Detection and quantification of the top-seven Shiga toxin-producing *Escherichia coli* serogroups in feces and on hides of feedlot cattle and whole genome sequence-based analysis of O103 serogroup

by

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B.S., Kansas State University, 2007
M.S., Kansas State University, 2015

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine & Pathobiology
College of Veterinary Medicine

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Abstract

Cattle are a reservoir for major Shiga toxin-producing *Escherichia coli* (STEC), which includes STEC O157 and the top six non-O157 serogroups (STEC-6; O26, O45, O103, O111, O121, O145). Collectively known as the STEC-7, these organisms are harbored in the hindgut and shed in the feces of cattle, which can contaminate hides. The de-hiding step during beef cattle processing can introduce fecal contaminants from the hide onto the carcass surface, creating the potential for contaminated beef products. The STEC-7 have been declared by the USDA-Food Safety and Inspection Service as adulterants in ground beef and non-intact beef products, and are monitored during beef cattle processing. However, many of the culture- and PCR-based tests for detection and/or quantification of the STEC, particularly of the STEC-6, are not established or require improvement and also virulence characteristics of STEC strains from cattle have not been fully analyzed. Therefore, the following studies were conducted: 1. Immunomagnetic separation (IMS)-based culture-method for detection of STEC-6 in cattle feces was developed and compared to a PCR-based method; 2. Detection sensitivity of pooled vs. individual IMS beads for isolation STEC-6 from cattle feces was evaluated; 3. Real-time PCR assay, based on the clustered regularly interspaced short palindromic repeat sequence polymorphisms (CRISPR), was developed and validated for serotype-specific detection and quantification of STEC O157:H7 in cattle feces; 4. Virulence gene profiles of bovine enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype *E. coli* O103 strains were examined with whole genome sequence (WGS)-based comparative analysis; 5. Prevalence and concentration of STEC-7 of fed-beef, cull beef and cull dairy cattle were determined. The culture and PCR methods detected all six serogroups in samples negative by the other method. Based on noninferiority tests, detection with pooled IMS beads was not

inferior to detection with individual beads. Detection limits of the CRISPR-based qPCR assay for cattle feces spiked with pure cultures were 2.1×10^3 and 2.3×10^0 colony-forming units/g before and after enrichment, respectively. WGS-based analysis of *E. coli* O103 strains revealed key differences in the virulomes and mobilomes of EHEC, EPEC, and putative non-pathotype strains. The prevalence study revealed that a significantly higher ($P < 0.01$) proportion of hide samples from fed beef cattle (4.8%) were positive for STEC O157:H7, compared to samples from cull beef (1.6%) or cull dairy (0.2%); the majority of quantifiable STEC O157:H7 from each cattle type was at concentrations between 3 to 4 log CFU/100 cm^2. These data contribute to a knowledge gap on prevalence and concentration of STEC-7 and surrogate bacteria on cattle hides and carcasses, respectively. Furthermore, the development and refinement of culture- and PCR-based screening assays may lead to increased surveillance of major STEC serogroups, especially if the potential of WGS-based comparative genomics in identifying novel gene targets can be harnessed.

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inferior to detection with individual beads. Detection limits of the CRISPR-based qPCR assay for cattle feces spiked with pure cultures were 2.1×10^3 and 2.3×10^0 colony-forming units/g before and after enrichment, respectively. WGS-based analysis of *E. coli* O103 strains revealed key differences in the virulomes and mobilomes of EHEC, EPEC, and putative non-pathotype strains. The prevalence study revealed that a significantly higher ($P < 0.01$) proportion of hide samples from fed beef cattle (4.8%) were positive for STEC O157:H7, compared to samples from cull beef (1.6%) or cull dairy (0.2%); the majority of quantifiable STEC O157:H7 from each cattle type was at concentrations between 3 to 4 log CFU/100 cm^2. These data contribute to a knowledge gap on prevalence and concentration of STEC-7 and surrogate bacteria on cattle hides and carcasses, respectively. Furthermore, the development and refinement of culture- and PCR-based screening assays may lead to increased surveillance of major STEC serogroups, especially if the potential of WGS-based comparative genomics in identifying novel gene targets can be harnessed.

Table of Contents

List of Figures

List of Tables

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Dedication

This work is dedicated to my wife, Ashley Renee Noll, for her unwavering support and endless encouragement. Every day, you inspire me to be a better man, husband and father.

Chapter 1 - A Comparison of Culture- and PCR-Based Methods to Detect Six Major Non-O157 Serogroups of Shiga Toxin-Producing *Escherichia coli* in Cattle Feces

Introduction

Cattle are asymptomatic reservoirs for Shiga toxin-producing *Escherichia coli* (STEC), which are major foodborne pathogens (Karmali *et al*., 2010). The organisms reside in the hindgut of cattle and are shed in feces, which can serve as a source of contamination of beef, produce, and water for infections in humans (Mathusa *et al*., 2010; Ferens *et al*., 2011). Human illness from a STEC infection can result in clinical manifestations ranging from mild to bloody diarrhea to potentially life-threatening complications, such as hemolytic uremic syndrome primarily in children, but also in other individuals. *Escherichia coli* O157:H7, which was declared an adulterant in 1994 by the USDA Food Safety and Inspection Service (FSIS), has been the focus of many studies, because of the serotype's predominance in human STEC infections (Rangel *et al*., 2005; Scallan *et al*., 2011). As a result, detection methods for STEC O157 in cattle feces are well established (Omisakin *et al*., 2003; LeJeune *et al*., 2006; Fox *et al*., 2008; Jacob *et al*., 2010). In recent years, STEC belonging to six O groups, O26, O45, O103, O111, O121, and O145, often referred to as 'non-O157 STEC' have been recognized as a growing public health concern (Scallan *et al*., 2011; Brooks *et al*, 2005). According to the CDC, the six serogroups account for a majority of non-O157 STEC infections in the U.S. (Scallan *et al*., 2011; CDC 2011). In 2011, the FSIS declared the six serogroups carrying *stx*1 and/or *stx*2

with *eae* genes as adulterants in ground beef and non-intact beef products (Pihkala *et al*., 2012; USDA, 2013).

Due to the relatively recent recognition of the six non-O157 serogroups, studies on the methodology and standardization of the procedure for detection and isolation, particularly in fecal samples of cattle are limited. PCR-based methods, including commercially available automated methods such as BAX System (DuPont, Wilmington, DE) have been developed for detection of the six non-O157 serogroups and/or virulence genes from cattle feces (Burns *et al*., 2011; DebRoy *et al*., 2011; Bai *et al*., 2012; Paddock *et al*., 2012; Baltasar *et al*., 2014). Although PCR methods are sensitive and are of high-throughput, the inherent limitation is that the presence of virulence genes cannot be associated with any specific serogroups. Also, unlike the culture method, there is no isolate obtained for follow-up analysis and PCR does not discriminate based on viability of cells, which can result in amplification of DNA from dead cells. Despite these advantages over PCR method, development of culture methods for detection of non-O157 STEC has lagged. Identification of O157 STEC has relied on the organism's inability to ferment sorbitol (March and Ratnam, 1986); however, no such unique phenotypic characteristic is associated with all six non-O157 serogroups, making culture-based identification problematic. A few differential media based on a chromogenic compound to detect β-galactosidase activity and one or more fermentative sugars combined with or without inhibitory compounds (novobiocin, tellurite, etc.) have been developed to detect non-O157 STEC in cattle feces (Possé *et al*., 2008; Kalchayanand *et al*., 2013; Ekiri *et al*., 2014). However, differentiation of the six non-O157 serogroups based on colony phenotype is not reliable.

We have utilized an 11-plex PCR (Bai *et al*., 2012) assay that targeted the seven serogroups and four major virulence genes (*stx*1, *stx*2, *eae*, and *ehxA*) to determine prevalence of the O157

and six non-O157 serogroups in cattle feces collected from a commercial feedlot (Cernicchiaro *et al*., 2013). In that study, a culture method that included an immunomagnetic separation (IMS) procedure and plating on a relatively non-selective medium (MacConkey agar) was utilized to determine fecal prevalence of only three serogroups, O26, O103 and O111, because IMS beads for the other three serogroups, O45, O121, and O145, were not available at that time. Some studies have determined the prevalence of non-O157 STEC on beef carcasses (Arthur *et al*., 2002) and in commercial ground beef (Bosilevac *et al*., 2011; Ju *et al*., 2012). Only a few have investigated fecal shedding of one or more of the non-O157 serogroups in cattle by culture method (Baltasar *et al*., 2014; Kalchayanand *et al*., 2013; Ekiri *et al*., 2014; Monaghan *et al*., 2011; Ennis *et al*., 2012; Paddock *et al*., 2014). Our objectives of this study were to develop a culture-based method to detect the six non-O157 (O26, O45, O103, O111, O121, and O145) STEC serogroups in cattle feces and compare the detection of the six serogroups with a PCR method. The culture method described here involved an enrichment step, followed by IMS with serogroup-specific beads for each target serogroup, plating on a chromogenic selective medium and confirming the serogroup and major virulence genes by a multiplex PCR. Although PCR detected significantly higher proportions of samples positive for O26 and O121 serogroups compared to the culture method, each method detected all six serogroups in some samples that were negative by the other method, indicating that both methods are required to provide an accurate detection of the presence of non-O157 STEC in cattle feces.

Materials and Methods

Animals and fecal sample collection and enrichment

Fecal samples of crossbred finishing cattle were collected from pens in a commercial feedlot in the central US. The permission to collect pen-floor samples was granted by the feedlot Manager and the Nutritionist responsible for feeding and management of the feedyard. The feedlot's standard operating procedures were followed for care and management of cattle throughout the study period. Kansas State University Institutional Animal Care and Use Committee approved the study (IACUC # 3172). Fecal samples were collected during a 12-week period from June through August 2013. Each week, 24 pen-floor fecal samples were collected from each of two pens that housed an average of 270 cattle per pen. Samples (approximately 100 g) were collected from freshly defecated fecal pats using a plastic spoon, and care was taken to avoid ground contamination. The spoon with feces was placed into a Whirl-pak bag (Nasco, Ft. Atkinson, WI), and once samples were collected, they were placed in a cooler with ice packs and transported to the laboratory in cold storage for processing within 36 h. The sample collection was completed in the late afternoon 12 h prior to transportation of cattle for slaughter. In the laboratory, the sample was mixed by kneading the Whirl-pak bag and approximately 2 g of feces were added to 18 ml of *Escherichia coli* broth (EC medium; Difco™, Becton, Dickinson Co., Sparks, MD), vortexed for 30 s and incubated at 40° C for 6 h (Paddock *et al*., 2012).

Culture-based detection and isolation

Enriched fecal suspensions were subjected to IMS procedure and plated onto a selective medium to detect and isolate O26, O45, O103, O111, O121, and O145 serogroups of *E. coli*.

Each fecal sample was individually subjected to six serogroup-specific IMS beads. Nine-hundred and eighty microliters of enriched sample were mixed with 20 µl of serogroup-specific IMS beads (Abraxis, Warminster, PA) in 96-well microtiter plates. The IMS procedure was carried out in a Kingfisher™ Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA) according to the protocol provided by the manufacturer. Then, 50 µl of the sample bead suspensions of the non-O157 serogroups were spread-plated onto Possé agar medium (Possé *et al*., 2008) modified to include novobiocin at 5 mg/l and potassium tellurite at 0.5 mg/l. Plates were incubated for 20 to 24 h at 37° C. Six chromogenic colonies (mauve, green, blue or purple) from the modified Possé medium were picked, inoculated onto blood agar (Remel, Lenexa, KS) plates, and incubated at 37° C for 24 h. Six colonies from each sample were pooled in distilled water, boiled for 10 min, centrifuged at 9,300 x g for 5 min, and the lysate containing the DNA was tested by a multiplex PCR assay that targeted genes specific to the six serogroups (*wzx* gene for O26, O45, O103, O111, and O145 and *wbqE* and *wbq* for O121). Primers for the O26, O103, O121, and O145, serogroups were according to Bai *et al*. (2012), while the O45 serogroup primers were according to Paddock *et al*. (2012). The primers for the *wzx* gene of O111 were modified from Bai *et al*. (2012) and sequences were as follows: O111-F3, ACA AGA GTG CTC TGG GCT TC and O111-R3, AAA CTA AGT GAG ACG CCA CCA. If the pooled colonies were positive for any of the six non-O157 serogroups, then each of the six isolates were tested by a ten-plex PCR targeting six serogroups and four virulence genes (*stx*1, *stx*2, *eae*, and *ehxA*) (Bai *et al*., 2012). The primers for the four virulence genes were according Bai *et al*. (2012), except for *eae*, which were changed to: *eae*-F2, TAC GCG AAA GAT ACC GCT CT and *eae*-R2, CAT GCG GAA ATA GCC GTT A. Colonies isolated from any serogroup-specific IMS beads that tested positive for a different serogroup were considered positive by non-serogroup specific IMS

beads. If pooled colonies were PCR negative for any of the six serogroups, the sample was considered negative. Isolates confirmed to be positive for one of the six serogroups were stored in cryogenic beads (CryoCare™, Key Scientific Products, Round Rock, TX).

PCR-based detection

One milliliter aliquots of the fecal suspension in EC broth before (pre-enrichment) and after incubation (post-enrichment) were boiled for 10 min, then centrifuged at 9,300 x g for 5 min. One hundred µl of crude DNA, from pre- and post-enriched samples, were then purified with GeneClean® Turbo Kits (MP Biomedicals, Solon, OH) and subjected to a multiplex PCR assay (Bai *et al*., 2012) to detect six O serogroups (O26, O45, O103, O111, O121, and O145) and four virulence genes (*stx*1, *stx*2, *eae* and *ehxA*).

Data analysis

Fecal samples were considered positive for one or more of the six non-O157 serogroups if the enriched sample, subjected to IMS and plated on modified Possé medium, tested positive by the multiplex PCR of the pooled colonies (culture method) or by the direct multiplex PCR (PCR method) of the enriched fecal sample. Isolates of serogroups from the culture method testing positive for Shiga toxin genes (*stx*1 and/or *stx*2) were considered STEC. The proportion of fecal samples tested positive by culture- or PCR-based method was computed by dividing the number of samples testing positive for each O serogroup by the total number of samples tested (n=576). A two-sample test of proportions was performed to determine whether the proportions of positive samples differed significantly between the two detection methods. In addition, the proportion of samples that tested positive for the three predominant serogroups, O26, O45, and

O103, detected by culture only, PCR only, culture or PCR, and culture and PCR methods were compared. Furthermore, to assess overall agreement between the two methods for detection of O26, O45, and O103 serogroups, the Cohen's Kappa statistic (and 95% confidence intervals) and McNemar's chi-square test were calculated using STATA MP 11.0 (kap and mcc commands, StataCorp, College Station, TX). Interpretations of the kappa statistic were based on the scale proposed by Landis and Koch (1977).

Results

Culture method of detection of six non-O157 serogroups

A total of 428 samples (74.3%) tested positive for one or more of the six serogroups of *E. coli*. Fecal samples were identified as positive for non-O157 serogroups, if pooled colonies (up to six per sample) from either serogroup-specific or non-serogroup specific IMS beads were positive by the multiplex PCR (Table 1.1). Based on serogroup-specific IMS beads, O103 was detected in 37.5% of the samples (216/576), followed by O26 (17.0%), O45 (15.6%), O145 (2.1%), O121 (1.7%), and O111 (0.2%). The proportion positive for each serogroup, except O111, which was present in only one fecal sample, increased when samples that tested positive for those serogroups from non-serogroup specific IMS beads were included (Table 1.1). The O103 and O26 beads were less specific as they identified only 62.2% (216/347) and 74.8% (98/131) of the total samples positive for their target O serogroups, respectively. The most common serogroup identified using non-serogroup specific IMS beads was O103. Nearly 38% (131/347) of all O103 positive samples were detected by non-O103 IMS beads, but were tested as negative with the O103 bead. The O26 serogroup was identified by non-O26 beads in 25.2% (33/131) of all O26 positive samples. Only 6.3% (6/96) of all O45-positive samples were

identified by non-O45 IMS beads. The O145 and O121 serogroups were identified by non-target beads in 29.4% (5/17) and 23.1% (3/13) of positive samples, respectively.

After accounting for redundancy in samples that were identified as positive for a serogroup by multiple IMS beads, the O103 was the most predominant serogroup with 60.2% (347/576) of samples testing positive. The O26 and O45 serogroups were detected in 22.7% (131/576) and 16.7% (96/576) of samples, respectively. The other three serogroups, O145 (3.0%), O121 (2.3%) and O111 (0.2%), were detected in lower proportions. The proportion of multiple serogroups detected within a fecal sample by the culture-based method is shown in Table 1.2. None of the fecal samples tested positive for five or six serogroups and the majority (50.7%) tested positive for a single serogroup.

Major virulence genes in isolates of six non-O157 serogroups

A total of 640 non-O157 isolates were obtained from 576 fecal samples in the study (Table 1.3). Isolates with identical virulence gene profiles, recovered from the same fecal sample from one or more IMS beads, were recorded once. Isolates with differing virulence gene profiles from the same fecal sample, were recorded separately, regardless from which IMS bead they were recovered. All O121 (n = 12) and O111 (n = 1) isolates tested negative for *stx*1, *stx*2, *eae* and *ehxA* genes. Only small proportions of O26 (7.6%; 10/132) and O145 (5.3%; 1/19) isolates were negative for all four virulence genes (Table 1.3). Conversely, high proportions of O103 and O45 were negative for all four virulence genes (79.8% [304/381] and 58.9% [56/95] of O103 and O45, respectively). A total of 23 *stx*-positive isolates were obtained, which included serogroups O103 (10), O26 (7), and O145 (6) (Table 1.3). The *stx*1 gene was detected in 20 of the 23 (87.0%) isolates, and *stx*2 was identified only in isolates of serogroups O103 (1) and O145 (2).

None of the non-O157 STEC isolates contained both *stx* genes and all were positive for *eae*. High proportions of O26 (122/132; 92.4%) and O145 (17/19; 89.5%) isolates tested positive for *eae* compared with 19.7% (75/381) for O103 isolates. None of the O45 isolates (n=95) tested positive for *eae.* A high proportion of O145 isolates (89.5%) tested positive for the *ehxA* gene compared with O26, (9.8%), O45 (41.1%) and O103 (19.9%) isolates (Table 1.3). A total of 99 *stx*-positive isolates were recovered that were not associated with any of the six non-O157 or O157 serogroups, and only a small proportion of those (22.2%) tested positive for *eae* (data not shown).

PCR detection of six non-O157 serogroups and four virulence genes

In pre-enriched samples (n=576), a total of 90 samples (15.6%) tested positive for one or more of the six serogroups, with O103 and O45 the most commonly detected serogroups (5.9 and 5.2%, respectively; Table 1.4) by the direct PCR method. None of the pre-enriched samples were positive for the O145 serogroup. In post-enriched samples, a total of 446 samples (77.4%) tested positive for one or more of the six serogroups. The O103 serogroup was the most frequently identified serogroup, with 56.6% of the samples (326/576) testing positive. The O26 serogroup (44.4%) was the second most predominant, followed by O121 (22.9%), and O45 (17.9%). Serogroups O111 and O145 were detected in only 0.7% and 1.9% of samples, respectively. Among the four virulence genes, *stx*2, *eae***,** and *ehxA* were detected in a high proportion (94.1 to 99.5%) of enriched samples (Table 1.4). A significantly greater ($P < 0.05$) proportion of fecal samples were positive for *stx*2 (94.1%) than *stx*1 (64.4%). Interestingly, before enrichment, a significantly greater ($P < 0.05$) proportion of samples tested positive for *stx*1 (35.9%) than for *stx*2 (23.1%). Before enrichment, a total of 270 (46.9%) samples tested

positive for at least one *stx* gene and 83 (30.7%) of those samples tested negative for any of the six non-O157 serogroups. After enrichment, a total of 552 (95.8%) samples tested positive for at least one *stx* gene, and 193 (35.0%) of those samples tested negative for any of the six non-O157 serogroups. The proportion of fecal samples testing positive for multiple serogroups by PCR is shown in Table 1.2. A high proportion of samples (73.2%) tested positive for up to three serogroups. None of the fecal samples tested positive for five or six serogroups.

Culture vs. PCR methods for the detection of six non-O157 serogroups

The culture and PCR methods did not differ in the proportions of fecal samples that tested positive (74.3 vs. 77.4%) or negative (25.7 and 22.6%) for one or more of the six non-O157 serogroups (Table 1.2). A higher proportion of samples were positive for O26, O45, O111, and O121 serogroups by PCR than by the culture method (Fig. 1.1); however, only differences in the proportions of O26 and O121 serogroups were statistically significant ($P < 0.001$) between the two methods. The O103 and O145 serogroups were detected slightly more often with the culture method than the PCR method, but these proportions were not significantly different. A total of 266 (46.2%) samples tested positive after enrichment for more than one serogroup (2 to 4 serogroups) by PCR compared with only 136 (23.6%) of samples by the culture method (Table 1.2). The PCR assay detected a higher proportion of fecal samples as positive for three or four serogroups than the culture method (16.7 and 6.3%, respectively; $P < 0.05$) and the culture method detected a higher percentage ($P < 0.05$) of samples positive for 1 or 2 serogroups (68.1%) compared with the PCR based method (60.8%; Table 1.2). Samples testing positive for a single serogroup by both PCR and culture-based method were further analyzed for predominance of a particular serogroup. For the PCR-based method, 35.5 % (50/141) of samples

that tested positive for a single serogroup were positive for the O103 serogroup. The culture-based method detected the O103 serogroup in 54.6% (119/218) of samples that were positive for a single serogroup. Samples positive for a single serogroup other than O103 were detected at similar levels with both detection methods.

Because high proportions of fecal samples were positive for O26, O45, and O103 serogroups, detection capabilities by either or both methods were compared (Table 1.5). A significantly ($P < 0.05$) higher proportion of samples tested positive for O26 and O121 serogroups by PCR only than by the culture-based detection only. Samples positive for the three serogroups were detected by both methods in only 28.6% (86/301), 30.1% (46/153), and 56.5% (243/430) for O26, O45, and O103 serogroups, respectively (Table 1.5). The PCR method detected O26 (56.5%), O45 (37.3%), and O103 (19.3%) in samples that were negative by the culture method. Conversely, the culture method detected O26 (15.0%), O45 (32.7%), and O103 (24.2%) in samples that were negative by the PCR method. There was a slight to fair agreement (Kappa range: 0.01–0.4) between the PCR only and culture-based only methods for detection of O26, O45, and O103 serogroups in fecal samples (Table 1.6); however, the McNemar's Chi-square tests for the comparison between tests for detection of the O26 serogroup was statistically significant ($P < 0.001$), indicating a disagreement between the proportions of positive samples detected by the two methods.

Discussion

Although the fecal shedding of the O157 serogroup has been studied extensively, only a few studies have examined fecal shedding of non-O157 STEC in cattle, particularly in the United States (Baltasar *et al*., 2014; Ekiri *et al*., 2014; Cernicchiaro *et al*., 2013; Paddock *et al*., 2014; Dargatz *et al*., 2013). This may be due in part to the lack of a validated culture method to detect

and isolate non-O157 STEC from cattle feces. The six non-O157 serogroups do not possess unique phenotypic markers (similar to non-sorbitol fermentation by O157) that allow differentiation from other *E. coli*, making detection of non-O157 STEC problematic. Some studies have relied on PCR to determine the prevalence of the six serogroups and virulence genes (Baltasar *et al*., 2014; Cernicchiaro *et al*., 2013; Dargatz *et al*., 2013). The inherent limitation of a PCR method is the inability to link virulence genes to target serogroups present in samples. Therefore, we utilized both PCR and culture methods to detect the six serogroups of non-O157 STEC in fecal samples. The culture method included IMS with serogroup-specific beads and plating the bead suspension onto a chromogenic Possé medium (Possé). The Possé medium is based on lactose-free MacConkey medium with a mixture of sugars (sucrose and sorbose), β-glucosidase activity and selective components (novobiocin and potassium tellurite), which allows color-based identification (blue to purple to mauve to green) of serogroups. The modification of the medium included lowering the concentration of novobiocin (from 8 to 5 mg/L) and potassium tellurite (from 2.5 to 0.5 mg/L) because certain strains of non-O157 serogroups in our culture collection did not grow on the medium with the original concentrations of these inhibitory compounds (Paddock *et al*., 2014). Although the differentiation of serogroups is supposedly based on colony color, distinguishing the phenotypes was difficult because of the shades of colors observed, particularly when the plate was crowded with colonies. Little association between the colors of the colonies and the target non-O157 serogroup was evident. Therefore, we chose to pick multiple "*E. coli*-like" colonies (six colonies per plate) with any shades of blue/purple/mauve/green colors. The pooling of colonies and testing by a multiplex PCR allowed us to identify samples that were positive for any of the six serogroups. Subsequent

testing of individual colonies permitted us to confirm the samples positive for any of the six serogroups and the virulence genes and to obtain pure cultures of the non-O157 serogroups.

