Detection, quantification and genetic characterization of six major non-O157 Shiga toxin-producing *Escherichia coli* serogroups and *E. coli* O104 in feedlot cattle feces

by

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AN ABSTRACT OF A DISSERTATION

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Abstract

Cattle feces are a major source of six Shiga toxin-producing *E. coli* (STEC) serogroups, O26, O45, O103, O111, O121, and O145, called non-O157 STEC, responsible for >70% of non-O157 STEC-associated human illnesses. Another *E. coli* serotype, O104:H4, a hybrid pathotype of enteroaggregative and STEC, was responsible for a large outbreak of foodborne illness in Germany. Studies were conducted to develop and validate culture- and PCR-based methods to detect and or quantify six non-O157 *E. coli* serogroups and *E. coli* O104 in cattle feces, and genetically assess their virulence potential, based on DNA microarray and whole genome sequencing (WGS). Two multiplex quantitative PCR (mqPCR) assays (assay 1: O26, O103 and O111; assay 2: O45, O121 and O145), targeting serogroup-specific genes, were developed and validated for the detection and quantification of six non-O157 *E. coli* in cattle feces and was compared to culture-based and end-point PCR methods. The mqPCR assays detected higher proportion of fecal samples as positive for one or more non-O157 *E. coli* serogroups compared to culture-based and end-point PCR methods. Spiral plating method was validated to quantify six non-O157 *E. coli* serogroups in cattle feces, and was compared to mqPCR assays. The mqPCR assays quantified higher proportion of fecal samples positive for one or more non-O157 *E. coli* serogroups compared to spiral plating method, however, unlike mqPCR, spiral plating method quantifies serogroups positive for virulence genes. Quantification by either mqPCR or spiral plating identified a subset of cattle that was shedding non-O157 *E. coli* at high concentrations (≥ 4 log CFU/g of feces), similar to *E. coli* O157. Identification of Shiga toxin subtypes associated with non-O157 *E. coli* serogroups isolated from cattle feces revealed a variety of subtypes, with *stx*1a and *stx*2a being the most predominant. Microarray-based analysis of six non-O157 *E. coli* serogroups isolated from cattle feces revealed the presence of *stx*, LEE-encoded, and other

virulence genes associated with human illnesses. Analysis of WGS of STEC O145 strains isolated from cattle feces, hide and human clinical cases revealed similarity in virulence gene profiles, suggesting the potential of cattle *E. coli* O145 strains to cause human illnesses. Shiga toxin 1a was the most common *stx* subtype, followed by *stx*2a, and *stx*2c. The strains also carried LEE-encoded, and plasmid-encoded virulence genes.

Model adjusted prevalence estimates of *E. coli* O104 in cattle fecal samples collected from feedlots (n=29) were 0.5% and 25.9% by culture and PCR methods, respectively. Cattle harbor O104 serotypes other than H4, with O104:H7 being the predominant serotype and only a small proportion of them carried *stx*. DNA microarray and WGS analysis revealed absence of LEE-encoded virulence genes in bovine and human O104 strains. *Escherichia coli* O104:H7 has the potential to be a diarrheagenic foodborne pathogen in humans, since they possess *stx*1c and genes that code for enterohemolysin and a variety of adhesins. Data on prevalence, concentration and virulence potential of non-O157 *E. coli* serogroups, including O104, isolated from cattle feces are essential to design effective intervention strategies to reduce the potential to cause human foodborne illness outbreaks.

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Dedication

I would like to dedicate this dissertation to the almighty for his blessings and support. I would also like to dedicate this dissertation to my husband, Vinay for his continuous support and love.

Chapter 1 - Detection, quantification and genetic characterization of non-O157 Shiga toxin-producing *Escherichia coli* serogroups in cattle feces

Introduction

Escherichia coli is an enteric Gram-negative bacteria belonging to the family *Enterobacteriaceae*. This organism was first described by a German pediatrician, Theodore Escherich (Escherich, 1885). They are facultatively anaerobic and non-sporulating organisms. Biochemically, they are positive for indole, methyl red, and lactose, and negative for Voges-Proskauer reaction and citrate test. It is a normal inhabitant of the gastrointestinal tract of humans and other warm-blooded animals (Kaper et al., 2004). The commensal *E. coli* strains reside on the mucus layer covering the epithelial cells in the cecum and colon, they are shed into the lumen of the intestinal tract along with mucus, and are subsequently excreted in feces (Poulsen et al., 1994). Commensal *E. coli* benefits the host by preventing the colonization of harmful pathogens in the intestine (Hudault et al., 2001).

During the course of evolution, some strains have acquired virulence attributes through mobile elements such as plasmids, pathogenicity islands, transposons and phages to adapt to different environments. These pathogenic *E. coli* can infect the gastrointestinal tract, urinary tract, enter the bloodstream and cause sepsis, or meningitis (Nataro and Kaper, 1998). Pathogenic *E. coli* are categorized into groups called pathotypes or virotypes based on the virulence factors they possess and mechanisms of their pathogenesis. Diarrheagenic *E. coli* are subdivided into six pathotypes: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*

(EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). Different pathotypes of *E. coli* are classified into serogroups (O antigen only) and serotypes (combination of O and H antigens) based on O (lipopolysaccharide) and H (flagellar) antigens (Nataro and Kaper, 1998). There are 174 O antigens (one to 181, with 31, 47, 67, 72, 93, 94, and 122 deleted), and 53 H antigens (Gyles, 2007; Scheutz et al., 2004).

Shiga toxin producing *E. coli* (STEC)

Pathogenic *E. coli* which carry the Locus of enterocyte effacement (LEE) pathogenicity island and Shiga toxins (1 and or 2) are termed as Enterohemorrhagic *E. coli* (EHEC), and those that carry only Shiga toxins are termed as Shiga toxin-producing *E. coli* (STEC) (Steiner, 2016), also called as 'Verocytotoxin producing *E. coli*' (VTEC). They are foodborne pathogens responsible for severe human illnesses characterized by bloody to non-bloody diarrhea, and life-threatening complications such as hemolytic uremic syndrome (HUS). Shiga toxins are the major virulence factors of STEC. There are two main types of Shiga toxins: Shiga toxin 1 and 2, encoded by *stx*1 and *stx*2 genes. They belong to the AB5 family of toxins, with a single A subunit involved in enzymatic activity and 5 B subunits involved in receptor binding. They inhibit protein synthesis by cleaving 28s rRNA (Brown et al., 1986; Sandvig et al., 2002). The two antigenically distinct Stx types, Stx1 and Stx2, share approximately 56% amino acid sequence identity (Strockbine et al., 1986; Weinstein et al., 1988). Variants exist within *stx*1 (*stx*1a, *stx*1c, *stx*1d) and *stx*2 (*stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f, *stx*2g) families based on differences in amino acid compositions of the A and B subunits and in cytotoxicity. Shiga toxin 2 is more commonly associated than Stx1 with complications such as HUS (Ethelberg et al.,

2004b). In addition to *stx*, some strains also carry intimin, and other virulence factors encoded by LEE Pathogenicity Island similar to EPEC. Intimin-positive STEC are called classical enterohemorrhagic *E. coli* (EHEC). Cattle are major reservoirs of STEC, which carry these organisms in their hindgut and shed them in their feces.

STEC infections in humans

Majowicz et al. (2014) have estimated that each year STEC cause 2.8 million cases of acute illness, leading to 3,890 cases of HUS, 270 cases of end-stage renal disease, and 230 deaths globally (Majowicz et al., 2014).. *Escherichia coli* O157 is most commonly associated with foodborne illness outbreaks. *Escherichia coli* O157 outbreak was first reported in 1982 in the United States (Pennington, 2010; Riley et al., 1983), and this serogroup has since led to several large outbreaks in the United States and other parts of the world (Bell et al., 1994; Tarr et al., 2005). A total of 390 STEC O157 outbreaks resulting in 4,928 illnesses, 1,272 hospitalizations, 299 cases of HUS and 33 deaths were reported during the period of 2003-2012 in the United States (Heiman et al., 2015). Most of the STEC illness outbreaks have been associated with STEC O157:H7, however, non-O157 STEC associated outbreaks have been increasing in recent years (Brooks et al., 2005b; Huang et al., 2016). Similar to O157, non-O157 STEC infections range from non-bloody to bloody diarrhea, and life-threatening complications like hemorrhagic colitis and hemolytic uremic syndrome (HUS). Approximately 380 serotypes of *E. coli* isolated from animals have also been isolated from humans (Karmali et al., 2010). STEC serogroups such as O26, O45, O103, O111, O121, and O145 account for more than 70% of non-O157 STEC infections in the United States (Brooks et al., 2005c). In 2011, the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) declared these six major non-

O157 STEC serogroups to be regulated as adulterants in ground beef and non-intact raw beef products (USDA-FSIS, 2011).

According to the Foodborne Diseases Active Surveillance Network's (FoodNet) report on the human illness cases caused by nine foodborne pathogens in 10 US states in 2016, STEC was the 4th most (next to *Shigella*) common cause of human foodborne illness with 1,399 confirmed cases, 326 hospitalizations, and three deaths. Non-O157 STEC (64% of confirmed cases) were more prevalent compared to O157 STEC (36% of confirmed cases) among the confirmed cases of foodborne illness due to STEC. The report suggested that there is an increase in the incidence of non-O157 STEC infections when compared to the previous report (2013-2015), while the incidence of STEC O157 infections remains unchanged. The most common non-O157 STEC serogroup responsible for foodborne illness is O26 (21%), followed by O103 (20%) and O111 (12%) (Huang et al., 2016).

Karmali et al. (2003) classified STEC serotypes into 5 seropathotypes based on their association with human illness. Serotypes, which are most commonly associated with severe human illness and outbreaks such as O157:H7 and O157:NM, were included in seropathotype A. Seropathotype B consists of O26:H11, O103:H2, O111:H8/NM, O121:H19, and O145:NM, which are less commonly associated with outbreaks and HUS compared to seropathotype A. Seropathotypes C and D are associated with sporadic cases of HUS and diarrhea, respectively. Seropathotype E includes strains isolated from animals and not involved in human illness (Karmali et al., 2003).

The outbreak that occurred in Germany in 2011involving contaminated sprouts resulted in nearly 4,000 illnesses, and about 900 cases of hemolytic uremic syndrome, leading to 54 deaths (Karch et al., 2012). The causative agent is a hybrid strain carrying characteristics of two

pathotypes: STEC and EAEC (Bielaszewska et al., 2011). Although cattle are the primary reservoirs of STEC, this German outbreak pathotype has not been isolated from cattle feces so far (Paddock et al., 2013; Shridhar et al., 2016b).

Prevalence of non-O157 STEC in cattle feces

Ruminants, especially cattle are the main reservoirs of STEC. They carry STEC in their hindgut and shed the organisms in their feces, which is the source of contamination of food and water. Consumption of contaminated food or water leads to human illness. Knowledge of the prevalence of non-O157 STEC in various sources is essential to design effective intervention strategies to prevent foodborne illness outbreaks in humans. There are several studies reporting the prevalence of non-O157 *E. coli* serogroups in cattle feces.

Blanco et al. (1996) determined the prevalence and characteristics of STEC in cattle fecal samples collected from multiple farms, where 84% of farms were found positive for STEC. The isolated STEC strains belonged to 25 serogroups of which seven serogroups (O8, O20, O22, O77, O113, O126 and O162) accounted for 46% of the strains, and only one strain belonged to top six non-O157 *E. coli* serogroup (O26) (Blanco et al., 1996). Prevalence of non-O157 *E. coli* serogroups was determined in cattle fecal samples collected from abattoirs, where O26 and O103 were more prevalent compared to O111 and O145 (Joris et al., 2011). Cernicchiaro et al. (2013) reported the prevalence of non-O157 *E. coli* serogroups, based on PCR assay, in feces of feedlot cattle that originated from multiple backgrounding operations in six Midwestern states of the United States, and finished in a feedlot operation in Nebraska. *Escherichia coli* O26 (20.3%) was the most prevalent non-O157 serogroup, followed by O103 (11.8%), O121 (10.7%), O45 (10.4%), O145 (2.8%), and O111 (0.8%) (Cernicchiaro et al., 2013). Prevalence of six major

non-O157 *E. coli* serogroups was determined in pooled fecal samples collected from feedlots in four Midwestern states in the United States by direct PCR, and they found that *E. coli* O45 (13.8%) was the most prevalent serogroup followed by O26 (9.9%), O103 (9.3%), O121 (5.5%), O145 (1.1%), and O111 (0.5%) (Dargatz et al., 2013).

Prevalence of non-O157 *E. coli* serogroups in fecal samples is influenced by several factors such as sampling methods, the amount of fecal sample used, detection methods used, and the detection limit of the methods. Several studies have reported the influence of seasons on the prevalence of non-O157 *E. coli* serogroups in cattle feces. Dewsbury et al. (2015) reported a statistically significant differences in the seasonal prevalence of *E. coli* O103 in cattle feces (Dewsbury et al., 2015). Seasonal variation in the prevalence of other non-O157 *E. coli* serogroups was also observed, with O26, O45, O103 and O121 being less prevalent and O111 and O145 being highly prevalent during winter months (Stanford et al., 2016). Cull et al. (2017) reported that *E. coli* O103 and O145 were the most prevalent non-O157 serogroups in cattle feces collected from commercial feedlots in Texas and Nebraska. They also identified sampling month, and sex composition of the pen as potential risk factors for O157 and non-O157 EHEC shedding in cattle feces (Cull et al., 2017). Prevalence of non-O157 *E. coli* serogroups determined based on less sensitive methods might result in underreporting of their prevalence. Development and validation of sensitive and specific methods for the detection of non-O157 *E. coli* serogroups are essential for the accurate estimation of their prevalence.

Detection of non-O157 STEC serogroups

Rapid and sensitive detection methods are required to estimate the prevalence of non-O157 *E. coli* serogroups in cattle feces. There are various culture-based, serological, and

molecular techniques for the detection of non-O157 *E. coli* serogroups. However, the detection of non-O157 *E. coli* serogroups poses special challenges due to lack of distinguishing phenotypic characteristics to differentiate among them and from *E. coli* O157 (Wang et al., 2013). Following is a brief review of various methods used for the detection of non-O157 STEC serogroups in cattle feces.

Culture-based detection method

Culture-based methods of detection involve suspending feces in selective enrichment broth, incubating the broth for a specified period to amplify target STEC populations, and plating the fecal broth on a selective medium. Putative STEC colonies are further subjected to serological or molecular techniques for confirmation of the serogroups. Additional steps such as utilization of immunomagnetic separation (IMS) are often included to increase the sensitivity of detection. Both selective and non-selective enrichment broths have been used for enrichment of fecal samples. Vimont et al. (2007) studied the effects of different basal media (*E. coli* broth and tryptic soy broth), the addition of novobiocin to the broth, and different incubation temperatures on the simultaneous growth of fecal background bacterial flora and two non-O157 STEC strains. Based on their results, *E. coli* (EC) broth was the most optimal enrichment medium for the detection of non-O157 STEC in cattle feces; they also found that the addition of novobiocin inhibited the growth of one of the STEC strains used in the study (Vimont et al., 2007). Paddock et al. (2012) compared different enrichment broths and incubation conditions for the growth of non-O157 STEC and reported that EC broth incubated at 40°C for 6 h was more sensitive than EC broth incubated at 40°C for 24 h, or TSB_{BRV} (TSB broth+bile salts+Rifamicin+Vancomycin) and TSB_{BRVN} (TSB_{BRV}+novobiocin) incubated at 42°C for 24h (Paddock et al., 2012). A study conducted by Stromberg et al. (2015) also revealed that EC broth and modified Trypticase soy

(TSB + bile salts; mTSB) broth were more efficient in increasing growth and isolation of non-O157 *E. coli* serogroups compared to non-selective TSB (Stromberg et al., 2015).

Immunomagnetic separation is a technique used to isolate *E. coli* serogroups from various sample matrices by using paramagnetic beads coated with antibodies specific to serogroup specific antigens. Immunomagnetic beads specific to individual serogroups are available for the major six non-O157 STEC serogroups. Pooling of immunomagnetic beads for the detection of non-O157 *E. coli* serogroups has proven to be equally sensitive when compared with individual beads, thus, saving time, labor and expense (Noll et al., 2016). Immunomagnetic separation is followed by plating IMS beads on the agar media. A variety of selective and non-selective media have been used for the isolation of non-O157 STEC serogroups. Posse et al. (2008) developed a differential agar media containing sucrose, sorbose, and X-gal (chromogenic substrate, which indicates β-D-galactosidase activity) and selective components for the isolation of non-O157 *E. coli* serogroups. Colonies of different non-O157 *E. coli* serogroups were differentiated based on fermentation of specific carbohydrates which resulted in different colored colonies: O26 colonies were bright red to dark purple, O103 and O111 colonies were blue-purple, O145 colonies were green color with dark center (Possé et al., 2008). Rhamnose MacConkey agar supplemented with cefixime and tellurite (CT-RMAC) was found to be more efficient in isolating *E. coli* O26 from cattle and sheep feces when compared to tryptone bile X-glucuronide (TBX) agar, however, few strains isolated from TBX were not isolated from CT-RMAC. Hence the authors suggested the use of both CT-RMAC and TBX for the efficient isolation of *E. coli* O26 from cattle and sheep feces (Evans et al., 2008). Kalchayanand et al. (2013) evaluated USMARC chromogenic agar medium for the detection and isolation of non-O157 STEC from fresh beef, and cattle feces. A combination of carbohydrates, β-galactosidase

activity, and resistance to selective agents was used for the detection of non-O157 STEC. There was variability in the colony morphology of some strains within a serogroup, and the medium allowed the growth of both STEC and non-STEC serogroups. Hence, the authors suggested testing representative colonies by PCR to confirm the presence of serogroup-specific and virulence genes (Kalchayanand et al., 2013).

Six chromogenic agar media (Tryptone Bile X-glucuronide agar (TBX), Rainbow® Agar O157 (RB), Rapid *E. coli* O157:H7 (RE), Modified MacConkey Agar (mMAC), CHROMagar™ STEC (CHR- STEC) and chromID™ EHEC (CHR-ID) were compared for the isolation of non-O157 STEC. Use of a combination of a more selective agar like CHR-STEC, and less selective agar like TBX or CHR- ID which allows growth of all STEC strains was found to be the most efficient method for the isolation of non-O157 STEC (Verhaegen et al., 2015). Stromberg et al. (2016) compared CHR- STEC, Posse differential agar (Posse´), Posse´ modified by the reduction or addition of antimicrobials (MP), STEC heart infusion washed blood agar with mitomycin C (SHIBAM), and SHIBAM modified by the addition of antimicrobials for the detection and quantification of O157 and non-O157 STEC in cattle feces. The recovery of STEC serogroups from inoculated fecal samples was significantly higher using MP medium containing decreased concentrations of novobiocin and potassium tellurite when compared to SHIBAM agar. CHROMagar STEC recovered a significantly higher number of STEC-positive samples compared to Posse´ and MP agar (Stromberg et al., 2016). Blood agar supplemented with vancomycin, cefixime, and cefsulodin was used for the isolation of hemolysin-producing STEC serotypes (O5:H-, O26:H-, O26:H11, O91:H21, O11:H-, O111:H8, O104:H11, O113:H21 and O157:H8) in cattle fecal samples, and suspect colonies were further characterized by multiplex PCR (Hornitzky et al., 2001). The inclusion of mitomicin C to the washed blood agar medium

improved the detection and isolation of non-O157 STEC producing enterohemolysin (Sugiyama et al., 2001). Isolates obtained by culture method of detection should be further tested by serological and molecular methods to confirm the serotype and virulence genes carried by the organisms.

