	2

Table 6.6. Isolation of *Escherichia coli* O26 from pooled colonies that were PCR-positive for serogroup O26 but did not yield O26 when plated on sorbose MacConkey agar (n = 47)

Item	PCR positive for O26	Virulence genes			
		*stx*1	*stx*2	*eae*	*ehx*A
Pooled colonies[1]	47	1	9	9	12
Isolates from each agar[2]:					
Sorbose MacConkey agar[3]	0	0	7	0	1
Sorbose MacConkey agar + Novobiocin (8 mg/l)	3	1	6	2	2
Rhamnose MacConkey agar[4] + Novobiocin (8 mg/l)	2	0	11	6	3
Non-O157 Shiga toxin-producing *E. coli* differential agar[5] + Novobiocin (8 mg/l)	1	0	1	3	1

[1]Pooled colonies isolated from MacConkey agar, previously indicated to be positive for *E. coli* O26 by mPCR, but did yield *E. coli* O26 when an initial attempt was made by streaking onto sorbose MacConkey agar.

[3]Sorbose agar was a lactose-free MacConkey agar base with sorbose added (10.0 g/l).

[4]Rhamnose agar is lactose-free MacConkey agar base with rhamnose (10.0 g/l).

[5]Composition according to Posse et al., 2008.

Table 6.7. Pathotypes and virulence gene profiles of isolated *Escherichia coli* O26 (n=260).

Pathogroup	No. of isolates	Virulence genes							
		*stx*1	*stx*2	*eae*β1	*ehx*A	*bfp*A	*tir*	*fli*C$_{H11}$	*esp*P
Shiga toxin-Producing *E. coli* O26	7	6	1	7	3	0	7	6	3
Profile 1	2	+	-	+	+	-	+	+	+
Profile 2	1	+	-	+	-	-	+	+	+
Profile 3	1	+	-	+	-	-	+	+	-
Profile 4	1	+	-	+	+	-	+	+	-
Profile 5	1	+	-	+	-	-	+	-	-
Profile 6	1	-	+	+	-	-	+	+	-
Atypical enteropatho-genic[1] *E. coli* O26	236	0	0	236	7	0	234	197	3
Profile 1	187	-	-	+	-	-	+	+	-
Profile 2	37	-	-	+	-	-	+	-	-
Profile 3	7	-	-	+	+	-	+	+	-
Profile 4	3	-	-	+	-	-	+	+	+
Profile 5	2	-	-	+	-	-	-	-	-
Avirulent[\] *E. coli* O26	17	0	0	0	0	0	0	1	0
Profile 1	16	-	-	-	-	-	-	-	-
Profile 2	1	-	-	-	-	-	-	+	-

[1]Atypical enteropathogenic classification is based on the presence of *eae* and absence of *bfp*A and *stx* genes.