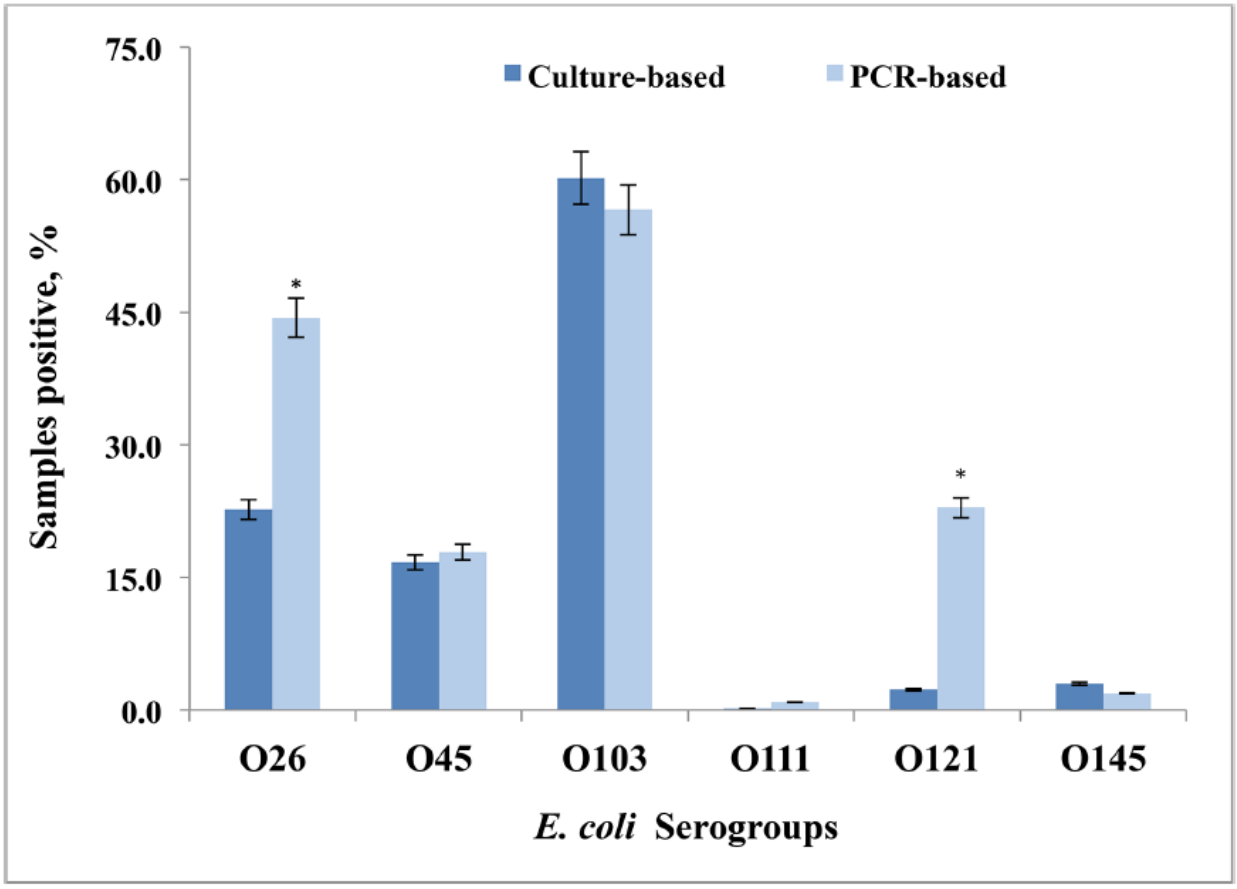
Based on the culture method, O103 was the most commonly detected serogroup; however, the O103 O group was the most common non-specific target of the IMS beads with nearly 38% of O103-positive samples identified using non-O103 IMS beads. The high affinity of non-O103 IMS beads toward the O103 O group could have overestimated the relative proportion of samples that were positive for the O103 serogroup, but PCR-based detection also identified O103 as the predominant serogroup. Both PCR and culture-based detection methods identified the O26 serogroup as the second most common serogroup. However, PCR identified the O26 and O121 serogroups in more samples than the culture-method, likely because PCR detects nonviable cells. Interestingly, Paddock *et al*. (2014) reported detection of the O26 serogroup in cattle feces more often by the culture-based method (22.7%) compared with PCR (10.5%), highlighting the overall lack of agreement between the two detection methods. Both detection methods agreed in identifying the O45 serogroup. In addition, the O45 IMS beads exhibited the most specificity (93.8% of the samples positive for O45) towards the target serogroup, not considering the single O111 serogroup detected by the O111 IMS beads. Other studies have reported difficulty in isolating the O111 serogroup after IMS, possibly owing to a low affinity between the O111 IMS bead antibody and the O111 antigen (Verstraete *et al*., 2010; Fratamico *et al*., 2011), but in our study, the O111 serogroup-specific gene also was detected in a low proportion of samples by the PCR assay. Other studies have had difficulty recovering certain serogroups from feces (Verstraete *et al*., 2010) and ground beef (Fratamico *et al*., 2011) after IMS. The degree of non-specificity may depend on the source of IMS beads. Cernicchiaro *et al*. (2013) reported a degree of cross-reactivity with Dynabeads (Invitrogen, Carlsbad, CA),

suggesting non-specific binding to the IMS beads. Our results confirm the non-specificity of certain IMS beads, possibly contributing to differences in identification of certain serogroups observed between detection methods.

Based on the culture method, only 4.0% (23/576) of fecal samples tested were positive for at least one of the six non-O157 serogroups carrying the *stx* gene, with a higher proportion harboring the *stx*1 gene. Interestingly, Paddock *et al*. (2014) reported a higher proportion of pooled colonies that tested positive for the six non-O157 isolates recovered from cattle feces testing positive for *stx*2 rather than *stx*1. Cernicchiaro *et al*. (2013) reported similar findings, although a higher proportion of O26 isolates were positive for the *stx*1 gene. Interestingly, 31.6% (6/19) of the total O145 isolates identified in the study were STEC. Conversely, the O103 and O26 STEC isolates contributed to only 2.6% (10/381) and 5.3% (7/132) of the total isolates identified for these serogroups, respectively. Numerous non-O157 isolates that tested negative for Shiga toxin genes were isolated in the current study, and the majority (60%) of these isolates were also negative for the *eae* and *ehxA* genes. Nearly 80% (304/381) of all O103 isolates identified were negative for the four virulence genes. Hence, although the O103 serogroup is present in cattle feces at a relatively high proportion, only a small percentage of isolates contained any of the four virulence genes. Conversely, 92.4% (122/134) of O26 isolates and 89.5% (17/19) of O145 isolates were positive for the *eae* gene, suggesting that a majority of these isolates in the cattle feces are capable of intimin-based attachment. Unlike the non-O157 STEC that tested mostly positive for *stx*1, 80.8% (80/99) of the isolates that were not one of the six non-O157 (or O157) serogroups were positive for *stx*2. Considering the low prevalence of non-O157 STEC isolates belonging to the six serogroups, it is possible that serogroups from other non-O157 STEC are more predominant in the cattle feces of this study population.

Although, numerous isolates belonged to the O103, O26 and O45 serogroups, the majority did not carry Shiga toxin genes. However, if STEC from the six serogroups as well as from undetermined serogroups (not including O157 serogroup) were included, 18.8% (108/576) of samples tested in the study were positive for STEC. In conclusion, serogroups O103 and O26 were the predominant serogroups in feces of cattle sampled in this study, and PCR detected higher proportions of fecal samples as positive for O26 but not O103 than the culture method. Only a small proportion of the non-O157 serogroup isolates carried the Shiga toxin gene. More importantly, each method (culture and PCR) detected the six non-O157 serogroups in fecal samples that were negative by the other method. This is an important observation because the FSIS method (USDA, 2013) for detecting non-O157 STEC in beef samples is based on PCR detection of *stx* and *eae* genes first, followed by detection of serogroups, and a sample positive for both is then subjected to culture method. Therefore, it will be of interest to compare the culture method and PCR method to detect the six non-O157 serogroups in beef samples. Our data on fecal sample analysis suggest that a sample should be subjected to both culture and PCR method to get an accurate estimate of the presence of the six non-O157 STEC.

Figure 1.1 Detection of the six major non-O157 serogroups of *Escherichia coli*, based on a culture- or multiplex PCR-based method, in fecal samples of feedlot cattle (n=576)



*Denotes significant difference in proportions within serogroups ($P < 0.05$).

Table 1.1 Detection of six serogroups of non-O157 *Escherichia coli* in feces (576) of feedlot cattle by culture-based method involving enrichment, immunomagnetic bead separation (IMS) and plating on a selective medium

	Number of samples positive (%)[†, ‡]					
IMS beads	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
O26	**98 (17.0)**	5	128	0	0	3
O45	18	**90 (15.6)**	118	0	0	0
O103	20	4	**216 (37.5)**	0	1	2
O111	22	9	99	**1 (0.2)**	1	1
O121	22	4	117	0	**10 (1.7)**	3
O145	12	7	110	0	2	**12 (2.1)**
	***Total non-redundant positives**					
	131 (22.7)	**96 (16.7)**	**347 (60.2)**	**1 (0.2)**	**13 (2.3)**	**17 (3.0)**

[†]Feces were enriched in *Escherichia coli* broth for 6 h at 40ºC.
[‡]Enriched samples, subjected to serogroup-specific IMS beads, were plated onto modified Possé medium (Possé *et al*., 2008) modified to include novobiocin at 5 mg/l and potassium tellurite at 0.5 mg/l and then up to six chromogenic colonies were pooled and tested by a multiplex PCR assay (Paddock *et al*., 2012) targeting serogroup-specific genes.
*Non-redundant positive data include samples that were positive for a serogroup by serogroup-specific and non-specific IMS beads.

Table 1.2 Number and percentage of samples testing positive to multiple non-O157 *Escherichia coli* serogroups in fecal samples (n=576) of feedlot cattle based on culture method and multiplex PCR method of detection

Detection method	Total positive	Number of serogroups (%)						
		0	1	2	3	4	5	6
Culture-based	428 (74.3)	148 (25.7)	292 (50.7)	100 (17.4)	31 (5.4)	5 (0.9)	0	0
PCR-based	446 (77.4)	130 (22.6)	180 (31.3)	170 (29.5)	72 (12.5)	24 (4.2)	0	0

Table 1.3 Distribution of major virulence genes in six non-O157 serogroups of *Escherichia coli* isolated from fecal samples (n=576) of feedlot cattle

Virulence genes (*stx*1, *stx*2, *eae*, *ehxA*)	Number of six serogroups of non–O157 isolates (%)						
	O26 (n=132)	**O45 (n=95)**	**O103 (n=381)**	**O111 (n=1)**	**O121 (n=12)**	**O145 (n=19)**	**Total (n=640)**
None	10 (7.6)	56 (58.9)	304 (79.8)	1 (100)	12 (100)	1 (5.3)	384 (60.0)
***stx*1**	7 (5.3)	0	9 (2.4)	0	0	4 (21.1)	20 (3.1)
***stx*2**	0	0	1 (0.3)	0	0	2 (10.5)	3 (0.5)
eae	122 (92.4)	0	75 (19.7)	0	0	17 (89.5)	214 (33.4)
ehxA	13 (9.8)	39 (41.1)	76 (19.9)	0	0	17 (89.5)	145 (22.7)
***stx*1 + *stx*2**	0	0	0	0	0	0	0
stx*1 + *eae	7 (5.3)	0	9 (2.4)	0	0	4 (21.1)	20 (3.1)
stx*2 + *eae	0	0	1 (0.3)	0	0	2 (10.5)	3 (0.5)
stx*1 or *stx*2 + *eae	7 (5.3)	0	10 (2.6)	0	0	6 (31.6)	23 (3.6)

Table 1.4 Detection of six major serogroups and virulence genes of non-O157 Shiga toxin-producing *Escherichia coli*, based on multiplex PCR assay, in fecal samples (n=576) of feedlot cattle

Enrichment[†]	Total positives[‡]	Serogroup-specific genes, no. positive (%)						Virulence genes, no. positive (%)			
		O26	O45	O103	O111	O121	O145	*stx*1	*stx*2	*eae*	*ehxA*
Before	90 (15.6)	19 (3.3)	30 (5.2)	34 (5.9)	2 (0.3)	21 (3.6)	0 (0)	207 (35.9)	133 (23.1)	122 (21.2)	327 (56.8)
After	446 (77.4)	256 (44.4)	103 (17.9)	326 (56.6)	4 (0.7)	132 (22.9)	11 (1.9)	371 (64.4)	542 (94.1)	561 (97.4)	573 (99.5)

[†]Feces were enriched in *Escherichia coli* broth for 6 h at 40º C.

[‡] Samples positive for one or more of the six serogroups of *E. coli*

Table 1.5 Detection of *Escherichia coli* O26, O45, and O103 serogroups, based on culture method and/or multiplex PCR method of detection, in fecal samples

Serogroups	Total negatives[†] (n=576)	Total positives[‡] (n=576)	Number positive of the total samples positive (%)			
			Culture only	PCR only	Culture or PCR	Culture and PCR
O26	275 (47.7)	301 (52.3)	45 (15.0)[a]	170 (56.5)[b]	215 (71.4)[c]	86 (28.6)[d]
O45	423 (73.4)	153 (26.6)	50 (32.7)[a]	57 (37.3)[a]	107 (69.9)[b]	46 (30.1)[a]
O103	146 (25.3)	430 (74.7)	104 (24.2)[a]	83 (19.3)[a]	187 (43.5)[b]	243 (56.5)[c]

[†]Total negatives include samples negative by culture- and/or PCR-based methods.

[‡] Total positives include samples positive by culture- and/or PCR-based methods

[a,b,c,d] Numbers within the same row with different superscripts are statistically different ($P < 0.001$)

Table 1.6 Agreement between culture and PCR methods for detection of *Escherichia coli* O26, O45, and O103 serogroups in fecal samples of feedlot cattle (n=576)

Serogroup	Statistics	Comparison of detection methods				
		Culture only vs. PCR only	**Culture only vs. PCR and culture**	**PCR only vs. PCR and culture**	**Culture only vs. PCR or culture**	**PCR only vs. PCR or culture**
O26	*Kappa	-0.14	-0.11	-0.25	0.25	0.83
	(95% CI)	(-0.18 – -0.10)	(-0.15 – -0.08)	(-0.30 – -0.20)	(0.19 – 0.31)	(0.78 – 0.87)
	McNemar's χ^2 (*P*-value)	72.7 (< 0.001)	12.8 (< 0.001)	27.6 (< 0.001)	170.0 (< 0.001)	45.0 (< 0.001)
O45	Kappa	-0.10	-0.09	-0.10	0.59	0.65
	(95% CI)	(-0.13 – -0.07)	(-0.12 – -0.06)	(-0.13 – -0.07)	(0.50 – 0.68)	(0.56 – 0.74)
	McNemar's χ^2 (*P*-value)	0.5 (0.5)	0.2 (0.7)	1.2 (0.3)	57.0 (< 0.001)	50.0 (< 0.001)
O103	Kappa	-0.19	-0.34	-0.27	0.63	0.52
	(95% CI)	(-0.23 – -0.15)	(-0.40 – -0.28)	(-0.33 – -0.22)	(0.56 – 0.70)	(0.45 – 0.59)
	McNemar's χ^2 (*P*-value)	2.4 (0.12)	55.7 (< 0.001)	78.5 (< 0.001)	83.0 (< 0.001)	104.0 (< 0.001)

*Kappa statistic values were interpreted based on the scale proposed by Landis and Koch (Landis and Koch, 1977).

Chapter 2 - Pooling of Immunomagnetic Separation Beads Does Not Affect Detection Sensitivity of Six Major Serogroups of Shiga Toxin-Producing *Escherichia coli* in Cattle Feces

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) of the serogroups O26, O45, O103, O111, O121, and O145, often called non-O157 STEC, are foodborne pathogens recognized with increasing frequency (Gould *et al*., 2013). Similar to the O157:H7 serotype, six serogroups of STEC cause sporadic cases and outbreaks of diarrhea, hemorrhagic colitis, hemolytic uremic syndrome in children and thrombotic thrombocytopenic purpura in the elderly (Brooks *et al*., 2002; Mead and Griffin, 1998). Cattle are asymptomatic reservoirs for STEC, which are normal inhabitants of the hindgut and are shed in feces. The feces serves as a source of contamination of beef and dairy products and water.

The culture method of non-O157 STEC detection and isolation involves enrichment of feces in a selective broth, which is then subjected to immunomagnetic separation (IMS), followed by plating on a selective medium. The putative colonies are picked based on a phenotypic characteristic of the serogroup with the final confirmation by immuno- or PCR assay (Cernicchiaro *et al*., 2014; Paddock *et al*., 2014; Verstraete *et al*., 2010). Immunomagnetic separation increases sensitivity of detection of STEC, particularly from complex matrices like feces (Chapman *et al*., 1994). Detection of six serogroups of non-O157 STEC requires treating each sample individually with six serogroup-specific beads, which makes the culture method a labor-intensive, time-consuming procedure. Pooling IMS beads would allow for fewer IMS

cycles to test samples for the six serogroups resulting in reduced labor, time and expense. The objective of this study was to determine whether pooling of IMS beads affects detection sensitivity of the six major serogroups of non-O157 STEC in cattle feces compared to individual beads.

Materials and Methods

Three experiments were performed, two with fecal samples spiked with pure cultures of STEC (experiments 1 and 2) and one with feces from naturally-shedding cattle (experiment 3). In each experiment, fecal samples were subjected to individual IMS beads and combinations of pooled IMS beads to compare detection of sensitivity.

Fecal samples spiked with individual or pooled pure cultures of STEC

Strains of seven STEC (Table 2.1) from our culture collection were used to spike pen-floor fecal samples collected from feedlot cattle in the University Beef Cattle Research Center. Strains from frozen protect beads (CryoCare, Key Scientific Products, Round Rock, TX) were grown individually on blood agar (BA; Remel, Lenexa, KS) plates. Single colonies were inoculated into 10 mL Luria Bertani (LB) broth (Becton Dickinson, Inc., Sparks, MD) and incubated at 37º C for 16 h. Then, 100 µl was inoculated into 10 mL LB and incubated at 37º C until an absorbance of 0.4 (600 nm) was achieved (approx. ~10^8 CFU/mL). Ten-fold serial dilutions (10^{-1} to 10^{-7} dilutions) were prepared in buffered peptone water (BPW) for each cultured STEC strain. Viable cell counts (CFU/mL) were determined by spread-plating 100 µl of 10^{-5}, 10^{-6}, and 10^{-7} dilutions on four BA plates per dilution. In experiment 1, feces suspended in *Escherichia coli* broth (EC; Difco, ThermoFisher, Sparks, MD; Paddock *et al*., 2012) were

inoculated with individual or pooled STEC at 10 CFU/mL of each serogroup and enriched (6 h at 40° C) before the IMS step. In experiment 2, feces suspended in *E. coli* broth were inoculated with individual or pooled STEC at 100 CFU/mL of each serogroup and then subjected to the IMS step without enrichment.

Experiment 1. Feces spiked with pure cultures and subjected to enrichment

Approximately 30 g of each fecal sample (n=6) were suspended in 270 mL of EC broth and a uniform suspension was achieved with a magnetic stirrer. Fecal suspensions were transferred in 18 mL aliquots to tubes, which were inoculated with individual or a pool of three (O26, O45, and O103; O111, O121, and O145), four (O26, O45, O103 and O157; O111, O121, O145 and O157) or seven (O26, O45, O103, O111, O121, O145, and O157) STEC cultures (Table 2.2) to obtain a final concentration of 10 CFU/mL of each STEC. The final volume of inoculated fecal suspension was adjusted to 20 mL with BPW. Fecal suspensions were enriched (40º C for 6 h) and subjected to IMS in a Kingfisher Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA) according to the protocol provided by the manufacturer. Enriched fecal suspensions not spiked with pure cultures and subjected to individual and a pool of seven IMS beads (seven serogroups; Abraxis, Warminster, PA) served as control to detect whether the feces were positive for any of the serogroups before inoculation. Eight hundred and sixty microliters of enriched sample were mixed with 20 µL of individual IMS beads or 20 µL of each pooled serogroup IMS beads (60 µL of pool of three IMS beads, 80 µL of pool of four IMS beads or 140 µL of pool of seven IMS beads). Total volume of each sample was adjusted to 1 mL with the addition of sterile EC broth. Fecal suspensions inoculated with individual or a pool of three, four, or seven STEC (10 µl/mL of each serogroup) were subjected to IMS with corresponding

individual or pool of three, four, or seven IMS beads (Table 2.2). Following the IMS procedure, 50 µL of each non-O157 bead suspension were spread-plated onto chromogenic Possé agar (Possé *et al*., 2008) modified to include novobiocin at 5 mg/L and potassium tellurite at 0.5 mg/L (Paddock *et al*., 2014). It has been shown that compared to STEC O157, many non-O157 STEC have a reduced tolerance to novobiocin (Kanki *et al*., 2011; Vimont *et al*., 2007) and potassium tellurite (Catarame *et al*., 2003; Orth *et al*., 2007). As a result, we modified the composition of the Possé medium by decreasing the concentrations of novobiocin (8.0 mg/L to 5.0 mg/L) and potassium tellurite (2.5 mg/L to 0.5 mg/L). The O157 bead suspensions were spread-plated onto sorbitol MacConkey agar containing cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L; CT-SMAC). Plates were incubated for 20-24 h at 37° C and up to six for individual IMS or up to 10 for pooled three, four, or seven IMS bead treatments of chromogenic colonies (mauve, green, blue or purple,) from modified Possé were randomly picked, inoculated onto BA plates, and incubated at 37° C for 24 h. Similarly, from CT-SMAC plates, up to 6 (for individual beads) or 10 (for pooled three, four, or seven IMS bead treatments) sorbitol-negative colonies were randomly picked, inoculated onto BA plates, and incubated at 37° C for 24 h. The colonies (6 or 10) from each non-O157 sample were pooled in distilled water, boiled for 10 min, and centrifuged at 9,300 x g for 5 m, and the lysate containing the DNA was tested by a 7-plex PCR (Paddock *et al*., 2012) that targeted the seven serogroup-specific genes (*wzx* gene for O26, O45, O103, O111, and O145; *wbqE* and *wbq* for O121, and *rfbE* for O157). The amplified DNA was separated on a capillary electrophoresis system in a QIAxcel Advanced System (Qiagen, Valencia, CA) and analyzed by QIAxcel Screengel software. For the O157 serogroup, the non-sorbitol-fermenting colonies were tested for the O157 antigen by latex agglutination (Oxoid) and if positive, a spot-indole test was performed. Colonies positive for agglutination and indole

production were tested by a 6-plex PCR (*1*) that targeted *rfbE*, $fliC_{H7}$, *eae*, *stx*1, *stx*2 and *ehxA* genes.

Experiment 2. Feces spiked with pure cultures and not subjected to enrichment

Twenty pen-floor fecal samples were collected from the University Beef Cattle Research Center, and aliquots of samples were enriched in EC broth and subjected to a 7-plex PCR assay that targets serogroup specific genes of the seven STEC serogroups (Paddock *et al*., 2012). Six fecal samples that were negative for all of the seven serogroups were used to spike with pure cultures of STEC. The procedures to prepare the inoculum and determine the concentration of inoculum were as in experiment 1. The final concentration of inoculated fecal suspension in EC broth was adjusted to 100 CFU/mL of each serogroup and subjected to the IMS procedure without enrichment. Different combinations of pooled IMS beads, O26+O45+O111 and O103+O121+O145, were used in this experiment. Fecal suspensions not spiked with pure cultures and subjected to individual IMS beads served as the control to detect natural presence of any of the six serogroups. Eight hundred and eighty microliters of samples were mixed with 20 µL of individual IMS beads or 20 µL of each serogroup IMS beads for pooled bead treatments. The total volume of each sample was adjusted to 1 mL with the addition of sterile EC broth. Fecal suspensions inoculated with individual or pools of three (O26+O45+O111 and O103+O121+O145) or six STEC (O26, O45, O111, O103, O121, and O145) were subjected to IMS with corresponding individual or pools of three or six IMS beads (Table 2.3). The IMS beads were spread-plated on to modified Possé medium and colonies picked and tested for serogroups by PCR as previously described.

Experiment 3. Feces from naturally-shedding dairy and feedlot cattle

Pen-floor fecal samples were collected from dairy cows (n=48) at the University Dairy Teaching and Research Center, from feedlot cattle (n=48) at the Beef Cattle Research Center and from feedlot cattle (n=288) from six commercial feedlots in Nebraska and Texas. Approximately 2 g of feces were suspended in 18 mL of EC broth, vortexed for 30 sec and incubated at 40° C for 6 h. Enriched fecal suspensions were then subjected to individual and two pools of IMS beads (Table 2.4). The pooled IMS beads were O26+ O45+O111 and O103+O121+O145. For individual IMS beads, 980 µL of enriched sample were mixed with 20 µL of individual IMS beads and subjected to IMS procedure. For pooled IMS bead treatments, 940 (pool of three) µL of enriched sample were mixed with 20 µL of each IMS beads in the pool and subjected to the IMS procedure. Cultural procedures following IMS to identify the serogroups were as previously described. For fecal samples (n=288) from the six commercial feedlots, if pooled colonies were positive for any of the six serogroups, then each colony was tested individually by an 11-plex PCR (Bai *et al*., 2012) to identify the seven serogroups (O157 and six non-O157) and the four major virulence genes (*stx*1, *stx*2, *eae*, and *ehxA*).

Statistical analysis

We applied a non-inferiority test for each serogroup to test the hypothesis that pooling IMS beads was not inferior to the use of individual IMS beads for detection of STEC O serogroups in fecal samples subjected to the culture method. A total of 384 (96 and 288 samples from experiment 3) observations corresponding to individual fecal samples were used in the analysis. Non-inferiority margins (δ) or the acceptable amount by which the pooling test may differ from the individual IMS testing and still not be considered practically inferior, of 20, 10, and 5% were

defined to test the following hypotheses: $Pp - Pi \geq \delta$ vs. $Ha: Pp - Pi < \delta$, where Pp represents the proportion that was positive as determined by pooling IMS beads and Pi the proportion positive based on use of individual IMS beads. Non-inferiority tests were performed using the *binomial* and *noninferiority* options in PROC FREQ in SAS 9.3 (SAS Institute Inc., Cary, NC). The identification of samples with pooled IMS beads was based on the use of two pools: pool 1 combined O26, O45, and O111 beads, and pool 2 combined O103, O121, and O145 beads. We have observed a degree of cross reactivity with IMS beads that suggest binding of non-target serogroups to serogroup-specific antibody (Cernicchiaro *et al*., 2013; Dewsbury *et al*., 2015). As a result, a sample was defined as positive for O26 based on pooling, if it was positive for O26 based on pool 1 and/or pool 2. For individual IMS, a sample was identified as positive for O26 if the sample was positive for O26 based on any individual IMS bead. Same definitions were set across all serogroups.

Results

Experiment 1

In the control (feces not spiked with STEC), five of the six fecal samples, subjected to individual IMS beads, were negative and one sample was positive for the O45 serogroup. However, with pooled beads of all six serogroups, three of the six control samples were positive for O45 and two samples were positive for O103 (Table 2.2). For feces (n=6) spiked with individual or pool of three or four STEC and subjected to individual or pool of three or four IMS beads, detection of serogroups of O26, O103, O111, and O157 were six of six, or in a few cases five of six samples (Table 2.2). With feces spiked with individual or pooled STEC, the detection of O121 and O145 serogroups was less frequent and ranged from zero to five; however, for the

most part the recovery was similar or even higher with pooled beads compared to individual beads. With O45, the serogroup was detected in only one of the six spiked fecal samples, although three of the six samples not spiked (control) were positive for the O45 serogroup.

Experiment 2

For feces (n = 6) spiked with individual STEC and subjected to individual or pools of three or six IMS beads, detection of serogroups of O26, O121 and O145 were five of six or six of six. For feces spiked with O103, the detection with a pool of three beads was three of six, but with pool of six beads detection was six of six. With O45, the detection was only one of six spiked samples with individual or pool of six beads and none with pool of three beads (Table 2.3). For feces spiked with a pool of three STEC subjected to individual or a pool of three IMS beads, detection of serogroups O26, O111, O103 and O145 was four, five or six of six. The recovery of O121 was one of six with pool of three beads and five of six with pool of six beads. The O45 was not detected with either individual or pool of three beads.

Experiment 3

A total of 384 fecal samples were subjected to individual beads and two pools of three beads (O26+O45+O111 and O103+O121+O145). All six serogroups were detected in the fecal samples, with O26 and O103 being the two predominant serogroups (Table 2.4). Because the detection of serogroups was based on the PCR assay of six (for individual beads) or 10 (for pooled beads) pooled colonies, serogroups other than the targeted group were also detected. Of the six serogroups, O26 and O103 were detected in a substantial number of fecal samples subjected to other IMS beads. With O26, 24.7% (95/384) of fecal samples were positive with

O26 beads, but with the inclusion of non-redundant fecal samples that tested positive with the other five beads, the proportion of samples testing positive increased to 38.5% (148/384). Similarly, with O103, an additional 17 fecal samples were detected as positive with non-O103 IMS beads. With the other four serogroups, only a few (zero to five) additional fecal samples were detected as positive with non-specific IMS beads. A similar identification of serogroups with nonspecific beads was observed with pooled beads; however, only a few additional fecal samples were identified as positive with non-specific beads. For example, with O26, only seven additional fecal samples were detected as positive with non-O26 beads. The proportions of fecal samples positive for the six serogroups detected by individual or pooled beads were very similar (within 4%; Figure 2.1). The O26 serogroup was detected in 38.5% (148/384) of samples tested by individual IMS beads compared with 42.2% (162/384) of samples subjected to pooled IMS beads. Likewise, the O103 serogroup was detected in 53.6% (206/384) of samples subjected to the pooled IMS beads compared with 51.8% (199/384) detected with individual IMS beads. Individual IMS beads detected a slightly higher number of O45 (52 vs. 46), O121 (30 vs. 24) and O145 (32 vs. 26) serogroups than pooled beads. Application of non-inferiority tests indicated that detection of the six serogroups with pooled IMS beads was not substantially inferior to individual IMS beads ($P < 0.05$) based on non-inferiority margins as low as 5%. The significance of the test was verified by the lower limit of the 90% confidence interval being greater than the non-inferiority limit in all tests.