Serological methods

The conventional method of identifying *E. coli* serotypes involves using antibodies specific to O and H antigens. Hegde et al. (2012) developed a rapid and sensitive enzyme-linked immunosorbent assay (ELISA) for detection of the top six non-O157 *E. coli* serogroups. A total of 174 reference O groups (O1 through O181) were tested in the study. The sensitivity of the assay was reported to be 5 $x10^5$ CFU/ml, and the detection limit was 1-10 CFU/25 g of ground beef after enrichment. The limitation of this assay was that the antibodies against the O26 serogroup cross-reacted with O146, O156, O167 and O168 serogroups, the O45 antibodies with O146, O153, O156, and O168 serogroups, and the O145 antibodies with O150 and O151 serogroups. Also, this assay has to be used along with PCR assays to identify the virulence genes associated with the serogroups (Hegde et al., 2012).

Several studies have targeted Shiga toxins for the detection of STEC in human clinical samples and other matrices. Vero cell cytotoxicity assay has been used for the detection of Shiga toxins; the assay is based on the cytopathic effect of Shiga toxins on the Vero cells (African green monkey kidney cells) (Konowalchuk et al., 1977). A latex agglutination assay and an immunoblotting method were developed for the detection of STEC using polyclonal antibodies; rabbit antiserum was raised against the whole-cell antigen of STEC strain VT3 (positive for Stx1, Stx2, and intimin). They reported that the specificity of immunoblotting was higher than that of the latex agglutination assay, however, the latter was less expensive (Hajra et al., 2007).

Sepehriseresht et al. (2008) compared PCR and a latex agglutination method targeting Shiga toxins for the detection of STEC in cattle feces. They found 100% agreement between PCR and latex agglutination test results (Sepehriseresht et al., 2009). There are several commercial enzyme immunoassay kits for the detection of STEC in human fecal samples, such as the Premier EHEC kit (Teel et al., 2007), Shiga Toxin Quik Chek (Chui et al., 2015b) and ImmunoCard STAT kit (Chui et al., 2013). Latex agglutination tests are also available for the detection of the top six non-O157 *E. coli* serogroups (Medina et al., 2012). Serotyping based on agglutination reactions is the standard method, however, it is laborious, time-consuming, and more prone to cross-reactions (Orskov et al., 1977).

PCR-based detection

Several PCR-based methods have been developed and validated for the detection of non-O157 *E. coli* serogroups in cattle feces. Based on the nucleotide sequence analyses of O-antigen biosynthesis gene clusters of 184 *E. coli* serogroups, it has been reported that *wzx*/*wzy* and *wzm*/*wzt* O-AGC genes are the most frequently used genes that can differentiate various serogroups due to their sequence diversity, hence these genes can be used to design methods for the detection of various non-O157 *E. coli* serogroups (Iguchi et al., 2014). Various PCR-based methods have been developed to identify the *E. coli* serogroups and the associated virulence genes. Iguchi et al. (2015) designed 162 primer pairs for the detection of *E. coli* O groups in 20 multiplex end-point PCR reactions. Of the total 162 primer pairs, 147 of them were used to identify 142 individual serogroups, and 15 pairs for 15 groups of strains (GP1 to GP15). The assays were validated with pure cultures of 184 O-group reference strains, and were compared with the results obtained by serotyping based on agglutination reaction. They reported that the serogroups of 13 strains identified by PCR did not match with that identified by agglutination

reactions, and also serogroups of 39 strains belonging to 20 O groups were not identified by PCR due to lack of PCR products. They attributed this to variation in O-antigen gene sequence among the strains which belong to same serogroup (Iguchi et al., 2015). A multiplex end-point PCR was developed for the detection of *E. coli* belonging to seropathotypes A (O157:H7 and O157:NM) and B (O26:H11, O111:NM, O121:H19, O145:NM and O103:H2). The assay targeted *stx*, *eae*, 16s rRNA internal amplification control, and O-antigen genes (*wzx*) of O26, O103, O111, O121, O145, and O157. This assay correctly identified the serogroups and virulence genes of the 40 strains tested in the study (Monday et al., 2007).

Clustered regularly interspaced short palindromic repeat (CRISPR) sequences were used as targets for the detection of *E. coli* serotypes such as O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and O157:H7 using a TaqMan-based real-time PCR. The assays were validated using pure cultures of 958 *E. coli* strains; the sensitivity and specificity of the assays were 95.7 to 100% and 97.5 to 100%, respectively. This study showed that CRISPR polymorphisms were correlated with *E. coli* serotypes (O:H) and also the presence of major virulence genes, such as *stx* and *eae*. They concluded that the assays based on CRISPR sequences were more specific than the assays based on O-antigen genes (Delannoy et al., 2012b). Microbead-based suspension array was used to identify clinically relevant STEC serogroups such as O26, O45, O91, O103, O111, O113, O121, O128, O145, and O157 simultaneously using Luminex xMAP® technology. The assay was evaluated using 114 STEC isolates in multiple laboratories, and two different instruments (Bio-Plex 200 and MAGPIX). The assay was found to be specific without any false positive results, and the results were consistent among all the laboratories and the instruments (Lin et al., 2011a). Clotilde et al. (2015) compared conventional slide agglutination method, Luminex-based multiplex PCR assay, and antibody-based microbead

assay using Luminex technology for serotyping STEC. They compared the three methods by using 162 STEC isolates, the Luminex PCR assay serotyped 22 more strains which were not detected by conventional serotyping based on agglutination, and 10 additional strains which were not detected by Luminex antibody-based assay (Clotilde et al., 2015). In addition, commercial real-time PCR assays are also available for rapid and high throughput detection of STEC. Beutin et al (2009) have evaluated GeneDiscs for simultaneous detection of Shiga toxin genes (*stx*1, *stx*2), *E. coli* serogroups such as O26, O103, O111, O145, O157 and flagellar antigen genes (*fliC*$_{H7}$). However, the above mentioned assays have only been validated using pure cultures, but not feces spiked with pure cultures.

Paddock et al. (2011) developed a multiplex end-point PCR assay targeting O-antigen genes of *E. coli* O26, O45, O103, O111, O121, O145, and O157 serogroups in cattle feces. The assay was validated with pure cultures, spiked and naturally-shedding cattle fecal samples. The sensitivity of the assay was 4.1×10^5 CFU/g before enrichment and 2.3×10^2 CFU/g after enrichment of spiked fecal samples. This assay was also reported to detect a higher proportion of fecal samples that were positive for at least one of the tested *E. coli* serogroups compared to the culture-based method (Bai et al., 2012). However, this assay only detected O157 and non-O157 *E. coli* serogroups targeting O-antigen genes, but not the virulence genes. To address this issue, the researchers developed multiplex end-point PCR assay to detect seven major *E. coli* serogroups (O26, O45, O103, O111, O121, O145, and O157) targeting O-antigen genes and four major virulence genes (*stx*1, *stx*2, *eae*, *ehx*A) in cattle feces (Bai et al., 2012). The assay was validated with pure cultures, cattle fecal samples spiked with pure cultures, and naturally-shedding cattle fecal samples. The detection limit of the assay was 10^5 CFU/g before enrichment

and 10^2 CFU/g after enrichment. This assay was also reported to be more sensitive in detecting STEC when compared to the culture-based method.

End-point PCR assays are less sensitive compared to real-time PCR assays. Hence, several real-time PCR assays have been developed and validated for the detection of non-O157 STEC. Real-time PCR-based detection of non-O157 STEC is based on several available chemistries such as SYBR green, TaqMan, molecular beacon probes, fluorescence resonance energy transfer probes, and LUX (light upon extension). A SYBR green-based real-time PCR assay was developed for the simultaneous detection and quantification of *stx*1, *stx*2, and *eae* using spiked cattle fecal samples and naturally-shedding cattle fecal samples using a culture-based method as reference. The sensitivity and specificity of the assay were 83% and 77%, respectively, when compared with the culture method (Verstraete et al., 2014). Multiplex real-time PCR assays were developed for simultaneous detection and quantification of EHEC O157, O26, and O111 in cattle feces and beef. Two sets of TaqMan-based multiplex real-time PCR assays were developed for the simultaneous detection and quantification of EHEC O157, O26, and O111; one assay targeted *eae* genes specific to *E. coli* O26, O111, and O157 serogroups, and another targeted *stx*1 and *stx*2. The detection limits of the assays were 1-10 CFU/g of feces and ground beef after enrichment (Sharma, 2002). Anklam et al. (2012) developed four TaqMan multiplex real-time PCR assays for the detection of *E. coli* O157 and six major non-O157 *E. coli* serogroups (O26, O45, O103, O111, O121, O145) and four major virulence genes (*eae*, *stx*1, *stx*2, and *ehx*A). Assay 1 targeted O26, O103 and O145; assay 2 targeted O45, O111 and O121, assay 3 targeted O157; and assay 4 targeted *stx*1, *stx*2, *eae* and *ehx*A. The detection limits of the assays were 10^3, 10^4, and 10^0 CFU/ml for pure cultures, spiked fecal samples before enrichment, and after enrichment, respectively (Anklam et al., 2012). However, the applicability of the assays

for the detection of STEC in naturally-shedding fecal samples was not evaluated. Hence, we developed two multiplex real-time PCR assays (assay 1: O26, O103, O111 and assay 2: O45, O121, O145) to detect and quantify six major non-O157 *E. coli* serogroups in cattle feces. The assays were validated with pure cultures and cattle fecal samples spiked with pure cultures. The detection limit of the assays were 3, 4, and 2 CFU/ml or g for pure cultures, fecal samples spiked with pure cultures before and after enrichment, respectively. Additionally, the applicability of these two assays along with another multiplex PCR assay targeting *stx*1, *stx*2, *eae* and $rfbE_{O157}$ to detect seven *E. coli* serogroups and three virulence genes in feedlot cattle fecal samples was determined, and were compared to conventional PCR and culture-based methods. These assays detected a higher proportion of samples positive for one or more *E. coli* serogroups compared to conventional PCR and culture-based methods (Shridhar et al., 2016a).

Fecal concentrations of non-O157 STEC serogroups

The concentration of STEC in cattle feces plays a major role in contamination of hide and subsequent contamination of carcasses during slaughter operations (Arthur et al., 2009; Jacob et al., 2010). Contaminated meat, fresh produce, flour, juices and milk have resulted in several outbreaks and sporadic cases of human foodborne illnesses. Hence, estimating the concentration of STEC in various sources of contamination of food products is important in estimating the risk of human illness associated with them. It is also useful in designing intervention strategies to prevent foodborne illnesses in humans. There are many studies on the shedding pattern of *E. coli* O157 (duration and season of shedding, concentration, and factors affecting shedding, etc.) in cattle feces. However, there is lack of studies on the shedding patterns of non-O157 *E. coli* serogroups in cattle feces.

Escherichia coli O157 has been shown to shed at variable concentrations in cattle feces, which fluctuates within the same animal over time (Munns et al., 2014; Robinson et al., 2009). However, most of the cattle have been shown to shed STEC at a concentration less than 10^2 CFU/g (Lahti et al., 2003; Omisakin et al., 2003; Pearce et al., 2004). A subset of cattle, termed super shedders has been reported to shed *E. coli* O157 at concentrations $\geq 10^4$ CFU/g in their feces(Arthur et al., 2009; Chase-Topping et al., 2008; Matthews et al., 2006; Munns et al., 2014). Super-shedding cattle play a major role in the transmission of *E. coli* O157 within pens, during transportation and lairage, and subsequent contamination of carcasses during slaughter (Fox et al., 2008; Jacob et al., 2010; Omisakin et al., 2003). Arthur et al. (2009) suggested that pre-harvest intervention strategies should be aimed at high-level shedders to reduce the overall prevalence of STEC in cattle (Arthur et al., 2009).

There are several factors that affect the fecal concentrations in cattle. Host genetic and physiological factors including age, rumen microflora, breed, and sex have been reported to influence the super shedding event in cattle (Cray and Moon, 1995; Jeon et al., 2013; Nielsen et al., 2002; Zhao et al., 1998). Environmental factors, such as diet, temperature, humidity, and high cattle density within pens, also affect super shedding in cattle (Jeon et al., 2013; Williams et al., 2014). Cobbold et al. (2007) showed that the colonization of *E. coli* O157:H7 in the recto-anal junction influences super shedding in feedlot cattle (Cobbold et al., 2004). Bacterial strain diversity has also been reported to influence super shedding in cattle. Stanford et al. (2012) have shown that the PFGE profiles of *E. coli* O157:H7 strains isolated from super shedding cattle were different from profiles of low shedders housed in the same pen (Stanford et al., 2012). Cote et al. (2015) analyzed the whole genome sequence of an *E. coli* O157:H7 super shedder strain, and reported that the non-synonymous single nucleotide polymorphisms in the adherence and

other virulence genes contribute to the super shedding phenomenon in cattle. They also observed LEE-independent strong aggregative adherence of super shedder strains to bovine RAJ stratified squamous epithelial (RSE) cells (Cote et al., 2015). Identification of super-shedders depends on the several factors such as methods of sampling, time elapse between sample collection and processing, and quantification method used for identification of super-shedders.

There are several studies on *E. coli* O157 super shedders in cattle (Arthur et al., 2013; Arthur et al., 2009; Cobbold et al., 2007; Stephens et al., 2009). However, there is a lack of reports on the super shedders of non-O157 *E. coli* serogroups; this is due to lack of validated methods for the quantification of non-O157 *E. coli* serogroups in cattle feces. Below is the review of commonly used quantification methods used to estimate the concentration of O157 and non-O157 *E. coli* in various sample matrices. Since there is a lack of reports on the concentration data of non-O157 *E. coli* serogroups, most of the following studies focus on *E. coli* O157 quantification.

Direct plating method

Direct plating method involves inoculating dilutions of the sample matrix directly onto selective media without prior enrichment or any processing of sample matrix, followed by colony counting to determine the concentration of target organisms. This method allows enumeration of viable organisms. Omisakin et al. (2003) determined the concentration of *E. coli* O157 in cattle feces using a direct plating method by spread plating serially diluted feces onto CT-SMAC media, and reported that 9% of the cattle were super shedders of *E. coli* O157 (Omisakin et al., 2003). LeJuene et al. (2006) quantified a nalidixic acid resistant strain of *E. coli* O157 by direct plating on MacConkey agar supplemented with nalidixic acid (MACnal); the detection limit of direct plating method was >100 CFU/g of feces. Fox et al. (2007) reported a

sensitivity and specificity of 74.4% and 68.8%, respectively when quantifying nalidixic acid resistant *E. coli* O157 at concentrations >3.0 log CFU/g in artificially inoculated cattle feces by direct plating (Fox et al., 2007).

Direct plating method of bacterial enumeration does not require any special equipment. However, it is labor intensive and time-consuming, since it requires performing multiple dilutions and plating. Hence, it is not a high throughput method. Additionally, the number of dilutions required may vary from one sample to another depending on the concentration of bacteria in the sample. High concentrations of target and/or background flora may result in overcrowding of plates, making it difficult to count the colonies, necessitating performing further dilutions of the samples.

Most probable number technique (MPN)

The most probable number technique (MPN) is another method of enumerating viable organisms. It involves performing serial dilutions of the sample and the concentration is determined based on the most diluted sample that is positive. The MPN technique has been used for the quantification of several bacteria including *E. coli* O157 in different types of sample matrices. Guy et al. (2014) quantified *E. coli* O157 in enriched samples using a modified most probable number method, which allowed quantification of low levels of *E. coli* O157 that was below the detection limit of qPCR (Guy et al., 2014). The samples carrying O157 at levels below the detection threshold of direct qPCR were subjected to immunomagnetic separation to isolate O157, and the resulting isolates were detected by qPCR (Guy et al., 2014). Similarly, Widiasih et al. (2004) used a combination of MPN and IMS methods for the quantification of *E. coli* O157 and O26 in naturally shedding cattle fecal samples; the concentration of *E. coli* O157 ranged from 4 to > 110,000 CFU/10 g, and O26 ranged from 3 to 2400 CFU/10 g (Widiasih et al.,

2004). Fegan et al. (2004) also used a combination of IMS and MPN to quantify *E. coli* O157 in feces of pasture-fed and grain-fed cattle, the concentration ranged from <3 MPN to 2.4 x 10^4 MPN/g feces (Fegan et al., 2004). Stephens et al. (2007) compared direct plating with MPN/IMS method for the quantification of *E. coli* O157 in artificially inoculated cattle fecal samples. There was a correlation between the two methods, and MPN/IMS method was suggested as the most effective method for the quantification of *E. coli* O157 in naturally shedding cattle fecal samples (Stephens et al., 2007). This method has also been used for the quantification of STEC, most commonly O157, in other sample matrices such as fresh produce (Russo et al., 2014), cattle hides and carcasses (Brichta-Harhay et al., 2007), milk (Jamshidi et al., 2011), and meat products (Picozzi et al., 2004). Fox et al. (2007) compared the concentrations of *E. coli* O157 in artificially inoculated cattle feces determined by direct plating of diluted fecal samples (gold standard method) with post-enrichment direct streak-MPN and IMS-MPN methods. There was a good correlation between the gold standard method and post-enrichment direct streak-MPN and IMS-MPN methods, but the sensitivity and specificity of both the methods were too low (Fox et al., 2007).

The most probable number technique has been widely used for the quantification of STEC, since it can detect low numbers of bacteria. However, it has to be coupled with other methods such as IMS and qPCR to increase its sensitivity, which makes it labor intensive, expensive and time-consuming. The major disadvantage of this method is determining the appropriate dilution for the samples, which makes it labor intensive and expensive (Luedtke and Bosilevac, 2015). Another disadvantage is that this technique assumes that the organisms are evenly distributed throughout the sample, which is not true especially when the sample contains particulate matter (Oblinger and Koburger, 1975). It also requires enrichment of fecal samples to

detect low levels of target organisms, which also promotes the growth of background flora that interferes with the quantification of target organisms.

Spiral plating method

Spiral plating is a method used to enumerate bacteria in a sample using a machine, which deposits the inoculum in the form of an Archimedes spiral on a rotating agar plate. It was first described by Gilchrist et al (Gilchrist et al., 1973). Following incubation, colonies are counted using a specialized counting grid that relates colonies on the agar plate to the volume deposited in the area. Jarvis et al. (1977) evaluated the spiral plating method to enumerate bacteria in four types of food. They also compared it to other methods (pour plate, drop count, spread plate), they reported that the spiral plating method can effectively replace other methods for the enumeration of viable microorganisms (Jarvis et al., 1977).