Individual isolates (n = 288) from samples from the six commercial feedlots were tested by 11-plex PCR, if DNA from pooled isolates tested positive for one or more of the six serogroups and *stx*1 and/or *stx*2 genes. A total of 75 and 74 pure cultures of isolates positive for one of the six serogroups were obtained with individual or pooled beads procedure, respectively. The three

predominant serogroups were O103, O26 and O145 (Table 2.5). The total number of isolates obtained that carried either or both Shiga toxin genes, i.e., STEC, were similar between the individual and pooled IMS procedures (22 vs. 19; Table 2.5). The majority of STEC isolates detected by individual and pooled IMS beads were from O103 (10 vs. 8) and O145 (7 vs. 6) serogroups. All STEC isolates were also positive for the intimin gene. None of the O45 and O121 isolates carried Shiga toxin genes. In addition to the six non-O157 serogroups, a total of 36 and 40 Shiga toxigenic isolates of undetermined serogroups were obtained from individual and pooled IMS procedures, respectively. Only a small number of these STEC isolates carried the intimin gene (Table 2.5).

Discussion

It is well established that inclusion of an IMS step before plating onto a selective medium in the culture-based detection method improves STEC detection sensitivity in feces or other complex sample matrices (Chapman *et al*., 1994; LeJeune *et al*., 2006; Paddock *et al*., 2012; Widiasih *et al*., 2004). In studies requiring detection of the six serogroups of non-O157 STEC, the culture method becomes time-consuming and labor-intensive because each sample is subjected to six individual IMS procedures, followed by plating onto six plates of selective medium. Therefore, our intent was to determine whether pooling of beads affects the detection sensitivity for the six major serogroups of non-O157 STEC in cattle feces. In two experiments conducted with inoculated feces, the detection of the six serogroups, for the most part, was comparable between the individual and pooled IMS procedures. In the first experiment, the inoculated concentration was low (10 CFU per mL), thus requiring enrichment before the IMS step. Of the six serogroups, the detection of O45 was lower regardless of the IMS procedure. The strain of inoculated O45 may not have survived or grown in the fecal suspension during 6 h

of incubation for enrichment. Therefore, in the second experiment, we used a different strain of O45 and increased the concentration of inoculum from 10 to 100 CFU per mL of the fecal suspension and eliminated the enrichment step. Despite the changes, the recovery of the inoculated strain did not improve. It is possible that O45 beads were not specific, although the same beads detected O45 in the un-inoculated feces in experiment 1 and in feces of naturally-shedding cattle in experiment 3.

Serogroups O26, O103, and O111 showed high recoveries from inoculated feces with both individual and pooled IMS beads procedures. The other three serogroups, O45, O121, and O145 were detected less frequently, but showed similar recovery between individual and pooled IMS beads. The data generated in the study suggest that pools of 3 or 4 beads have similar recovery to that of individual beads. The addition of O157 IMS beads to all pooled treatments showed no change in detection. Different combinations of IMS beads (O26+O45+O103, O26+O45+O111, O111+O145+O121, and O103+O121+O145) showed no change in detection. Because O26 and O103 are the predominant non-O157 serogroups in cattle feces (Cernicchiaro *et al*., 2014; Dargatz *et al*., 2013; Dewsbury *et al*., 2015), we chose to separate the two serogroups into different pooled IMS combinations, as done in experiments 2 and 3. The separation also resulted in less crowded colonies on inoculated plates, which increased the likelihood of selecting a more complete representation of non-O157 colonies present on the agar plate. Although a pool of six or seven beads had similar recovery to that of pool of three or four beads, pool of three beads resulted in less crowded colonies on the medium. Also, based on our previous study (Dewsbury *et al*., 2015), a majority of the fecal samples (60 to 70%) from feedlot cattle had three or fewer serogroups per sample. Traditionally, we and others have picked five to six colonies after plating IMS beads on a selective medium (Fegan *et al*., 2004; Fox *et al*., 2007; Jacob *et al*., 2010;

LeJeune *et al*., 2006; Omisakin *et al*., 2003). With the pooled beads, we chose to increase the number of colonies picked to 10 to enhance the chance for detection of the non-O157 serogroups. Overall, pooling of the beads reduces the number of colonies to be tested by latex agglutination or PCR for serogroup confirmation (18 vs. 10 for three serogroups for individual or pooled IMS beads, respectively).

Further evaluation with naturally-shedding cattle showed that pools of three IMS beads detected relatively equal number of non-O157 serogroups compared with individual IMS beads. We did not include O157 serogroup in the pooled combinations because the O157 serogroup traditionally has been cultured on its own selective medium (for example, CT-SMAC or CHROMagar O157) and in some cases enriched in a different medium (for example, Gram negative broth). Therefore, fecal samples could be tested by individual O157 IMS beads and two pools of three (O26+O45+O111 and O103+O121+O145) IMS beads for the detection of seven STEC. Although a high proportion of fecal samples from feedlot cattle were positive for one or more of the six serogroups, only a small proportion of the isolates obtained in pure culture carried Shiga toxin genes, which were predominantly *stx*1. All *stx*-carrying isolates were positive for intimin genes suggesting they were the enterohemorrhagic *E. coli* pathotype. A majority of the non-O157 serogroups shed in cattle feces are not Shiga toxigenic, which is in agreement with our previous findings (Cernicchiaro *et al*., 2013; Dewsbury *et al*., 2015; Paddock *et al*., 2014).

Based on feces inoculated with pure cultures and feces from naturally-shedding cattle, pooling of IMS beads did not affect detection sensitivity of six serogroups of non-O157 STEC in cattle feces. The prospect of a higher throughput test because of a less laborious, timesaving,

and less expensive protocol, coupled with the statistical evidence of non-inferiority, make pooling a desirable option for detecting STEC serogroups in bovine fecal samples.

Table 2.1 Virulence gene profiles of Shiga toxin-producing *Escherichia coli* strains used to spike fecal samples

Serogroup	Strain	Source	*stx*1	*stx*2	*eae*	*ehxA*
E. coli O26	2013-3-6D	Bovine feces	+	-	+	+
E. coli O45*	1.2622	Bovine feces	+	-	-	-
E. coli O45*	CDC-96-3285	Human	+	-	+	+
E. coli O103	2013-3-174C	Bovine feces	+	-	+	+
E. coli O111	2013-3-390E	Bovine feces	+	-	+	+
E. coli O121	KDHE 48	Human	-	+	+	-
E. coli O145	2013-3-86C	Bovine feces	+	-	+	+
E. coli O157	2013-376D	Bovine feces	+	+	+	+

*Strain 1.2622 was used in experiment 1, and strain CDC-96-3285 was used in experiment 2.

Table 2.2 Detection of Shiga toxin-producing *Escherichia coli* (STEC) with individual or pooled immunomagnetic beads (IMS) in cattle feces (n=6) spiked with individual or pooled 3, 4 or 7 STEC

Fecal samples and IMS beads	Serogroups detected, no. positive						
	O26	O45	O103	O111	O121	O145	O157
Control (not spiked)							
Individual beads	0	1	0	0	0	0	0
Pooled beads: O26+O45+O103 +O111+O1211+O145+O157	0	3	2	0	0	0	0
Spiked with individual STEC							
Individual beads	6	0	6	6	2	3	6
Pooled beads:							
O26+O45+O103	6	0	5				
O111+O121+O145				5	4	2	
O26+O45+O103+O157	5	0	5				6
O111+O121+O145+O157				6	1	5	6
O26+O45+O103+O111+O121+ O145+O157	6	1	6	6	3	4	6
Spiked with STEC O26+O45+O103							
Individual beads	6	0	6				
Pooled beads: O26+O45+O103	6	0	6				
Spiked with STEC O111+O121+O145							
Individual beads				6	3	3	
Pooled beads: O111+O121+O145				6	3	2	
Spiked with STEC O26+O45+O103+O157							
Individual beads	6	0	5				6
Pooled beads: O26+O45+O103+O157	5	1	5				5
Spiked with STEC O111+O121+O145 +O157							
Individual beads				5	2	2	5
Pooled beads: O111+O121+O145+O157				6	1	2	5
Spiked with STEC O26+O45+O103+ O111+O121+O145+O157							
Individual beads	6	0	5	5	4	3	6
Pooled beads: O26+O45+O103+ O111+ O121+O145+O157	6	0	5	4	1	0	3

Table 2.3 Culture-based detection of six serogroups of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in cattle feces (n=6) spiked with six serogroups of STEC with individual or pooled immunomagnetic beads (IMS)

Fecal samples and IMS beads	Serogroups detected, No. of samples					
	O26	O45	O111	O103	O121	O145
Spiked with individual STEC						
Individual beads	5	1	6	6	5	6
Pooled beads:						
O26+O45+O111	6	0	2			
O103+O121+O145				3	6	6
O26+O45+O103+O111+O121+O145	6	1	6	6	5	6
Spiked with STEC O26+O45+O111						
Individual beads	6	0	6			
Pooled beads:						
O26+O45+O111	6	0	4			
O26+O45+O111+O103+ O121+O145	6	0	6			
Spiked with STEC O103+O121+O145						
Individual beads				6	6	6
Pooled beads:						
O103+O121+O145				6	1	4
O26+O45+O111+O103+ O121+O145				5	5	4

Table 2.4 Culture-based detection of six serogroups of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in cattle feces (n=384) with individual and pooled immunomagnetic beads (IMS)

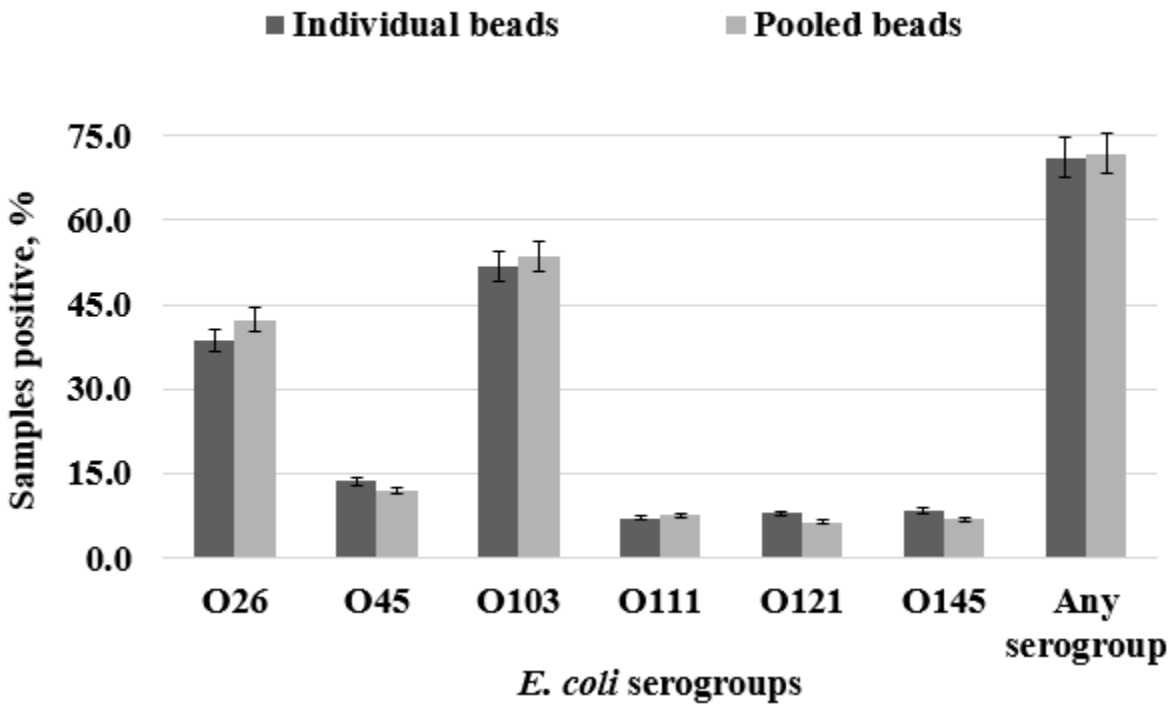
IMS beads	Serogroups, no. of samples positive (%)					
	O26	O45	O103	O111	O121	O145
Individual beads						
O26	95 (24.7)	3	21	0	0	1
O45	40	47 (12.2)	24	3	1	1
O103	25	2	182 (47.4)	1	1	1
O111	36	2	38	27 (7.0)	1	1
O121	31	4	28	1	28 (7.3)	2
O145	36	2	27	1	0	32 (8.3)
Positive with any individual bead*	148 (38.5)	52 (13.5)	199 (51.8)	27 (7.0)	30 (7.8)	32 (8.3)
Pooled beads:						
O26+O45+O111	155 (40.4)	42 (10.9)	39 (10.2)	29 (7.6)	0	1
O103+O121+O145	51	7	206 (53.6)	2	24 (6.3)	26 (6.8)
Positive with any pooled beads*	162 (42.2)	46 (12.0)	206 (53.6)	29 (7.6)	24 (6.3)	26 (6.8)

*Denotes non-redundant samples positive for each serogroup by any individual or pooled IMS beads

Table 2.5 Detection of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) and STEC isolates of undetermined serogroups by individual and pooled immunomagnetic separation (IMS) procedures in fecal samples (n=288) of feedlot cattle

Virulence genes (*stx*1, *stx*2, *eae*)	Non-O157 *E. coli* Serogroups							STEC of undetermined serogroups
	O26	O45	O103	O111	O121	O145	Total	
Total serogroups isolated								
Individual IMS	19	6	33	3	3	11	75	36
Pooled IMS	16	5	35	4	4	10	74	40
Shiga toxin 1 (*stx*1)								
Individual IMS	1	0	10	3	0	4	18	3
Pooled IMS	0	0	8	4	0	5	17	4
Shiga toxin 2 (*stx*2)								
Individual IMS	1	0	0	3	0	3	7	34
Pooled IMS	1	0	0	4	0	1	6	36
Intimin (*eae*)								
Individual IMS	19	0	24	3	0	11	57	5
Pooled IMS	13	0	28	4	0	10	55	1
***stx*1 + *stx*2**								
Individual IMS	0	0	0	3	0	0	3	1
Pooled IMS	0	0	0	4	0	0	4	0
stx*1 or *stx*2 + *eae								
Individual IMS	2	0	10	3	0	7	22	5
Pooled IMS	1	0	8	4	0	6	19	1

Figure 2.1 Proportions and 95% confidence intervals (error bars) of fecal samples positive for the six serogroups of non-O157 Shiga toxin-producing *Escherichia coli* in cattle feces (n=384) detected with individual or pooled immunomagnetic beads (O26+O45+O111 and O103+O121+145) (Experiment 3)



Chapter 3 - Development and Validation of a Real-time PCR Assay, Based on the Clustered Regularly Interspaced Short Palindromic Repeat Sequence Polymorphisms (CRISPR), for Serotype-Specific Detection and Quantification of Enterohemorrhagic *Escherichia coli* O157:H7 in Cattle Feces

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are a major foodborne pathogen in humans, however not all EHEC strains pose an equal risk of infecting humans (Brooks *et al*., 2005; Jiang *et al*., 2015). Like other *E. coli*, EHEC serogroups are defined based on the O (Ohne) antigen of the cell wall lipopolysaccharide. Within a given serogroup, there are several serotypes, which are defined by the H (Hauch) flagellar antigen. However, a high proportion of EHEC, including O157 serogroup, are non-motile mutants that lack H antigens (Gyles, 2007). Although over 380 Shiga toxin-producing *E. coli* (STEC) serotypes have been isolated from humans with gastrointestinal illnesses (Karmali *et al.,* 2010), *E. coli* O157:H7 remains the most frequently associated serotype with foodborne outbreaks and severe complications of the disease (Karmali *et al.,* 2010).

More than 435 Shiga-toxigenic *E. coli* serotypes, including O157:H7, have been recovered from cattle, which represent a major EHEC reservoir (Gyles, 2007). The organisms colonize the hindgut of cattle, and are shed in the feces, which serves as a source of food and water contamination (Gyles, 2007). Cattle typically shed *E. coli* O157:H7 at concentrations below the limit of PCR detection ($\leq 10^2$ CFU/g of feces), therefore an enrichment step is required for

detection (Chapman, 2000). Several quantitative PCR (qPCR) assays have been developed for detection and quantification of *E. coli* O157:H7 in a fecal matrix by targeting genes for serogroup specific O-antigen (*rfb*E_{O157}), H7 antigen (*fli*C_{H7}), and one or more major virulence factors, including Shiga toxins (*stx*1 and *stx*2) and intimin (*eae*) (Ibekwe and Grieve, 2003; Hsu *et al*., 2005; Anklam *et al*., 2012; Jacob *et al*., 2012; Noll *et al*., 2015). The major limitation of these qPCR assays is that co-amplification of *rfb*E_{O157}, *stx*1, *stx*2, and *eae* genes in a sample does not signal association of the serogroup-specific gene with virulence genes because multiple STEC and EHEC serotypes can be present in a fecal sample.

The clustered regularly interspaced short palindromic repeats (CRISPR) loci consist of highly conserved direct DNA repeats (~29 base pair in *E. coli*) separated by non-repetitive spacer sequences (~32 base pair in *E. coli*) variable in nucleotide composition but with sequence identity matching that of prior invading nucleic acids (Marraffini and Sontheimer, 2010; Touchon and Rocha 2010; Delannoy *et al*., 2016). Interestingly, CRISPR polymorphisms are highly correlated with certain *E. coli* O:H serotypes, including O157:H7, and presence of Shiga toxin and intimin genes, and have been used as a typing method for EHEC (Toro *et al*., 2014; Delannoy *et al*., 2016; Jiang *et al*., 2015). Delannoy *et al*. (2012) previously examined the CRISPR loci of EHEC O157:H7 strains, and based on a small degree of sequence heterogeneity, developed a panel of singleplex qPCR assays targeting three CRISPR regions (A, B, C). Delannoy *et al*. (2012) reported highest sensitivity (100%) and specificity (99.7%) from combined test results from CRISPR B and C singleplex assays. The singleplex qPCR assays have only been validated with pure cultures and the applicability of the assays to detect and quantify O157:H7 in a complex matrix has not been evaluated. Therefore, our objective was to

develop and validate a qPCR assay, based on the CRISPR array targets (Delannoy *et al*., 2012), for the detection and quantification of *E. coli* O157:H7 serotype in cattle feces.

Materials and Methods

PCR optimization

Initial assay development and optimization were performed using DNA extracted from *E. coli* O157:H7 strain ATCC 43894 (American Type Culture Collection, Manassas, VA), positive for *rfbE*$_{O157,}$ *stx*1, *stx*2, *eae* and *fli*C$_{H7}$. Primer and probe sequences of the three CRISPR target loci were according to Delannoy *et al*., (2012), and fluorescent dyes and quenchers used in the assays are in Table 3.1. First, target DNA was tested in a singleplex format using pairwise concentrations of individual primer (4.0 and 10.0 pmol/µl) and probe targets (2.5, 5.0 and 10.0 pmol/µl). Optimum primer and probe concentrations for each target were 5.0 and 10.0 pmol/µl, respectively. Target DNA was then serially diluted in ddH_2O, each dilution was subjected to singleplex (A, B, C), duplex (B+C) and triplex (A+B+C) assay running conditions, then standard curve data were evaluated. The same limit of detection was achieved for the duplex and CRISPR B and C singleplex assays; PCR efficiencies were between 90 to 110% and R^2 values of > 0.99. However, due to poor PCR efficiencies (~70.0%) observed for the triplex assay, CRISPR A target was abandoned and the duplex assay targeting CRISPR B and C were further evaluated.

Duplex qPCR running conditions

The working concentrations of all primers were 10 pM/µl. Working concentrations of CRISPR B and C probes were 5.0 pM/µl and 2.5 pM/µl, respectively. The PCR reaction consisted of 1 µL of primer mix and each probe, 10 µL of BioRad iQ multiplex powermix, 5 µL

of sterile PCR grade water, and 2 µL of DNA template (total reaction volume= 20 µL). Assay running conditions consisted of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 30 s. Standard curve data, PCR efficiency, and R^2 values were generated from samples, ran in triplicate, using the BioRad (Hercules, CA) CFX96 Real-Time System.

Analytical Specificity of the duplex qPCR assay

Analytical specificity of the qPCR assay was tested with 24 strains of EHEC O157:H7, 26 strains of major "top-six" non-O157 EHEC (O26, O45, O103, O111, O121, and O145), 2 strains of enteropathogenic *E. coli* (EPEC) (O55:H7 and O55:NM serotypes), and 5 strains of non-*E. coli* enteric bacteria (*Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Salmonella enterica*, and *Serratia marcescens*).

The analytical specificity of the assay was also assessed with cattle feces spiked with EHEC O157:H7, *E. coli* O157 non-H7 strains and "top-six" non-O157 Shiga-toxigenic serogroup strains (O26, O45, O103, O111, O121, O145) (Table 3.2). Pen-floor fecal samples (n=10) were collected from Kansas State University Beef Cattle Research Center. Samples were tested by three mqPCR assays: assay 1 (Noll *et al*., 2015) targeted *rfb*E_{O157}, *stx*1, *stx*2 and *eae* genes, assay 2 (Shridhar *et al*., 2015) targeted *wzx*$_{O26}$, *wzx*$_{O103}$, and *wzx*$_{O111}$ genes and assay 3 (Shridhar *et al*., 2015) targeted *wzx*$_{O45}$, *wbq*E_{O121}+*wbq*F_{O121} and *wzx*$_{O145}$ genes. Fecal samples that tested negative for the 10 genes were spiked with pure cultures: spike 1: *E. coli* O157:H7, spike 2: *E. coli* O157 non-H7 strains + STEC O26, O103 and O111, and spike 3: *E. coli* O157 non-H7 strains + STEC O45, O121 and O145 strains. Strains were grown on blood agar (Remel, Lenexa, KS) and a single colony of each strain was individually inoculated into 10 mL Luria-Bertani (LB; Becton Dickinson Co., Sparks, MD) broth. Inoculated broth was incubated at 37°C for 16 h, then 100

µL of broth culture was inoculated into 10 mL LB and incubated at 37° C until (~3 h) a target absorbance of 0.4 (600 nm) was achieved (~10^8 CFU/mL). A bulk fecal suspension was prepared by adding 20 g of PCR-negative feces to 180 mL *Escherichia coli* (EC) broth. Then, 9 mL of fecal suspension was added to four sterile glass tubes; 100 µL of the cultured strain(s) assigned to each spike treatment was added to the three tubes. The fourth tube served as an uninoculated fecal control suspension. Tubes were vortexed for 30 s, then 1 mL from each spiked fecal suspension was boiled for 10 min and centrifuged at 9,000 RCF for 5 min to obtain a crude DNA preparation. Extracted DNA was then purified using the GeneClean® Turbo Kit (MP Biomedicals LLC, Solon, OH). Purified DNA from each of the spiked samples was subjected to the $CRISPR_{O157:H7}$ qPCR assay and to the three previously mentioned mqPCR assays.

Analytical Sensitivity of the duplex qPCR assay

The analytical sensitivity of the assay was individually assessed with EHEC O157:H7 ATCC strains (43888, 43889, 4389 and 43894) variable for three major virulence genes (*stx*1, *stx*2, and *eae*; Table 3.3). Individual strains were cultured on blood agar and then in LB broth culture to reach target growth (~10^8 CFU/mL), as previously described. Serial ten-fold dilutions of broth culture were prepared in LB broth and 1 mL from each of 10^{-1} to 10^{-8} dilutions was boiled for 10 min and centrifuged at 9,000 RCF for 5 min to obtain a crude DNA preparation. Extracted DNA (2 µL) was then subjected to the duplex qPCR assay. Broth cultures (100 µL) from 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spread-plated onto blood agar plates, incubated overnight at 37°C and colonies were counted to determine concentration (CFU/mL). Two replications of the assay were performed with each of the four strains.

The analytical sensitivity of the assay was also assessed with cattle feces spiked with the same four strains (43888, 43889, 4389 and 43894) (Table 3.4). Pen-floor fecal samples (n=10) were collected and tested by end-point PCR (Bai *et al*., 2010) that targeted *rfb*E_{O157}, *fli*C_{H7}, *stx*1, *stx*2 and *eae* genes, and samples that tested negative for *rfb*E_{O157} and *fli*C_{H7} genes were spiked with pure cultures. A bulk fecal suspension was prepared by adding 40 g of PCR-negative feces to 320 mL *Escherichia coli* broth (EC medium; Difco, Becton, Dickinson Co., Sparks, MD). Serial ten-fold dilutions of each *E. coli* O157:H7 strain were prepared as before and 1 mL from each of 10^{-1} to 10^{-8} dilutions of *E. coli* O157:H7 was added to 9 mL of fecal suspension. One milliliter of each spiked fecal sample suspended in EC broth was removed before and after 6 h enrichment at 40° C. Purified DNA, prepared as before, from each dilution of spiked sample was subjected to the duplex qPCR assay. Two replications of the assay were performed with each of the four strains. Average end-point cycle threshold (Ct) values and minimum detection limits were determined for each strain tested, both in pre- and post-enriched pure culture and spiked fecal samples.

Application of duplex qPCR assay to detect *E. coli* O157:H7 in fecal samples from naturally shedding feedlot cattle

A total of 576 fecal samples were collected from a research feedlot in the Southwest that housed cattle in 48 pens with 10 to 12 cattle per pen. Pen-floor fecal samples (6 samples per pen) were collected on three different occasions, approximately 20 to 25 days apart during July and August 2015. Fecal samples were enriched in *EC* broth for 6 h at 40° C and DNA was extracted and purified as before for detecting *E. coli* O157 with the CRISPR$_{O157:H7}$ qPCR assay and 4-plex qPCR assay (Noll *et al*., 2015) targeting *rfb*E_{O157}, *stx*1, *stx*2 and *eae* genes (Table

3.5). The qPCR results were then compared to a culture-method of detection, based on immunomagnetic separation (IMS), plating of IMS beads on a selective medium, and PCR confirmation of suspect *E. coli* O157 colonies (Dewsbury *et al*., 2015).

Statistical analyses

The overall agreement between the $CRISPR_{O157:H7}$ and 4-plex qPCR assays and between the $CRISPR_{O157:H7}$ and culture method were assessed by the Cohen's Kappa statistic and 95% confidence intervals using the Kappa calculator (http://vassarstats.net/kappa.html) (Table 3.5). Kappa statistic values were interpreted based on the scale proposed by Landis and Koch (Landis and Koch, 1977).

Results

Analytical specificity

Analytical specificity of the duplex qPCR assay was tested with strains of EHEC O157:H7 (n=25), "top-six" non-O157 Shiga-toxigenic *E. coli* (n=26), EPEC O55 (n=2), and *E. coli* related Gram negative bacteria (*Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Salmonella enterica*, and *Serratia marcescens*). The $CRISPR_{O157:H7}$ assay correctly detected the presence of the CRISPR B and C loci in all 25 strains of *E. coli* O157:H7 and none of the other strains of *E. coli* and other Gram negative bacteria yielded amplifications (data not shown).