The spiral plating method has been used for the quantification of *E. coli* O157 in cattle feces, hides, carcasses and manure (Berry and Wells, 2008; Brichta-Harhay et al., 2007; Robinson et al., 2004). Robinson et al. (2004) evaluated the spiral plating method for the quantification of *E. coli* O157 in artificially inoculated cattle feces, there was a good agreement between the concentration of inoculum and the concentration recovered from spiked cattle feces, especially when the concentration was $>10^2$ CFU/g of feces (Robinson et al., 2004). Berry et al (2008) evaluated the spiral plating method for the quantification of *E. coli* O157 in aged bovine manure, bovine manure compost, and manure-amended soil. There was a good correlation between inoculated and recovered levels of *E. coli* O157, and the lower limit of detection in manure and manure-containing samples was 200 CFU/g (Berry and Wells, 2008). Fox et al. (2007) evaluated various culture methods (direct plating, MPN, spiral plating method) for the quantification of *E. coli* O157 in cattle feces. The sensitivity and specificity estimates of the

spiral plating method were 79% and 63.2%, respectively when 3 log CFU/g was used as a threshold; using 4 log CFU/g as threshold resulted in sensitivity and specificity estimates of 34.2% and 88.1%, respectively. There was a strong linear relationship between the concentration determined by direct plating and that determined by spiral plating (Fox et al., 2007). However, the spiral plating method has not been validated for the quantification of non-O157 *E. coli* serogroups.

Arthur et al. (2009) used the spiral plating method to investigate the effect of super-shedders of *E. coli* O157 on the prevalence and concentration of *E. coli* O157 on the hide of the cattle housed in the same pen. Based on their results, they suggested that the contamination of hides could be reduced by decreasing the prevalence and concentration of *E. coli* O157:H7 to < 20% and < 200 CFU/g of feces, respectively (Arthur et al., 2009). The spiral plating method was used to investigate the variation in the concentration of *E. coli* O157 within bovine fecal pats. They reported up to two-log difference in the concentration of *E. coli* O157 in different areas of fecal pats. Hence, they suggested pooling of samples collected from different areas within the fecal pats in order to get a good representative sample (Robinson et al., 2005). Brichta-Harhay et al. (2007) evaluated the spiral plating method for the quantification of *E. coli* O157:H7 in various sample matrices (ground beef, carcasses, hides and fecal samples) from cattle using artificially inoculated samples. The lower limit of detection was $8X10^{-1}$ CFU/100 cm^2 for carcass, 4×10^1 CFU/100 cm^2 for hide, and 2×10^2 CFU/100 cm^2 for fecal samples. They also evaluated the method using naturally contaminated samples; the median observed values were 1.6×10^0 CFU/100 cm^2, 8×10^1 CFU/100 cm^2, and 1.6×10^3 CFU/g for carcass, hide and fecal samples respectively. They also reported that the determined concentration values were more accurate when the levels were above the detection limit of the method (Brichta-Harhay et al.,

2007). The spiral plating method was also used for evaluating the interventions used in the slaughter plant for the reduction of EHEC and indicator microorganisms on veal calf hides (Wang et al., 2014). It was also used for evaluating the antimicrobial intervention strategies in fresh beef using samples inoculated with STEC serotypes such as O26, O45, O103, O111, O121, O145, and O157:H7 (Kalchayanand et al., 2012). Berry et al. (2015) used the spiral plating method to determine the effect of proximity of feedlot on *E. coli* O157:H7 contamination of leafy greens. The results suggested air-borne transmission of *E. coli* O157:H7 from feedlots leading to contamination of leafy greens (Berry et al., 2014).

The spiral plating method has been widely used for the quantification of *E. coli* O157:H7 in various sample matrices, and has been reported to be an effective method for enumeration of *E. coli* O157 (Brichta-Harhay et al., 2007; Fox et al., 2007; Robinson et al., 2004). It is also a high throughput method to quantify STEC in any sample matrix. It is also less expensive, and less labor intensive compared to other conventional enumeration methods, since it requires fewer dilutions. This method has been reported to be reproducible and the concentration values are more reliable at higher concentrations (Robinson et al., 2004). The major disadvantage of this method is the initial equipment cost, since it requires special equipment for spiral plating (Fox et al., 2007). Another disadvantage is the growth of background flora on the agar plate leading to overcrowding, which might result in false negative results.

We validated the spiral plating method for the quantification of non-O157 *E. coli* (O26, O45, O103, O111, O121, and O145) serogroups in cattle feces. The method was validated with individual, pooled pure cultures, and cattle fecal samples spiked with pooled pure cultures. Additionally, the applicability of the method to quantify non-O157 *E. coli* serogroups in feedlot cattle feces was determined, and was compared to mqPCR assays. The quantification limit of the

spiral plating method was 3, 3-4, 3-5 log CFU/ml or g for individual, pooled pure cultures, and cattle fecal samples spiked with pooled pure cultures, respectively. Quantification of virulence genes associated with serogroups was performed by screening the presumptive colonies from the spiral plate by PCR targeting serogroup specific and virulence genes (Bai et al., 2012). The mqPCR assays quantified a higher proportion of samples compared to spiral plating method, however, unlike mqPCR assays, the spiral plating method could quantify virulence genes associated with serogroups.

Real-time quantitative PCR (qPCR)

Real-time PCR has been widely used for the quantification of microorganisms including STEC in various sample matrices. There are two methods of quantification by real-time PCR: absolute (standard curve method) and relative quantification. Relative quantification is most commonly used for gene expression studies, where results are expressed as fold change or fold difference of expression levels. The absolute method of quantification is performed using standard curve developed using templates of known concentrations. The concentration of unknown sample is determined based on the standard curve (Brankatschk et al., 2012).

Although several assays have been developed for the detection of non-O157 *E. coli* serogroups, there is a lack of validated qPCR assays for the quantification of non-O157 *E. coli* serogroups. There are several assays validated for the quantification of *E. coli* O157:H7 and other virulence genes such as *stx*1, *stx*2, *eae*, and *ehxA*. Verstraete et al. (2014) developed a qPCR assay for the detection and quantification of *stx*1, *stx*2, and *eae* in cattle fecal samples. The limit of quantification of the assay was <2.7 log copies/g feces for *stx*1, *stx*2 and *eae*. Further, the qPCR assay was also evaluated using naturally contaminated fecal sample using culture method as a reference. There was no correlation between the concentration of STEC determined by

qPCR, and the contamination status of the farms determined by culture method, since the concentration range of STEC in culture positive farms was same as culture negative farms (Verstraete et al., 2014). Jacob et al. (2012) evaluated multiplex quantitative real-time PCR (mqPCR) assays for the quantification of *E. coli* O157 and major virulence genes (*stx*1 and *stx*2) in cattle feces. The assays were validated with pooled pure cultures, culture spiked cattle feces, and naturally contaminated cattle feces; they also compared the assay to a culture-based method using feces from experimentally inoculated cattle. The detection limit of the assay was 3.6×10^3 CFU/mL, and 3.6×10^4 CFU/g for pooled pure cultures, and culture spiked fecal samples, respectively. There was one log difference between the concentration determined by mqPCR and culture-based method. This assay was useful in identifying super shedders ($\geq 10^4$ CFU/g) but not the low shedders (Jacob et al., 2012). Lawal et al. (2015) developed a qPCR assay to quantify *E. coli* O157 and O26 in bovine recto-anal swabs. The method was validated using bovine recto-anal junction swab samples inoculated with *E. coli* O157 and O26. The limit of quantification was 1.1 and 1.8 log CFU/swab for *E. coli* O157 and *E. coli* O26, respectively following enrichment of swab samples (Lawal et al., 2015). However, the concentration of the target bacteria determined following enrichment of samples does not represent true levels of the pathogen in the sample. Noll et al. (2015) developed a mqPCR assay for the simultaneous detection of *E. coli* O157:H7 (*rfb*E) and major virulence genes (*stx*1, *stx*2, and *eae*). The assay was validated using pure cultures, and cattle fecal samples spiked with pure cultures. The detection limit of the assay was 3.1×10^3 CFU/ml and 2.8×10^4 CFU/g for pure cultures and cattle fecal samples spiked with pure cultures, respectively. However, the assay was not validated for quantification of *E. coli* O157 and associated virulence genes in naturally-positive fecal samples (Noll et al., 2015b). Jacob et al. (2014) determined the applicability of mqPCR and

end-point PCR to distinguish between low (~10^2 CFU/g) and high (≥10^2 CFU/g) shedders of *E. coli* O157:H7 using cattle feces collected from feedlots. Fecal samples initially tested for *E. coli* O157 by culture method were classified as high shedders, IMS positive after enrichment, and culture negative; pre and post enrichment samples from each category were further subjected to end-point PCR and mqPCR for the detection of *E. coli* O157, *stx*1, and *stx*2. There was a significant difference between the proportion of high shedder and culture positive samples between culture- and PCR-based methods. Based on their results, they suggested that PCR-based methods are useful in identifying high-shedders; however, they cannot replace culture-based methods (Jacob et al., 2014). Omisakin et al. (2003) determined the prevalence and concentration of *E. coli* O157 in cattle fecal samples collected at the time of slaughter. Samples were initially subjected to enrichment followed by IMS, positive samples were further subjected to serial dilutions, and an aliquot from each dilution was plated onto Harlequin SMAC 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) supplemented with cefixime and tellurite (Harlequin CT-BCIG) and CT-SMAC media. The concentration of *E. coli* O157 was determined by counting the presumptive colonies and testing randomly selected colonies by biochemical and agglutination tests. The concentration of *E. coli* O157 in cattle feces ranged from 10^2 to 10^5 CFU/g, with 9% of the cattle shedding *E. coli* O157 were super shedders (Omisakin et al., 2003). Menrath et al. (2010) investigated the presence of super-shedders of non-O157:H7 STEC in dairy cows. Fecal samples (n=1,646) were collected from 133 dairy cows during a 12-month period. Enriched fecal samples were initially screened for the presence of *stx* genes by PCR. Shiga toxin gene positive samples (pre-enriched) were plated onto LB-agar along with two ten-fold dilutions, and resultant colonies on the incubated plates were examined by colony hybridization and 20 representative colonies were tested by PCR targeting *stx*1, *stx*2, *eae* and EHEC *hlyA*. Of the 133 cows sampled,

14 of them were super shedders, 61 STEC isolates from the super-shedder cows belonged to 24 non-O157 serotypes (Menrath et al., 2010).

There are a few reports on the concentration of non-O157 STEC in cattle feces, based on qPCR. However, most of the qPCR assays targeted virulence genes. There are very few studies targeting serogroup-specific genes. Sharma et al. (2002) developed two multiplex real-time PCR assays for the simultaneous detection and quantification of *E. coli* O157, O111, and O26 in ground beef and cattle feces. One of the assays targeted *stx*1 and *stx*2 genes, another assay targeted the *eae* gene specific to EHEC O26, O157, and O111 serogroups. The detection limit of both assays ranged from 1-10 CFU/g of beef and cattle feces following 16-h enrichment in modified trypticase soy broth (Sharma, 2002). Luedtke et al. (2014) developed a real-time PCR targeting EHEC-specific *ecf1* (*E. coli* attaching and effacing gene-positive conserved fragment 1) gene encoded on pO157 and pO157-like plasmids, and *eae* for the quantification of EHEC in cattle feces. The assay was initially validated using isolates belonging to the top seven serotypes (O26, O45, O103, O111, O121, O145, and O157) isolated from cattle feces. Relative quantification method and direct sequencing were used to determine the plasmid copy number. Additionally, the EDL 932 strain with or without cattle feces background was used to generate standard curves, and five cattle fecal samples positive for characterized EHEC isolates were also used for validation of the assay. The average copy number ranged from 3-5 copies/genome and 1-2.5 copies/genome by qPCR and sequencing methods, respectively. The limit of quantification of the assay determined using spiked cattle feces was 1.25×10^3 CFU/ml. However, this assay could not determine the load of individual non-O157 *E. coli* serogroups in cattle feces.

There are several assays developed for the quantification of *E. coli* O157:H7 in cattle feces. However, there is a lack of qPCR assays for the quantification of non-O157 *E. coli*

serogroups in cattle feces. Real-time PCR assays are more sensitive and rapid compared to culture-based methods for the detection and/or quantification of any pathogens in various sample matrices. However, the major limitation of qPCR assays is that they detect/quantify both viable and dead cells. Also, qPCR assays cannot associate any virulence gene with a particular serogroup, hence, cannot differentiate pathogenic from non-pathogenic serogroups. Unlike culture-based methods, isolates are not available by qPCR assays for further characterization.

Characterization of non-O157 *E. coli* serogroups

Characterization of pathogenic *E. coli* is essential to determine their pathotype based on their virulence attributes, identification of molecular markers for the development of diagnostic methods for their detection and or quantification, understand host-pathogen interaction, and identification of vaccine and therapeutic targets. *Escherichia coli* serogroups isolated from various sources can be characterized by phenotypic and genotypic methods. Genotypic characterization is based on the genetic material of the organisms, while phenotypic characterization is based on the metabolic properties and chemical composition of the organisms (Emerson et al., 2008). Phenotypic methods include biochemical tests, antimicrobial susceptibility tests, culture-based methods, serology, in vitro cell culture adhesion assays, cytotoxicity assays, and phenotype microarrays. Genotypic characterization methods include PCR, PCR-restriction fragment length polymorphism (RFLP), Random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), microarray, and whole genome sequencing (WGS).

Physiological and functional properties of microorganisms can be identified by phenotypic characterization at the protein level. Most of the conventional phenotypic methods

are based on the identification of activity of enzymes, such as catalase and oxidase, and metabolic functions (Emerson et al., 2008). Phenotypic methods can also be used to determine the virulence properties of pathogens, for example, cell culture adhesion assay, Vero cell cytotoxicity assay, biofilm formation assay, hemolysis on blood agar, and antimicrobial susceptibility tests. Large-scale analysis of proteins is also possible by using proteomics tools such as one or two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), electrospray ionization mass spectrometry (ESI-MS), liquid chromatography tandem mass spectrometry (LC/MS/MS), and surface-enhanced laser desorption ionization mass spectrometry (SELDI). Although phenotypic methods are useful in the characterization of pathogens, genotypic characterization has been found to be more sensitive (Moraes et al., 2013). Also, phenotypic methods are more time consuming compared to genotypic methods (Castro-Escarpulli et al., 2015). Many studies have reported a lack of correlation between genotypic and phenotypic methods for the detection and or characterization of various bacteria (Moraes et al., 2013; Velasco et al., 2004). Phenotypic methods are also limited by the environmental conditions which influence the expression of genes (Moraes et al., 2013).

Several genotypic methods are available to overcome the limitations of phenotypic methods. Genotypic methods allow sensitive and rapid detection and/or characterization of pathogens. Genotypic methods such as PCR, PCR-RFLP, PFGE, microarray, and WGSare available for the characterization of non-O157 *E. coli* serogroups isolated from various sources. These methods can be used to determine the virulence potential of organisms, and also to determine the genetic relatedness among the strains isolated from various sources, which is useful in tracking the source of human foodborne illness outbreaks. Following is a brief review

on the most commonly used methods for the genetic characterization of non-O157 *E. coli* serogroups.

Polymerase chain reaction (PCR)

Several multiplex PCR assays are available for the simultaneous detection of an array of virulence genes and or serogroup-specific genes, which are used for their characterization. Multiplex qPCR assays were developed for the detection and characterization of STEC isolated from cattle feces. A total of 22 assays targeting *stx*1, *stx*2, *ehxA*, *katP* (catalase-peroxidase), *espP* (serine protease), *etpD* (type II secretion protein), *saa* (STEC autoagglutinating adhesin), seven subtypes of *eae*, four subtypes of *tir* (translocated intimin receptor), and three subtypes of *espD* (type three secretory system protein) were validated using reference strains and *E. coli* strains isolated from cattle feces. Fifteen different combinations of serogroup and virulence gene profiles were observed in isolates obtained from 16 calves, of which a combination of *stx*1, *eae*-ζ, *tir*-α, *ehxA*, and *espP* was the most common virulence gene profile (Nielsen and Andersen, 2003b). *Escherichia coli* O103:H2 strains isolated from cattle and human sources have been characterized by genotypic and phenotypic methods. Virulence gene profiles were determined by multiplex (*stx*1, *stx*2, *eae*, and *ehxA*) and individual PCR (*eae*-ε, *hlyA*, *katP*, *espP*, and *etp*) assays, and phenotypic methods were used to determine the production of hemolysins and colicins, and to determine the antimicrobial resistance. Additionally, PFGE patterns and plasmid profiles were also determined. All the O103:H2 strains were highly diverse as shown by their PFGE patterns, plasmid profiles, antimicrobial resistance, colicin production and plasmid encoded genes, however, all the strains carried *stx*1, *eae* and *ehx*A genes (Karama et al., 2008).

Perera et al. (2015) characterized non-O157 *E. coli* serogroups isolated from ruminant feces in Malaysia. Enriched fecal samples were initially screened by PCR for the presence of

major virulence genes (*stx*1, *stx*2, *eae*, and e*hxA*), and samples positive for *stx* and *eae* were cultured on chromocult-TBX agar and coliformen agar. Up to 50 colonies were screened by PCR targeting *stx*1, *stx*2, *eae*, and *ehxA*, and those that were positive for *stx* and *eae* were further tested by PCR targeting serogroup-specific genes of the top six non-O157 *E. coli* serogroups (O26, O45, O103, O111, O121, O145). Additionally, enriched fecal samples were directly tested by PCR targeting serogroup-specific genes of six major non-O157 *E. coli* serogroups, and those which were positive for serogroup-specific genes were subjected to IMS using serogroup specific beads. Post-IMS bead suspensions were plated onto chromocult-TBX agar and coliformen agar, representative colonies were further screened by PCR targeting serogroup-specific genes. The serogroup-positive colonies were subsequently screened by PCR targeting major virulence genes. Isolates were also subjected to biochemical tests for the confirmation of *E. coli* and PFGE to determine the genetic relatedness among the isolates. Shiga toxin and intimin subtypes were also determined by PCR, and *stx* gene expression was determined using ELISA. Only 1.5% (2/136) of the samples were positive for STEC isolates, one of which carried *stx*1a, *stx*2a, *stx*2c, and *ehxA*, and the other isolate carried *stx*1a. The two isolates showed two different PFGE patterns (Perera et al., 2015). Monaghan et al. (2011) determined the prevalence and characterized the non-O157 *E. coli* isolated from cattle feces and soil samples collected from beef and dairy farms. Enriched fecal samples were initially screened by PCR targeting *stx*1 and *stx*2, and samples positive for *stx* were plated onto Chromocult tryptone bile X-glucuronide agar for further characterization. Following incubation, representative colonies were subjected to serotyping and multiplex PCR assays to determine their virulence gene profile: Assay 1 targeted *stx*1, *stx*2, *eaeA*, and *hlyA*; assay 2 targeted *katP*, *etpD*, *tir*, *saa*, *toxB*, *iha*, $lpfA_{O157/OI-141}$, $lpfA_{O113}$, and $lpfA_{O157/OI-154}$; and assay 3 targeted *espA*, *espB*, *espP*, and *sab* genes. Additionally, *stx* and

eae subtypes were determined using PCR and PCR-RFLP protocols. A total of seventeen STEC serotypes were isolated from fecal and soil samples, with O113:H4 being the most commonly isolated STEC serotype, majority of them carried *stx*2a and *stx*2d subtypes. Few isolates (16.8%) carried *eae* gene of varying subtypes (β1, γ and θ). They also carried other virulence genes such as *hlyA, saa, tir, espA, espB, lpf*$_{O157}$, *lpf*$_{O113}$, *espB, toxB,* and *iha* (Monaghan et al., 2011).