The analytical specificity of the assay was also assessed with cattle feces spiked with *E. coli* O157:H7 (spike 1), *E. coli* O157 non-H7 strains (O157:NM, O157:H43, O157:H45) plus STEC O26, O103 and O111 (spike 2) or plus STEC O45, O121 and O145 strains (spike 3) (Table 3.2). The $CRISPR_{O157:H7}$ qPCR correctly detected *E. coli* O157:H7 in spike 1 and no amplification was observed for spikes 2 or 3, which contained *E. coli* O157 non-H7 strains and the six non-O157 *E.*

coli strains. The 4-plex qPCR assay amplified *rfb*E_{O157}, *stx*1 and/or *stx*2 and *eae* genes in all three spiked samples that contained *E. coli* O157 H7 or non-H7 indicating samples were positive for *E. coli* O157, Shiga toxin, and intimin (Table 3.2). The two non-O157 mqPCR assays detected the presence of the six non-O157 *E. coli* serogroups in spikes 2 and 3.

Analytical sensitivity with pure cultures

The initial concentrations of all strains subjected to 10-fold serial dilutions ranged from 1.3 to 1.8 x 10^8 CFU/mL. For all strains tested, the average endpoint threshold cycle (Ct) ranged from 39.4 to 39.9 and from 39.4 to 39.7 for CRISPR B and C targets, respectively. Correlation coefficients were all > 0.99 and PCR amplification efficiencies were between 94.7 and 105.1% (Table 3.3). The average minimum detection limit of the $CRISPR_{O157:H7}$ qPCR assay was 10^2 CFU/mL for each strain tested.

Analytical sensitivity with cattle feces spiked with EHEC O157:H7

The detection limit of the assay for *E. coli* O157:H7 with DNA extracted directly from spiked cattle feces was 10^3 CFU/g for all *E. coli* O157:H7 strains tested; correlation coefficients were all > 0.99 and PCR amplification efficiencies were 102.4-108.1% (Table 3.4). For all strains tested, the average endpoint Cts ranged from 37.4 to 37.5 and from 37.2 to 37.6 for CRISPR B and C targets, respectively. After a six-hour enrichment, sensitivity increased to 10^0 CFU/g for all O157:H7 strains tested. Correlation coefficients were all > 0.99 and average endpoint Cts ranged from 37.2 to 37.8 and from 37.2 to 37.9 for CRISPR B and C targets, respectively (Table 3.4).

Application of CRISPR$_{O157:H7}$ qPCR assay and comparison with 4-plex qPCR and culture method for detection of EHEC O157 in naturally shedding cattle fecal samples.

A sample positive for CRISPR B and/or C targets was considered positive for EHEC O157:H7 serotype by the CRISPR qPCR assay. A sample positive for *rfb*E$_{O157}$ was considered positive for *E. coli* O157 serogroup by the 4-plex assay. In the culture method, a fecal sample from which an isolate positive for *rfb*E$_{O157}$, *stx*1 and/or *stx*2 and *eae* was obtained was considered as positive. Of the 576 fecal samples subjected to the CRISPR$_{O157:H7}$ qPCR assay, 243 were positive (Table 3.5) and had Ct values below the maximum threshold for CRISPR B (37.7) and/or CRISPR C (37.9) targets. Nearly all samples (560/576; 97.2%) were positive for *rfb*E$_{O157}$ by 4-plex PCR, compared to 30.4% (175/576) of sample positive for STEC O157:H7 by culture-method. The Cohen's Kappa statistic indicated a fair agreement between the CRISPR$_{O157:H7}$ qPCR and culture method ($\kappa = 0.334$) but only a slight agreement between CRISPR$_{O157:H7}$ qPCR and 4-plex qPCR ($\kappa = 0.005$). The majority of CRISPR$_{O157:H7}$ qPCR positive samples were also positive for *rfb*E$_{O157}$ by the 4-plex qPCR assay (237/243; 97.5%); nearly half of these samples (119/243; 49.0%) were positive for *E. coli* O157:H7 by culture-method of detection. The *rfb*E$_{O157}$ gene was detected in 97.0% (323/333) of samples negative for *E. coli* O157:H7 by CRISPR$_{O157:H7}$ qPCR; 16.8% (56/333) of CRISPR$_{O157:H7}$ qPCR negative samples were positive by the culture-method of detection.

An effort was made to further examine why the 56 fecal samples from which EHEC O157:H7 was isolated were negative by the CRISPR$_{O157:H7}$ qPCR. First, the 56 EHEC O157:H7 strains isolated from these samples were cultured, DNA were extracted and tested by the CRISPR$_{O157:H7}$ qPCR. All 56 strains were positive for CRISPR B and C targets. Fecal DNA

from the 56 samples were then re-tested by the CRISPR$_{O157:H7}$ qPCR using a temperature gradient (48 to 62°C) option during the annealing-extension step (normally set at 60°C). Although some amplification was observed, Ct values (~39-43) were all above the cut-off threshold for CRISPR B (37.7) and/or CRISPR C (37.9) targets. Fecal DNA from the 56 samples were then re-tested using a 2-step annealing PCR with assay running conditions consisting of 95°C for 5 min followed by 35 cycles of 95°C for 15 sec, 64°C for 10 sec and 60°C for 30 sec. As before, amplification was observed for a small proportion of samples (8/56), but Ct values were all above cut-off thresholds. Additionally, a touchdown PCR method was used to test the fecal DNA, which consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 15 sec, 65°C for 30 sec (then 1°C incremental every cycle thereafter for 15 cycles) and 60°C for 30 sec. Again, only a small proportion of samples (6/56) amplified, but all Ct values were above cut-off thresholds. To test whether a fecal matrix may be inhibiting amplification of DNA from these samples, serially diluted DNA prepared from pure cultures of a randomly selected subset of the 56 strains (n=6) were used to spike aliquots of the unthawed enriched fecal suspensions from which the strains were originally isolated from. Three DNA dilutions of each strain, previously generating Ct values between 26 to 34 in pure-culture testing, were used in the spike. Spiked tubes were vortexed for 30 s, and a crude DNA was prepared then purified, as previously described, for testing with the CRISPR$_{O157:H7}$ qPCR. Positive test results were achieved for each CRISPR target for all sample dilutions tested. Whole genome sequences of the 56 strains were available from another study (Yang *et al*., unpublished data). CRISPR regions of a randomly selected subset of EHEC O157:H7 strains (n=20) isolated from CRISPR$_{O157:H7}$ qPCR positive (n=10) and negative (n=10) fecal samples were examined in BioEdit version 7.1.3.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). None of the twenty strains displayed any

sequence heterogeneity at the CRISPR regions targeted by the qPCR, and a 100% match was observed between the nucleotide sequence of all CRISPR B and C primers/probes and their binding sites.

Discussion

Several multiplex qPCR assays have been developed for detection of *E. coli* O157:H7, many of which have targeted the O157 serogroup specific antigen gene (*rfb*E_{O157}), in combination with other major *E. coli* O157:H7 virulence genes, including the flagellar H7 antigen (*fli*C_{H7}), Shiga toxin (*stx*1 and/or *stx*2) and intimin (*eae*) genes. However, these genes are also found in other non-O157 EHEC or STEC and non-EHEC or non-STEC that can be present in cattle feces (Dewsbury *et al*., 2015; Noll *et al*., 2015; Shridhar *et al*., 2016). Therefore, the qPCR assays do not distinguish between a strain of EHEC O157:H7 carrying these genes and multiple strains of non-pathogenic *E. coli*, each carrying one or more of these genes. Luedtke *et al*. (2014) have developed an assay targeting the pO157 plasmid carried *ecf*1 gene for detection of EHEC pathotype from cattle feces, but this assay identifies all EHEC, including O157:H7, the "top-six" non-O157 EHEC (O26, O45, O103, O111, O121, O145) and EHEC serogroups other than the top 7. Although the top-six non-O157 EHEC serogroups have become important food-borne pathogens (Brooks *et al*., 2005), among EHEC, O157:H7 serotype represents the majority of foodborne illnesses in the U.S. (Mead *et al*., 1999; Scallan *et al*., 2011). Furthermore, O157:H7 remains the only EHEC serotype that United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) has declared as an adulterant in ground beef and non-intact beef products, whereas the top-six EHEC serogroups that were declared as adulterants in 2012 include multiple serotypes. The results of this study indicate that the $CRISPR_{O157:H7}$ qPCR is a

sensitive and specific method for detection and quantification of EHEC O157:H7 in cattle feces. Minimum detection limit for pure cultures of EHEC O157:H7 was 10^2 CFU/mL, one log lower than results from previous studies basing sensitivity on quantification of *rfb*E_{O157} gene (Jacob *et al*., 2012, Noll *et al*., 2015). The detection limit of the assay with DNA extracted directly from cattle feces was ~10^3 CFU/g, compared to 10^4 CFU/g minimum detection limit reported in previous studies (Jacob *et al*., 2012, Noll *et al*., 2015). It is possible that the CRISPR targets allowed for increased amplification in a fecal matrix, compared to *rfb*E_{O157} gene, however, differences in the preparation of spiked cattle feces for testing may have also played a role. After a six-hour enrichment, sensitivity of the assay increased to ~10^0 CFU/g, which is in agreement with previous studies (Jacob *et al*., 2012, Noll *et al*., 2015).

Extracted DNA from fecal samples (n=576) from naturally-shedding cattle after 6 h enrichment were tested with the $CRISPR_{O157:H7}$ qPCR assay and results were compared to 4-plex qPCR and culture-method. When comparing agreement among tests, Cohen's Kappa statistic indicated a disagreement, beyond that due to chance, between $CRISPR_{O157:H7}$ qPCR and 4-plex, which may not be surprising after considering the target for each assay. While 42.2% (243/576) of samples were positive for EHEC O157:H7 by $CRISPR_{O157:H7}$ qPCR, the 4-plex qPCR detected the O157 serogroup-specific *rfb*E gene in 97.2% (560/576) of samples. Examination of other 4-plex gene targets revealed that 7.9% (44/560) of *rfb*E_{O157} positive samples were negative for Shiga toxin and/or intimin genes, making it unlikely that EHEC O157:H7 was present in these samples, and as previously mentioned and demonstrated in the fecal spike specificity assay, these major virulence genes can originate from a variety of other non-EHEC and non-O157 organisms. Cohen's Kappa statistic indicated a fair agreement, beyond that due to chance, between $CRISPR_{O157:H7}$ qPCR and culture-method; 16.8% (56/175) of samples that were culture positive

for EHEC O157:H7 were negative by $CRISPR_{O157:H7}$ qPCR. A minimum detection limit of between 5-100 CFU/g cattle feces has been reported on for IMS-based EHEC O157:H7 culture-method (Omisakin *et al.*, 2003; LeJeune *et al.*, 2006), which is comparable to the post-enrichment minimum detection limit of the $CRISPR_{O157:H7}$ qPCR (~10^0 CFU/g). However, similar to the results of the current study, we have previously reported on only a slight to fair agreement between PCR and culture-methods for detection of major non-O157 EHEC from cattle feces (Noll *et al*., 2015). In fact, 51.0% (124/243) of $CRISPR_{O157:H7}$ qPCR positive samples in this study were negative by culture-method, which may also be explained, in part, by qPCR detection of nonviable cells. Surprisingly, 56 of the fecal samples that were culture positive were negative by the $CRISPR_{O157:H7}$ qPCR assay. Extracted DNA from pure cultures of the 56 strains isolated from the fecal samples produced positive test results by $CRISPR_{O157:H7}$ qPCR. Whole genome sequences of EHEC O157:H7 isolated from a randomly selected subset of $CRISPR_{O157:H7}$ qPCR positive (n=10) and negative (n=10) fecal samples confirmed no sequence heterogeneity occurring at any of the CRISPR B and C primer/probe binding sites. Possibly, a 1-2 base pair mismatch between primers/probes and target may not affect amplification in pure culture, but could impact amplification in a fecal matrix, replete with PCR inhibitors (Hamner *et al.,* 2013; Hsu *et al.,* 2005). Furthermore, changes in PCR assay running conditions (temperature gradient, 2-step annealing and touchdown option) resulted in no new test-positive samples by the $CRISPR_{O157:H7}$ qPCR. It is likely that EHEC O157:H7 concentrations in the 56 samples were simply below the limit of detection for the $CRISPR_{O157:H7}$ qPCR assay.

Unlike other multiplex qPCR assays that have traditionally targeted the O157 serogroup-specific somatic antigen gene (*rfb*E) and other major EHEC virulence genes (*stx*1, *stx*2, *eae*), the

$CRISPR_{O157:H7}$ qPCR assay is novel in that it can detect and quantify EHEC O157:H7, the most clinically relevant EHEC serotype shed in cattle feces. The lack of strong agreement between IMS-based culture-method and the $CRISPR_{O157:H7}$ qPCR highlights the need to subject samples to both methods for more accurate EHEC O157:H7 detection. However, the assay targeting the CRISPR array is a sensitive and high-throughput method for serotype-specific detection and quantification of *E. coli* O157:H7 in cattle feces.

Table 3.1 Primers and probes[†] used to develop the multiplex quantitative PCR assays targeting clustered regularly interspaced short palindromic repeat sequences (CRISPR) loci for the detection and quantification of enterohemorrhagic *Escherichia coli* O157:H7

Target	Primer/probe	Sequence	Fluorescent dye	Quencher
CRISPR A locus	Forward primer	GAACACAAACCGAAACACACG		
	Reverse primer	ATAAACCGTCACCAAAACAGTG		
	Probe	ACAAAAACTGTCACCAAAGTGTTC	FAM	BHQ-1
CRISPR B locus	Forward primer	GGGAACACAAACCGAAACACA		
	Reverse primer	CTTAGTGTGTTCCCCGCGC		
	Probe	CGATCAATCCGAATATGAGCGGT	VIC	BHQ1
CRISPR C locus	Forward primer	GAACACTTTGGTGACAGTTTTTGT		
	Reverse primer	CTTAGTGTGTTCCCCGCGC		
	Probe	CACTGTTTTGGTGACGGTTTATCC	Texas Red	BHQ-2

[†]Primers and probes used to develop the multiplex qPCR assay were from a previous study (Delannoy *et al*., 2012)

Table 3.2 Cycle threshold (Ct) values and resulting PCR profile of spiked feces tested with CRISPR$_{O157:H7}$ qPCR assay and other assays targeting serogroup-specific antigens and or major virulence genes of major *E. coli* serogroups

Spike Treatment	Serotype	Strain	Major virulence gene profile				Target Ct and resulting PCR profile of each qPCR assay used to test spiked feces									
							CRISPR qPCR		†4-plex qPCR				‡non-O157 mqPCR-1			‡non-O157 mqPCR-2		
			*rfb*E$_{O157}$	*stx*1	*stx*2	*eae*	B locus	C locus	*rfb*E	*stx*1	*stx*2	*eae*	O26	O103	O111	O45	O121	O145
Spike 1	O157:H7	ATCC 43894	+	+	+	+	26.2	25.8	24.9	25.4	24.8	23.2	-	-	-	-	-	-
qPCR assay result							*E. coli* O157:H7$^+$		O157$^+$, *stx*1/2$^+$, *eae*$^+$				Negative for all targets			Negative for all targets		
Spike 2	O157:NM	08-4-553-F	+	-	-	-	-	-	24.6	24.5	-	22.1	24.5	24.8	24.7	-	-	-
	O157:H43	DEC7D	+	-	-	-												
	O157:H45	RN587/1	+	-	-	+												
	O26:H11	DEC10E	-	+	-	+												
	O103:H2	2014-5-7C	-	+	-	+												
	O111:H11	88-4110-nh	-	+	-	+												
qPCR assay result							Negative for all targets		O157$^+$, *stx*1$^+$, *eae*$^+$				O26$^+$, O103$^+$, O111$^+$			Negative for all targets		
Spike 3	O157:NM	08-4-553-F	+	-	-	-	-	-	24.7	25.5	24.7	22.7	-	-	-	24.6	24.6	24.5
	O157:H43	DEC7D	+	-	-	-												
	O157:H45	RN587/1	+	-	-	+												
	O45:H2	B8227-C8	-	+	-	-												
	O121:H19	8-084	-	-	+	+												
	O145:H28	IHIT0304	-	-	+	+												
qPCR assay result							Negative for all targets		O157$^+$, *stx*1/2$^+$, *eae*$^+$				Negative for all targets			O45$^+$, O121$^+$, O145$^+$		

†Four-plex qPCR assay (Noll *et al*., 2015)

‡Non-O157 mqPCR assays (Shridhar *et al*., 2015)

Table 3.3 Average detection limits, end-point cycle threshold (Ct) values, correlation coefficients and PCR amplification efficiencies of quantitative PCR of pure cultures of enterohemorrhagic *Escherichia coli* O157:H7 cultured in Luria Bertani broth

E. coli O157:H7 strain (virulence gene profile)	*Average end-point threshold cycle (Ct)		Detection limit (CFU/mL)	Correlation coefficients	PCR efficiency (%)
	CRISPR B array	CRISPR C array			
ATCC 43888 ($stx1^-$, $stx2^-$, eae^+)	39.9	39.5	$1.8x10^2$	> 0.99	94.7-101.1
ATCC 43889 ($stx1^-$, $stx2^+$, eae^+)	39.6	39.7	$1.5x10^2$	> 0.99	95.2-103.9
ATCC 43890 ($stx1^+$, $stx2^-$, eae^+)	39.4	39.4	$1.3x10^2$	> 0.99	101.2-105.1
ATCC 43894 ($stx1^+$, $stx2^+$, eae^+)	39.6	39.6	$1.7x10^2$	> 0.99	100.8-104.9

*Data are shown as means from two independent experiments

Table 3.4 Average detection limits, end-point cycle threshold (Ct) values, correlation coefficients and PCR amplification efficiencies of multiplex quantitative PCR of cattle feces spiked with enterohemorrhagic *Escherichia coli* O157:H7

E. coli O157:H7 Strain	*Average end-point threshold cycle (Ct)		Detection limit (CFU/g)	Correlation coefficients	PCR efficiency (%)
	CRISPR B array	CRISPR C array			
Before enrichment					
ATCC 43888	37.5	37.2	$2.1x10^3$	> 0.99	103.5-105.6
ATCC 43889	37.4	37.4	$2.0x10^3$	> 0.99	106.9-108.1
ATCC 43890	37.4	37.7	$2.2x10^3$	> 0.99	102.4-105.3
ATCC 43894	37.4	37.6	$2.2x10^3$	> 0.99	106.0-106.5
After enrichment (40° C for 6 h)					
ATCC 43888	37.2	37.8	$2.1x10^0$	> 0.99	107.1-126.7
ATCC 43889	38.3	38.2	$2.0x10^0$	> 0.99	109.1-110.0
ATCC 43890	37.5	37.7	$2.8x10^0$	> 0.99	95.2-96.8
ATCC 43894	37.8	37.9	$2.2x10^0$	> 0.99	109.9-117.3

*Data are shown as means from two independent experiments

Table 3.5 Detection of *Escherichia coli* O157:H7 from enriched cattle fecal samples (n=576) by CRISPR$_{O157:H7}$ qPCR, and agreement of samples positive and negative for O157 and O157:H7 by four-plex qPCR and culture-method, respectively

CRISPR$_{O157:H7}$ qPCR	Number of total samples positive and negative (%) by detection method			
	Culture method[†]		4-plex qPCR[‡]	
	Positive	Negative	Positive	Negative
Positive (n=243)	119 (49.0)	124 (51.0)	237 (97.5)	6 (2.5)
Negative (n=333)	56 (16.8)	277 (83.2)	323 (97.0)	10 (3.0)
Kappa Statistic (95% CI)	0.334 (0.258-0.411)		0.005 (-0.018-0.027)	
*Strength of Agreement	Fair		Slight	

[†]Based on immunomagnetic separation and plating on selective medium

[‡]Based on detection of *rfb*E$_{O157}$ gene (Noll et al., 2015)

*Based on the scale proposed by Landis and Koch (1977)

Chapter 4 - Whole Genome Sequencing-Based Identification and Comparative Analysis of Virulence Genes of *Escherichia coli* O103 of Bovine Fecal Origin

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) carry one or both phage-encoded Shiga toxin genes (*stx*1 and *stx*2) and the attaching and effacing gene (*eae*), which is harbored in the chromosomal-encoded locus of enterocyte effacement (LEE) pathogenicity island. Among EHEC pathotypes, O157:H7 serotype is most frequently associated with human foodborne illness, while O103 is ranked by the Centers for Disease Control and Prevention (CDC) as the second most common serogroup (next to O26) identified in laboratory confirmed non-O157 EHEC infections in the U.S. (CDC, 2012). In human EHEC infections, disease outcomes can range from mild to bloody diarrhea (hemorrhagic colitis) to more serious complications, such as hemolytic uremic syndrome (HUS), and even death (Abdullah *et al*., 2014). Differences in disease-causing potential, particularly the ability to cause serious complications, are attributed to differences in virulence of EHEC strains (Karmali, 1989). In addition to the major virulence factors, which include Shiga toxins and LEE gene-encoded proteins, other virulence attributes, including resistance to antimicrobials (da Silva and Mendonça, 2012) and known putative virulence factors, contribute to the development, progression, and outcome of the disease (Ewers *et al*., 2002; Jordan *et al*., 2004; Stevens *et al*., 2004). Enteropathogenic *E. coli* (EPEC), including EPEC O103, do not carry *stx* genes; however, they possess *eae* and other virulence genes to cause attaching and effacing lesions that can result in mild to severe diarrhea, or even

death, particularly in children (Trabulsi *et al*., 2002; Donnenberg *et al*., 2013). Strains within the EPEC pathotype are further characterized as typical or atypical, depending on presence or absence, respectively, of the EPEC adherence factor (EAF) plasmid (Nataro *et al*., 1998). The loss of the *stx* gene(s), a frequently reported event (Mellmann *et al*., 2008; Bielaszewska *et al*., 2008), can make an EHEC into an EPEC pathotype. These major pathotype defining mobile virulence genes have been well studied, but less is known about how other mobile elements contribute to the overall virulence diversity in O103 serogroup. Some strains of *E. coli* O103 carry neither Shiga toxin nor intimin genes, possibly a non-pathotype; even less is known about the virulence profiles of these strains. Cattle have been shown to harbor EHEC, EPEC and putative non-pathotype O103 in the hindgut and shed them in the feces (Noll *et al*., 2015). We hypothesize that the diversity of O103 pathotypes harbored and shed in the feces of cattle is reflective of the loss or acquisition of genes carried on mobile genetic elements.

Whole genome sequencing (WGS) has been used to characterize the virulence gene profiles of EHEC O157 (Perna *et al*., 2014), identify phylogenetic relationships between EHEC O157 and non-O157 serotypes (Ogura *et al*., 2009; Ison *et al*., 2015; Norman *et al*., 2015; Gonzalez-Escalona *et al*., 2016; Carter *et al*., 2015) as well as discover novel virulence determinants (Hayashi *et al*., 2001). However, differences in virulence gene profiles and phylogenetic relationships of O103 pathotypes are less characterized (Söderlund *et al*., 2016). Therefore, our objectives were to utilize WGS to identify and compare major and putative virulence genes and antimicrobial resistance genes, particularly genes located on mobile elements, of bovine and human clinical EHEC O103, bovine EPEC O103, and putative non-pathotype O103 strains and analyze phylogenetic relationships among the strains.

Materials and Methods

Strains

The bovine EHEC strains investigated in this study were isolated from cattle feces from several feedlots in the Midwest region of the US (Noll *et al*., 2015; Dewsbury *et al*., 2015; Cull *et al*., 2017). A total of 69 bovine O103 strains, previously identified by end-point PCR (Bai *et al*., 2012) as positive for *stx*1 (Shiga toxin 1) and *eae* (intimin) (bovine EHEC; n=43), negative for *stx*1 and positive for *eae* (bovine EPEC; n=13) and negative for both *stx*1 and *eae* (bovine putative non-pathotype; n=13) were used in the study. Human clinical O103 strains positive for *stx*1 and *eae* (human EHEC; n=6) were included in the study for comparison. The strains were cultured onto Tryptone soy agar (TSA; BD Difco, Sparks, MD) slants and shipped overnight in cold storage to the University of Maryland for whole genome sequencing.

DNA preparation and whole genome sequencing

The O103 strains from TSA slants were streaked onto blood agar (Remel, Lenexa, KS) and then subcultured in tryptone soy broth (BD Difco, Sparks, MD). Bacterial DNA from overnight culture was extracted from each strain using the DNeasy Blood and Tissue Kit with the QIAcube robotic workstation (Qiagen, Germantown, MD). The genomes were sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA) at approximately 37x average coverage. Genomic libraries were constructed using Nextera XT DNA Library Preparation Kit and MiSeq Reagent Kits v2 (500 Cycles) (Illumina, Inc.). *De novo* genome assembly was performed using SPAdes 3.6.0.

Genomic analysis

Draft genomes were annotated using Rapid Annotation using Subsystem Technology (RAST version 2.0 - http://rast.nmpdr.org/; (Aziz *et al*., 2008)), a web-based service commonly used for annotation of draft bacterial genomes (Kwon *et al*., 2016; Ferdous *et al*., 2015). Average number of genes located on mobile elements (prophages, transposable elements and plasmids), and genes related to virulence, disease and defense were determined, using RAST, for each of the O103 subgroups (bovine EHEC, human EHEC, bovine EPEC and bovine putative non-pathotype). Genomic sequencing data in this study exceeded the minimum criteria for analysis that RAST requires of each genome: at least 10x coverage (using 454 pyrosequencing) and 70% of assembled sequences in contigs > 20,000 base pairs. Serotype identity, virulence and AMR genes and plasmid make-up of the 75 strains were determined using default parameters of Center for Genomic Epidemiology Serotype-Finder 1.1 (https://cge.cbs.dtu.dk/services/SerotypeFinder/) (Joensen *et al*., 2015),Virulence Finder 1.4 (https://cge.cbs.dtu.dk//services/VirulenceFinder/) (Joensen *et al*., 2014), ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari *et al*., 2012), and PlasmidFinder 1.3 (Carattoli *et al*., 2014) programs, respectively. The number of prophage sequences in the 75 strains were determined using Phage Search Tool Enhanced Release (PHASTER; http://phaster.ca/) (Zhou *et al*., 2011; Arndt *et al*., 2016); intact, questionable and incomplete prophage sequences were defined by PHASTER scores >90, 70-90 and <70, respectively. The complete genome of EHEC O103:H2 strain 12009 (GenBank accession no. AP010958.1; https://www.ncbi.nlm.nih.gov/nuccore/AP010958.1) and 12009 plasmid pO103 DNA (GenBank accession no. NC_013354.1; https://www.ncbi.nlm.nih.gov/nuccore/NC_013354.1), a classical O103 reference strain of clinical origin used in many O103 genomic studies (Ogura *et al*., 2009;

Iguchi *et al*., 2012; Nadya *et al*., 2016), was tested with Virulence Finder 1.4, ResFinder 2.1, Plasmid Finder 1.3 and PHASTER as a control for comparison. The complete genomes EHEC O157:H7 Sakai (GenBank accession no. BA000007.2; https://www.ncbi.nlm.nih.gov/nuccore/BA000007.2) and EHEC O157:H7 EDL933 (GenBank accession no. CP008957.1; https://www.ncbi.nlm.nih.gov/nuccore/CP008957.1) and their associated plasmids (Sakai plasmid pO157: GenBank accession no. NC_002128.1, https://www.ncbi.nlm.nih.gov/nuccore/NC_002128.1; Sakai plasmid pOSAK1: GenBank accession no. NC_002127.1, https://www.ncbi.nlm.nih.gov/nuccore/NC_002127.1; EDL933 plasmid pO157: GenBank accession no. AF074613.1, https://www.ncbi.nlm.nih.gov/nuccore/AF074613.1) were also tested for comparison. Parsnp v1.2 (http://harvest.readthedocs.io/en/latest/content/parsnp.html) (Treangen *et al*., 2014) was used for core genome alignment of the 75 strains and subsequent construction of a maximum likelihood tree. For improved visualization, an equal branch transformation of the output file (.tree) from Parsnp was performed using FigTree 1.4 software (http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut, 2014) and bootstrap values were reported for each branch.