High-throughput qPCR assay systems are available for the simultaneous detection of a panel of virulence genes. A High throughput OpenArray® System is available targeting a panel of 28 genes including virulence genes (*stx*1, *stx*2, *eae*, *hly*$_{933}$, and *tir*), O-antigen genes (*rfbE*$_{O157}$, *wbdI*$_{O111}$, *wzx*$_{O121}$, *wzx*$_{O26}$, *wzx*$_{O145}$, *wzx*$_{O45}$, and *wzx*$_{O103)}$, H7 flagellar antigen (*fliC*$_{H7}$), stress associated genes (*rpos*) and prophage genes. The assays were validated with pure cultures of 65 EHEC strains, and the detection limit of the assay was 10^4 CFU/ml (Gonzales et al., 2011). High-throughput qPCR assay was developed for the characterization of *E. coli* O26 strains isolated from feedlot cattle feces. A combination of high throughput real-time PCR platforms targeting 25 genes was used for the study. Target genes included were: O26 antigen flippase (*wzx*$_{O26}$), Shiga toxins (*stx*1 and *stx*2), flagellar antigens (*fliC*H11, *fliC*H32, *fliC*H8), type three secretory system effectors (*esp*K, *esp*V, and *esp*N), EHEC hemolysin (*ehxA*), bundle-forming pili (*bfpA*), iron-repressible protein Irp2 (*irp2*), tellurite resistance protein (*terE*), urease (*ureD*), open reading frames of OI-57, Z2098 and Z2099, CRISPR-associated markers such as SP_O26-C, SP_O26-D, and SP_O26-E, aerobic respiratory control protein A allele 2 (*arcA* allele 2), and reference genetic marker for *E. coli* (*wecA*). *Escherichia coli* O26 strains (n=178) isolated from cattle feces were subjected to multiple real-time PCR assays to identify the gene profile, and CRISPR (clustered regularly interspaced short palindromic repeat) typing by sequencing. Strains were classified as different marker types based on their gene profile. A combination of marker

types and CRISPR types were used to define diversity types. Based on these results, a hierarchical clustering dendrogram was constructed. Strains were classified into 18 marker types, and 24 CRISPR types, a combination of which resulted in 37 diversity types. This study suggested that cattle shed a highly diverse *E. coli* O26 serogroup in their feces (Ison et al., 2015).

PCR-based characterization of non-O157 *E. coli* serogroups is rapid and sensitive. However, the major demerit of PCR-based methods is that they cannot associate any serogroup with particular virulence genes present in complex samples. Hence, characterizing the isolates obtained from the sample is the most efficient way of characterizing non-O157 *E. coli* serogroups. Comprehensive characterization of non-O157 *E. coli* serogroups is accomplished by a set of multiplex PCR assays, which are time-consuming and laborious. Hence, other platforms such as microarray and whole genome sequencing are preferred over PCR-based methods for comprehensive characterization of non-O157 *E. coli* serogroups.

Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Pulsed-field gel electrophoresis involves digestion of the entire genome of the organism with restriction enzymes resulting in DNA fragments of various sizes, which can be separated by electrophoresis by switching the direction of electric current periodically. The PFGE patterns of the target strains are compared to determine their genetic relatedness. The method has been used extensively to characterize O157 strains from various sources (Bohm and Karch, 1992; Davis et al., 2003; Scott et al., 2006; Shima et al., 2006). This method has also been used for characterization of non-O157 *E. coli* serogroups. It is considered as the gold standard method for typing several foodborne pathogens by PulseNet, the national foodborne illness outbreak surveillance network.

Multilocus sequence typing (MLST) is another method to determine the genetic relatedness among the strains, which is based on the sequences of seven housekeeping genes. Any variation in the sequence of the housekeeping genes is designated as allele, and are assigned with an allele number. Based on the allelic profile, each strain is assigned a sequence type (ST). MLST has been used for the characterization of *E. coli* O157 (Lindstedt et al., 2003; Lupindu et al., 2014) and non-O157 *E. coli* serogroups (Monaghan et al., 2012; Palanisamy et al., 2017; Timmons et al., 2016) from various sources. In most studies, these methods are used in combination with other genetic and phenotypic methods for the characterization of *E. coli* serogroups and to determine their clonal relatedness.

Beutin et al. (2005) characterized the *E. coli* O103 strains isolated from animal, human and meat sources based on their virulence gene profiles, PFGE and multilocus sequence typing (MLST). The strains were classified as STEC and EPEC based on their virulence gene profile. Seven MLST profiles were determined using seven housekeeping genes. Based on PFGE and MLST profile of STEC and EPEC O103 strains, they reported that alterations in PFGE patterns are caused by acquiring virulence genes and DNA rearrangements (Beutin et al., 2005). Dong et al. (2017) determined the prevalence of STEC in farm environments in South Korea. They also determined virulence gene profiles and the genetic relatedness among the STEC isolates. The non-O157 *E. coli* serogroups were isolated from samples using culture-based methods. Shiga toxin-producing *E. coli* isolates were subjected to serotyping, antimicrobial susceptibility tests, multiplex PCR to determine the virulence gene profile, and PFGE. STEC O108 was the most prevalent non-O157 STEC followed by O8, O84, O15 and O119. Shiga toxin 2a and 2c were the most prevalent Shiga toxin subtypes, and isolatesshowed resistance to tetracyclin, ampicillin, and cefotaxime. The non-O157 *E. coli* serogroups showed a high degree of genetic diversity,

however, strains belonging to same serogroups clustered together (Dong et al., 2017). Amezquita-Lopez et al. (2012) characterized the non-O157 *E. coli* serogroups isolated from fecal samples of cattle, sheep, and chicken. Non-O157 STEC isolated from the samples were subjected to a combination of multiplex PCR assays and oligonucleotide microarrays to determine the serogroup (O26, O45, O91, O103, O104, O111, O113, O121, O128, and O145) specific genes, flagellar genes (*fliC*$_{H1}$, *fliC*$_{H2}$, *fliC*$_{H4}$, *fliC*$_{H7}$, *fliC*$_{H8}$, *fliC*$_{H19}$, *fliC*$_{H21}$, *fliC*$_{H28}$) and virulence genes (*stx*1, *stx*2, *eae*, *ehxA*). The strains were also subjected to PFGE and MLVA (multiple-locus variable number tandem repeat analysis) to determine the clonal relationship among the strains. Serotypes were also identified by agglutination method. The non-O157 STEC isolated from fecal samples belonged to O8:H19, O75:H8, O111:H8 and O146:H21 serotypes. The non-O157 *E. coli* serotypes were grouped into 23 PFGE types and 14 MLVA types, thus demonstrating greater genetic diversity (Amézquita-López et al., 2012).

Pulsed-field gel electrophoresis and MLST have been the most commonly used typing methods for O157 and non-O157 *E. coli* serogroups. However, these methods have several limitations; they are time consuming and laborious, and they are less sensitive compared to other methods such as whole genome sequencing.

DNA microarray

DNA microarray is another method which is extensively used for genetic characterization of *E. coli* O157 and non-O157 *E. coli* serogroups from various sources. Microarray techniques can be used for the detection of gene targets through the detection of fluorescent signal generated by hybridization of fluorescently labeled target DNA with oligonucleotide probes immobilized on a solid support such as nitrocellulose, glass or silicon (Karama and Gyles, 2010). Microarray has been used for characterization of *E. coli* serogroups isolated from animal, human, food and

environmental sources (Anjum et al., 2007; Feng et al., 2014; Jackson et al., 2012; Jin et al., 2005; Lacher et al., 2014; Lacher et al., 2016; Patel et al., 2016).

Quinones et al. (2012) developed DNA microarrays with the ampliPHOX colorimetric method for the detection of 11 *E. coli* serogroups (O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, and O157), *fliC*H7 and 11 virulence genes (*stx*1, *stx*2, *eae, nleA, espL2, espP, katP, saa, subA, ehxA,* and *per*). This method was validated with STEC strains isolated from animal, human and environmental sources. It was found to be a rapid and accurate method for the genetic characterization of STEC from various sources (Quiñones et al., 2012). Kruger et al. (2015) characterized O26:H11 strains isolated from cattle, human, meat and farm environments in Argentina. The virulence gene profiles of O26:H11 strains were determined using a combination of oligonucleotide microarrays and multiplex PCRs, and their antibiotic susceptibility was determined using a disk diffusion method. The strains were also characterized using MLVA. *Escherichia coli* O26:H11 strains were positive for genes associated with the type III secretory system (*eae-β, tir, espB, espA, espF, espJ*), *nle* genes (*nleA, nleB, nleC*), plasmid encoded genes (*ehxA, espP, katP*), Shiga toxins (*stx*1a, *stx*2a), and antimicrobial resistance genes (bla_{TEM}, *strA*-strB, and *sul2*). *Escherichia coli* O26:H11 strains were grouped into three different populations, which differed by genes carried by mobile genetic elements (Krüger et al., 2015).

Whole genome microarrays developed using whole genome sequences of reference strains incorporate a multitude of genes, thus enabling comprehensive characterization of non-O157 *E. coli* serogroups. The *E. coli* Identification microarray developed by the U.S. Food and Drug Administration (FDA-ECID) is a custom Affymetrix DNA microarray that detects more than 40,000 gene targets of *E. coli* enabling rapid genome level characterization of *E. coli* strains. It was designed using 368 whole genome sequences of *E. coli* and *Shigella*, for the

detection of 152 O types, 53 H-types, *stx* and *eae* subtypes, and other virulence genes. Additionally, they selected 9,985 unique single nucleotide polymorphisms (SNP) from the whole genome sequences of reference strains, which were used to determine the phylogenetic relationship among the strains using whole genome sequence and microarray data. The array was used to analyze strains whose whole genome sequences were available. The array was found to be rapid and accurate in determining the genetic characteristics of non-O157 *E. coli* serogroups. There was 99.7% correlation between the phylogenetic relationship determined based on SNP by whole genome sequencing and microarray data (Patel et al., 2016). Moxley et al. (2015) characterized *E. coli* O165:H25 strains isolated from colonic mucosa of a one-year-old feedlot heifer which exhibited neurological signs and bloody diarrhea before death. Histopathological examination revealed *E. coli* O165 antigen positive rods adhering to necrotic colonic mucosal epithelial cells along with attaching and effacing lesions. The strain was positive for genes associated with type III secretion system (T3SS) structure and regulation (*cesD, cesT, escD, escF, escN/escV, escR, escT, ler, sepL, sepQ*), T3SS effectors (*espA, espB, espC, espD, espD, espF, espH, espJ, nleB, nleC, nleD, nleH, tir*), serine proteases (*eatA, espC, espP*), Shiga toxin (*stx*2), EHEC-haemolysin (*ehxA*), adhesins (intimin-ϵ (*eae-ϵ*), type 1 fimbria (*fimA, fimB, fimH*), type IV pili (*pilA, pilB, pilC, pilM, pilP, pilQ*) and non-fimbrial adhesin (*efa1/lifA*) (Moxley et al., 2015).

Microarray-based genetic characterization enables rapid and comprehensive characterization of non-O157 *E. coli* serogroups. It is also less expensive and less laborious compared to whole genome sequencing. However, the major limitation of microarray is that it can only detect known genes that are included in the array. Also, it cannot differentiate between closely related genes such as *stx*2 subtypes, and a few H-types (Patel et al., 2016).

Whole genome sequencing

Whole genome sequencing (WGS) technique is currently the most reliable method for studying pathogens at the genome level. It has several applications in the field of food safety: outbreak surveillance and investigation (Dallman et al., 2015; Mellmann et al., 2011), to determine the phylogenetic relationship among the target strains, to determine the source of outbreaks (Jenkins et al., 2015), and to identify the molecular markers that can be used to design assays for the rapid detection of foodborne pathogens (Underwood et al., 2013). Several public health agencies (FDA, Public Health England, Serum Staten Institute in Denmark) have employed WGS for the surveillance of foodborne pathogens (Franz et al., 2016). Another potential application of WGS would be for microbial risk assessments. For example, understanding the genomes of non-O157 *E. coli* serogroups shed in cattle feces will help in estimating the risk of human illness associated with contamination of meat or any food products with cattle feces. There are very few studies on the WGS of non-O157 *E. coli* serogroups isolated from cattle feces. Following is a brief review on the studies employing WGS as a method for characterizing non-O157 *E. coli* serogroups.

Ju et al. (2012) determined the phylogenetic relationship among the non-O157 *E. coli* serotypes isolated from animal (cattle and pig) and human sources using WGS data. Shiga toxin-producing *E. coli* strains (n=15) were subjected to WGS using a 454 pyrosequencing system. Whole genome sequencing data of the 15 STEC strains, along with 28 published genomes, were used to generate a phylogenetic tree. Additionally, 15 STEC strains were subjected to PFGE and MLST to determine the phylogenetic relationship among the strains. Based on the phylogenetic relationship determined by all three methods, they hypothesized that STEC serotypes with same H-types have common ancestors. They also hypothesized that the similar genetic background

shared by the strains with same H-types might facilitate the uptake of virulence genes such as *stx*, which are involved in STEC pathogenesis (Ju et al., 2012a). Gonzalez-Escalona et al. (2016) investigated the whole genome sequences of O26:H11 strains isolated from feedlot cattle in the United States. They subjected 42 O26:H11 strains isolated from cattle feces to WGS, and compared them to 37 published WGS of O26:H11 strains. Based on the phylogenetic relationship determined by the whole genome MLST and virulence gene profile, they showed that US cattle are reservoirs of two different lineages of O26:H11 strains: Lineage 1- consisted of O26:H11 EHEC-like (ST29) (4 strains) and O26:H11 EHEC (ST21) (2 strains); and Lineage 2- consisted of O26:H11 EPEC (ST29; 36 strains) (Gonzalez-Escalona et al., 2016). Norman et al. (2015) compared the differences in genotypes identified based on nucleotide polymorphisms and virulence gene profiles of O26 strains isolated from human and animal sources using WGS data. Phylogenetic trees were constructed using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) genotyping derived using a subset of SNPs identified in the WGS data. Of the 64 genotypes derived from polymorphisms, 21 were common to human and animal strains, 24 were found only in human strains, and 19 were found in only animal strains. Nighbor-Joining trees constructed using 64 genotypes identified seven clusters that differed in their virulence gene profile. Both human and cattle strains clustered together depending on their virulence gene profiles. This study provides evidence for the presence of similar strains in humans and animals, which suggests transmission of the O26 strains between animals and humans (Norman et al., 2015). Yan et al. (2015) compared the whole genome sequences of *E. coli* O104:H21 (Montana outbreak strain) and O104:H7 (isolated from cattle feces) to determine the basis of their evolution and pathogenicity, a few published WGS of O104:H4 strains were also included in the analysis. The analysis of WGS of O104 strains revealed the absence of the LEE pathogenicity

island. The *E. coli* O104:H7 strain was more closely related to O104:H21 than to other *E. coli* O104:H4 strains. They suggested that pathogenic STEC O104:H7 and O104:H21 strains might have evolved by acquiring *stx* and other virulence genes from other STEC strains (Yan et al., 2015).

There are several published whole genome sequences of non-O157 *E. coli* strains isolated from human outbreaks and sporadic cases and several studies have analyzed the WGS of these strains (Brzuszkiewicz et al., 2011; Cooper et al., 2014; Haugum et al., 2014; Kwon et al., 2016). However, there are a few studies on the WGS of non-O157 *E. coli* serogroups isolated from cattle feces.

The whole genome sequencing method enables in-depth analysis of the genomes of pathogens, which helps in understanding their pathogenic potential and basis of evolution. However, it is time-consuming, expensive, and requires personnel with bioinformatics skills for data handling and analyzing.

Escherichia coli O104

Escherichia coli O104:H4 is the major non-O157 *E. coli* serotype responsible for a large outbreak of foodborne illness in Germany, 2011, responsible for nearly 4000 cases, with 900 of them developing HUS leading to 54 deaths (Karch et al., 2012). It was determined to be hybrid pathotype possessing characteristics of enteroaggregative and enterohemorrhagic *E. coli* pathotypes (Bielaszewska et al., 2011). *Escherichia coli* O104:H21 is another STEC serotype responsible for an outbreak of hemorrhagic colitis in Helena, Montana in 1994 with 11 confirmed and seven suspected cases (CDC, 1995). Other *E. coli* O104 serotypes are also responsible for sporadic cases of human illness (Hussein, 2007; Miko et al., 2013). Unlike other

STEC serotypes, *E. coli* O104:H4 has not been detected in cattle feces so far (Auvray et al., 2012; Paddock et al., 2013; Shridhar et al., 2016b; Wieler et al., 2011). However, other O104 serotypes such as O104:H2, O104:H11, O104:H12, O104:H21, and O104:H27 have been detected in cattle feces (Miko et al., 2013; Rump et al., 2012; Shridhar et al., 2016b) have been found in cattle feces. *Escherichia coli* O104:H7 was the most predominant serotype isolated from cattle feces, with a proportion of isolates carrying the *stx*1c gene (Shridhar et al., 2016b). There are several published studies characterizing the genetic profile of the *E. coli* O104:H4, German outbreak strain based on microarray and WGS (Ahmed et al., 2012; Brzuszkiewicz et al., 2011; Jackson et al., 2012; Mellmann et al., 2011). There are very few studies on the comprehensive genetic characterization of O104 serotypes other than *E. coli* O104:H4. Yan et al. (2015) analyzed the whole genome sequence of an *E. coli* O104:H7 strain isolated from cattle feces and compared it to the *E. coli* O104:H21 Montana outbreak strain. *Escherichia coli* O104:H7 was more similar to *E. coli* O104:H21 than to O104:H4. They attributed the variation between O104 serotypes to loss or gain of mobile genetic elements such as genomic islands, plasmids and prophages (Yan et al., 2015). More studies to comprehensively characterize *E. coli* O104 serotypes other than O104:H4 are required to elucidate their virulence potential.

Conclusions

Foodborne illnesses associated with non-O157 STEC serogroups has increased in recent years. Well-designed intervention strategies are required to mitigate the incidence of human foodborne illness associated with non-O157 STEC serogroups. Having a prior knowledge about the prevalence, concentration and the pathogenic potential of non-O157 *E. coli* serogroups helps in designing effective intervention strategies and conducting risk assessments to protect public

health. Hence, it is critical to develop and validate sensitive and accurate methods for detection, quantification, and characterization of non-O157 *E. coli* serogroups.

There are culture-based, serological, and molecular methods for the detection of non-O157 *E. coli* serogroups. Culture-based detection methods for non-O157 *E. coli* serogroups in feces are challenging because of lack of distinguishing characteristics between pathogenic non-O157 *E. coli* and generic *E. coli* and complex fecal matrix which harbors a lot of background flora. They are also laborious and time-consuming. Hence, there are several end-point and qPCR assays available for the detection of non-O157 *E. coli* serogroups. Six non-O157 STECserogroups (O26, O45, O103, O111, O121, and O145) are responsible for most human illness; however, these serogroups can be pathogenic or non-pathogenic. Hence, multiplex PCR assays for the simultaneous detection of serogroup-specific genes and virulence genes are critical in detecting the pathogenic *E. coli* serogroups. The major demerit of PCR-based methods is that virulence genes present in the sample cannot be associated with a particular serogroup, and isolates are not available for further characterization. While each method has its own merits and demerits, the choice of methods mainly depends on the purpose of detection of non-O157 *E. coli* serogroups.