Statistical analysis

A single factor analysis of variance (ANOVA) test was performed to determine whether average genome size, and average number of extra-chromosomal genes and virulence, disease and defense genes were significantly different among the four subgroups (bovine EHEC, human EHEC, EPEC and putative non-pathotype). If means were significantly different ($P < 0.01$),

Tukey adjustment for multiple comparisons was performed, using SAS 9.4 with Proc Glimmix, to test each pairwise comparison for significant differences ($P < 0.01$).

Nucleotide sequence accession numbers

Draft genome sequences of the 75 *E. coli* O103 strains are available in GenBank and their accession numbers are listed in tables A.1, A.2, and A.3.

Results

All bovine EHEC strains (43/43; 100%) and a majority of EPEC (12/13; 92.3%) and putative non-pathotype strains (12/13; 92.3%) were O103:H2 serotype. The two remaining strains of EPEC (1/13) and putative non-pathotype (1/13) were O103:H11 and O103:H16 serotypes, respectively. Four of the six human EHEC strains were O103:H11 and two were O103:H2 serotype.

RAST subsystem summary

Genome size range of bovine (5.32-5.79 Mb) and human EHEC (5.43-5.77 Mb) subgroups were similar (Table 4.1; P=0.98). However, both EHEC subgroups had significantly larger average genome sizes ($P < 0.0001$) compared to EPEC and putative non-pathotype subgroups. Average genome size was similar between EPEC and putative non-pathotype subgroups. However, the bovine EPEC O103:H11 strain (2013-3-492A) had a similar genome size (5.67 Mb) to that of other EHEC strains. A bovine putative non-pathotype O103:H16 strain (2013-3-111C) had the smallest genome (4.76 Mb) of all the strains analyzed.

Overall, the number of genes in the category of virulence, disease and defense was comparable for all 75 strains tested (Table 4.1), with no significant differences observed among the mean number of genes of O103 subgroups. However, the number of genes on mobile elements (prophages, transposable elements, and plasmids) varied considerably among O103 subgroups and among serotypes within subgroups. Strains belonging to bovine and human EHEC subgroups had a significantly higher ($P < 0.001$) number of mobile genes compared to EPEC and putative non-pathotype subgroups. Average number of mobile genes was not significantly different between bovine and human EHEC subgroups or between EPEC and putative non-pathotype subgroups. The bovine EHEC strains possessed the widest range in the number of genes on mobile elements (221-351). Similarly, wide ranges were observed in bovine EPEC strains (137-289 genes) and bovine putative non-pathotype strains (100-157 genes), but not in human EHEC strains (256-292 genes). Mobile gene counts above 300 were only observed in a few bovine EHEC strains (4/43), and one bovine EHEC strain (2014-5-933A) had 351 mobile genes, nearly 60 more than the highest number in strains of the human EHEC subgroup. Furthermore, the one bovine EPEC O103:H11 (strain 2013-3-492A) had 289 mobile genes and 76 more mobile genes than the highest number in strains within the EPEC O103:H2 subgroup.

A strong correlation (R^2=0.70) was observed between genome size vs. number of genes on mobile elements for the 75 strains (Figure 4.1). The EHEC strains had larger genome size and higher number of genes on mobile elements compared to EPEC and putative non-pathotype strains. The EPEC O103:H11 strain (2013-3-492A) appeared to be an EPEC outlier, with genome size and number of genes on mobile elements closer to those of the EHEC O103 strains (Figure 4.1).

Virulence genes

Virulence genes with >90% sequence homology were considered to be positively identified in a genome. The complete virulence gene profiles of each genome are shown in supplementary tables A.1, A.2, and A.3. All EHEC strains were positive for Shiga toxin 1a (*stx*1a) subtype. On average, bovine and human EHEC strains were positive for more virulence genes than EPEC strains; putative non-pathotype strains were negative for all LEE encoded, non-LEE encoded, and pO157 plasmid-encoded genes (Table 4.2).

Among LEE-encoded genes, all EHEC and EPEC strains were positive for *eae*, translocated intimin receptor protein (*tir*), and type III secretion effectors (*esp*A and *esp*B), but a small number of bovine EHEC (4/43) and EPEC O103:H2 strains (3/12) were negative for type III secretion effector gene, *esp*F (Table 4.2). All EHEC/EPEC O103:H2 and O103:H11 serotype strains were positive for *eae*-epsilon and *eae*-beta1 subtypes, respectively. Other phage-encoded type III secretion effector genes (*cif*, *esp*J, and *tcc*P) were present in all human EHEC O103:H2 strains but observed at varying proportions for other EHEC and EPEC O103 subgroups. Non-LEE encoded effectors A (*nle*A) and B (*nle*B) were present in all EHEC strains, in the EPEC O103:H11 strain, but also in a majority of EPEC O103:H2 strains (6/12 for *nle*A and 10/12 for *nle*B). The *nle*C gene, absent in two human EHEC O103:H2 strains, was present in all human EHEC O103:H11 strains (4/4) and also in over half of bovine EHEC O103:H2 strains (23/43; 53.5%).

Among pO157 plasmid-encoded genes (*ehx*A, *esp*P, *etp*D, *kat*P and *tox*B), enterohemolysin (*ehx*A) and extracellular serine protease (*esp*P) were present in most, but not all EHEC and EPEC strains (Table 4.2). Conversely, toxin B gene (*tox*B), a homolog of EHEC factor for adherence gene (*efa*1), was found in only 2/6 (33.3%) human clinical EHEC and in only one

bovine EHEC strain (2014-5-941B). The *efa*1 gene, not encoded on the pO157 plasmid, was present in a higher proportion of EHEC strains (41/49; 83.7%), compared to *tox*B; interestingly, bovine EPEC O103:H11 strain was also positive for *efa*1 gene (Table 4.3). All EPEC strains in this study were negative for the EAF plasmid.

The putative virulence genes that were present in the O103 strains are shown in Table 4.3. Of all adherence-based genes in EHEC and EPEC strains (Tables 4.2 and 4.3), only long polar fimbriae gene (*lpf*A) was present in putative non-pathotype strains. The *lpf*A gene was also present in all human EHEC O103:H11 strains (n=4) and in the EPEC O103:H11 strain, but was not detected in O103:H2 strains within bovine and human EHEC and bovine EPEC subgroups or within any of the human EHEC control strains (O103:H2 12009, O157:H7 Sakai, O157:H7 EDL933). ABC transporter protein MchF (*mcf*F), MchC protein (*mch*C), Microcin H47 part of colicin H (*mch*B) and Microcin M part of colicin H (*mcm*A) genes were present in 5/12 (41.7%) bovine putative non-pathotype O103:H2 strains but absent in all other strains. The colicin M gene (*cma*) was found in 5 of 12 putative non-pathotype O103:H2 strains, but also in one bovine EHEC O103:H2 (strain 2014-5-1565C). Glutamic acid decarboxylase (*gad*) was present in all 75 strains. EAST-1 toxin gene (*ast*A), encoding for an enterotoxin, was in all O103:H11 strains (human EHEC and bovine EPEC) in the study, and in a majority of bovine EPEC O103:H2 strains (9/12), but not in any of the EHEC O103:H2 strains. Endonuclease colicin E2 gene (*celb*) was present in nearly half (20/43) of all bovine EHEC strains, and in the bovine EPEC O103:H11 strain, but absent from all other subgroups.

Plasmid and prophage sequences

The complete plasmid profiles and number of prophage sequences from each genome are shown in supplementary tables A.4, A.5, and A.6. Plasmid profiles exhibited some commonality among strains within an O103 subgroup but varied dramatically between subgroups. Five plasmids, including sequences of IncFIA(HI1), IncFII(pRSB107), IncFII(pSE11), IncX1 and IncY were present at varying proportions in bovine EPEC O103:H12 strains, but absent from all other subgroups (Table 4.4). Similarly, strains from bovine EHEC were positive for IncA/C2, IncFII(pCoo) (enterotoxigenic *E. coli* associated plasmid), IncI2 and IncN plasmid sequences, while other subgroups were negative for these plasmids sequences. A high proportion of bovine EHEC (19/43; 44.2%) and the bovine EPEC O103:H11 strains were positive for Col156 plasmid sequence, while strains from all remaining subgroups were negative for this plasmid sequence. Among the nineteen total plasmid types identified in the strains used in this study, nearly half (9/19; 47.4%) belonged to the IncF incompatibility family. The IncFIB (*E. coli* K-12) plasmid sequence was most prevalent among the 75 strains, found in 39/43 (90.7%) bovine EHEC strains and in all human EHEC (6/6) and O103:H2 putative non-pathotype strains (12/12). The IncFIB plasmid sequence was present in the bovine EPEC O103:H11 strain, but absent from all EPEC O103:H2 strains.

The number of intact, questionable and incomplete prophage sequences for all 75 strains ranged from 0 to 6, 0 to 5 and 0 to 15, respectively (Table 4.5). Bovine EHEC were positive for a slightly higher average number of intact and incomplete prophage sequences (3.3), compared to human EHEC (2.8), bovine EPEC (2.3) or bovine putative non-pathotype (2.5) subgroups, while bovine EPEC were positive for the highest average number of questionable prophage sequences (2.6).

Antimicrobial resistance genes

Resistance genes with >90% sequence homology were considered to be positively identified in a genome. The complete resistance gene profiles of strains that were positive for AMR genes are listed in table A.7. A relatively high proportion (10/43; 23.3%) of bovine EHEC strains were positive for at least one AMR gene, and among these strains, 3/10 were positive for ten AMR genes representing six drug classes (aminoglycoside, beta-lactam, macrolide, phenicol, sulphonamide and tetracycline; Table A.7). Human EHEC O103:H11 (strain KSU-75) was positive for 8 AMR genes representing seven drug classes. Interestingly, none of the bovine EPEC strains were positive for AMR genes, yet 5/12 (41.7%) bovine putative non-pathotype O103:H2 strains tested positive for AMR genes representing aminoglycoside and tetracycline drug classes; three of these strains were also positive for a sulphonamide resistance gene (*sul*2) (Table A.6). None of the human EHEC control strains (O103:H2 12009, O157:H7 Sakai, O157:H7 EDL 933) were positive for AMR genes.

Phylogenetic relationships

A maximum likelihood phylogenetic tree, based on core genome alignment of all 75 strains, was constructed using Parsnp v.1.2. The output file was equal branch transformed using FigTree 1.4 (Figure 4.2). Overall, strains clustered according to pathotypes, with one notable exception: bovine EPEC O103:H11 strain was more closely related to a human EHEC O103:H11 (strain KSU-74) than to any of the other bovine EPEC strains included in the study (Figure 4.2). All EPEC O103:H2 strains clustered together and putative non-pathotype strains exhibited a similar clustering. One human EHEC O103:H2 strain (KSU-72) was more closely related to two bovine

EHEC O103:H2 strains (2014-5-330A and 2014-5-332A) than to the other human EHEC O103:H2 strain (KSU-71) included in the study.

Discussion

Serogroup O103 is the third most common EHEC (next to O157 and O26) implicated in human STEC infections (CDC, 2012; Brooks *et al*., 2005), but based on our studies, the second most prevalent STEC (next to O157) shed in cattle feces (Noll, *et al*., 2015; Dewsbury *et al*., 2015). Brooks *et al.* (2005) have reported that 117 human clinical O103 isolates, submitted to CDC from 1983 to 2002, were positive for *stx*1 and negative for *stx*2, and included only four flagellar types, H2, H11, H25 and non-motile. Similarly, all Shiga toxin-producing strains of cattle origin in this study (n=43) were positive for *stx*1 gene only, however, all possessed the H2 flagellar type. The predominance of the H2 flagellar type in bovine strains is in agreement with previous reports of O103 strains in cattle and sheep (Söderlund *et al*., 2016; Blanco *et al*., 2004; Padola *et al*., 2004; Sekse *et al*., 2013). The majority of EHEC strains (48/49; 98.0%) in our study had Shiga toxin 1a (*stx*1a) gene. Söderlund *et al.* (2015) report Shiga toxin 1a (*stx*1a) subtype present in five EHEC O103:H2 isolated from Swedish cattle. Similar to findings from previous studies (Söderlund *et al*., 2015; Iguchi *et al*., 2012), all EHEC/EPEC O103:H2 and O103:H11 strains carried epsilon and beta1 *eae* subtypes, respectively. All EPEC strains included in this study were considered atypical, as indicated by the absence of the EAF plasmid, a finding also in agreement with previous studies (Söderlund *et al*., 2015; Sandu *et al*, 1999; Paddock *et al*., 2014).

All EHEC O103 strains in this study (43 bovine and 6 human strains) had a higher number of genes on mobile elements (prophages, transposable elements, and plasmids) compared to the

bovine EPEC (except for one O103:H11 strain) and putative non-pathotype strains. Significant differences in the genome size observed between the O103 subgroups are reflective of the number of genes from mobile elements. However, one bovine EPEC O103:H11 strain (2013-3-492A) was an exception as its genome size and number of genes on mobile elements were more comparable to EHEC strains (Figure 4.1); furthermore, this strain was more closely related to a human EHEC O103:H11 strain (KSU-74) than to any of the EPEC strains (Figure 4.2). Also, the virulence gene profile of the EPEC O103:H11 strain 2013-3-492A more closely resembled the virulence gene profiles of the EHEC O103 subgroup than that of the bovine EPEC O103 subgroup. Furthermore, the strain is positive for *stx*1 bacteriophage insertion site (*yehV*) and bacteriophage-*yehV* right and left junctions (Shaikh *et al*., 2003), suggesting that the EPEC O103:H11 strain may be capable of acquiring and/or had once acquired but lost *stx* gene(s). This suggests that much of the genetic diversity in *E. coli* O103 strains shed in cattle feces can be attributed to the loss or to acquisition of mobile genetic elements (Ochman *et al*., 2000).

Although overall number of genes implicated in virulence, disease and defense was comparable among all 69 bovine strains, a closer examination revealed key differences in virulence gene profiles of O103 subgroups and serotypes within subgroups.

LEE effector genes

The chromosomal LEE pathogenicity island carries genes that encode for intimin (*eae*), translocated intimin receptor protein (*tir*), and type III secretion system effector proteins (*esp*A and *esp*B). Studies have shown that without any one of these genes (*eae*, *tir*, *esp*A, *esp*B), attaching and effacing (A/E) *E. coli* are unable to produce their characteristic A/E lesions (McDaniel *et al*., 1995; McDaniel and Kaper, 1997; Abe *et al*., 1998). The *esp*F gene is also

LEE encoded, but unlike the other LEE genes that were present in all EHEC and EPEC strains, a small number of bovine EPEC (3/13) and EHEC (4/43) strains were *esp*F-negative. Although *esp*F contributes to the disruption of intestinal barrier function during attachment, McNamara *et al*. (2001) have shown that the gene is not required for A/E lesion formation. Other type III effector genes (*cif*, *esp*J, and *tcc*P) were variably present in the EHEC and EPEC strains, possibly, because they are prophage-encoded genes. The cycle inhibiting factor gene (*cif*) is responsible for the arrestment of the host eukaryotic cell cycle during attachment (Marchés *et al*., 2003), while *esp*J has been shown to contribute to higher levels of *in vivo* colonization in inoculated mice, compared to *esp*J mutants (Dahan *et al*., 2005). Although *cif* and *esp*J genes enhance attachment, *in vivo* and/or *in vitro* studies have shown that A/E lesions are not significantly inhibited in the absence of either gene (Marchés *et al*., 2003; Dahan *et al*., 2005). Both genes were present in all six human EHEC strains and in a majority (43/43 for *cif* and 38/43 for *esp*J) of bovine EHEC. Garmendia *et al*. (2004) have shown that *tir*-cytoskeleton coupling protein gene (*tcc*P) assists in the translocation of the intimin receptor protein during bacterial attachment. In the same study, *tcc*P mutants were unable to trigger A/E lesions on *in vitro*-inoculated HeLa epithelial cells. Considering its seemingly critical importance in type III secretory system-related disease outcomes, it is surprising that not all human clinical EHEC were positive for the *tcc*P gene. Garmendia *et al*. (2004) did report that *tir* translocation was not affected in *tcc*P mutants, therefore, it is possible that bacterial attachment and expression of other virulence factors in *tcc*P-negative EHEC could contribute to A/E lesions.

Non-LEE effector genes

Non-LEE effector (*nle*) genes, including *nle*A, *nle*B and *nle*C, have been associated with HUS-causing strains of EHEC (Bugarel *et al*., 2011) and were present in varying proportions within EHEC and EPEC O103 subgroups in this study. In two independent studies, Δ*nle*A (Gruenheid *et al*., 2004) and Δ*nle*B mutant strains of *Citrobacter rodentium* (Wickham *et al*., 2006) were unable to cause mortality in inoculated mice. Wickham *et al*. (2006) also reported a three-log decrease (10^6 vs. 10^3) in infectious dose for *nle*B wildtype- compared to Δ*nle*B-mutant, which highlights the importance of *nle*B gene as it relates to the low infectious dose of EHEC strains. Interestingly, *nle*A and *nle*B genes were present in all human and bovine EHEC but in fewer bovine EPEC strains. The *nle*C gene serves to down-regulate host NF-κB signaling pathway in efforts to disrupt immune clearance of invading bacteria (Yen *et al*., 2010). Although *nle*C has also been significantly associated with HUS-causing strains (Bugarel *et al*., 2011), it was present only in 4 of 6 human clinical EHEC strains, but in 53.5% (23/43) of bovine EHEC strains.

pO157 plasmid encoded virulence genes

The pO157 plasmid (~93 kb) carries a number of virulence genes implicated in EHEC virulence (Makino *et al*., 1998) and is present in nearly all clinical O157:H7 strains (Schmidt *et al*., 1996). Major pO157 plasmid-encoded genes, *ehx*A, *esp*P, *etp*D, *kat*P and *tox*B, were present in many EHEC and EPEC O103 strains. The enterohemolysin gene (*ehx*A), present in all EHEC (49/49) and nearly all EPEC (12/13) strains in this study, encodes for a pore-forming toxin, which elicits *in vivo* production of IL-1β from human mononuclear cells, a commonly expressed cytokine during HUS infections (Taneike *et al*., 2002). The extracellular serine protease gene

(*esp*P) was found in almost all EHEC and EPEC strains and is considered to contribute to hemorrhagic colitis via the cleavage of pepsin A and human coagulation factor V (Brunder *et al*., 1997).

The *etp*D, *kat*P and *tox*B genes, located on the pO157 plasmid, were less frequently present in EHEC and EPEC strains, compared to *ehx*A and *esp*P genes. Schmidt *et al*. (1997) report that EHEC type II secretion pathway (*etp*) genes are not commonly detected (~10%) in bovine EHEC isolated from feces. In this study, *etp*D gene was present in 9 of 43 (20.9%) of bovine EHEC strains, but absent in the other subgroups. Brunder *et al*. (1996) report a close association between the presence of *ehx*A and the catalase peroxidase gene (*kat*P) in EHEC O157:H7 strains. We observed a similar trend for bovine and human EHEC; however, *ehx*A was present in a majority (11/12) of bovine EPEC O103:H2, whereas *kat*P was absent in all of those strains. The *tox*B gene, identified by Tatsuno *et al.* (2001), is a homolog of EHEC factor for adherence gene (*efa*1), carried on the pO157 plasmid and is commonly present in clinical EHEC O157:H7. In a study examining the prevalence of *tox*B in O157 and major non-O157 EHEC and EPEC of clinical and animal origin, Tozzoli *et al*. (2005) report all O103 strains used in their study were negative for the gene. In the current study, 3 of 6 human EHEC strains were positive for *tox*B. Yet, the gene was present in only 1/43 bovine EHEC strains and in the single bovine EPEC O103:H11 strain. Although *tox*B is not required for formation of A/E lesions, Tatsuno *et al*. (2001) showed that expression of *tox*B does lead to enhanced virulence by increasing expression of major LEE-encoded effector genes including *esp*A, *esp*B and *tir*.

Other virulence genes

Studies (Stevens *et al*., 2004; Deacon *et al*., 2010) have shown that *efa*1 gene, harbored on the O122 pathogenicity island, plays a significant role in bovine intestinal colonization. Although some have reported a strong association of the *efa*1 gene with non-O157 EHEC and EPEC strains (Bardiau *et al*., 2009; Bardiau *et al*., 2010), 8 of 43 bovine EHEC strains and all bovine EPEC O103:H2 strains (n=12) were negative for *efa*1. Enteroaggregative *E. coli* (EAEC) heat-stable enterotoxin gene (*ast*A), traditionally associated with EAEC strains (Savarino *et al*., 1996), has been shown to be present in EPEC and EHEC strains (de Sousa *et al*., 2001). This was true for O103 strains in this study; however, while *ast*A gene was present in a majority of EPEC (10/13), among EHEC, the gene was only found in O103:H11 human strains. This is not surprising, considering that the enterotoxin is linked to diarrheagenic disease outcomes in EPEC infected children (Trabulsi *et al*., 2002).

The long polar fimbriae (*lpf*A) gene has been associated with increased colonization rates of O157:H7-inoculated sheep and pigs (Jordan *et al*., 2004). Interestingly, *lpf*A was the only adherence-based virulence gene present in the bovine putative non-pathotype O103:H2 strains (n=12). Yet the gene was absent in all EHEC (n=43) and EPEC O103:H2 (n=12) strains, suggesting possible loss of *lpf*A gene by O103:H2 serotype at some point during the course of acquiring new genetic elements. The *irg*A homolog adhesion gene (*iha*) was present in all O103:H11 serotype of EHEC and EPEC strains in this study, but absent in many of the O103:H2 serotype of EHEC and EPEC strains.

The glutamic acid decarboxylase gene (*gad*) catalyzes the α-decaboxylation of glutamic acid to produce γ-aminobutyric acid and CO_2 (Smith *et al*., 1992), and was the only virulence gene present in all 75 strains in this study. The conserved nature of this gene is likely a reflection of

its important biological function, which is to maintain pH homeostasis of the cell, particularly during passage through the acidic environment of the gut (De Biase *et al*., 1999). The gene for increased serum survival (*iss*) was the second most prevalent virulence gene among all 75 strains. Interestingly, the *iss* gene is often associated with avian pathogenic *E. coli* (APEC) that cause colibacillosis in poultry, and serves as a genetic marker for APEC strains (Ewers *et al*., 2002). Expression of the *iss* gene allows for increased resistance to bactericidal effects of serum, thereby enabling rapid bacterial cell growth in the host (Ewers *et al*., 2002). The *iss* gene was present in all human EHEC strains in this study, but is not considered to play a major role in human infection (Johnson *et al*., 2008). Among APEC, the *iss* gene is carried by a ColV plasmid (Johnson *et al*., 2006) that in addition to conferring increased virulence and fitness traits, also encodes for multidrug resistance (Johnson *et al*., 2008). Interestingly, the APEC ColV plasmid bears a similar sequence identity to an *iss*-carrying ColV plasmid commonly found in *Salmonella* serovar Kentucky, a strain that has only recently emerged in both poultry and cattle populations (Johnson *et al*., 2008). Johnson *et al*. (2008) have speculated that *S*. Kentucky may have horizontally acquired the APEC ColV plasmid which as a result, has increased its virulence potential.

The *E. coli* secreted protease island encoded gene (*esp*I) is considered part of the family of extracellular proteases known as SPATE, or serine protease autotransporters of *Enterobacteriaceae* (Dautin, 2010). The *esp*I gene is harbored on the O91:H^{-} pathogenicity island and was first described by Schmidt *et al*. (2001). Based on their findings, Schmidt *et al*. (2001) concluded that the O91:H^{-} island occurs exclusively in a LEE-negative subgroup of STEC that carry a *stx*2d gene variant. Krüger *et al*. (2015) also report detection of *esp*I gene exclusively in *stx*2- (but not *stx*1) positive *E. coli* O26:H11 strains of clinical, bovine and food

origin. In our study, *esp*I gene was present in more than half (23/43; 53.5%) of all bovine EHEC O103:H2 that were *stx*1a positive; *esp*I gene was also present in three of 12 bovine EPEC O103:H2 strains. These results are in contrast with previous studies linking the *esp*I gene to *stx*2-carrying EHEC only (Schmidt *et al*., 2001; Krüger *et al*., 2015) and may be the first time *esp*I gene has been reported in bovine EHEC and EPEC O103 strains. It is worth noting that ABC transporter protein MchF (*mcf*F), MchC protein (*mch*C), Microcin H47 part of colicin H (*mch*B), and Microcin M part of colicin H (*mcm*A) genes were present in only 5 of 12 bovine putative non-pathotype O103:H2 strains. Considering the absence of these genes in all other O103 subgroups included in the study, it is possible that horizontal acquisition of additional genes may impact the occurrence of *mcf*F, *mch*C, *mch*B, and *mcm*A genes in EHEC/EPEC O103:H2 and O103:H11.

Plasmid and prophage sequences

Considering their highly mobile nature, it is not surprising that plasmid make-up differed between O103 subgroups, and also to some extent within a subgroup. Some of these plasmid sequences are originally associated with non-*E. coli* bacteria, including *Klebsiella pneumoniae* (ColRNAI and IncA/C2), *Salmonella typhi* (IncFIA(HI1)), *Salmonella typhimurium* (IncN) and *Pseudomonas aeruginosa* (IncP), which further highlights the mobility of these genetic elements. Many of the plasmids, including IncA/C2, IncFII, IncFII(pHN7A8), IncFII(pRSB107), IncN and IncX1 have also been associated with AMR determinants and/or other putative virulence-associated functions, that in some cases have been the causative genetic element behind human outbreaks (Boyd *et al*., 2004). The IncF incompatibility family represents the majority of virulence-associated plasmids carried by *E. coli* (Johnson *et al*., 2009), therefore it may not be

surprising that IncF plasmids represented nearly half (96/218; 44.0%) of all total plasmids identified in the strains used in this study.