Apart from the prevalence, estimating the concentration of non-O157 STEC is also important in estimating the risk of human illness associated with various sources. There are several reports on the concentration of *E. coli* O157 in various sample matrices. However, there is a paucity of concentration data of non-O157 *E. coli* serogroups because of lack of validated quantification methods. There are several culture-based and PCR–based methods used for quantification of several bacteria including *E. coli* O157. These methods have to be validated for the quantification of non-O157 *E. coli* serogroups in cattle feces. However, the concentration

data estimated by culture and PCR-based methods might be different since the PCR-based methods quantify both live and dead cells, while the culture methods quantify only live cells.

Characterization of non-O157 *E. coli* serogroups is essential to determine their pathogenic potential. There are several genotypic and phenotypic methods that can be used for their characterization. Genotypic methods determine the genomic features of the pathogens, while the phenotypic methods characterize the pathogens based on the products of gene expression. Both methods have pros and cons, hence the choice of method mainly depends on the purpose of characterization.

In conclusion, detection, quantification, and characterization of non-O157 *E. coli* serogroups shed in cattle feces are essential to design effective intervention strategies. However, there is a limited data on the prevalence and concentration of non-O157 *E. coli* serogroups in cattle feces, because of lack of validated methods. Rapid, sensitive, and high throughput detection methods are required to estimate the prevalence and concentration of non-O157 *E. coli* serogroups in cattle feces. Also, comprehensive characterization of non-O157 *E. coli* serogroups isolated from cattle feces is required to determine their virulence potential.

General Introduction

Shiga toxin-producing *E. coli* (STEC) are major foodborne pathogens responsible for human illnesses, characterized by non-bloody to bloody diarrhea, and hemolytic uremic syndrome (HUS) (Gyles, 2007). Cattle are major reservoirs of STEC, harboring them in the hindgut and shedding them in feces. Cattle usually shed STEC at a concentration of $\leq 10^2$ CFU/g feces, however, a subset of cattle, referred to as super shedders, shed O157 at a concentration of $\geq 10^4$ CFU/g feces (Chase-Topping et al., 2008; Chase-Topping et al., 2007). Although *E. coli*

O157:H7 is the most common STEC serotype associated with human illnesses, non-O157 STEC serogroups are gaining more attention in recent years because of the increased incidence of non-O157 STEC-associated human illnesses. Six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) are responsible for a majority of the non-O157 STEC associated human illnesses (Scallan et al., 2011a). The U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) has declared the six non-O157 STEC serogroups to be adulterants in ground beef and non-intact raw beef products in 2011 (USDA-FSIS, 2011). Methodologies to detect, isolate, and quantify non-O157 STEC in cattle feces have not been well established. Rapid, sensitive, and high throughput detection and quantification methods are required to estimate the prevalence and concentration of non-O157 *E. coli* serogroups in cattle feces. Determining the concentration of non-O157 *E. coli* in cattle feces is important for estimating the risk of human illness associated with cattle feces and for assessing the efficacy of intervention strategies to reduce STEC in various matrices. Additionally, genetic characterization and assessment of virulence potential of non-O157 cattle fecal strains are lacking.

Escherichia coli O104:H4, a hybrid pathotype possessing characteristics of enteroaggregative and enterohemorrhagic *E. coli* (Bielaszewska et al., 2011), was responsible for a large outbreak of hemorrhagic colitis and HUS during summer of 2011 in Germany (Karch et al., 2012). Studies conducted in Europe (Auvray et al., 2012; Wieler et al., 2011) and a study in the US (Paddock et al., 2013) reported that none of the cattle fecal samples carried *E. coli* O104:H4 hybrid pathotype. However, cattle do harbor and shed serogroup O104 in the feces, but prevalence, serotype, and virulence potential of *E. coli* O104 have not been determined.

Investigations on six non-O157 STEC and O104 in cattle feces included the following studies:

1. Development, validation, and applicability of two sets of multiplex quantitative PCR (mqPCR) assays to detect and quantify six non-O157 STEC serogroups in cattle feces.
2. Validation and applicability of a spiral plating method to quantify six non-O157 STEC serogroups in cattle feces
3. Prevalence and isolation of *E. coli* O104 in cattle feces
4. Genetic characterization of six non-O157 *E. coli* serogroups and *E. coli* O104 based on Shiga toxin subtyping, pulsed-field gel electrophoresis, DNA microarray, and whole genome sequencing.

The studies have resulted in the following published manuscripts and manuscripts in preparation for submission:

1. Shridhar, P.B., Noll, L.W., Shi, X., An, B., Cernicchiaro, N., Renter, D.G., Nagaraja, T.G., Bai, J., 2016. Multiplex Quantitative PCR Assays for the Detection and Quantification of the Six Major Non-O157 *Escherichia coli* Serogroups in Cattle Feces. Journal of Food Protection 79, 66-74.
2. Shridhar, P.B., Noll, L.W., Cull, C.A., Shi, X., Cernicchiaro, N., Renter, D.G., Bai, J., Nagaraja, T.G., 2017. Spiral Plating Method To Quantify the Six Major Non-O157 *Escherichia coli* Serogroups in Cattle Feces. Journal of Food Protection, 848-856.
3. Shridhar, P.B., Noll, L.W., Shi, X., Cernicchiaro, N., Renter, D.G., Bai, J., Nagaraja, T.G., 2016. *Escherichia coli* O104 in Feedlot Cattle Feces: Prevalence, Isolation and Characterization. PloS One 11, e0152101.
4. Shridhar, P.B., Siepker, C., Noll, L.W., Shi, X., Nagaraja, T.G., Bai, J., 2017. Shiga Toxin Subtypes of Non-O157 *Escherichia coli* Serogroups Isolated from Cattle Feces. Frontiers in Cellular and Infection Microbiology 7, 121.

5. Shridhar, P.B., Patel, R., Gangiredla, J., Noll, L. W., Shi, X., Elkins, C. A., Bai, J., and Nagaraja, T.G. Genotypic characterization of top six non-O157 *Escherichia coli* serogroups isolated from feces of feedlot cattle based on DNA Microarray (*In preparation*).

6. Shridhar, P.B., Worley, J. N., Yang, X., Noll, L. W., Shi, X., Bai, J., Meng, J., and Nagaraja, T.G. Analysis of Virulence Potential of *Escherichia coli* O145 Isolated from Cattle Feces and Hide Samples Based on Whole Genome Sequencing (*In preparation*).

7. Shridhar, P.B., Patel, R., Gangiredla, J., Noll, L. W., Shi, X., Elkins, C. A., Bai, J., and Nagaraja, T.G. DNA Microarray-Based Assessment of Virulence Potential of Shiga Toxigenic *Escherichia Coli* O104:H7 Isolated from Feedlot Cattle Feces (*In preparation*).

8. Shridhar, P.B., Patel, R., Gangiredla, J., Noll, L. W., Shi, X., Elkins, C. A., Bai, J., and Nagaraja, T.G. Whole Genome Sequence-Based Analysis of Virulence Potential of *Escherichia coli* O104 Serotypes Isolated from Cattle Feces (*In preparation*).

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Chapter 2 - Multiplex Quantitative PCR Assays for the Detection and Quantification of the Six Major Non-O157 *Escherichia coli* Serogroups in Cattle Feces

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) serogroups O26, O45, O103, O111, O121, and O145, called non-O157 STEC, are important foodborne pathogens. Cattle, a major reservoir, harbor the organisms in the hindgut and shed them in the feces. Although limited data exist on fecal shedding, concentrations of non-O157 STEC in feces have not been reported. The objectives of our study were (i) to develop and validate two multiplex quantitative PCR (mqPCR) assays, targeting O-antigen genes of O26, O103, and O111 (mqPCR-1) and O45, O121, and O145 (mqPCR-2); (ii) to utilize the two assays, together with a previously developed four-plex qPCR assay (mqPCR-3) targeting the O157 antigen and three virulence genes (*stx*1, *stx*2, and *eae*), to quantify seven serogroups and three virulence genes in cattle feces; and (iii) to compare the three mqPCR assays to a 10-plex conventional PCR (cPCR) targeting seven serogroups and three virulence genes and culture methods to detect seven *E. coli* serogroups in cattle feces. The two mqPCR assays (1 and 2) were shown to be specific to the target genes, and the detection limits were 4 and 2 log CFU/g of pure culture-spiked fecal samples, before and after enrichment, respectively. A total of 576 fecal samples collected from a feedlot were enriched in *E. coli* broth and were subjected to quantification (before enrichment) and detection (after enrichment). Of the 576 fecal samples subjected, before enrichment, to three mqPCR

assays for quantification, 175 (30.4%) were quantifiable (≥4 log CFU/g) for at least one of the seven serogroups, with O157 being the most common serogroup. The three mqPCR assays detected higher proportions of postenriched fecal samples ($P < 0.01$) as positive for one or more serogroups compared with cPCR and culture methods. This is the first study to assess the applicability of qPCR assays to detect and quantify six non-O157 serogroups in cattle feces and to generate data on fecal concentration of the six serogroups.

Introduction

Shiga toxin–producing *Escherichia coli* (STEC) is a major foodborne pathogen that causes human illnesses characterized by non-bloody and bloody diarrhea, with hemolytic uremic syndrome as a potential complication (Gyles, 2007). Serotype O157:H7 is the most common STEC responsible for the majority of foodborne STEC illnesses; however, other STEC serogroups, particularly O26, O45, O103, O111, O121, and O145, have gained more recognition in recent years because they account for more than 70% of non-O157 STEC infections in the United States (Scallan et al., 2011a). In 2011, the U.S. Department of Agriculture, Food Safety and Inspection Service declared these six non-O157 STEC serogroups to be adulterants in ground beef and non-intact raw beef products (USDA-FSIS, 2011). Cattle, a major reservoir of STEC, harbor the organisms in the hindgut and shed them in their feces; this serves as a major source of contamination of food and water (Gyles, 2007).

Not much is known about fecal shedding of the six non-O157 *E. coli* pathogens in cattle because detection methods, PCR- and culture-based, have been developed and validated only recently (Bai et al., 2012; Baltasar et al., 2014; Dewsbury et al., 2015; Kalchayanand et al., 2013; Paddock et al., 2012). In addition to the presence of STEC pathogens in cattle feces, the

concentration of these organisms plays a role in the spread between animals and subsequent hide and carcass contamination. A subset of cattle, known as ‘‘super shedders,’’ shed the STEC O157:H7 organism at high concentrations ($\geq 10^4$ CFU/g of feces) (Chase-Topping et al., 2008). Super-shedding cattle have been reported to be a major source of transmission of O157:H7 among cattle within the herd (Matthews et al., 2006) and of subsequent contamination of hides and carcasses (Arthur et al., 2009; Fox et al., 2008; Jacob et al., 2010). Because there are no data on fecal concentration of non-O157 STEC in cattle, it is not known whether a subset of cattle that are super shedders of non-O157 STEC (as with O157) exists in a population. Fecal concentration data of the six non-O157 serogroups (O26, O45, O103, O111, O121, and O145) in cattle, when factored in microbial risk assessment models, allow estimation of the potential contamination burden that fecal shedding represents in upstream production stages. Although real-time PCR assays have been developed to detect non-O157 STEC in food matrices (Fratamico et al., 2011; Fratamico et al., 2014; Lin et al., 2011b), the applicability of the real-time PCR assays to detect and quantify non-O157 *E. coli* in cattle feces has not been evaluated. Anklam et al. (Anklam et al., 2012) have developed four separate multiplex qPCR assays to target the seven serogroups (O26, O45, O103, O111, O121, O145, and O157) and four virulence genes (*stx*1, *stx*2, *eae*, and *ehxA*) in cattle feces. The assays were validated using pure cultures and culture-spiked cattle feces, but applicability of the assays for the detection and quantification of *E. coli* serogroups and associated virulence genes in feces of naturally shedding cattle was not evaluated. Luedtke et al. (Luedtke et al., 2014) developed a multiplex qPCR to target enterohemorrhagic *E. coli* (EHEC)-specific attaching and effacing gene positive conserved fragment 1, *ecf1*, and *eae* for enumeration of EHEC directly from cattle feces; however, the assay does not quantify individual serogroups. The objectives of our study were (i) to develop

and validate two sets of multiplex quantitative PCR (mqPCR) assays to target O antigen genes of O26, O103, and O111 (mqPCR-1) and O45, O121, and O145 (mqPCR-2) and (ii) to evaluate the applicability of the two assays, together with a previously developed four-plex qPCR assay (mqPCR-3) targeting the O157 antigen gene and three virulence genes (*stx*1, *stx*2, and *eae* (Noll et al., 2015), to quantify six non-O157 and O157 serogroups and three virulence genes in cattle feces (n=576) from a commercial feed yard. Additionally, the detection of the seven STEC serogroups and three virulence genes in cattle feces by the three mqPCR assays was compared with detection by a 10-plex conventional PCR (cPCR) that targets the same genes (seven serogroup-specific and three virulence genes) and by culture methods.

Materials and Methods

Design of the assays

Two sets of mqPCR assays (mqPCR-1 and mqPCR-2) were developed to detect and quantify O26, O45, O103, O111, O121, and O145 serogroups. The serogroups targeted by mqPCR-1 were O26, O103, and O111; those targeted by mqPCR-2 were O45, O121, and O145. The target gene for O26, O103, O111, O45, and O145 was *wzx* (Bai et al., 2012), which encodes for flippase involved in O-polysaccharide export (Liu et al., 1996). The target genes for O121 were *wbqE* and *wbqF*, which encode for putative glycosyl and acetyl transferases, respectively (Bai et al., 2012). The reporter dyes, FAM, VIC/MAX, and Texas Red, were conjugated at 5′ ends, and Black Hole Quencher dyes I and II were conjugated at 3′ ends of the probes to detect amplification products specific to each gene target. Primers and probes (Integrated DNA Technologies, Inc., Coralville, IA) designed to target O-antigen genes of the six non-O157 *E. coli* serogroups are shown in Table 2.1.

Optimization of the assays

The assays were optimized and validated with pure cultures of one strain each of six serogroups of non-O157 STEC (Table 2.2) individually and, subsequently, with pooled mixtures of two different combinations (O26, O103, and O111 for mqPCR-1 and O45, O121, and O145 for mqPCR-2). The strains of non-O157 STEC, stored at -80° C on cryobeads (CryoCare, Key Scientific Products, Round Rock, TX), were streaked onto blood agar plates (Remel, Lenexa, KS). Single colonies of each serogroup were inoculated and grown overnight in Luria-Bertani broth (Difco, BD, Sparks, MD) at 37° C, and then 100 µl of the culture was added to 10 ml of the broth and incubated at 37° C for 3 to 4 h until an absorbance of 0.4 at 600 nm (approximately 8 log CFU/ml) was reached. Equal volumes of the cultures of each serogroup were mixed into two combinations as described before. One milliliter of the culture suspension (individual serogroup and pooled mixtures) was boiled for 10 min and centrifuged at 9,300 x g for 5 min; the supernatant was used as DNA template for mqPCR assays. DNA was also subjected to 10-fold serial dilutions (10^{-1} to 10^{-7}), and standard curves were generated with the mqPCR assays.

Running conditions of the assays

The working concentrations of all primers in the primer mixture were 10 pmol/µl. The working concentrations of probes were 5 pmol/µl for O26 and O103, 3 pmol/µl for O121, 2.5 pmol/µl for O111 and O45, and 1.25 pmol/µl for O145. The PCR reaction mixture contained 10 µl of 2X Bio-Rad iQ Multiplex Powermix (Bio-Rad Laboratories, Life Science Group, Hercules, CA), 4 µl of nuclease-free water, 1 µl of primer mixture, 1 µl of each probe, and 2 µl of DNA template, making a total reaction volume of 20 µl. PCR was performed with the BioRad CFX96 mqPCR system, and data were analyzed using CFX Manager software version 3.1 (Bio-Rad

Laboratories). The optimized PCR amplification protocol included a 10-min denaturation at 95° C, followed by 45 cycles of 95° C for 15 s, 56° C for 20 s, and 72° C for 40 s.

Specificity of the assays

Specificity of the assays was evaluated with a number of *E. coli* and non-*E. coli* strains from our culture collection. A total of 170 strains (human clinical and bovine origin) belonging to six non-O157 *E. coli* serogroups (35 strains of O26, 40 strains of O103, 40 strains of O111, 25 strains of O45, 12 strains of O121, and 18 strains of O145) were used as a positive control, and another 100 strains representing 42 *E. coli* serogroups other than the six non-O157 serogroups and other bacterial species (*Klebsiella pneumoniae*, *Proteus mirabilis*, and *Serratia marcescens*) were used as a negative control.

In addition, pure cultures of target serogroups for each assay were pooled by mixing each serogroup at equal and different concentrations. Each serogroup of the assay was added at equal concentration and at decreasing concentrations ($1:10^{0}$, $1:10^{-1}$, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, $1:10^{-6}$, $1:10^{-7}$) to a mixture containing the other two targets at fixed concentration and was inoculated into a cattle fecal sample that tested negative for the six target non-O157 serogroups. One gram of spiked fecal samples was added to 9 ml of *E. coli* (EC) broth (Difco, BD), vortexed, and incubated at 40° C for 6 h. Extracted DNA from pre- and postenrichment fecal samples was subjected to mqPCR assays.

Sensitivity of the assays using pure cultures of non-O157 STEC

Sensitivity of the assays was determined by using 10-fold serial dilutions of pure cultures of the six serogroups. Three different combinations of pooled pure cultures were prepared as described before: O26, O103, and O111 for mqPCR-1; O45, O121, and O145 for mqPCR-2; and O26, O45, O103, O111, O121, and O145 for both assays. Ten-fold serial dilutions (10^{-1} to 10^{-7})

of each pooled pure culture grown in Luria-Bertani broth were performed, and 100-µl aliquots of the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were spread plated onto blood agar plates (four plates per dilution) to determine initial cell concentrations (CFU per milliliter). One milliliter from each dilution (10^{0} to 10^{-7}) was removed, boiled for 10 min, and centrifuged at 9,300 x g for 5 min. Supernatants were used as DNA templates for mqPCR, and standard curves were generated to determine correlation coefficients, amplification efficiencies, and detection limits for each assay. Both mqPCR assays (targeting three serogroups each) were compared to the corresponding single (targeting a single serogroup) and duplex (targeting two serogroups) assays. The experiment was repeated on a different day.

Sensitivity of the assays using feces spiked with pure cultures of non-O157 STEC

Fifteen pen-floor fecal samples from cattle housed in the Kansas State University feedlot were collected and tested by both sets of mqPCR assays (1 and 2), and a sample that was negative for the six serogroups was selected to spike with pure cultures. Three different combinations of pooled pure cultures were prepared as described before: O26, O103, and O111 (mqPCR-1); O45, O121, and O145 (mqPCR-2); and O26, O45, O103, O111, O121, and O145 (both assays). Ten-fold serial dilutions (10^{0} to 10^{-7}) of each mixture were performed in Luria-Bertani broth, and initial concentrations of each were determined. Aliquots of 10 g of fecal sample were inoculated with 1 ml of different dilutions (10^{0} to 10^{-7}) of pooled pure cultures and were mixed as thoroughly as possible; 1 g of the spiked fecal sample was added to 9 ml of EC broth. The fecal suspension was vortexed and incubated at 40° C for 6 h. One-milliliter samples of pre- and post-enrichment fecal suspensions were boiled for 10 min and centrifuged at 9,300 x g for 5 min. DNA cleanup of pre- and post-enrichment fecal suspensions was performed using the GeneClean Turbo Kit (MP Biomedicals LLC, Solon, OH). Purified DNA from pre- and post-

enriched fecal suspensions and boiled DNA from pooled pure cultures used to spike the fecal sample were subjected to mqPCR, and standard curves were generated to determine the correlation coefficients, amplification efficiencies, and detection limits of each assay. The experiment was repeated with a different fecal sample.

Application of mqPCR assays to quantify *E. coli* serogroups and virulence genes in fecal samples from feedlot cattle

A total of 576 pen-floor fecal samples from cattle housed in 24 pens (24 samples per pen) at a commercial feedlot in the central United States were collected the day before transportation of cattle to slaughter in the summer of 2013. Details regarding the design of the study and characteristics of the study population are available in Dewsbury et al. (Dewsbury et al., 2015). Fecal samples were suspended in EC broth, and DNA was extracted as described above. DNA was subjected to the two mqPCR assays (mqPCR-1 and mqPCR-2) and to a previously developed four-plex qPCR assay (mqPCR-3) targeting O157 serogroup (*rfbE*$_{O157}$) and the three major virulence genes, *eae*, *stx*1, and *stx*2 (30). Cycle threshold (C_T) values were determined to quantify seven major *E. coli* serogroups and three virulence genes. Concentration of each serogroup and virulence gene was determined based on standard curves obtained using cattle fecal samples spiked with known concentrations of pure cultures of *E. coli* O157 and non-O157 STEC serogroups.

Application of mqPCR assays and comparison with cPCR and culture methods for the detection of *E. coli* O157 and six non-O157 *E. coli* serogroups in fecal samples from feedlot cattle

Fecal samples (n =576) suspended in EC broth were enriched at 40° C for 6 h and then were subjected to three mqPCR assays (mqPCR-1, mqPCR-2, and mqPCR-3), 10-plex cPCR

assay targeting the same genes (seven serogroups and three virulence genes) (Bai et al., 2012), and culture-based methods (Dewsbury et al., 2015) for the detection of the seven serogroups. For the culture-based detection method, postenriched fecal samples were subjected to immunomagnetic separation with seven serogroup-specific beads (Abraxis, Warminster, PA), and 50 µl of post–immunomagnetic separation bead suspensions were spread plated onto chromogenic Posse′ medium (Possé et al., 2008) that was modified to include novobiocin at 5 mg/liter and potassium tellurite at 0.5 mg/liter for non-O157 serogroups, and sorbitol MacConkey with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter) for *E. coli* O157. The plates were then incubated at 37° C for 18 to 24 h, and six chromogenic colonies (shades of blue, purple, mauve, or green) from modified Posse′ medium and sorbitol-negative colonies from sorbitol MacConkey with cefixime and potassium tellurite were picked and streaked onto blood agar and incubated for 18 to 24 h. For non-O157 serogroups, colonies from each of the six streaks on the blood agar plate were pooled in 50 µl of distilled water, boiled for 10 min, and used as a DNA template for seven-plex cPCR targeting the seven serogroups (Paddock et al., 2012). For O157, colonies on blood agar were subjected to indole test, and indole-positive colonies were tested for O157 antigen using a latex agglutination assay (*E. coli* O157 latex test kit, Oxoid Ltd., Cheshire, England). Agglutination-positive colonies were then subjected to a six-plex cPCR assay (*rfbE*$_{O157}$, *fliC*$_{H7}$, *stx*1, *stx*2, *eae*, and *ehxA*) to confirm the O157 serogroup, H7 flagellar gene, and virulence genes (Bai et al., 2010).

Statistical analyses

The proportion of positive samples for each serogroup and virulence gene based on the three detection methods (mqPCR, cPCR, and culture method) was calculated as the number of samples detected as positive for each gene by each detection method divided by the total number

of samples tested by each detection method. The Cohen's kappa statistic was used to evaluate the agreement beyond that due to chance among mqPCR, cPCR, and culture methods for the detection of seven *E. coli* serogroups and three virulence genes. Interpretation of the kappa statistic was done based on the scale proposed by Landis and Koch (Landis and Koch, 1977). The McNemar's chi-square test was used to compare the proportion of positive samples obtained by the three detection methods (McNemar, 1947). When the *P* value of McNemar's test is not significant ($P > 0.05$), there is little evidence to conclude that the tests are different; and, when the *P* value is significant ($P < 0.05$), there is a significant disagreement between tests, indicating that there is little value in further assessing agreement by Cohen's kappa statistic. In the latter case, Cohen's kappa statistics are provided for reference only. A receiver operating characteristic curve was generated by plotting the true positive against the false positive proportions across a range of reciprocal CT values ($1/C_T$ value) to determine the C_T value that yields optimum sensitivity and specificity and the highest proportion of correctly classified samples by cPCR in relation to mqPCR. Statistical analyses were performed in Stata/MP version 12.0 (StataCorp LP, College Station, TX).

Results

Specificity of the assays

All 170 strains belonging to O26, O103, O45, O111, O121, and O145 serogroups were detected by the corresponding mqPCR-1 and mqPCR-2 assays (data not shown). No cross-amplification occurred with nontargeted serogroups. None of the *E. coli* strains belonging to serogroups other than the six non-O157 serogroups were detected by the mqPCR assays, nor were other bacterial species. Both assays (mqPCR-1 and mqPCR-2) correctly detected the target

serogroups when performed with pooled pure cultures and spiked fecal samples of two different combinations containing equal or different concentrations of target serogroups (data not shown).

Sensitivity of the assays with pure cultures and pure culture–spiked fecal samples

The detection limits of the two assays, mqPCR-1 and mqPCR-2, with pure cultures were 3 log CFU/ml, with mean C_T values of 37.1 and 37.4 and amplification efficiencies from 99 to 104% and 99 to 102%, respectively. The correlation coefficient was 0.99 for both assays (Table 2.3). Detection limits, correlation coefficients, and amplification efficiencies of both assays were similar to the corresponding single (targeting a single serogroup) or duplex (targeting two serogroups) assays (data not shown). In fecal samples spiked with serially diluted, pooled pure cultures, detection limits of both assays (mqPCR-1 and mqPCR-2) were 4 log CFU/g of feces, with mean CT values of 37.2 and 37.4, respectively. After enrichment, detection limits of both assays improved to 2 log CFU/g of feces, with mean CT values of 37.6 and 37.9 for mqPCR-1 and mqPCR-2, respectively. The correlation coefficients and amplification efficiencies are shown in Table 2.3.

Application of mqPCR assays to quantify *E. coli* serogroups and virulence genes in cattle feces

Pre-enriched fecal samples that yielded C_T values less than or equal to the mean cut-off C_T value were considered positive (37.2 for mqPCR 1, 37.4 for mqPCR-2, and 38.3 for mqPCR-3) for the serogroups. Of the 576 fecal samples (before enrichment), 175 (30.4%) were quantifiable for at least one of the seven serogroups. Serogroup O157 (n=66; 11.5%) was the predominant serogroup quantified by mqPCR, followed by O45 (n=41; 7.1%), O103 (n =41; 7.1%), O121 (n =37; 6.4%), O26 (n=29; 5%), O111 (n=2; 0.3%), and O145 (n=2; 0.3%) (Table 2.4). The concentrations of *E. coli* serogroups ranged from 4 to 7 log CFU/g of feces. A greater

proportion of fecal samples tested positive for *E. coli* serogroups at concentrations of 4 (19.3%) than 5 log CFU/g (17.4%), and none of the non-O157 *E. coli* serogroups had concentrations >6 log CFU/g. Seven fecal samples (1.2%) were positive for O157 at concentrations of 6 to <7 log CFU/g. Of the 175 fecal samples that tested positive for at least one of the seven *E. coli* serogroups at quantifiable concentrations (≥4 log CFU/g), 141 (80.6%) were positive for one serogroup, 28 (16%) for two serogroups, and three (1.7%) each for three and four serogroups. The concentrations of *stx*1 and *eae* ranged from 4 to 8 log CFU/g, and that of *stx*2 ranged from 4 to 7 log CFU/g (Table 2.4).

Application of mqPCR assays to detect *E. coli* serogroups and virulence genes in cattle feces

Post-enriched fecal samples that yielded C_T values less than or equal to the mean cut-off C_T value were considered positive (37.6 for mqPCR-1, 37.9 for mqPCR-2, and 37.9 for mqPCR-3) for serogroups. Of 576 samples, 566 (98.3%) were positive for at least one of the seven serogroups, and all except one sample were positive for at least one of the three virulence genes. Serogroup O157 (89.8%) was the predominant *E. coli* detected, followed by O103 (84.7%), O26 (59.0%), O121 (57.8%), O45 (55.9%), and O145 (5.9%). Only four samples (0.7%) tested positive for serogroup O111. Among the virulence genes, *eae* (99.7%) was predominant, followed by *stx*1 (95.7%) and *stx*2 (94.6%) (Table 2.5).

Based on cPCR assay, 484 (84.0%) of the 576 fecal samples tested positive for at least one of the seven serogroups, and 571 (99.1%) tested positive for at least one of the three virulence genes. Of the 10 samples that tested negative for any of the seven serogroups by three mqPCR assays, one sample was positive for O157 by cPCR. *E. coli* O103 (56.6%) was the most commonly detected serogroup by cPCR, followed by O157 (54.7%), O26 (44.4%), O121

(22.9%), O45 (17.9%), O145 (1.9%), and O111 (0.7%). Among the virulence genes, *eae* (97.4%) was predominant, followed by *stx*2 (94.1%) and *stx*1 (64.4%) (Table 2.5).

Based on the culture method, 481 (83.5%) fecal samples tested positive for at least one of the seven serogroups. *E. coli* O103 (60.2%) was the most commonly detected serogroup, followed by O157 (43.1%), O26 (22.7%), O45 (16.7%), O145 (3.0%), O121 (2.3%), and O111 (0.2%) (Table 2.5). Because the pooled colonies were screened by a seven-plex PCR targeting only the seven major serogroups, virulence gene detection was not part of the culture detection method.

The McNemar's test indicated a significant ($P < 0.01$) difference between the proportions of positive fecal samples detected by mqPCR and cPCR for all the target genes except for wzx_{O111} and *stx*2. There was also a significant ($P < 0.01$) difference in the proportion of positive samples detected by mqPCR and the culture method for all target genes, except wzx_{O111}. In both cases, the kappa statistics were provided for reference only (Table 2.5). The receiver operating characteristic curve analysis showed that a mean C_T value of 32.3 yielded optimum sensitivity (83.3 to 100%), specificity (94.0 to 100%), and the highest number of correctly classified samples (93.0 to 100%) by cPCR in relation to mqPCR for all the seven serogroups.

Table 2.6 shows the number and proportion of fecal samples that tested positive or negative for each serogroup and virulence gene by three mqPCR assays, which tested positive or negative by cPCR or the culture method. Of the fecal samples that tested positive by three mqPCR assays for the seven serogroups, 0 to 68% tested negative by the cPCR, and 32.0 to 100% tested negative by the culture method, depending on the serogroup. Of the fecal samples that tested negative by the three mqPCR assays, the proportion of samples that were also negative by cPCR or culture method ranged from 83.0 to 100%. However, a few fecal samples

that tested negative by mqPCR were positive by cPCR (4 for O157, 15 for *stx*2, 3 for *stx*1, and 1 for *eae*). Similarly, the culture method detected a few samples as positive that tested negative based on mqPCR (16 for O26, 15 for O103, 10 for O45, 8 for O145, 7 for O157, 1 for O111, and 1 for O121) (Table 2.6).

Discussion

We have developed two multiplex qPCR assays that target serogroup-specific O-antigen genes to detect and quantify *E. coli* O26, O103, O111, O45, O121, and O145 serogroups in cattle feces. Both assays were specific to their corresponding target genes, and the detection limits of both assays in pure cultures were 3 log CFU/ml. The detection limit increased to 4 log CFU/g when fecal samples were spiked with known concentrations of pure cultures. The enrichment of spiked fecal samples in EC broth for 6 h at 40° C improved the detection limit of both assays to 2 log CFU/g feces. These two assays, in combination with the four-plex assay targeting O157 serogroup and the three major virulence genes (Noll et al., 2015b), can be used to quantify (before enrichment) and detect (after enrichment) seven *E. coli* serogroups and three major virulence genes in cattle feces.

Real-time PCR assays have been developed for the rapid detection of non-O157 *E. coli* in food matrices (Fratamico et al., 2011; Fratamico et al., 2014; Lin et al., 2011b). Also, several commercially available real-time PCR-based detection systems have been evaluated for detection of non-O157 *E. coli* serogroups in beef and beef products (Fratamico and Bagi, 2012; Fratamico et al., 2014; Wasilenko et al., 2014). Conventional PCR assays for the detection of non-O157 *E. coli* serogroups in cattle feces have been reported (Bai et al., 2012; Cernicchiaro et al., 2013; Dargatz et al., 2013; Paddock et al., 2012), but, to date, there has been no published study on the

utility of mqPCR assays to detect or quantify the six non-O157 *E. coli* serogroups in feces of naturally shedding feedlot cattle. Quantitative PCR assays targeting serogroup-specific virulence genes have been developed to detect non-O157 *E. coli* serogroups in cattle feces. Sharma (Sharma, 2002) developed two multiplex qPCR assays that target a region of *eae* gene specific to O26, O111, and O157 serogroups (assay 1) and *stx*1 and *stx*2 (assay 2). Luedtke et al. (Luedtke et al., 2014) developed a four-plex qPCR to detect EHEC in cattle feces by targeting *ecf1*, an EHEC-specific gene, and the three major virulence genes, *eae*, *stx*1, and *stx*2. That assay was designed to detect EHEC but not individual serogroups of EHEC. Anklam et al. (Anklam et al., 2012) developed four separate mqPCR assays to target O-antigen genes of seven (O26, O45, O103, O111, O121, O145, and O157) serogroups and four virulence genes (*stx*1, *stx*2, *eae*, and *ehxA*) in cattle feces. One assay targeted O26 (wzy_{O26}), O103 (wzx_{O103}), and O145 (wzx_{O145}); the second assay targeted O45 (wzy_{O45}), O111 ($manC_{O111}$), and O121 (wzx_{O121}); the third assay targeted O157 ($rfbE_{O157}$); and the fourth assay targeted *stx*1, *stx*2, *eae*, and *ehxA* (enterohemolysin). Our two mqPCR assays targeting the six non-O157 serogroups are, to some extent, similar to those of Anklam et al. (Anklam et al., 2012): the same genes were targeted for two serogroups (O103 and O145), but for the other four serogroups (O26, O45, O111, and O121), the targeted genes were different (wzx_{O26} instead of wzy_{O26}; wzx_{O45} instead of wzy_{O45}; wzx_{O111} instead of $manC_{O111}$; and *wbqE* and *wbqF* instead of wzx_{O121}). We chose *wzx* genes for O26, O45, and O111 mainly because they worked better with the primers and probes of the other two O-antigen targets (O103 and O145) in the same reaction. As to O121, a *Shigella dysenteriae* strain (GenBank accession:AY380835.1) has an O-antigen gene cluster nearly identical to that of *E. coli* O121. We used minor differences in *wbqE* and *wbqF* genes to differentiate O121 from the *Shigella* strain. We used target regions of the *wzx* gene for O103 and O145 to make sure that

there was no secondary structure or interference with each other; hence, the primers are different. Also, different combinations of the serogroups were included in the two assays, compared with Anklam et al. (Anklam et al., 2012). We did not include *ehxA* in our assay because the gene is present in many of the non–Shiga toxigenic *E. coli* pathogens (Cookson et al., 2007; Lorenz et al., 2013). Anklam et al. (Anklam et al., 2012) validated their assays in detecting *E. coli* serogroups and virulence genes by using pure cultures and cattle fecal samples spiked with pure cultures, but they did not evaluate the applicability of these assays to detect and quantify *E. coli* serogroups and virulence genes in feces of naturally shedding cattle. The detection limits of the assays reported by Anklam et al. (Anklam et al., 2012) were 10^3 and 10^4 CFU/ml for pure cultures and spiked fecal samples, respectively, which improved to 10^0 after a 6-h enrichment of fecal samples. The detection limits of the pure cultures and spiked fecal samples are in agreement with the detection limits of our two assays. However, the detection limit for enriched samples was lower in their study than ours (10^0 versus 10^2), possibly because the fecal samples they used to inoculate with pure cultures were diluted 1:50 compared with the 1:10 dilution used in our assays.

To our knowledge, this is the first study to assess the applicability of mqPCR assays for detection and quantification of the six non-O157 *E. coli* serogroups in fecal samples from naturally shedding cattle. All three mqPCR assays detected more samples as positive for one or more of the serogroups compared with detection by cPCR and the culture method. McNemar's test indicated disagreement between the proportion of positive samples detected by mqPCR, cPCR, and the culture method. The disagreement between mqPCR and cPCR is explained by receiver operating characteristic curve analysis of C_T values, which indicated that cPCR is less sensitive than mqPCR. It is known that real-time PCR assay is more sensitive than conventional

PCR- or culture-based testing methods (Lemmon and Gardner, 2008). As with any PCR assay, there is also a possibility of false positives because of amplification of DNA from nonviable cells in the feces. However, a few samples that were negative by mqPCR (16 for O26, 10 for O45, 15 for O103, 1 for O111, 1 for O121, 8 for O145, and 7 for O157) were positive by cPCR or the culture method. A likely reason for the difference could be the uneven distribution of target genes or serogroups in each aliquot that was subjected to the different methods of detection. Also, the misidentification of culture-positive samples by mqPCR is likely reflective of the difference in detection limits between the two methods. The mqPCR requires a concentration of 10^4 CFU/g for detection, whereas the culture method, which uses immunomagnetic separation beads, may randomly capture *E. coli* cells at lower concentrations. A similar disagreement between culture method and mqPCR assay has been reported for *E. coli* O157 in cattle feces (Jacob et al., 2014; Noll et al., 2015b). The number of fecal samples testing positive for intimin and Shiga toxin genes was generally higher than the number positive for the seven *E. coli* serogroups, which is likely due to the presence of STEC serogroups other than the seven targeted in this study as well as free bacteriophages carrying Shiga toxin genes in fecal samples (Conrad et al., 2014).