Antimicrobial resistance genes

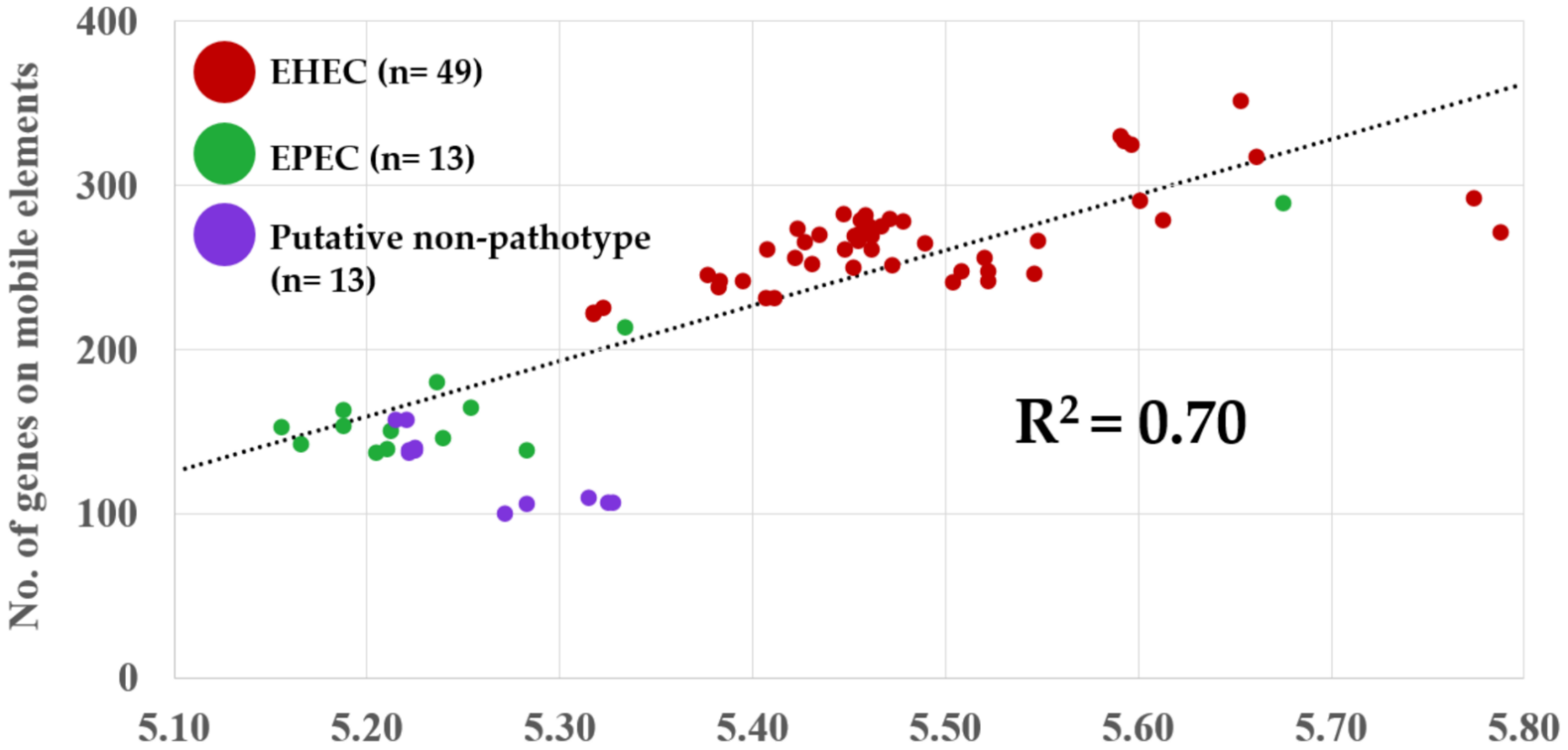
Considering that bovine putative non-pathotype O103:H2 strains were negative for many of the virulence genes common in strains from other O103 subgroups, it is surprising that nearly 42% (5/12) of these strains were positive for AMR genes, compared to a much lower proportion of AMR prevalence in bovine EHEC (10/43; 23.3%), human EHEC (1/6; 16.7%) or bovine EPEC strains (0/13). Although these are *in silico* results, they are in contrast with a study (Beier *et al*., 2016) that reported a high rate of AMR prevalence in human EHEC O103 strains (50%; 10/20) but none in animal EHEC O103 strains (n=11). A high degree of multi-drug resistance was observed in the gene profiles of three bovine EHEC strains (2014-5-841G, 2014-5-863D and 2014-5-1565C), and interestingly, all three were isolated from samples collected from the same feed yard (Table A.4). It is unclear why bovine EPEC were negative for AMR genes, especially considering that many of these strains were isolated from the same feed yard pens as other AMR positive bovine strains.

Conclusion

The virulence gene profiles of the bovine and human EHEC, bovine (atypical) EPEC and putative non-pathotype strains of *E. coli* O103 were quite diverse. The difference in the number of strains tested within each subgroup and lack of publicly available O103 genome sequences may have limited the strength of comparison. Although the *in silico* analysis performed here does not provide phenotypic evidence of virulence contributions, a number of major and putative

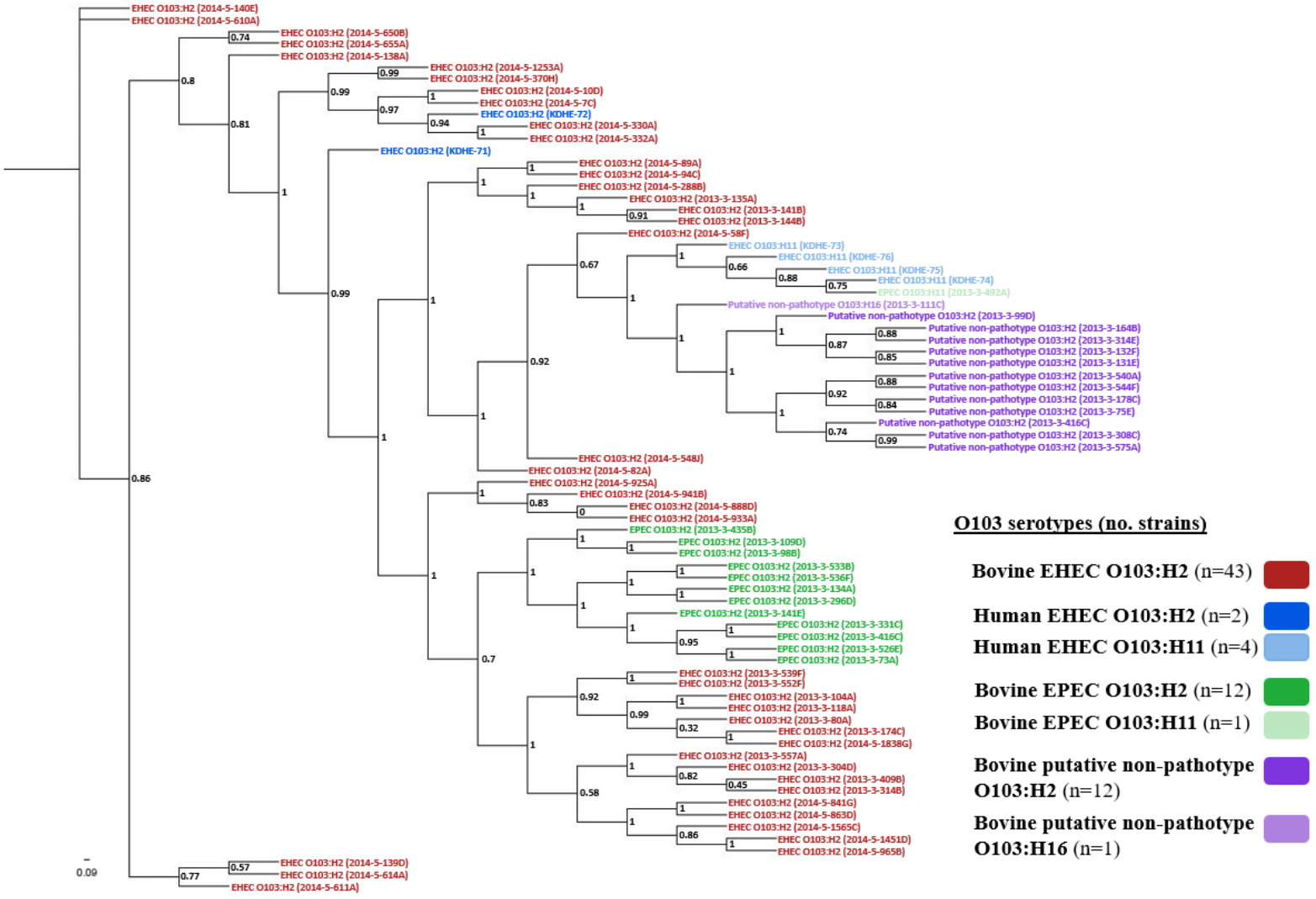
virulence genes were comparable among bovine and human EHEC O103 strains, which may indicate the potential for bovine EHEC O103 to cause human infection. The bovine EPEC O103:H11 strain also shared similar virulence gene and plasmid profiles with human EHEC O103:H11 strains, raising the possibility that the EPEC may have lost its *stx* prophage. Regardless, the *in silico* data highlight the numerous virulence genes carried on mobile genetic elements (prophages, transposable elements, and plasmids) that contribute to the plasticity of bovine EHEC or EPEC. Genome size and number of genes from mobile elements were strongly correlated between the O103 subgroups. The putative non-pathotype strains had the smallest genome size and were positive for the fewest overall number of mobile genes and perhaps related to this, lacked any specific major or putative mobile virulence genes. The EPEC strains in this study had larger genomes and were positive for a higher number of specific virulence genes compared to putative non-pathotype strains. Excluding the outlying EPEC O103:H11 strain, the EHEC overshadowed EPEC, and putative non-pathotype subgroups in both these categories, which raises the question whether progenitor EHEC bacteria are more genetically predisposed toward acquiring certain mobile elements that could confer virulence. Conversely, putative virulence genes that allow for increased EHEC survival within the environment or within a host may afford EHEC with increased opportunity to acquire mobile genetic elements. We believe that the diversity of pathotypes of *E. coli* O103 harbored and shed in the feces of cattle is reflective of the loss or acquisition of genes carried on mobile genetic elements. The environmental and biological mechanisms that allow for loss or acquisition of virulence genes by EHEC and EPEC and putative non-pathotype strains remain an exciting frontier for the whole-genome sequence-based analysis of *E. coli* pathotypes.

Figure 4.1 Scatterplot of genome sizes and number of genes on mobile elements[†] of 75 strains of enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103



[†]Genome sizes and number of genes located on mobile elements (prophages, transposable elements and plasmids) were determined using Rapid Annotation Using Subsystem Technology (RAST).

Figure 4.2 Equal branch transformed phylogenetic tree[†] of 75 strains of enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 of bovine and human origin using FigTree 1.4



[†]Numbers on the branches correspond to bootstrap values.

Table 4.1 Average genome size, guanine-cytosine (GC) content, and number of contigs and average number of extra-chromosomal genes, virulence, disease and defense genes and plasmids of enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin

Genome size and gene categories[†]	Host origin, pathotype and serotype (no. of strains tested)						
	Bovine EHEC	Human EHEC		Bovine EPEC		Bovine putative non-pathotype	
	O103:H2 (n=43)	O103:H2 (n=2)	O103:H11 (n=4)	O103:H2 (n=12)	O103:H11 (n=1)	O103:H2 (n=12)	O103:H16 (n=1)
Genome size (Mb)	5.47	5.45	5.61	5.22	5.67	5.26	4.76
	(5.32-5.79)	(5.43-5.46)	(5.52-5.77)	(5.16-5.33)		(5.21-5.33)	
GC content (%)	50.58	50.6	50.43	50.54	50.5	50.44	50.8
	(50.5-50.6)	(50.6-50.6)	(50.4-50.5)	(50.5-50.6)		(50.4-50.5)	
Contigs	312	350	432	204	406	140	132
	(181-398)	(339-360)	(412-453)	(137-268)		(99-172)	
Virulence, disease and defense	113	111	112	114	109	115	110
	(111-124)	(111)	(109-121)	(113-117)		(115-116)	
Prophages, transposable elements and plasmids	260	265	273	157	289	128	101
	(221-351)	(260, 270)	(256-292)	(137-213)		(100-157)	
Plasmids	2.9	2.5	4	2	5	3	1
	(1-6)	(2-3)	(3-6)	(0-4)		(3-4)	

[†]Genome sizes, GC content, contigs, virulence, disease and defense and mobile element (prophages, transposable elements and plasmids) data were determined using Rapid Annotation Using Subsystem Technology (RAST). Plasmid data was determined using PlasmidFinder 1.3.

Table 4.2 Major chromosomal-, phage-, and plasmid-encoded virulence genes in enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin[†]

Origin	Protein and gene		Host origin, pathotype and serotype (no. of strains)						
			Bovine EHEC	Human EHEC		Bovine EPEC		Bovine putative non-pathotype	
			O103:H2 (n=43)	O103:H2 (n=2)	O103:H11 (n=4)	O103:H2 (n=12)	O103:H11 (n=1)	O103:H2 (n=12)	O103:H16 (n=1)
Locus of enterocyte effacement (LEE) encoded	Intimin	***eae***	43	2	4	12	1	0	0
	Translocated intimin receptor	***tir***	43	2	4	12	1	0	0
	Type III secretions system	***espA***	43	2	4	12	1	0	0
	Secreted protein B	***espB***	43	2	4	12	1	0	0
	Type III secretion system	***espF***	39	2	4	9	1	0	0
Non-LEE encoded	Effector A	***nleA***	43	2	4	6	1	0	0
	Effector B	***nleB***	43	2	4	10	1	0	0
	Effector C	***nleC***	23	0	4	0	0	0	0
Phage-encoded Shiga toxin	O157 FLY16, variant a	***stx*1**	42	2	4	0	0	0	0
	Shigella dysenteriae 3818T	***stx*1**	1	0	0	0	0	0	0
	Shiga toxin 1, subunit A, variant a	***stx*1A**	43	2	4	0	0	0	0
	Shiga toxin 1, subunit B, variant a	***stx*1B**	43	2	4	0	0	0	0
Phage-encoded type III secretion effectors	Cycle inhibiting factor	***cif***	43	2	4	0	1	0	0
	Type III secretion system effector	***espJ***	38	2	4	0	1	0	0
	Tir-cytoskeleton coupling protein	***tccP***	41	2	3	12	0	0	0
pO157 plasmid-encoded	Enterohaemolysin	***ehxA***	43	2	4	11	1	0	0
	Extracellular serine protease	***espP***	37	2	4	11	1	0	0
	Type II secretion protein	***etpD***	9	0	0	0	0	0	0
	Catalase peroxidase	***katP***	29	2	4	0	1	0	0
	Toxin B	***toxB***	1	1	1	0	1	0	0

[†]Virulence genes were determined using Virulence Finder 1.4.

Table 4.3 Putative virulence genes in enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin[†]

Protein and gene		Host origin, pathotype and serotype (no. of strains)						
		Bovine EHEC	Human EHEC		Bovine EPEC		Bovine putative non-pathotype	
		O103:H2 (n=43)	O103:H2 (n=2)	O103:H11 (n=4)	O103:H2 (n=12)	O103:H11 (n=1)	O103:H2 (n=12)	O103:H16 (n=1)
EHEC factor for adherence	***efa1***	35	2	4	0	1	0	0
Adherence protein	***iha***	19	1	4	2	1	0	0
EAST-1 heat-stable toxin	***astA***	0	0	4	9	1	0	0
SPATE	***espI***	23	0	0	3	0	0	0
Glutamic acid decarboxylase	***gad***	43	2	4	12	1	12	1
Increased serum survival	***iss***	43	2	4	9	1	12	1
Long polar fimbriae	***lpfA***	0	0	4	0	1	12	1
Endonuclease colicin E2	***celb***	20	0	0	0	1	0	0
Colicin B	***cba***	29	0	4	0	1	5	0
Colicin M	***cma***	1	0	0	0	0	5	0
ABC transporter protein MchF	***mchF***	0	0	0	0	0	5	0
MchC protein	***mchC***	0	0	0	0	0	5	0
Microcin H47 part of colicin H	***mchB***	0	0	0	0	0	5	0
Microcin M part of colicin H	***mcmA***	0	0	0	0	0	5	0

[†]Virulence genes were determined using Virulence Finder 1.4.

Table 4.4 Number of enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin positive for plasmids[†]

Plasmid	Host origin, pathotype and serotype (no. isolates tested)						
	Bovine EHEC	Human EHEC		Bovine EPEC		Bovine putative non-pathotype	
	O103:H2 (n=43)	O103:H2 (n=2)	O103:H11 (n=4)	O103:H2 (n=12)	O103:H11 (n=1)	O103:H2 (n=12)	O103:H16 (n=1)
Col156	19	0	0	0	1	0	0
ColRNAI	36	0	4	0	1	12	1
IncA/C2	3	0	0	0	0	0	0
IncB/O/K/Z	20	2	4	0	1	0	0
IncFIA(HI1)	0	0	0	2	0	0	0
IncFIB(AP001918)	39	2	4	0	1	12	0
IncFIC(FII)	0	0	0	0	0	12	0
IncFII	1	0	1	0	0	0	0
IncFII(29)	0	1	0	0	0	0	0
IncFII(pCoo)	3	0	0	0	0	0	0
IncFII(pHN7A8)	0	0	0	2	0	5	0
IncFII(pRSB107)	0	0	0	2	0	0	0
IncFII(pSE11)	0	0	0	9	0	0	0
IncI2	1	0	0	0	0	0	0
IncN	2	0	0	0	0	0	0
IncP	0	0	1	0	0	0	0
IncX1	0	0	0	2	0	0	0
IncY	0	0	0	7	0	0	0
p0111	2	0	2	0	1	0	0

[†]Plasmids were determined from whole genome sequences of strains using Plasmid Finder 1.3.

Table 4.5 Average number and range of intact, questionable and incomplete prophage sequences[†] of enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin

	Host origin, pathotype and serotype (no. isolates tested)						
	Bovine EHEC	Human EHEC		Bovine EPEC		Bovine putative non-pathotype	
Prophage region completeness	O103:H2 (n=43)	O103:H2 (n=2)	O103:H11 (n=4)	O103:H2 (n=12)	O103:H11 (n=1)	O103:H2 (n=12)	O103:H16 (n=1)
Intact	3.3 (2-6)	4.5 (3-6)	2 (0-3)	2.3 (0-5)	2	2.4 (1-3)	4
Questionable	1.7 (0-3)	1 (1-1)	0	2.8 (0-5)	0	1.8 (0-4)	0
Incomplete	9.8 (4-15)	6 (5-7)	8.8 (7-13)	8.3 (5-12)	11	3.6 (2-7)	0

[†]Number of prophage sequences were determined from whole genome sequences of strains using Phage Search Tool Enhanced Release (PHASTER)

Table 4.6 Number of enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin positive for antimicrobial resistance genes[†]

Antibiotic class	Resistance gene	Host origin, pathotype and serotype (no. isolates tested)						
		Bovine EHEC	Human EHEC		Bovine EPEC		Bovine putative non-pathotype	
		O103:H2 (n=43)	O103:H2 (n=2)	O103:H11 (n=4)	O103:H2 (n=12)	O103:H11 (n=1)	O103:H2 (n=12)	O103:H16 (n=1)
Aminoglycoside	*str*B	7	0	0	0	0	5	0
	*str*A	7	0	0	0	0	5	0
	*aad*A1	3	0	0	0	0	0	0
	aac(3)-VIa	3	0	0	0	0	0	0
	*aad*A2	0	0	1	0	0	0	0
Beta-lactam	*blaTEM*-1B	2	0	0	0	0	0	0
	blaCMY-2	3	0	0	0	0	0	0
	blaTEM-1C	0	0	1	0	0	0	0
Macrolide	*mph*(A)	5	0	1	0	0	0	0
Phenicol	*floR*	5	0	1	0	0	0	0
Sulphonamide	*sul*2	7	0	0	0	0	3	0
	*sul*1	3	0	1	0	0	0	0
Tetracycline	*tet*(A)	5	0	1	0	0	5	0
	tet(B)	4	0	0	0	0	0	0
	tet(C)	1	0	0	0	0	0	0
	tet(M)	0	0	1	0	0	0	0
Trimethoprim	*dfr*A12	0	0	1	0	0	0	0

[†]Resistance genes were determined from whole genome sequences of strains using ResFinder 2.1

Chapter 5 - Detection and Quantification of Seven Major Serogroups of Shiga toxin-producing *Escherichia coli* on Hides of Cull Dairy, Cull Beef, and Fed Beef Cattle at Time of Slaughter

Introduction

Cattle represent a major reservoir for Shiga toxin-producing *Escherichia coli* (STEC), including STEC O157 and the top six major non-O157 serogroups (STEC-6; O26, O45, O103, O111, O121, O145). Collectively known as the STEC-7, these organisms reside in the hindgut of cattle and are shed in the feces, which can subsequently contaminate the cattle hide during transportation and lairage (Gyles, 2007). The removal of cattle hides during harvest can allow for hide to carcass transfer of STEC via aerosol or direct contact between a fecal contaminated hide and carcass surfaces. Improper handling of carcasses and/or knives and other equipment used during the de-hiding step may also allow opportunities for cross-contamination to occur (Elder *et al*., 2000). Unless otherwise removed or killed by a downstream intervention procedure, STEC that has been transferred to a carcass surface may become incorporated into a final beef product, creating the potential for human illness (Koohmaraie *et al*., 2005).

The majority of approximately 265,000 annual cases of STEC infection in the U.S. are foodborne and have been largely associated with one of the major STEC-7 serogroups (Scallan *et al*., 2011; Brooks *et al*., 2005). Among foodborne STEC illnesses, the consumption of contaminated ground beef represents the most common food vehicle of STEC transmission (Rangel *et al*., 2005). Although fed beef cattle contribute to the majority of beef consumed in the

U.S., a sizable percentage (15-20%) of beef is produced from cull cattle (Kansas Livestock Association, 2010). The sale of cull beef and cull dairy cattle account for approximately 16% and 4% of total returns for the average U.S. beef cow/calf and dairy operations, respectively (National Market Cow and Bull Beef Quality Audit, 2007). Although multiple cattle production types contribute to the national beef supply, the majority of STEC prevalence studies have focused on fed beef cattle. Fecal prevalence and concentration of O157 and major non-O157 STEC serogroups has been well studied in fed beef cattle (Omisakin *et al*., 2003; LeJeune *et al*., 2004; Cernicchiaro *et al*., 2013; Dewsbury *et al*., 2015), and to a lesser extent in dairy cattle (Cobbold *et al*., 2000; Dunn *et al*., 2004), with one study reporting that cattle production type has a significant impact on fecal prevalence of STEC (Cobbold *et al*., 2004). Although STEC fecal prevalence has been reported as a significant indicator of the risk of carcass contamination (Omisakin *et al*., 2003), another study reports that STEC hide prevalence is a more accurate predictor of carcass contamination (Barkocy-Gallagher *et al*., 2003). Furthermore, fecal contaminated hides have been specifically identified as the primary source of contamination of beef carcasses (Koohmaraie *et al*., 2005). Therefore, data on prevalence and concentration of STEC-7 on cattle hides are critical for risk assessments and management.

Most reports on STEC hide prevalence have focused on O157 serogroup in fed beef cattle (Elder *et al*., 2000; Keen and Elder 2002; Fegan *et al*., 2005; Nastasijevic *et al*., 2008; Arthur *et al*., 2009), with few examining STEC O157:H7 prevalence on hides of cull cattle (Brichta-Harhay et al., 2008 Stromberg *et al*., 2016). Studies on non-O157 STEC hide prevalence are limited (Barkocy-Gallagher *et al*., 2003; Thomas *et al*., 2012; Stromberg *et al*., 2015; Stromberg *et al*., 2016) and to date have focused mostly on fed beef cattle. Some of these studies have determined concentration of non-O157 STEC on cattle hides (Stromberg et al., 2016; Thomas *et*

al., 2012); however, data are extremely limited due to undetectable STEC concentrations for a majority of samples tested. Unlike STEC, coliforms can be reliably detected from a variety of cattle sample matrices, including hide (Bacon *et al*., 2000). Considering their high prevalence on cattle hides and role as an indicator organism for STEC contaminants (Brown *et al*., 2000), spiral-plate method was also used in this study to enumerate coliforms from hide samples.

It is well established that STEC prevalence, particularly for O157 serogroup, are known to vary tremendously by time and place (Robinson *et al*., 2009). Thus, previous disparate studies of fed beef, cull dairy and cull beef are not very useful for direct comparisons among cattle types, because they vary by time, place, and usually diagnostic methods. Therefore, our objective was to utilize established IMS-based detection and spiral-plate quantification methods to compare the prevalence and concentration of STEC-7 on hides of cull dairy, cull beef and fed beef cattle harvested during the same days, at a single processing plant, immediately prior to hide removal.

Materials and Methods

Sample collection

Hide sponge samples were collected at a commercial beef processing facility in Texas once a week for four and six weeks in the summers of 2015 and 2016, respectively. A convenience sample of animals (n=50) of each cattle type (fed, cull beef, cull dairy) was selected for sampling based on animals present for harvest on that sampling date. Ante-mortem, the processing plant personnel used water to wet the cattle hides, then applied a commercial dishwashing detergent, and then rinsed prior to cattle entering the stun box. After shackling, hides were then washed vigorously by two personnel operating high volume/pressure water hoses. Samples were collected at the stage immediately before hide removal. Sampling materials consisted of a Speci-

Sponge® suspended in 25 mL of buffered peptone water contained in a Whirl-Pak® sampling bag. During each visit, an approximate 2,730 cm^2 (1ft x 3ft) area from the brisket to the umbilicus was sampled from cull dairy (n=50), cull beef (n=50) and fed beef cattle hides (n=50). Samples were transported in cold storage to Kansas State University Pre-Harvest Food Safety laboratory for processing within 24 h.

Quantification of STEC-7 and coliforms by spiral-plate method

Upon arrival at the laboratory, the liquid contents of the Speci-Sponges® were thoroughly expressed into the Whirl-Pak® bags, then the sponges were discarded. One-hundred microliters of hide sample was added to a micro-centrifuge tube containing 900 µl of EC broth, representing a 1:10 pre-enriched hide sample dilution for spiral plating. Then, using an Eddy Jet Spiral Plater (IUL Instruments; Barcelona, Spain), 100 µl of the sample dilution was spiral plated on 3 culture media: i) Sorbitol MacConkey agar containing cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l; CT SMAC) for quantification of STEC O157, ii) Possé agar medium modified to include novobiocin at 5 mg/l and potassium tellurite at 0.5 mg/l (MP) for quantification of STEC-6 and, iii) MacConkey agar (Difco; catalog# 212123) for quantification of coliforms. Plates were incubated at 37°C for 20-24h, then the resulting colony growth was enumerated. Five non-sorbitol fermenting colonies from CT-SMAC medium, ten chromogenic colonies (mauve, green, blue or purple) from MP medium and ten lactose fermenting colonies from MacConkey medium were picked, inoculated onto blood agar (Remel, Lenexa, KS) plates, and incubated at 37° C for 24 h. Non-sorbitol-fermenting colonies from CT-SMAC were tested for the O157 antigen by latex agglutination (Oxoid, Basingstoke, United Kingdom); if positive, a spot-indole test was performed. Colonies positive for agglutination and indole production were tested by a 6-plex

PCR (Bai *et al*., 2010) that targets the O157 somatic O-antigen (*rfb*E_{O157}), flagellar antigen (*fliC*$_{H7}$), Shiga-toxin (*stx*1 and *stx*2), intimin (*eae*) and enterohemolysin (*ehx*A) genes. Colonies positive for *rfb*E_{O157}, *fliC*$_{H7}$, *eae* and Shiga toxin genes (*stx*1 and/or *stx*2) were considered a STEC O157:H7 isolate. Chromogenic colonies from MP plates were pooled in distilled water, boiled for 10 min then centrifuged (9,300 x g) for 5 min. The boil-prep DNA was then tested by a 10-plex PCR targeting 6 serogroups and 4 virulence genes (*stx*1, *stx*2, *eae*, and *ehx*A; Bai *et al*., 2012). If the pooled colonies were positive for any of the six non-O157 serogroups, then each isolate was individually tested by the 10-plex PCR. Colonies positive for one of the six non-O157 somatic O-antigens and Shiga toxin genes (*stx*1 and/or *stx*2) were considered a STEC-6 strain. Lactose fermenting colonies from MacConkey agar were considered coliforms if they tested positive by spot-indole. Counts were adjusted based on number of colonies positive out of six for STEC O157 and out of ten for non-O157 STEC and coliforms.