Apart from estimating the prevalence of *E. coli* serogroups and their virulence genes in cattle feces, determining their concentrations is essential for estimating the risk of foodborne illnesses associated with fecal shedding of *E. coli* serogroups and their associated virulence genes. Estimating the concentration of *E. coli* serogroups is beneficial for evaluating the efficacy of intervention strategies employed to reduce the pathogen load in feces so as to reduce hide and carcass contaminations (Arthur et al., 2009). Data on the concentration of non-O157 *E. coli* serogroups in cattle feces are nonexistent. Quantitative PCR has been used to quantify *E. coli*

O157:H7 and virulence genes (Guy et al., 2014; Jacob et al., 2014; Noll et al., 2015b; Verstraete et al., 2014) in cattle feces. In our study, 175 (30.4%) of 576 fecal samples were positive for at least one of the seven *E. coli* serogroups, with a concentration of ≥4 log CFU/g of feces. A majority of the samples (80.6%) that were quantifiable were positive for one of the seven serogroups, with *E. coli* O157 being the most common serogroup. However, an inherent limitation of the mqPCR assay, similar to cPCR, is that the presence of virulence genes cannot be directly associated to any particular serogroup in the sample.

In conclusion, the two sets of mqPCR assays are rapid diagnostic tools for the detection and quantification of six major non-O157 *E. coli* serogroups in cattle feces. These two assays, together with the four-plex assay targeting *E. coli* O157 and three virulence genes (*stx*1, *stx*2, and *eae*), can be used to detect and quantify seven major *E. coli* serogroups and three virulence genes in cattle feces. This is the first study to provide data on concentrations of non-O157 *E. coli* serogroups in cattle feces and to identify the existence of a subset of cattle, similar to super shedders of O157, that shed non-O157 at high concentrations.

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Table 2.1. Primers and probes used in the multiplex quantitative PCR assays[a]

Target genes	Primers / Probes	Sequence	Reference
***wzx*O26**	Forward	GTGCGAATGCCATATTTCCT	This study
	Reverse	TGCTAGTACTTCACCAACAGCAA	
	Probe	FAM-CCTTAGGGGAAAACATCTTTTCAA-BHQ1	
***wzx*O103**	Forward	AAAGGCGCATTAGTGTCTGC	This study
	Reverse	GAGGAGTATGTCCCACCCAGT	
	Probe	MAX-ATGCGGTTGCAGGTGTCTG-BHQ1	
***wzx*O111**	Forward	CCGGTTGTTTCATCAATCCT	This study
	Reverse	AAACTAAGTGAGACGCCACCA	
	Probe	TexasRed-TGAGTCAAAATGGATGTTTCTTCAA-BHQ2	
***wzx*O45**	Forward	GGTGTTGCCATATGGTGTTG	This study
	Reverse	TCCGGAAATTTTACCTTCCA	
	Probe	MAX-TGATCATGCAACAGGTCACTATGA-BHQ1	
***wbqE*O121+ *wbqF*O121**	Forward	TGGATATGCTTCGAATGAACC	This study
	Reverse	CAATGCGAGTTTTGTCTCCA	
	Probe	FAM-TTCCTACACCTACTCGATATACTCCCA-BHQ1	
***wzx*O145**	Forward	CATTGTTTTGCTTGCTCGAC	This study
	Reverse	CAACGAAAATACCATATCCTACAGC	
	Probe	TexasRed-CCATCAACAGATTTAGGAGTGTATGG-BHQ2	
***rfb*EO157**	Forward	CTGTCCACACGATGCCAATG	Jacob et al., 2012
	Reverse	CGATAGGCTGGGGAAACTAGG	
	Probe	FAM-TTAATTCCACGCCAACCAAGATCCTCA-Iowa Black FQ	
***stx*1**	Forward	CAAGAGCGATGTTACGGTTTG	Jacob et al., 2012
	Reverse	GTAAGATCAACATCTTCAGCAGTC	
	Probe	TexasRed-ACATAAGAACGCCCACTGAGATCATCCA-BHQ2	
***stx*2**	Forward	GCATCCAGAGCAGTTCTGC	Jacob et al., 2012
	Reverse	GCGTCATCGTATACACAGGAG	
	Probe	Cy5-TGTCACTGTCACAGCAGAAGCCTTACG-BHQ2	

	Forward	AAAGCGGGAGTCAATGTAACG	
eae	Reverse	GGCGATTACGCGAAAGATAC	Noll et al.,
	Probe	MAX-CTCTGCAGATTAACCTCTGCCG-ZEN	2015

[a] BHQ, Black Hole Quencher dye.

Table 2.2. Sources and virulence gene profiles of *Escherichia coli* strains used for optimization of multiplex quantitative PCR assays

Serogroup	Strains	Host	*stx*1	*stx*2	*eae*
***E.coli* O26**	TW 8569	Human	-	+	+
***E.coli* O103**	TW 8103	Human	+	-	+
***E.coli* O111**	7726-1	Bovine	+	+	+
***E.coli* O121**	KDHE 48	Human	-	+	-
***E.coli* O45**	KDHE 22	Human	+	-	+
***E.coli* O145**	KDHE 53	Human	+	+	+

Table 2.3. Detection limits, correlation coefficients, and amplification efficiencies of the multiplex quantitative PCR assays for pure cultures and cattle fecal samples spiked with pure cultures of non-O157 Shiga toxin-producing *Escherichia coli* strains[a]

Template	Detection limit[b] (average cycle threshold value)	Correlation coefficients	Amplification efficiencies, %
Pure cultures	Log CFU/ml		
Assay 1: O26, O103, O111	3 (37.1)	> 0.99	99-104
Assay 2: O45, O121, O145	3 (37.4)	> 0.99	99-102
Cattle feces spiked with pure cultures:	Log CFU/g		
Before enrichment			
Assay 1: O26, O103, O111	4 (37.2)	> 0.96	90-95
Assay 1: O26, O103, O111, O45, O121, O145	4 (37.8)	> 0.96	92-94
Assay 2: O45, O121, O145	4 (37.4)	> 0.95	88-92
Assay 2: O26, O103, O111, O45, O121, O145	4 (37.5)	> 0.95	89-91
After enrichment			
Assay 1: O26, O103, O111	2 (37.6)	> 0.98	94-101
Assay 1: O26, O103, O111, O45, O121, O145	2 (37.7)	> 0.98	92-101
Assay 2: O45, O121, O145	2 (37.9)	> 0.98	91-98
Assay 2: O26, O103, O111, O45, O121, O145	2 (37.3)	> 0.98	90-96

[a]Cattle fecal samples spiked with pure cultures of non-O157 STEC were suspended in *E. coli* broth (before enrichment) and incubated at 40° C for 6 h (after enrichment).

[b]Values represent means of two individual experiments

Table 2.4. Quantification of seven major *Escherichia coli* serogroups and three virulence genes in pre-enriched cattle feces using multiplex quantitative PCR assays

Serogroup and virulence genes	Total no. (%) of negative samples	Total no. (%) of positive samples	Log CFU/g, no. (%) of samples positive			
			4 - < 5	5 - < 6	6 - < 7	7 - < 8
O26	547 (95.0)	29 (5.0)	19 (3.3)	10 (1.7)	0	0
O45	535 (92.9)	41 (7.1)	21 (3.6)	20 (3.5)	0	0
O103	535 (92.9)	41 (7.1)	27 (4.7)	14 (2.4)	0	0
O111	574 (99.7)	2 (0.3)	0	2 (0.3)	0	0
O121	539 (93.6)	37 (6.4)	21 (3.6)	16 (2.8)	0	0
O145	574 (99.7)	2 (0.3)	2 (0.3)	0	0	0
O157	516 (89.6)	66 (11.5)	21 (3.6)	38 (6.6)	7 (1.2)	0
Shiga toxin 1 (*stx*1)	401 (69.6)	183 (31.8)	50 (8.7)	90 (15.6)	39 (6.8)	4 (0.7)
Shiga toxin (*stx*2)	435 (75.5)	134 (23.3)	31 (5.4)	88 (15.3)	15 (2.6)	0
Intimin (*eae*)	362 (62.8)	257 (44.6)	75 (13.0)	125 (21.7)	56 (9.7)	1 (0.2)

Table 2.5. Number of cattle fecal samples that tested positive for seven major *Escherichia coli* serogroups and three virulence genes (n = 576) by mqPCR, cPCR, and culture methods of detection, and agreement between tests[d]

Serogroup and virulence genes	No. (%) of samples positive			mqPCR vs. cPCR		mqPCR vs. culture method	
	mqPCR	cPCR	Culture method	Kappa statistic (95% CI)	*P*-value for Kappa	Kappa statistic (95% CI)	*P* value for Kappa
O26	340 (59.0)	256 (44.4)	131 (22.7)	0.71 (0.66 - 0.77)	< 0.01	0.24 (0.18 – 0.29)	< 0.01
O45	322 (55.9)	103 (17.9)	96 (16.7)	0.29 (0.24 - 0.35)	< 0.01	0.21 (0.16 – 0.26)	< 0.01
O103	488 (84.7)	326 (56.6)	347 (60.2)	0.38 (0.32 - 0.44)	< 0.01	0.31 (0.24 – 0.38)	< 0.01
O111	4 (0.7)	4 (0.7)	1 (0.2)	1.00 (1.00 - 1.00)	< 0.01	-0.003 (-0.008 – 0.003)	0.53
O121	333 (57.8)	132 (22.9)	13 (2.3)	0.36 (0.30 – 0.41)	< 0.01	0.03 (0.009 – 0.05)	< 0.01
O145	34 (5.9)	11 (1.9)	17 (3.0)	0.47 (0.29 – 0.65)	< 0.01	0.33 (0.16 – 0.50)	< 0.01
O157	517 (89.8)	315 (54.7)	248 (43.1)	0.21 (0.16 – 0.27)	< 0.01	0.12 (0.07 – 0.16)	< 0.01
Shiga toxin 1 (*stx*1)	551 (95.7)	371 (64.4)	-	0.12 (0.07 – 0.18)	< 0.01	-	-
Shiga toxin (*stx*2)	545 (94.6)	542 (94.1)	-	0.46 (0.31 – 0.62)	< 0.01	-	-
Intimin (*eae*)	574 (99.7)	561 (97.4)	-	0.11 (-0.10 – 0.32)	< 0.01	-	-

[a]mqPCR, multiplex quantitative PCR; cPCR, conventional PCR. n=576

Table 2.6. Comparison of mqPCR, cPCR, and culture methods for the detection of seven *Escherichia coli* serogroups and three virulence genes in enriched fecal samples[a]

Serogroup and virulence genes[b]	mqPCR, no. (%) positive	cPCR[c]		Culture method[c]		mqPCR, no. (%) negative	cPCR[d]		Culture method[d]	
		No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative		No. (%) positive	No. (%) negative	No. (%) positive	No. (%) negative
O26	340 (59.0)	256 (75.3)	84 (24.7)	115 (33.8)	225 (66.2)	236 (41.0)	0	236 (100)	16 (6.8)	220 (93.2)
O45	322 (55.9)	103 (32.0)	219 (68.0)	86 (26.7)	236 (73.3)	254 (44.1)	0	254 (100)	10 (3.9)	244 (96.1)
O103	488 (84.7)	326 (66.8)	162 (33.2)	332 (68.0)	156 (32.0)	88 (15.3)	0	88 (100)	15 (17.0)	73 (83.0)
O111	4 (0.7)	4 (100)	0	0	4 (100)	572 (99.3)	0	572 (100)	1 (0.2)	571 (99.8)
O121	333 (57.8)	132 (39.6)	201 (60.4)	12 (3.6)	321 (96.4)	243 (42.2)	0	243 (100)	1 (0.4)	242 (99.6)
O145	34 (5.9)	11 (32.4)	23 (67.6)	9 (26.5)	25 (73.5)	542 (94.1)	0	542 (100)	8 (1.5)	534 (98.5)
O157	517 (89.8)	311 (60.2)	206 (39.8)	241 (46.6)	276 (53.4)	59 (10.2)	4 (6.8)	55 (93.2)	7 (11.9)	52 (88.1)

Shiga toxin 1 (*stx*1)	551 (95.7)	368 (66.8)	183 (33.2)	-	-	25 (4.3)	3 (12.0)	22 (88.0)	-	-
Shiga toxin 2 (*stx*2)	545 (94.6)	527 (96.7)	18 (3.3)	-	-	31 (5.4)	15 (48.4)	16 (51.6)	-	-
Intimin (*eae)*	574 (99.7)	560 (97.6)	14 (2.4)	-	-	2 (0.3)	1 (50.0)	1 (50.0)	-	-

[a]mqPCR, multiplex quantitative PCR; cPCR, conventional PCR. n=576

[b]McNemar's test indicated a significant ($P < 0.01$) difference between the proportion of positive fecal samples detected by mqPCR and cPCR for all the target genes except for wzx_{O111} and *stx*2.

McNemar's test indicated a significant ($P < 0.01$) difference in the proportion of positive samples detected by mqPCR and the culture method for all the target genes except for wzx_{O111}.

[c]Number of fecal samples that were positive or negative by cPCR or culture method from the samples that were positive by mqPCR for the respective serogroups and virulence genes.

[d]Number of fecal samples that were positive or negative by cPCR or culture method from the samples that were negative by mqPCR for the respective serogroups and virulence genes.

Chapter 3 - Spiral Plating Method To Quantify the Six Major Non-O157 *Escherichia coli* Serogroups in Cattle Feces

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Abstract

Cattle are a major reservoir of the six major Shiga toxin–producing non-O157 *Escherichia coli* (STEC) serogroups (O26, O45, O103, O111, O121, and O145) responsible for foodborne illnesses in humans. Besides prevalence in feces, the concentrations of STEC in cattle feces play a major role in their transmission dynamics. A subset of cattle, referred to as super shedders, shed *E. coli* O157 at high concentrations (≥ 4 log CFU/g of feces). It is not known whether a similar pattern of fecal shedding exists for non-O157. Our objectives were to initially validate the spiral plating method to quantify the six non-O157 *E. coli* serogroups with pure cultures and culture-spiked fecal samples and then determine the applicability of the method and compare it with multiplex quantitative PCR (mqPCR) assays for the quantification of the six non-O157 *E. coli* serogroups in cattle fecal samples collected from commercial feedlots. Quantification limits of the spiral plating method were 3 log, 3 to 4 log, and 3 to 5 log CFU/mL or CFU/g for individual cultures, pooled pure cultures, and cattle fecal samples spiked with pooled pure cultures, respectively. Of the 1,152 cattle fecal samples tested from eight commercial feedlots, 122 (10.6%) and 320 (27.8%) harbored concentrations ≥ 4 log CFU/g of one or more of the six serogroups of non-O157 by spiral plating and mqPCR methods, respectively. A majority of quantifiable samples, detected by either spiral plating (135 of 137, 98.5%) or mqPCR (239 of 320, 74.7%), were shedding only one serogroup. Only one of the

quantifiable samples was positive for a serogroup carrying Shiga toxin (*stx*1) and intimin (*eae*) genes; 38 samples were positive for serogroups carrying the intimin gene. In conclusion, the spiral plating method can be used to quantify non-O157 serogroups in cattle feces, and our study identified a subset of cattle that was super shedders of non-O157 *E. coli*. The method has the advantage of quantifying non-O157 STEC, unlike mqPCR that quantifies serogroups only.

Introduction

Shiga toxin–producing *Escherichia coli* (STEC) are major foodborne pathogens responsible for human illnesses that range from mild-to-severe bloody diarrhea with serious complications to death. *E. coli* O157 is the most common serogroup implicated in foodborne STEC outbreaks. However, serogroups other than O157 are gaining more recognition in recent years. Major serogroups responsible for a majority of the non-O157 STEC-associated illnesses in humans are O26, O45, O103, O111, O121, and O145. FoodNet sites have reported an increase in the number of non-O157 STEC infections from an incidence of 0.12 in 2000 to 0.95 per 100,000 in 2010 in the U.S. population (Gould et al., 2013).

Cattle, a primary reservoir, harbor STEC in the hindgut and shed them in the feces. A few studies have reported the prevalence of non-O157 STEC in cattle feces (Cernicchiaro et al., 2013; Dargatz et al., 2013; Dewsbury et al., 2015; Noll et al., 2015a). In addition to the prevalence, the concentration of STEC plays an important role in the spread of these bacteria between animals within a pen or herd, and cattle hide and carcass contaminations (Chase-Topping et al., 2008). Estimating the concentration of STEC in cattle feces is also important for assessing the risk of hide and subsequent carcass contamination associated with fecal shedding and for estimating the efficacy of various interventions used to reduce STEC in feces, hide, and

carcass (Guy et al., 2014). *E. coli* O157 is shed at concentrations ranging from < 2 to > 6 log CFU/g of feces (Chase-Topping et al., 2007). Cattle that shed *E. coli* O157 at high concentrations (≥ 4 log CFU/g) are called super shedders (Bach et al., 2005). It is not known whether super shedders of non-O157 STEC serogroups exist among the populations. Data on the concentration of *E. coli* O157:H7 in cattle feces and environmental samples are available because of validated quantification methods (Fegan et al., 2004; Fox et al., 2007; Ibekwe and Grieve, 2003; LeJeune et al., 2006). However, data on fecal concentration of non-O157 STEC in cattle are limited. We have developed two multiplex quantitative PCR (mqPCR) assays - mqPCR-1 targeting O26, O103, and O111 and mqPCR-2 targeting O45, O121, and O145—for detection and quantification of six non-O157 *E. coli* serogroups in cattle feces (Shridhar et al., 2016a). The limitation of the mqPCR assay is that it detects and quantifies the serogroups, but it does not distinguish between serogroups with or without *stx* and intimin (*eae*) genes. Even if an assay that concurrently detects and quantifies the *stx* and *eae* genes is included (Noll et al., 2015b), the presence of virulence genes cannot be associated to any particular serogroup in the sample. Spiral plating is a culture-based method initially developed to enumerate bacteria in food products (Gilchrist et al., 1973). The spiral plating method has been used for enumeration of *E. coli* O157 in cattle feces, hide, and carcass swab samples (Berry and Wells, 2008; Brichta-Harhay et al., 2007; Fox et al., 2007; Robinson et al., 2004). Because individual colonies are available on a spiral plate and isolates can be obtained to detect virulence genes (Shiga toxin and intimin genes), the spiral plating method can be used to quantify STEC, thereby offering an advantage over mqPCR assays. However, the spiral plating method has not been validated to detect multiple serogroups of non-O157 in cattle feces or in other sample matrices. Therefore, the objectives of our study were to initially validate the spiral plating method with individual and

pooled pure cultures and cattle feces spiked with pooled pure cultures to quantify six major serogroups of non-O157 *E. coli*-O26, O45, O103, O111, O121, and O145-and then determine the applicability of the spiral plating method and compare it with mqPCR assays to quantify six non-O157 serogroups in cattle feces collected from commercial feedlots.

Materials and methods

Spiral plating methodology

Fecal samples were suspended in *Escherichia coli* broth (EC medium; Difco, BD, Sparks, MD) in the ratio of 1:10 and vortexed thoroughly. Fecal suspensions were further diluted (1:10) in EC broth, and 100 µL of the diluted fecal suspension was spiral plated onto selective media using an Eddy Jet v.1.23 spiral plater (IUL Instruments, S.A, Barcelona, Spain). Plates were then incubated at 37° C for 18 to 24 h. After incubation, colonies were counted using a counting grid that relates colonies on the spiral plate to the volume deposited in the area. Colonies were counted according to the manufacturer's instructions and guidelines outlined in the Bacteriological Analytical Manual (Maturin and Peeler, 2001).