Detection of STEC-7 by Immunomagnetic Separation (IMS)-based culture method

A sterile transfer pipette was used to remove the entire liquid contents of a Whirl-Pak® sampling bag and then 5 mL was returned to the bag. Forty-five milliliters of *E. coli* (EC) broth (Oxoid Ltd., Hampshire, UK) was added to each bag. Sample suspensions were mixed by gently massaging each bag, incubated at 37° C for 6 h, then subjected to immunomagnetic separation (IMS) using Kingfisher Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA). Three IMS runs were performed on enriched hide sample suspensions: i) 980 µl of sample mixed with 20 µl O157 IMS beads (Abraxis®, Warminster, PA), ii) 940 µl of sample mixed with 20 µl each of O26, O45 and O111 IMS beads (Pool 1; Abraxis®), iii) 940 µl of sample mixed with 20 µl each of O103, O121 and O145 IMS beads (Pool 2; Abraxis®). Following IMS, 50 µl of IMS

bead suspension was spread-plated onto selective media for detection and isolation of STEC-7, as previously described (Dewbury *et al*., 2015, Noll *et al*., 2015). Briefly, O157 IMS bead suspensions were cultured onto CT-SMAC, while pooled combinations of non-O157 IMS bead suspensions were cultured onto MP agar. Following incubation, suspect colonies from selective media were picked and inoculated onto blood agar (Remel, Lenexa, KS) plates. Presumptive STEC O157 and STEC-6 colonies were tested and confirmed as described above.

Statistical analysis

All data analyses were performed using generalized linear mixed models (Proc Glimmix, SAS 9.4) with study week included as a random intercept term in all analyses to account for the lack of independence among samples within sampling week. Models with a binomial distribution and logit link were used to determine whether the proportions of hide samples positive for an individual STEC serogroup, or positive for at least one of the STEC-6 or STEC-7 serogroups, differed among cattle types. When convergence criterion was not satisfied for some STEC serogroups, Poisson or negative binomial distributions, offset by number of samples for the cattle type and week, were used to improve model fit. A multiplicative parameter was used to adjust for overdispersion, when it occurred. Data on the number of different STEC-6 and STEC-7 serogroups isolated from STEC-positive samples were analyzed using a Poisson distribution with an offset equal to the number of possible serogroups (6 and 7, respectively). For all analyses, pairwise comparisons among model-adjusted means of hide samples positive within cattle type categories were made when P values were ≤ 0.10 for the overall test (Type 3) of the fixed effect of cattle type (i.e., testing the null hypothesis that the outcomes were equal for all three cattle types). Differences among means were considered significant if P values were ≤ 0.05.

Results

Over the ten processing days at the commercial plant, a total of 1500 cattle hides were sampled comprised of 500 samples collected from each of the three cattle production types. Overall, 12.6% (189/1,500) of hide samples tested positive for at least one STEC-6; 14.0% (210/1500) of samples tested positive for at least one STEC-7 (Table 5.1). Overall prevalence of STEC O26 (82/1,500; 5.5%) was highest among the STEC-7 serogroups, followed closely by STEC O103 (81/1,500; 5.4%), then STEC O145 (47/1,500; 3.1%) and STEC O157:H7 (34/1,500; 2.3%) (Table 5.1); STEC O121 was the only STEC-7 serogroup not detected during the study.

A slightly higher proportion of cull beef (68/500; 13.6%) and fed beef hides (66/500; 13.2%) were positive for at least one STEC-6, compared to cull dairy hides (55/500; 11.0%), but differences were not significant (Table 5.2). Similarly, a slightly higher proportion of fed beef (77/500; 15.4%) and cull beef hides (76/500; 15.2%) were positive for at least one STEC-7, compared to cull dairy hides (57/500; 11.4%), but differences were not significant. However, the number of different STEC-7 serogroups isolated from a sample was significantly higher ($P < 0.05$) for fed beef cattle, compared to cull beef; number of different STEC-7 serogroups isolated from cull dairy hides were not significantly different from other cattle types (data not shown). Multiple STEC-7 serogroups (2 or more) were isolated from 39.0% (30/77) of fed beef samples positive for STEC-7, compared to in only 3.9% (3/76) and 10.5% (6/57) of STEC-7 positive cull beef and cull dairy samples, respectively.

No significant differences were observed in the prevalence of STEC O26 or STEC O111 between cattle types (Table 5.2). However, STEC O103 was detected on a significantly higher proportion of fed beef hides (34/500; 6.8%) compared to cull dairy hides (17/500; 3.4%), but not

compared to cull beef hides (30/500; 6.0%). No significant difference in STEC O103 prevalence was observed between cull dairy hides and cull beef hides. STEC O145 was detected on a significantly higher proportion of fed beef hides (20/500; 4.0%) compared to cull beef hides (9/500; 1.8%), but not compared to cull dairy hides (18/500; 3.6%). However, no significant difference in STEC O145 prevalence was observed between fed beef hides and cull dairy hides. Finally, fed beef hides were also positive for a significantly higher proportion of STEC O157:H7 (24/500; 4.8%) compared to both cull beef (8/500; 1.6%) and cull dairy hides (2/500; 0.4%), which were not significantly different from each other (Table 5.2). Due to overall low prevalence of STEC O45 (2/1500) and STEC O121 (0/1500) serogroups, analyses by cattle type could not be performed.

Major virulence genes (*eae*, *stx*1 and/or *stx*2) of STEC strains isolated from the hide samples are reported in Table 5.3. All STEC O103 (81/81) and nearly all STEC O26 strains (81/82) were positive for *stx*1 (but not *stx*2) and *eae* genes. All STEC O157:H7 strains were *eae* positive. All STEC O157:H7 strains isolated from cull dairy (2/2) and nearly all strains (23/24) isolated from fed beef were positive for *stx*2 only, while half the strains (4/8) from cull beef were positive for both Shiga toxins. Only a small proportion of STEC-7 strains, belonging to O111 serogroup isolated from cull beef and from O145 serogroup isolated from cull dairy and fed beef, were negative for the *eae* gene. However, only a small proportion of non-serogrouped STEC strains (not among the 7 serogroups tested) isolated from cull beef (4/23; 17.4%) and cull dairy (1/13; 7.7%) carried the *eae* gene, compared to a majority of strains (12/18; 66.7%) isolated from fed beef that were *eae* positive. Over half (12/23; 52.2%) of all non-serogrouped STEC strains isolated from cull beef were positive for both Shiga toxin genes, while only 15.4 (2/13) and

22.2% (4/18) of non-serogrouped STEC strains isolated from cull dairy and fed beef, respectively, were positive for both *stx*1 and *stx*2.

Spiral-plate data illustrate concentrations of the STEC-7 and coliforms (Table 5.4). Data were not analyzed for statistical differences, therefore only descriptive data are reported here. Again, O121 was the only STEC-7 serogroup not detected in samples from any of the cattle types. Highest overall proportions of quantifiable hide samples were observed for STEC O145 (32/1,500) and STEC O157 (31/1500) serogroups (Table 5.4). Despite STEC O145 being detected by the IMS-culture method in 3.6% of cull dairy samples, none were quantifiable by spiral plate-method. There were 4.8% (24/500) and 1.6% (8/500) of fed beef and cull beef hide samples, respectively, with quantifiable STEC O145, mostly at concentrations between 3 to 5 log CFU/100 cm^2. The majority of quantifiable STEC O157 from each cattle type was observed at concentrations between 3 to 4 log CFU/100 cm^2. Detectable concentrations of STEC O45, STEC O103 and STEC O111 were observed in less than 1.0% of total samples collected, with the majority of these samples at concentrations between 3 to 5 log CFU/100 cm^2. Coliforms were detected in nearly all samples from each cattle type, at a majority concentration of 4 to 6 log CFU/100 cm^2.

Discussion

This study is the first to directly compare both the prevalence and concentration of all major STEC-7 serogroups on the hides of cull beef, cull dairy, and fed beef cattle, harvested at the same place and times. To avoid potential confounding effects related to temporal or geographic differences in STEC, a single processing plant was selected for sampling, where an equal number of samples were collected from each cattle type during each of the ten processing days.

Although a couple of STEC serogroups were extremely rare (O45 and O121) on hides of all cattle types, and others (STEC O26 and O111) did not differ significantly among cattle types, fed beef cattle from this study population had a significantly higher prevalence of STEC O103, STEC O145, and STEC O157 on their hides, compared to cull cattle (dairy or beef). Many interrelated risk factors associated with these cattle types (Renter and Sargeant, 2002), including age, diet and production environment could be driving the differences in hide prevalence observed here.

Age of cattle has been shown to impact STEC O157 shedding, with higher estimates reported in yearlings (Van Donkersgoed *et al*., 1999) and weaned dairy calves (Zhao *et al*., 1995; Hancock *et al*., 1997) compared to adult cattle. Renter *et al*. (2004) have shown that feeder cattle are > 4 times more likely to shed STEC O157, compared to cow-calf and dairy cattle. Although cattle age data were not collected in this current study, the average slaughter age of fed beef cattle is between 18-22 months (Cattlemen's Beef Board and National Cattlemen's Beef Association, 2009), whereas the majority (52.9%) of cull beef cattle are ten years or older at the time of harvest (USDA, 2010). Cull dairy cattle, on average, are approximately 4 years of age at slaughter (Garcia-Peniche *et al*., 2006). Since higher STEC O157 prevalence estimates have typically been reported in younger cattle, age of fed beef cattle compared to cull cattle may be impacting STEC prevalence differences observed here.

Although the effects are not always consistent, it is well established that diet impacts STEC O157 populations in the gastrointestinal tract of cattle, and subsequently, STEC O157 concentrations shed in the feces (Jacob *et al*., 2009). Finishing beef and lactating dairy cattle that are raised in intensive production systems are typically fed high grain diets to increase weight gain or production efficiency (Callaway *et al*., 2010). Studies examining the impact of

grain- vs. forage-based diets on STEC O157 fecal shedding in cattle have had conflicting results (Hovde *et al*., 1999; Tkalcic *et al*., 2000; Van Baale *et al*., 2004). However, significant differences in STEC O157 fecal shedding in cattle have been associated with different grain types (Dargatz *et al*., 1997; Berg *et al*., 2004) and with different processing methods that prepare grains for cattle diets (Fox *et al*., 2007; Depenbusch *et al*., 2008). Although data on the diets of the cattle sampled in the current study were not collected, they likely differed by cattle type, and may contribute to observed differences in STEC hide prevalence.

Potential differences in production environments for the three cattle types also may be a driver of differences in STEC. Considering the many factors associated with pathogen transmission in a feedlot environment, including contaminated pen environments and high cattle density (Smith *et al*., 2001), it may not be surprising that fed cattle hides were positive for a significantly higher proportion of some major STEC serogroups. Smith *et al*. (2000) reported that STEC O157 is common among groups of feedlot cattle that share a pen. Further, muddy pen conditions were significantly associated with higher pen-level fecal prevalence estimates compared to cattle in normal pen conditions, which is likely reflective of an increased opportunity for STEC O157 transmission between pen-mates via fecal contaminated mud (Smith *et al*., 2001).

Because pathogens on the hide surface originate largely from fecal contamination, it may be reasonable to expect that fecal STEC prevalence trends reported among multiple cattle types may agree with the hide STEC prevalence reported here. Although results from many of the studies examining STEC fecal prevalence between cattle types are based only on PCR testing for *stx* genes (Cerqueria *et al*., 1999; Van Donkersgoed *et al*., 1999; Cobbold *et al*., 2004), interestingly none of these studies reported higher *stx* gene prevalence in fed cattle, compared to other cattle

types. Higher *stx* prevalence in fecal samples from dairy, compared to beef cattle, have been reported (Cerqueria *et al*., 1999; Cobbold *et al*., 2004), while another study found no difference in *stx* fecal prevalence among these cattle types (Van Donkersgoed *et al*., 1999). Considering the numerous (~435) STEC serotypes (each *stx*-carrying) that have been isolated from cattle feces (Gyles *et al*., 2007), coupled with the transient nature of STEC-shedding events in cattle (Robinson *et al*., 2009), it may not be surprising that results from these studies conflict. Furthermore, the lack of serogroup-specific STEC prevalence data from these studies makes it difficult to compare results to those presented here.

Cultural isolation of the STEC-7 serogroups, as performed in the current study, is tedious but allows for genetic characterization and comparison of potential disease causing STEC pathogens among multiple cattle types, and in turn, provides for a more adequate assessment of the potential STEC-related food safety risk. Stromberg *et al.* (2015) used culture-based techniques to determine STEC prevalence on hides (n=100) of cull dairy cattle and reported a similar prevalence of STEC O45 (0%), STEC O111 (1.0%), STEC O121 (1.0%) and STEC O26 (5.0%), compared to the current study. However, we report higher prevalence of STEC O103 (3.4% vs. 0%) and STEC O145 (3.6% vs. 0%), but not STEC O157 (0.4% vs. 9.0%) on hides of cull dairy cattle. Stromberg *et al.* (2015) determined STEC prevalence on hides of fed cattle, and reported low (0.2-0.6%) to zero prevalence of all STEC-7 serogroups. Thomas *et al*. (2012) determined prevalence of a subset of STEC-7 serogroups (O26, O103, O111, O145 and O157) on hides of fed cattle in Ireland and reported a much lower prevalence of STEC O26 (0.2%) and STEC O103 (0.2%) compared to prevalence reported in the current study. STEC O111 prevalence was similarly low in both studies, while Thomas *et al*. (2012) reported a much higher prevalence of STEC O157 (17.6%) compared to the current study (4.8% in fed beef). Similarly,

other studies have reported high prevalence of STEC O157 on hides of fed cattle (Elder *et al*., 2000; Keen and Elder 2002; Fegan *et al*., 2005; Nastasijevic *et al*., 2008), with estimates ranging from 28.2% (Nastasijevic *et al*., 2008) up to 73.4% (Keen and Elder 2002). It is unclear why STEC O157 prevalence, by comparison, was relatively low among this study population of cattle; however, sampling and diagnostic procedures, season, geographic location, and hide interventions (or lack of) are just a few of the potential contributing factors.

Differences in pathogen control systems between processing plants can contribute to the observed differences in prevalence of STEC. Numerous carcass-directed pathogen intervention strategies have been routinely employed at processing plants, following the USDA Food Safety and Inspection Service HACCP Systems-Final Rule" in 1996, and have been effective at reducing *E. coli* O157:H7 (Koohmaraie *et al*., 2005). Hide-directed intervention strategies have also been developed, including hide-washing cabinets, chemical dehairing, and application of antimicrobial compounds to hide surfaces (Koohmaraie *et al*., 2005; Bosilevac *et al*., 2004; Bosilevac *et al*., 2005), but their use in the beef-processing industry varies widely (Mies *et al*., 2004). During the current study, plant personnel wet and washed cattle ante- and post-mortem, respectively; a process that could have resulted in the relatively low prevalence of STEC on hides. The effectiveness of different hide-intervention techniques at removing or destroying bacterial contaminants can vary, along with their general adoption by processing facilities (Koohmaraie *et al*., 2005), which may contribute to differences in STEC hide prevalence reported in the literature. However, since most studies do not report on specific hide intervention strategies employed at a processing plant or whether interventions were used at all, it makes it difficult to speculate.

Interestingly, the numbers of different STEC-7 serogroups isolated from STEC positive fed beef hide samples were significantly higher than cull beef samples. Fecal shedding of STEC O157 in fed beef cattle has been previously associated with an increased probability of fecal shedding of other STEC serogroups, including O26, O45, O103 and O121 (Cernicchiaro *et al*., 2013). Therefore, it may be reasonable to expect a similar trend from a hide sample matrix, as reported here. The previously mentioned factors associated with pathogen transmission in a feedlot environment also could contribute to the higher numbers of different STEC serogroups isolated from fed beef cattle hides, compared to hides of other cattle types.

Many of the studies examining STEC O157 hide prevalence in fed cattle, have also sampled other matrices (feces, oral cavity, carcass surface) and have typically reported on overall distribution of major virulence genes among the strains isolated rather than for a specific matrix (e.g. hide). In one study (Elder *et al*., 2000), a majority (57.4%) of STEC O157 isolated from feces, hides and carcasses of fed beef cattle were positive for both Shiga toxin genes (*stx*1 and *stx*2) while 41.2% of remaining strains were *stx*2 positive only. In another study (Keen and Elder, 2002), a smaller percent of STEC O157 strains (6.9%) isolated from the hide surface and oral cavity of fed cattle were positive for both Shiga toxin genes, while a majority (93.1%) were *stx*2 positive only. Similarly, Fegan *et al*. (2005) reported a majority of STEC O157 strains (74.7%) isolated from feces, oral cavity, hide and carcass surfaces, as *stx*2 positive only, while only 5.7% of strains were positive for both Shiga toxins. Although distribution of *stx*2 only and *stx*1+*stx*2 STEC O157 strain profiles vary among these studies, all reported low prevalence of *stx*1 only strains and extremely high prevalence of intimin (*eae*) positive strains. None of the STEC O157 strains (n=34) isolated during the current study were positive for *stx*1 only, while all were *eae* positive. Similar to previous reports (Keen and Elder, 2000; Fegan *et al*., 2005; Dunn

et al., 2004), nearly all STEC O157 strains (23/24) isolated from fed beef cattle and all strains isolated from cull dairy (2/2) were *stx*2 positive only.

Few studies have reported major virulence gene profiles of STEC-6 serogroups isolated from hides of fed or cull dairy cattle (Thomas *et al*., 2012; Stromberg *et al*., 2015; Stromberg *et al*., 2016), and extremely low prevalence of most STEC serogroups in these studies limits potential comparisons to the current study. Thomas *et al*. (2012) reported *stx*1, but not *stx*2, in the single STEC O26 and STEC O103 strains isolated from fed beef hides during their study, just like all STEC O26 (32/32) and STEC O103 (34/34) isolated from hides of fed cattle in the current study. Thomas *et al*., (2012) did not isolate STEC O145 from cattle hides, however, Stromberg *et al*. (2015) characterized a single STEC O145 strain isolated from a fed cattle hide as *stx*2 + *eae* positive. However, the majority of STEC O145 strains isolated from each cattle type in the current study were *stx*1 + *eae* positive. Stromberg *et al*. (2016) isolated a single *stx*1 + *eae* positive strain of STEC O111 from the hide of a cull dairy cow. Only three STEC O111 strains were isolated from cull dairy hides during the current study but all were *stx*1 + *stx*2 + *eae* positive. Distribution of major virulence genes of non-serogrouped STEC seemed to vary within and between major cattle types; however, more strains isolated from fed beef (12/18) were positive for *eae* gene compared to strains from cull beef (4/23) or cull dairy (1/13). Considering the importance of the *eae* gene during a human STEC infection (Donnenberg *et al*., 1993), STEC strains isolated from fed beef hides during this study, may have higher virulence potential on average, based on genotype, compared to predominantly *eae*-negative strains isolated from other cattle types.

Although concentration data of STEC O157 on cattle hides has been reported in the literature (Fegan et al., 2005; Stromberg *et al*., 2016), due to a general lack of enumerable hide

samples for non-O157 STEC (Thomas *et al*., 2012; Stromberg *et al*., 2016), STEC-6 concentration data on hides of all cattle types have gone under-reported. Although STEC serogroups were not quantifiable in a majority of samples tested, among enumerable samples, most STEC serogroups were at concentrations between 3 to 5 log CFU/100 cm^2, which may be considered "super-shedder" concentrations in feces (Low *et al*., 2005; Chase-Topping *et al*., 2007). None of the samples were enumerable for STEC O121, nor was this serogroup detected by IMS-culture method, which may indicate its limited potential in all cattle types as a potential food safety risk.

Many factors, some of which are relevant to cattle types compared in this current study, have been associated with differences in STEC O157 fecal prevalence, including age of cattle at time of harvest, diet, and production environment. It is not certain whether risk factors from the studies discussed here extend to hide prevalence of the different STEC serogroups. However, we can conclude that a significantly higher number of major STEC serogroups (O103, O145, O157) were harbored on the hides of fed beef cattle compared to hides of cull beef and/or cull dairy cattle included in this study. Differences in age of cattle at slaughter, cattle diets, and production environments may have contributed to the observed differences in hide contamination. Contaminated pen surfaces and high cattle density are also factors conducive for pathogen transmission, which may explain why the number of different serogroups isolated from STEC-7 positive samples was highest for feedlot cattle. Although the data on enumerable STEC hide samples was relatively sparse, these data, particularly in combination with the unique hide prevalence data, help fill an important knowledge gap on the potential risks of STEC, and relative differences associated with different types of cattle presented for harvest and entry into the beef chain.

Table 5.1 Detection of seven major serogroups of Shiga toxigenic *Escherichia coli* (STEC) isolated from hide samples collected immediately prior to hide removal at a single processing plant for ten weeks

Sampling date	*Sampling week	No. samples positive for STEC serogroup (% prevalence)								
		O26	O45	O103	O111	O121	O145	O157	†STEC-6	‡STEC-7
5/4/2015	1	28 (18.7)	0	19 (12.7)	8 (5.3)	0	41 (27.3)	14 (9.3)	75 (50)	81 (54)
5/18/2015	2	27 (18)	2 (1.3)	42 (28)	0	0	0	8 (5.3)	58 (38.7	61 (40.7)
8/3/2015	3	1 (0.7)	0	3 (2)	1 (0.7)	0	0	2 (1.3)	5 (3.3)	7 (4.7)
8/24/2015	4	11 (7.3)	0	3 (2)	0	0	1 (0.7)	1 (0.7	14 (9.3)	15 (10)
6/20/2016	5	3 (2)	0	5 (3.3)	0	0	1 (0.7)	2 (1.3)	9 (6)	11 (7.3)
7/11/2016	6	1 (0.7)	0	1 (0.7)	0	0	0	0	2 (1.3)	2 (1.3)
7/18/2016	7	1 (0.7)	0	4 (2.7)	0	0	0	0	5 (3.3)	5 (3.3)
7/25/2016	8	8 (5.3)	0	4 (2.7)	1 (0.7)	0	4 (2.7)	2 (1.3)	16 (10.7)	18 (12)
8/8/2016	9	2 (1.3)	0	0	3 (2)	0	0	1 (0.7)	5 (3.3)	6 (4)
8/15/2016	10	0	0	0	0	0	0	4 (2.7)	0	4 (2.7)
Total (n=1500)		**82 (5.5)**	**2 (0.1)**	**81 (5.4)**	**13 (0.87)**	**0 (0)**	**47 (3.1)**	**34 (2.3)**	**189 (12.6)**	**210 (14)**

*****Each week, 150 hide samples were collected

†Samples positive for one or more of the 6 non-O157 STEC serogroups

‡Samples positive for one or more of the 7 STEC serogroups

Table 5.2 Detection of seven major serogroups of Shiga toxigenic *Escherichia coli* (STEC) isolated from hide samples collected from cull beef, cull dairy and fed beef cattle immediately prior to hide removal at a single processing plant

STEC serogroup	Overall no. samples positive (n/1500) (%)	No. samples positive (n/500) by cattle type (%)			*P* value for overall effect (Type III) of cattle type
		Cull Beef	Cull Dairy	Fed Beef	
O26	82 (5.5)	25 (5.0)	25 (5.0)	32 (6.4)	0.8
O45	2 (0.13)	0	0	2 (0.4)	NA[#]
O103	81 (5.4)	30 (6.0)[ab]	17 (3.4)[a]	34 (6.8)[b]	0.06
O111	13 (0.87)	7 (1.4)	3 (0.6)	3 (0.6)	0.34
O121	0	0	0	0	NA[#]
O145	47 (3.1)	9 (1.8)[a]	18 (3.6)[ab]	20 (4.0)[b]	0.09
O157	34 (2.3)	8 (1.6)[a]	2 (0.4)[a]	24 (4.8)[b]	<0.01
STEC6[†]	189 (12.6)	68 (13.6)	55 (11.0)	66 (13.2)	0.23
STEC7[‡]	210 (14.0)	76 (15.2)	57 (11.4)	77 (15.4)	0.19

#Data were too sparse for analysis

[†]Samples positive for one or more of the 6 non-O157 STEC serogroups

[‡]Samples positive for one or more of the 7 STEC serogroups

Values with different superscript letters within rows were significantly different ($P < 0.05$) from each other

Table 5.3 Distribution of major virulence genes of Shiga toxigenic *Escherichia coli* (STEC) isolated from hide samples collected from cull beef (n=500), cull dairy (n=500) and fed beef cattle (n=500) immediately prior to hide removal

STEC Serogroup	Cull Beef					Cull Dairy					Fed Beef				
	No. isolates	Virulence Genes				No. isolates	Virulence Genes				No. isolates	Virulence Genes			
		*stx*1	*stx*2	*stx*1 & *stx*2	*eae*		*stx*1	*stx*2	*stx*1 & *stx*2	*eae*		*stx*1	*stx*2	*stx*1 & *stx*2	*eae*
O26	25	25	0	0	25	25	24	0	1	25	32	32	0	0	32
O45	0	0	0	0	0	0	0	0	0	0	2	0	0	2	2
O103	30	30	0	0	30	17	17	0	0	17	34	34	0	0	34
O111	8	6	0	2	7	3	0	0	3	3	3	0	0	3	3
O121	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
O145	9	9	0	0	9	19	15	1	3	18	20	19	1	0	18
O157	8	0	4	4	8	2	0	2	0	2	24	0	23	1	24
Other STEC	23	4	7	12	4	13	5	6	2	1	18	4	10	4	12

Table 5.4 Spiral plate-based quantification of coliforms and seven major serogroups of Shiga toxigenic *Escherichia coli* (STEC) isolated from hide samples collected from cull beef (n=500), cull dairy (n=500) and fed beef cattle (n=500) immediately prior to hide removal

Organism	Cattle type	No. of quantifiable samples (% of total samples collected)	No. of quantifiable samples, Log CFU/100cm^2							
			0- < 1	1- < 2	2- < 3	3- < 4	4- < 5	5- < 6	6- < 7	GPLC[†]
STEC O26	Cull Beef	2 (0.4)	0	1	1	0	0	0	0	0
	Cull Dairy	8 (1.6)	0	0	2	2	4	0	0	0
	Fed Beef	3 (0.6)	0	0	1	1	1	0	0	0
STEC O45	Cull Beef	1 (0.2)	0	0	0	1	0	0	0	0
	Cull Dairy	0	0	0	0	0	0	0	0	0
	Fed Beef	0	0	0	0	0	0	0	0	0
STEC O103	Cull Beef	2 (0.4)	0	0	0	1	1	0	0	0
	Cull Dairy	2 (0.4)	0	0	1	1	0	0	0	0
	Fed Beef	4 (0.8)	0	0	0	3	1	0	0	0
STEC O111	Cull Beef	4 (0.8)	0	0	2	2	0	0	0	0
	Cull Dairy	2 (0.4)	0	0	0	0	2	0	0	0
	Fed Beef	4 (0.8)	0	0	0	3	1	0	0	0
STEC O121	Cull Beef	0	0	0	0	0	0	0	0	0
	Cull Dairy	0	0	0	0	0	0	0	0	0
	Fed Beef	0	0	0	0	0	0	0	0	0
STEC O145	Cull Beef	8 (1.6)	0	0	2	4	2	0	0	0
	Cull Dairy	0	0	0	0	0	0	0	0	0
	Fed Beef	24 (4.8)	0	0	0	12	12	0	0	0
STEC O157	Cull Beef	11 (2.2)	0	0	3	8	0	0	0	0
	Cull Dairy	3 (0.6)	0	0	0	2	0	0	0	1
	Fed Beef	17 (3.4)	0	0	5	11	1	0	0	0
Coliforms	Cull Beef	481 (96.2)	6	53	53	28	139	139	15	48
	Cull Dairy	488 (97.6)	3	19	44	17	131	130	17	127
	Fed Beef	497 (99.4)	4	22	32	6	110	219	28	76

[†]GPLC = Greater than 500,000 estimated spiral plate count per milliliter sample.

Conclusions

For study 1, IMS-based culture-method for detection of STEC-6 in cattle feces was developed and compared to a PCR-based method. Serogroups O103 and O26 were the predominant serogroups in feces of cattle sampled in this study, and PCR detected higher proportions of fecal samples as positive for O26 but not O103 than the culture method. Only a small proportion of the non-O157 serogroup isolates carried the Shiga toxin gene. More importantly, each method (culture and PCR) detected the six non-O157 serogroups in fecal samples that were negative by the other method. This is an important observation because the FSIS method (USDA, 2013) for detecting non-O157 STEC in beef samples is based on PCR detection of *stx* and *eae* genes first, followed by detection of serogroups, and a sample positive for both is then subjected to culture method. Detection sensitivity of pooled vs. individual IMS beads for isolation STEC-6 from cattle feces was evaluated in study 2. Based on feces inoculated with pure cultures and feces from naturally-shedding cattle, pooling of IMS beads did not affect detection sensitivity of six serogroups of non-O157 STEC in cattle feces. The prospect of a higher throughput test because of a less laborious, timesaving, and less expensive protocol, coupled with the statistical evidence of non-inferiority, make pooling a desirable option for detecting STEC serogroups in bovine fecal samples. For study 3, a real-time PCR assay, based on the CRISPR region, was developed and validated for serotype-specific detection and quantification of STEC O157:H7 in cattle feces. The detection limit of the assay with DNA extracted directly from cattle feces was ~10^3 CFU/g, a one log improvement compared to the minimum detection limit reported in previous studies (Jacob *et al*., 2012, Noll *et al*., 2015). Unlike other multiplex qPCR assays that have traditionally targeted the O157 serogroup-specific somatic antigen gene (*rfb*E) and other major EHEC virulence genes (*stx*1, *stx*2, *eae*), the

$CRISPR_{O157:H7}$ qPCR assay is novel in that it can detect and quantify EHEC O157:H7, the most clinically relevant EHEC serotype shed in cattle feces. The lack of strong agreement between IMS-based culture-method and the $CRISPR_{O157:H7}$ qPCR highlights the need to subject samples to both methods for more accurate EHEC O157:H7 detection. However, the assay targeting the CRISPR array is a sensitive and high-throughput method for serotype-specific detection and quantification of *E. coli* O157:H7 in cattle feces. In study 4, virulence gene profiles of bovine EHEC, EPEC and putative non-pathotype *E. coli* O103 strains were examined with WGS-based comparative analysis. Key differences in the virulomes and mobilomes of EHEC, EPEC, and putative non-pathotype strains were observed. Genome size and number of genes from mobile elements were strongly correlated between the O103 subgroups, with significantly larger genomes and higher mobile gene counts found in bovine EHEC and human EHEC compared to EPEC and putative non-pathotype strains. Although the *in silico* analysis performed here does not provide phenotypic evidence of virulence contributions, a number of major and putative virulence genes were comparable among bovine and human EHEC O103 strains, which may indicate the potential for bovine EHEC O103 to cause human infection. Finally, in study 5, prevalence and concentration of STEC-7 of fed-beef, cull beef and cull dairy cattle were determined. A significantly higher number of major STEC serogroups (O103, O145, O157) were harbored on the hides of fed beef cattle compared to hides of cull beef and/or cull dairy cattle included in this study, mostly at concentrations between 3 to 5 log CFU/100 cm^2. Differences in age of cattle at slaughter, cattle diets, and production environments may have contributed to the observed differences in hide contamination. These data, particularly in combination with the unique hide prevalence data, help fill an important knowledge gap on the

potential risks of STEC, and relative differences associated with different types of cattle presented for harvest and entry into the beef chain.

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Appendix A - Supplemental Tables

Supplemental Table A.1 Virulence gene profiles† of enterohemorrhagic *Escherichia coli* (EHEC) O103:H2 strains isolated from cattle feces collected from nine feedlots in the Midwest

State	Feedyard	Pen ID	Strain ID	Accession #	Phage-encoded Shiga toxin				Locus of Enterocyte Effacement (LEE) encoded					Phage-encoded Type III effectors			Non-LEE encoded			pO157 plasmid-encoded					Other													
					stx 1 FLY16	*stx* 1 3818T	*stx* 1A variant a	*stx* 1B variant a	*eae*	*tir*	*esp* A	*esp* B	*esp* F	*cif*	*esp* J	*tccP*	*nle* A	*nle* B	*nle* C	*ehx* A	*esp* P	*etp* D	*kat* P	*tox* B	*efa* 1	*iha*	*ast* A	*esp* I	*gad*	*iss*	*lpf* A	*celb*	*cba*	*cma*	*mch* F	*mch* C	*mch* B	*mcm* A
NE	A	D707	2013-3-304D	MVLT00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
		D716	2013-3-409B	MVLV00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■		■	■					
		D732	2013-3-539F	MVLW00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■		■	■					
			2013-3-552F	MVLX00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■		■	■					
		D736	2013-3-557A	MVLY00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
		D951	2013-3-80A	MVLM00000000	■		■	■	■	■	■	■	■	■		■	■	■	■	■	■		■	■			■	■	■								
		D976	2013-3-104A	MVLN00000000	■		■	■	■	■	■	■	■	■		■	■	■	■	■	■		■	■			■	■	■		■						
			2013-3-118A	MVLO00000000	■		■	■	■	■	■	■	■	■		■	■	■	■	■	■		■	■			■	■	■		■						
		D987	2013-3-174C	MVLS00000000	■		■	■	■	■	■	■	■	■		■	■	■	■	■	■		■	■			■	■	■		■	■	■				
		D989	2013-3-135A	MVLP00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■					
			2013-3-141B	MVLQ00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■					
			2013-3-144B	MVLR00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■					
		D991	2013-3-314B	MVLU00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
	C	28	2014-5-1253A	MVMX00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■						
		36	2014-5-58F	MVMB00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
		56	2014-5-89A	MVMD00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
			2014-5-94C	MVME00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
		80	2014-5-548J	MVMM00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
		90	2014-5-82A	MVMC00000000	■		■	■	■	■	■	■	■		■	■	■	■	■	■	■		■				■	■	■								
	E	2	2014-5-288B	MVMI00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
		44	2014-5-925A	MVMV00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■			■					
		51	2014-5-888D	MVMU00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■			■					
		53	2014-5-933A	MVMW00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■			■					
			2014-5-941B	MVNA00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■			■					
	G	36	2014-5-1838G	MVMZ00000000	■		■	■	■	■	■	■	■	■	■		■	■	■	■	■		■				■	■	■		■						
		151	2014-5-370H	MVML00000000		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
	I	19	2014-5-1451D	MVNC00000000				■	■	■	■	■		■	■	■	■	■	■	■	■		■				■	■	■		■	■					
TX	B	B12	2014-5-7C	MVLZ00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
			2014-5-10D	MVMA00000000	■		■	■	■	■	■	■	■		■	■	■	■	■	■	■		■				■	■	■		■	■					
	D	131	2014-5-650B	MVMQ00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
		131	2014-5-655A	MVMR00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
		234	2014-5-610A	MVMN00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
			2014-5-611A	MVMO00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
			2014-5-614A	MVMP00000000	■		■	■	■	■	■	■	■		■	■	■	■	■	■	■		■						■		■	■					
		237	2014-5-138A	MVMF00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
			2014-5-139D	MVMG00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
			2014-5-140E	MVMH00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
	F	D01	2014-5-965B	MVNB00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■					
		E05	2014-5-330A	MVMJ00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■						
			2014-5-332A	MVMK00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■						
	H	102	2014-5-841G	MVMS00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■								
		A20	2014-5-1565C	MVMY00000000	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■				■	■	■		■	■	■	■			
		A56	2014-5-863D	MVMT00000000	■		■	■	■	■	■	■	■	■	■		■	■	■	■	■		■				■	■	■								

†Virulence genes were determined using Virulence Finder 1.4.

Supplemental Table A.2 Virulence gene profiles[†] of enteropathogenic *Escherichia coli* (EPEC) O103 and *E. coli* O103 strains negative for Shiga toxin and intimin genes (putative non-pathotype) isolated from cattle feces collected from a Midwest feedlot

State	Feedyard	Pen ID	Pathotype	Serotype	Strain ID	Accession #	Phage-encoded Shiga toxin				Locus of Enterocyte Effacement (LEE) encoded					Phage-encoded Type III effectors			Non-LEE encoded			pO157 plasmid-encoded					Other													
							stx 1 FLY16	*stx* 1 3818T	*stx* 1A variant a	*stx* 1B variant a	*eae*	*tir*	*esp* A	*esp* B	*esp* F	*cif*	*esp* J	*tccP*	*nle* A	*nle* B	*nle* C	*ehx* A	*esp* P	*etp* D	*kat* P	*tox* B	*efa* 1	*iha*	*ast* A	*esp* I	*gad*	*iss*	*lpf* A	*celb*	*cba*	*cma*	*mch* F	*mch* C	*mch* B	*mcm* A
NE	A	D721	EPEC	O103:H11	2013-3-492A	MVLF00000000					■	■	■	■	■	■	■		■	■		■	■		■	■	■	■		■	■	■	■	■					
		D707		O103:H2	2013-3-296D	MVLG00000000					■	■	■	■	■			■				■	■					■		■	■								
		D716			2013-3-416C	MVLI00000000					■	■	■	■	■			■	■	■		■	■					■		■	■								
		D730			2013-3-526E	MVLL00000000					■	■	■	■				■	■	■		■	■					■		■	■								
		D732			2013-3-533B	MVLJ00000000					■	■	■	■	■			■		■		■	■					■											
					2013-3-536F	MVLK00000000					■	■	■	■	■			■		■		■	■					■											
		D951			2013-3-73A	MVKZ00000000					■	■	■	■	■			■	■	■		■	■					■		■	■								
		D976			2013-3-98B	MVLA00000000					■	■	■	■				■		■		■	■				■	■	■	■								
					2013-3-109D	MVLB00000000					■	■	■	■	■			■		■		■	■				■	■	■									
		D989			2013-3-141E	MVLC00000000					■	■	■	■				■	■	■		■	■					■	■								
					2013-3-134A	MVLD00000000					■	■	■	■	■			■				■	■					■	■								
		D991			2013-3-331C	MVLH00000000					■	■	■	■	■			■	■	■		■	■					■	■								
		D992			2013-3-435B	MVLE00000000					■	■	■	■	■			■	■	■								■	■								
NE	A	D976	Putative non-pathotype	O103:H16	2013-3-111C	MVNE00000000																							■	■	■							
		D707		O103:H2	2013-3-308C	MVNK00000000																							■	■	■							
		D716			2013-3-416C	MVNM00000000																							■	■	■							
		D732			2013-3-544F	MVNN00000000																							■	■	■							
					2013-3-540A	MVNO00000000																							■	■	■							
		D736			2013-3-575A	MVNP00000000																							■	■	■							
		D951			2013-3-75E	MVND00000000																							■	■	■							
		D976			2013-3-99D	MVNF00000000																							■	■	■		■	■	■	■	■	■
		D987			2013-3-178C	MVNJ00000000																							■	■	■							
		D989			2013-3-131E	MVNG00000000																							■	■	■		■	■	■	■	■	■
					2013-3-132F	MVNH00000000																							■	■	■		■	■	■	■	■	■
		D991			2013-3-314E	MVNL00000000																							■	■	■		■	■	■	■	■	■
		D995			2013-3-164B	MVNI00000000																							■	■	■		■	■	■	■	■	■

[†]Virulence genes were determined using Virulence Finder 1.4.

Supplemental Table A.3 Virulence gene profiles[†] of clinical human enterohemorrhagic *Escherichia coli* (EHEC) O103 strains

Location	Serotype	Strain ID	Accession #	Phage-encoded Shiga toxin							Locus of Enterocyte Effacement (LEE) encoded					Phage-encoded Type III effectors			Non-LEE encoded			pO157 plasmid-encoded					Other													
				stx 1 FLY16	*stx* 1 GPU96MM	*stx* 1A variant a	*stx* 1B variant a	*stx* 2 EDL933	*stx* 2A variant a	*stx* 2B variant a	*eae*	*tir*	*esp* A	*esp* B	*esp* F	*cif*	*esp* J	*tccP*	*nle* A	*nle* B	*nle* C	*ehx* A	*esp* P	*etp* D	*kat* P	*tox* B	*efa* 1	*iha*	*ast* A	*esp* I	*gad*	*iss*	*lpf* A	*celb*	*cba*	*cma*	*mch* F	*mch* C	*mch* B	*mcm* A
KS	O103:H2	KSU-71	TBD	■		■	■				■	■	■	■	■	■	■	■	■	■		■	■		■		■	■			■	■								
		KSU-72	TBD	■		■	■				■	■	■	■	■	■	■	■	■	■		■	■		■	■	■	■	■		■	■								
	O103:H11	KSU-73	TBD	■		■	■				■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■						
		KSU-74	TBD	■		■	■				■	■	■	■	■	■	■		■	■	■	■	■		■		■	■	■		■	■	■	■						
		KSU-75	TBD	■		■	■				■	■	■	■	■	■	■	■	■	■	■	■	■		■		■	■	■		■	■	■	■						
		KSU-76	TBD	■		■	■				■	■	■	■	■	■	■	■	■	■	■	■	■		■		■	■	■		■	■	■	■						
Japan	O103:H2	12009[‡]	AP010958.1	■		■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■		■		■	■			■	■								
Japan	O157:H7	Sakai[‡]	BA000007.2		■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■		■	■	■	■								
MI	O157:H7	EDL933[‡]	CP008957.1		■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■		■	■		■	■								

[†]Virulence genes were determined using Virulence Finder 1.4 (73).
[‡]Control strains were included for comparison and result from the testing of genomic and plasmid (O103:H2 12009, NC_013354.1; Sakai, NC_002128.1 and NC_002127.1; EDL933, AF074613.1) DNA sequences available at GenBank.

Supplemental Table A.4 Plasmid profiles[†] and number of prophage sequences[‡] of enterohemorrhagic *Escherichia coli* (EHEC) O103:H2 strains isolated from cattle feces collected from nine feedlots in the Midwest

State	Feedyard	Pen ID	Strain ID	Accession #	Plasmid profiles																			No. of prophage sequences		
					Col156	CoRNA1	IncA/C2	IncB/O/K/Z	IncFIA(HI1)	IncFIB(AP001918)	IncFIC(FII)	IncFII	IncFII(29)	IncFII(pCoo)	IncFII(pHN7A8)	IncFII(pRSB107)	IncFII(pSE11)	IncI2	IncN	IncP	IncX1	IncY	p0111	Intact	Questionable	Incomplete
NE	A	D707	2013-3-304D	MVLT00000000	■	■				■														3	1	11
		D716	2013-3-409B	MVLV00000000	■	■				■														3	2	12
		D732	2013-3-539F	MVLW00000000		■				■														3	1	15
			2013-3-552F	MVLX00000000		■				■														3	0	14
		D736	2013-3-557A	MVLY00000000	■	■				■														3	1	12
		D951	2013-3-80A	MVLM00000000		■				■														2	1	12
		D976	2013-3-104A	MVLN00000000	■	■				■				■					■					2	3	11
			2013-3-118A	MVLO00000000	■	■				■				■					■					2	2	11
		D987	2013-3-174C	MVLS00000000	■	■				■				■										2	3	10
		D989	2013-3-135A	MVLP00000000		■				■														3	1	10
			2013-3-141B	MVLQ00000000		■				■														4	1	10
			2013-3-144B	MVLR00000000		■				■														5	1	11
		D991	2013-3-314B	MVLU00000000	■	■				■														3	2	13
	C	28	2014-5-1253A	MVMX00000000				■		■														3	2	4
		36	2014-5-58F	MVMB00000000						■														2	3	7
		56	2014-5-89A	MVMD00000000						■														6	1	7
			2014-5-94C	MVME00000000						■														6	1	8
		80	2014-5-548J	MVMM00000000						■														2	3	6
		90	2014-5-82A	MVMC00000000						■														2	2	8
	E	2	2014-5-288B	MVMI00000000						■														3	0	6
		44	2014-5-925A	MVMV00000000		■		■																6	0	11
		51	2014-5-888D	MVMU00000000		■		■																5	2	10
		53	2014-5-933A	MVMW00000000		■		■																5	1	9
			2014-5-941B	MVNA00000000		■		■																4	1	10
	G	36	2014-5-1838G	MVMZ00000000	■	■				■														2	2	10
		151	2014-5-370H	MVML00000000		■		■		■														4	2	6
	I	19	2014-5-1451D	MVNC00000000	■	■				■								■						3	2	12
TX	B	B12	2014-5-7C	MVLZ00000000		■		■		■														3	2	8
			2014-5-10D	MVMA00000000		■		■		■														3	3	8
	D	131	2014-5-650B	MVMQ00000000	■	■		■		■														3	2	7
		131	2014-5-655A	MVMR00000000	■	■		■		■														3	2	8
		234	2014-5-610A	MVMN00000000	■	■		■		■														3	3	7
			2014-5-611A	MVMO00000000	■	■		■		■														3	2	7
			2014-5-614A	MVMP00000000	■	■		■		■														3	1	8
		237	2014-5-138A	MVMF00000000	■	■		■		■														3	2	11
			2014-5-139D	MVMG00000000	■	■		■		■														3	2	8
			2014-5-140E	MVMH00000000	■	■		■		■														3	3	13
	F	D01	2014-5-965B	MVNB00000000	■	■				■														3	1	9
		E05	2014-5-330A	MVMJ00000000		■		■		■													■	3	3	12
			2014-5-332A	MVMK00000000		■		■		■													■	6	1	9
	H	102	2014-5-841G	MVMS00000000		■	■			■														4	0	14
		A20	2014-5-1565C	MVMY00000000	■	■	■	■		■		■												3	1	15
		A56	2014-5-863D	MVMT00000000		■	■			■														3	2	11

[†]Plasmids were determined from whole genome sequences of strains using Plasmid Finder 1.3.

[‡]Number of prophage sequences were determined from whole genome sequences of strains using Phage Search Tool Enhanced Release (PHASTER). Intact, questionable and incomplete prophage counts based on PHASTER scores >90, 70-90 and <70, respectively.

Supplemental Table A.5 Plasmid profiles[†] and number of prophage sequences[‡] of enteropathogenic *Escherichia coli* (EPEC) O103 and *E. coli* O103 strains negative for Shiga toxin and intimin genes (putative non-pathotype) isolated from cattle feces collected from a Midwest feedlot

State	Feedyard	Pen ID	Pathotype	Serotype	Strain ID	Accession #	Plasmid profiles																			No. of prophage sequences		
							Col156	ColRNAI	IncA/C2	IncB/O/K/Z	IncFIA(HI1)	IncFIB(AP001918)	IncFIC(FII)	IncFII	IncFII(29)	IncFII(pCoo)	IncFII(pHN7A8)	IncFII(pRSB107)	IncFII(pSE11)	IncI2	IncN	IncP	IncX1	IncY	p0111	Intact	Questionable	Incomplete
NE	A	D721	EPEC	O103:H11	2013-3-492A	MVLF00000000	■	■		■		■													■	2	0	11
		D707		O103:H2	2013-3-296D	MVLG00000000													■				■			5	4	8
		D716			2013-3-416C	MVLI00000000													■				■			3	1	12
		D730			2013-3-526E	MVLL00000000													■							2	4	7
		D732			2013-3-533B	MVLJ00000000													■				■			0	4	7
					2013-3-536F	MVLK00000000													■				■			0	3	9
		D951			2013-3-73A	MVKZ00000000													■							2	3	8
		D976			2013-3-98B	MVLA00000000					■						■	■					■			3	1	8
					2013-3-109D	MVLB00000000					■						■	■					■			3	0	8
		D989			2013-3-141E	MVLC00000000													■					■		2	5	7
					2013-3-134A	MVLD00000000													■					■		4	3	11
		D991			2013-3-331C	MVLH00000000													■					■		2	4	9
		D992			2013-3-435B	MVLE00000000																				2	2	5
NE	A	D976	Putative non-pathotype	O103:H16	2013-3-111C	MVNE00000000		■																		4	0	0
		D707		O103:H2	2013-3-308C	MVNK00000000		■				■	■													3	0	7
		D716			2013-3-416C	MVNM00000000		■				■	■													3	1	4
		D732			2013-3-544F	MVNN00000000		■				■	■													3	1	4
					2013-3-540A	MVNO00000000		■				■	■													3	1	4
		D736			2013-3-575A	MVNP00000000		■				■	■													3	0	6
		D951			2013-3-75E	MVND00000000		■				■	■													3	1	4
		D976			2013-3-99D	MVNF00000000		■				■	■				■									2	3	2
		D987			2013-3-178C	MVNJ00000000		■				■	■													3	1	4
		D989			2013-3-131E	MVNG00000000		■				■	■				■									1	3	2
					2013-3-132F	MVNH00000000		■				■	■				■									2	3	2
		D991			2013-3-314E	MVNL00000000		■				■	■				■									1	4	2
		D995			2013-3-164B	MVNI00000000		■				■	■				■									2	3	2

[†]Plasmids were determined from whole genome sequences of strains using Plasmid Finder 1.3.

[‡]Number of prophage sequences were determined from whole genome sequences of strains using Phage Search Tool Enhanced Release (PHASTER). Intact, questionable and incomplete prophage counts based on PHASTER scores >90, 70-90 and <70, respectively.

Supplemental Table A.6 Plasmid profiles[†] and number of prophage sequences[‡] of clinical human enterohemorrhagic *Escherichia coli* (EHEC) O103 strains

Location	Serotype	Strain ID	Accession #	Plasmid profiles																			No. of prophage sequences		
				Col156	ColRNAI	IncA/C2	IncB/O/K/Z	IncFIA(HI1)	IncFIB(AP001918)	IncFIC(FII)	IncFII	IncFII(29)	IncFII(pCoo)	IncFII(pHN7A8)	IncFII(pRSB107)	IncFII(pSE11)	IncI2	IncN	IncP	IncX1	IncY	p0111	Intact	Questionable	Incomplete
KS	O103:H2	KSU-71	TBD				■		■			■											6	1	7
		KSU-72	TBD				■		■														3	1	5
	O103:H11	KSU-73	TBD		■		■		■														0	0	7
		KSU-74	TBD		■		■		■													■	3	0	7
		KSU-75	TBD		■		■		■		■								■			■	3	0	13
		KSU-76	TBD		■		■		■														2	0	8
Japan	O103:H2	12009[*]	AP010958.1						■														11	2	4
Japan	O157:H7	Sakai[*]	BA000007.2						■		■												12	4	1
MI	O157:H7	EDL933[*]	CP008957.1						■		■												12	2	5

[†]Plasmids were determined from whole genome sequences of strains using Plasmid Finder 1.3 (75)
[‡]Number of prophage sequences were determined from whole genome sequences of strains using Phage Search Tool Enhanced Release (PHASTER) (76, 77). Intact, questionable and incomplete prophage counts based on PHASTER scores >90, 70-90 and <70, respectively.
[*]Control strains were included for comparison and result from the testing of genomic and plasmid (O103:H2 12009, NC_013354.1; Sakai, NC_002128.1 and NC_002127.1; EDL933, AF074613.1) DNA sequences available at GenBank.

Supplemental Table A.7 Resistance gene profiles[†] of enterohemorrhagic (EHEC) and putative non-pathotype (*stx*/*eae* negative) *Escherichia coli* O103 strains of bovine and human origin

Location	Feedyard	Pen ID	Host Origin	Pathotype	Serotype	Strain ID	Accession #	Aminoglycoside					Beta-lactam			Macrolide	Phenicol	Sulphonamide		Tetracycline				Trimethoprim
								strB	*strA*	*aadA 1*	*aac(3)-VIa*	*aadA2*	*blaTEM-1B*	*blaCMY-2*	*blaTEM-1C*	*mph(A)*	*floR*	*sul 2*	*sul 1*	*tet(A)*	*tet(B)*	*tet(C)*	*tet(M)*	*dfrA 12*
NE	A	D976	Bovine	EHEC	O103:H2	2013-3-104A	MVLN00000000						■			■	■			■				
NE	A	D976				2013-3-118A	MVLO00000000						■			■	■			■				
TX	H	102				2014-5-841G	MVMS00000000	■	■	■	■			■		■	■	■	■	■				
TX	H	A56				2014-5-863D	MVMT00000000	■	■	■	■			■		■	■	■	■	■				
NE	E	51				2014-5-888D	MVMU00000000	■	■									■			■			
NE	E	44				2014-5-925A	MVMV00000000	■	■									■			■			
NE	E	53				2014-5-933A	MVMW00000000	■	■									■			■			
NE	E	53				2014-5-941B	MVNA00000000	■	■									■			■			
NE	I	19				2014-5-1451D	MVNC00000000															■		
TX	H	A20				2014-5-1565C	MVMY00000000	■	■	■	■			■		■	■	■	■	■				
NE	A	D976	Bovine	putative non-pathotype	O103:H2	2013-3-99D	MVNF00000000	■	■									■		■				
NE	A	D989				2013-3-131E	MVNG00000000	■	■											■				
NE	A	D989				2013-3-132F	MVNH00000000	■	■									■		■				
NE	A	D995				2013-3-164B	MVNI00000000	■	■											■				
NE	A	D991				2013-3-314E	MVNL00000000	■	■									■		■				
KS	NA	NA	Human	EHEC	O103:H11	KSU-75	TBD					■			■	■	■		■	■			■	■
Japan	NA	NA	Human	EHEC	O103:H2	12009[‡]	AP010958.1																	
Japan	NA	NA	Human	EHEC	O157:H7	Sakai[‡]	BA000007.2																	
MI	NA	NA	Human	EHEC	O157:H7	EDL933[‡]	CP008957.1																	

[†]Resistance genes were determined using ResFinder 2.1.
[‡]Control strains were included for comparison and result from the testing of genomic and plasmid (O103:H2 12009, NC_013354.1; Sakai, NC_002128.1 and NC_002127.1; EDL933, AF074613.1) DNA sequences available at GenBank