Validation of the spiral plating method by using pure cultures of non-O157 STEC

The validation was conducted with one strain of each of the six serogroups of non-O157 STEC individually or with pooled strains of two different combinations. The source and major virulence genes profile of the strains are shown in Table 3.1. A single colony of each serogroup from blood agar plates was grown overnight in Luria-Bertani broth (Difco, BD) at 37° C, and 100 µL of the culture was inoculated into 10 mL of Luria-Bertani broth and incubated at 37° C for 3 to 4 h until an absorbance of 0.4 at 600 nm (~10^8 CFU/mL) was achieved. Tenfold serial dilutions of each strain were performed in buffered peptone water, and three dilutions (10^{-5}, 10^{-6},

and 10^{-7}) were spread plated onto blood agar plates in quadruplicates to determine initial cell concentrations of each serogroup. One milliliter of the culture of each serogroup of each dilution was mixed in two different combinations, resulting in a threefold dilution of each serogroup: (i) O26, O103, and O111 and (ii) O45, O121, and O145. One hundred microliters of diluted cultures of individual and pooled serogroups was spiral plated onto modified Posse′ medium (MP) (Noll et al., 2015a; Stromberg et al., 2016) and incubated at 37° C for 18 to 24 h. Colonies (mauve, green, blue, or purple) were counted, and concentrations were determined as described above. Ten chromogenic colonies were randomly picked and subcultured on blood agar and were incubated for 18 to 24 h at 37° C. Each colony on the blood agar plate was then tested by a nine-plex PCR (Bai et al., 2012) targeting six major serogroups, namely O26, O45, O103, O111, O121, and O145, and the three virulence genes *stx*1, *stx*2, and *eae*. The concentration of each STEC or serogroup was determined based on the proportion of colonies testing positive for the serogroup. The experiment was done three times.

Application of spiral plating method to quantify non-O157 *E. coli* serogroups in cattle fecal samples collected from commercial feedlots

In total, 1,152 cattle fecal samples, collected from eight commercial feedlots located in Nebraska and Texas, were used. Twelve pen-floor fecal samples were collected from each of four pens per feedlot per visit once a month, for a total of three visits from June to August 2014. Samples were transported on ice in a cooler to the Pre-harvest Food Safety Lab at Kansas State University and processed within 24 h. Fecal samples were suspended in EC broth (1:10) and then mixed by vortexing. Next, 100 µL of fecal suspension was added to 900 µL of EC broth. One

hundred microliters of diluted fecal suspensions was spiral plated onto MP medium and incubated for 18 to 24 h at 37° C. Concentrations of each serogroup were determined as described above.

Quantification of non-O157 *E. coli* serogroups in cattle fecal samples by mqPCR assays

One milliliter of initial fecal suspension in EC broth (n=1,152), before incubation, was boiled for 10 min and then centrifuged at 9,300 x g for 5 min. The supernatant was purified using a GeneClean Turbo kit (MP Biomedicals, Solon, OH). Purified DNA was subjected to two mqPCR assays: mqPCR-1 targeting O26, O103, and O111, and mqPCR-2 targeting O45, O121, and O145 (Shridhar et al., 2016a). Cycle threshold values were recorded and concentration of each serogroup was determined based on the standard curves obtained with cattle fecal samples spiked with known concentrations of pure cultures of non-O157 *E. coli* serogroups (Shridhar et al., 2016a).

Detection of non-O157 *E. coli* serogroups in cattle fecal samples by culture method and mqPCR assays

Fecal samples (n=1,152) utilized for quantification of non-O157 STEC by spiral plating method and mqPCR assays were also subjected to a culture method and mqPCR assays to determine the prevalence of non-O157 STEC. The initial suspensions of feces in EC broth (1:10 dilution) were enriched at 40° C for 6 h and subjected to a culture method (Noll et al., 2015a) and two mqPCR assays (assay 1: O26, O103, and O111; assay 2: O45, O121, and O145) (Shridhar et al., 2016a) for the detection of samples positive for one or more of the six non-O157 *E. coli* serogroups. For the culture method, postenriched fecal samples were subjected to immunomagnetic separation (IMS) using combinations of pooled serogroup specific IMS beads

(Abraxis, Warminster, PA) (Noll et al., 2016) and 50 µL of post-IMS bead suspensions were spread plated onto MP medium (Noll et al., 2015a). The plates were then incubated at 37° C for 18 to 24 h, and 10 chromogenic colonies (shades of blue, purple, mauve, or green) (Noll et al., 2015a) from MP medium were subcultured on blood agar plates, and incubated for 18 to 24 h. Colonies from blood agar plates were pooled in 50 µL of distilled water, boiled for 10 min, and used as a DNA template for a nine-plex conventional PCR to identify the serogroup (Bai et al., 2012). For mqPCR, 1 mL of postenriched fecal suspensions (n=1,152) was boiled for 10 min, centrifuged at 9,300 x g for 5 min, and the supernatant was purified using GeneClean Turbo kit (MP Biomedicals, Solon, OH). Purified DNA was subjected to two mqPCR assays, assay 1 with O26, O103, and O111 and assay 2 with O45, O121, and O145, to detect the serogroup-specific genes (Shridhar et al., 2016a).

Statistical analysis

A two-sample mean comparison test for paired samples was performed to compare the mean concentrations, in log CFU per gram, of six non-O157 *E. coli* serogroups determined by the spiral plating method with the predetermined inoculum concentrations determined by spread plating for individual or pooled pure cultures and fecal samples spiked with pure cultures. The proportions of fecal samples (n=1,152) that tested positive for each serogroup based on spiral plating and mqPCR methods in preenriched fecal samples for quantification and on culture and mqPCR methods in postenriched fecal samples for detection were calculated as the number of samples positive by each method divided by the total number of samples tested. Cohen's kappa statistic was used to evaluate the agreement beyond that due to chance between the two methods for quantification or detection. Interpretation of the kappa statistic was based on the scale proposed by Landis and Koch (Landis and Koch, 1977). McNemar's chi-square test was used to

compare the proportion of positive samples determined by the different detection methods. When there is a significant disagreement between the diagnostic methods (*P* value of McNemar's test < 0.05), there is little value in assessing agreement, and Cohen's kappa statistic is provided for reference only.

The association between the presence of a super-shedding animal in the pen (shedding ≥ 4 log CFU/g) for each non-O157 *E. coli* serogroup (based on the spiral plating method or mqPCR of preenriched fecal samples) with the pen-level prevalence (based on culture method or mqPCR of postenriched fecal samples) of that serogroup was analyzed using generalized linear mixed models fitted with a binomial distribution, logit link, residual pseudo-likelihood estimation, and Kenward-Rogers degrees of freedom estimation using Proc Glimmix (SAS 9.4, SAS Institute Inc., Cary, NC). The outcomes consisted of the number of samples testing positive for each non-O157 serogroup within a pen divided by the total number of samples tested per pen. Explanatory variables consisted of the presence of a super-shedding animal in a pen, for each non-O157 serogroup, modeled as dichotomous variables: presence of at least one animal in the pen shedding > 4 log CFU/g =1; else=0. Independent models were fitted for each non-O157 *E. coli* serogroup.

Results

Validation of spiral plating method with pure cultures and culture-spiked fecal samples

The detection limit of the spiral plating method was 3 log CFU/mL when serial dilutions of each non-O157 *E. coli* serogroup were spiral plated individually (Table 3.2). The detection limits ranged from 3 to 4 log CFU/mL for each serogroup when serial dilutions of pooled

serogroups (pool 1: O26, O103, and O111 and pool 2: O45, O121, and O145) were spiral plated. Of the 10 chromogenic colonies that were picked from plates inoculated with pooled serogroups and tested individually by the nine-plex PCR assay to detect six serogroups and three virulence genes (*stx*1, *st*x2, and *eae*), the number of colonies that tested positive for each serogroup, across dilutions, ranged from zero to five for pooled pure cultures of the three serogroups. There was less than a 0.5-log difference between the concentrations of non-O157 *E. coli* serogroups determined by the spiral plating method and the concentration of the inoculum for each dilution determined by spread plating (Tables 3.2 and 3.3). There was no significant ($P > 0.05$) difference in the mean concentrations (log CFU per milliliter) of the non-O157 serogroups determined by the spiral plating method and the concentrations of the inoculum used (determined by spread plating) when pure cultures of individual serogroups were spiral plated. When pooled pure cultures were spiral plated, there was no significant ($P > 0.05$) difference in the mean concentrations (log CFU per milliliter) of the non-O157 serogroups determined by the spiral plating method and the concentrations of the inocula used (determined by spread plating), except for O45 and O111 (Table 3.3). For serogroups O45 and O111, the mean overall spiral plate counts were significantly lower ($P < 0.05$) than the mean concentrations of inocula used for spiral plating (Table 3.3).

When fecal samples spiked with serially diluted pooled pure cultures of non-O157 *E. coli* serogroups were spiral plated, detection limits ranged from 3 to 5 log CFU/g of feces. Generally, the spiral plate counts were similar to, or 0.5 to 2 log CFU higher than, the concentrations of inocula used to spike the feces (Table 3.4). There were significant ($P < 0.05$) differences in mean concentrations of the serogroups determined by the spiral plating of spiked fecal samples and the concentrations of the inocula used (determined by spread plating) for serogroups O45, O103,

O111, O121, and O145, but not O26. When 10 colonies were picked and tested individually by PCR, the number of colonies positive for each serogroup ranged from zero to six in fecal samples spiked with pooled pure cultures of the three serogroups.

Quantification of non-O157 *E. coli* serogroups in fecal samples from feedlot cattle by the spiral plating method

Of the total 1,152 fecal samples collected from eight commercial feedlots, 137 (11.9%) were quantifiable (≥3 log CFU/g) for one or more of the six non-O157 *E. coli* serogroups (Table 3.5). Of the 137 quantifiable samples, 122 (89.1%) were ≥ 4 log CFU/g of feces (i.e., super shedders). Among the super shedders, the majority (117 of 122, 95.9%) were at concentrations of 4 to ≤ 6 log CFU/g of feces and 5 (4.1%) were at concentrations of > 6 log CFU/g of feces (Table 5). *E. coli* O103 (72.3%) was the most predominant serogroup quantified, followed by O26 (13.9%), O45 (8.8%), O121 (5.1%), and O145 (1.5%). None of the quantifiable samples tested positive for serogroup O111. Except for two samples that were positive for both serogroups O26 and O103, all quantifiable samples were positive for only one serogroup. In all samples quantified by spiral plating method, the 10 colonies picked and tested individually by PCR were positive for the serogroup-specific gene only and negative for Shiga toxin and intimin genes. Only one of the 137 quantifiable samples that tested positive for serogroup O103 carried Shiga toxin (*stx*1) and intimin (*eae*) genes and 38 quantifiable samples were positive for serogroup-specific and intimin genes (Table 3.6). However, a few fecal samples (78 of 1,152) tested positive for virulence genes (*stx*1, *stx*2, *eae*, or combinations), but negative for any of the six serogroups based on the multiplex PCR of individual colonies from each sample (data not shown).

Based on the culture method of detection of fecal samples after enrichment, 787 (68.3%) samples were positive for at least one of the six serogroups. *E. coli* O103 (49.8%) was the predominant serogroup detected by the culture method followed by O26 (23.9%), O45 (16.9%), O121 (6.3%), O145 (3.1%), and O111 (0.8%) (Table 3.5). Of the fecal samples that were positive for one or more of the five serogroups, the proportion of super-shedders ($\geq 10^4$/g of feces) varied from 5.6 to 17.3% depending on the serogroup (Fig. 3.1 A). The presence of a super-shedder ($\geq 10^4$/g of feces) in a pen, determined by spiral plating, was significantly ($P < 0.05$) associated with the within-pen prevalence, determined by the culture method, for O26 and O45 serogroups, but not for O103, O121, and O145. The mean within-pen prevalence of *E. coli* O26 when a super shedder was present in a pen was 26.5% (95% confidence interval [CI]=13.3 to 46.1%) and 18.8% (95% CI=9.4 to 34.4%) when no super shedder was present. Mean within-pen prevalence for O45 was 30.4% (95% CI=18.8 to 45.1%) in the presence of at least one super shedder, compared with 14.1% (95% CI= 9.1 to 21.2%) when no super shedder was present in the pen.

Quantification of non-O157 *E. coli* serogroups in fecal samples from feedlot cattle by mqPCR assays

Of the 1,152 fecal samples subjected to quantification by two mqPCR assays (mqPCR-1 and mqPCR-2), 320 (27.8%) were quantifiable ($\geq$ 4 log CFU/g) for at least one of the six non-O157 *E. coli* serogroups. A majority of fecal samples (308 of 320) were positive for *E. coli* serogroups at concentrations between 4 and 6 log CFU/g of feces, and only a few samples (15 of 320) were positive at concentrations >6 log CFU/g of feces (Table 3.5). Of the six serogroups, *E. coli* O103 (65.6% of the quantifiable samples or 18.2% of the total number of fecal samples) was

the most predominant serogroup quantified by mqPCR assays, followed by O26 (25% of the quantifiable samples), O45 (20.3% of the quantifiable samples), O121 (11.9% of the quantifiable samples), O145 (3.4% of the quantifiable samples), and O111 (1.3% of the quantifiable samples). Of the fecal samples that tested positive for at least one of the six non-O157 *E. coli* serogroups at quantifiable concentrations (≥ 4 log CFU/g), 239 (74.7%) were positive for one serogroup, 75 (23.4%) for two serogroups, 5 for three serogroups (1.6%), and 1 (0.3%) for four serogroups. The proportions of positive fecal samples quantified by spiral plating method and mqPCR assays were significantly ($P < 0.05$) different for all six non-O157 serogroups. Hence, the kappa statistic is provided for reference only (Table 3.7).

Of the 1,152 fecal samples that were tested after enrichment by the two mqPCR assays, 989 (85.9%) were positive for at least one serogroup (Table 3.5). *E. coli* O103 (69.2%) was the predominant serogroup detected followed by O26 (38.4%), O121 (35.2%), O45 (34.5%), O145 (3.6%), and O111 (1.0%) (Table 3.5). Among the fecal samples that were positive for the six serogroups, the proportion of quantifiable samples (≥ 4 log CFU/g of feces) varied from 9.4 to 33.3% (Fig. 3.1 B). For the serogroup O111 12 fecal samples were positive and 4 (33.3%) of those were shedding at high concentrations. There was a significant ($P < 0.05$) difference between the proportions of positive postenriched fecal samples detected by mqPCR and culture method, except for O111 and O145. Kappa statistics are provided in Table 3.7. The presence of at least one super-shedder in a pen (based on mqPCR of preenriched samples) was significantly ($P < 0.05$) associated with the within-pen prevalence (based on mqPCR of postenriched fecal samples) of O26, O45, O121, and O145. In the presence of at least one super-shedder in the pen, mean within-pen prevalence of *E. coli* O26, O45, O121, and O145 was 45.2% (95% CI=18.9 to 74.5%), 37.6% (95% CI=21.4 to 57.1%), 39.9% (95% CI=22.6 to 60.1%), and 7.1% (95% CI=

1.7 to 2.5%), respectively. When no super-shedder was present in the pen, the mean within-pen prevalence was 26.2% (95% CI=9.1 to 55.7%) for *E. coli* O26, 28.9% (95% CI=15.7 to 47.0%) for O45, 27.9% (95% CI=14.7 to 46.5%) for O121, and 1.2% (95% CI=0.3 to 4.5%) for *E. coli* O145.

Discussion

To our knowledge, this is the first study to evaluate the spiral plating method for quantification of non-O157 *E. coli* serogroups in cattle fecal samples. Several studies have reported on the fecal prevalence of non-O157 *E. coli* in cattle (Baltasar et al., 2014; Cernicchiaro et al., 2013; Dargatz et al., 2013; Dewsbury et al., 2015; Ekiri et al., 2014; Ennis et al., 2012; Noll et al., 2015a; Paddock et al., 2014); however, fecal concentration data of non-O157 *E. coli* in cattle are limited. mqPCR assays have been developed for detection and quantification of non-O157 *E. coli* serogroups in cattle feces (Anklam et al., 2012; Shridhar et al., 2016), and the utility of mqPCR assays to quantify non-O157 serogroups in cattle feces has been reported (Shridhar et al., 2016). The major limitation of the PCR assay is that it allows quantification at serogroup level only, because detection of virulence genes in the sample cannot be associated with any particular serogroup. Previous studies have evaluated the applicability of spiral plating method for quantification of *E. coli* O157 in various sample matrices (Berry and Wells, 2008; Brichta-Harhay et al., 2007; Robinson et al., 2004).

In the present study, the validity of the spiral plating method was tested with pure cultures, fecal samples spiked with pooled pure cultures, and fecal samples from naturally

shedding cattle collected from commercial feedlots. The quantification limit of the spiral plating method with pure cultures of individual serogroups of non-O157 *E. coli* was 3 log CFU/mL, whereas the detection limit was 3 to 4 log CFU/g of feces when pooled pure cultures were spiral plated. The detection limit ranged from 3 to 5 log CFU/g of feces with cattle fecal samples spiked with pooled pure cultures. The numerical increase in quantification limits with pooled pure cultures or feces spiked with pooled pure cultures is because of the difficulty in distinguishing between serogroups based on colony morphology. The six targeted serogroups on the MP medium, which was used for spiral plating, are not distinguishable based on colony color (Noll et al., 2015a); therefore, when pooled pure cultures of three serogroups were spiral plated, 10 chromogenic colonies (blue, purple, mauve, green) were randomly picked for identification of serogroup and major virulence genes by multiplex PCR. The number of colonies positive for the three serogroups ranged from zero to six; hence, the detection limit of each serogroup in pooled pure cultures ranged from 3 to 5 log CFU/g of feces. Obviously, testing a higher number of colonies (perhaps 15 or 20 per sample) is likely to increase the sensitivity of detection. However, in cattle that are naturally shedding non-O157 serogroups, a vast majority (almost 92%) were shedding one or two serogroups (Noll et al., 2015a). Also, in the present study, a majority of the quantifiable samples (98.5%) were positive for only one serogroup. Robinson et al. (Robinson et al., 2004) evaluated the spiral plating method for quantification of *E. coli* O157 in cattle feces by estimating the coefficient of variation for the concentration of *E. coli* O157:H7 in fecal samples spiked with serial dilutions of the pure cultures. They reported that the concentration estimated by the spiral plating method was most precise when the inoculum concentrations were $>10^4$ CFU/g. In our study, concentrations of non-O157 *E. coli* serogroups in spiked fecal samples determined by the spiral plating method and concentrations of inocula used to spike fecal

samples were different. Likely, the difference is due to the MP medium being less selective for the detection of the six serogroups. The growth of background flora with colony morphology (color) similar to the six non-O157 serogroups makes the modified PM not as selective as sorbitol MacConkey agar with cefixime and potassium tellurite or Chromagar generally used for quantification of *E. coli* O157.

The applicability of the spiral plating method to quantify the six serogroups of non-O157 *E. coli* was evaluated with fecal samples (n=1,152) from naturally shedding cattle from commercial feedlots. Based on spiral plating method, 10.6% of the fecal samples tested or 15.5% of the samples that were culture positive were super-shedders (≥ 4 log CFU/g) of one or more of the six non-O157 serogroups. Based on mqPCR assays, 27.8% of the fecal samples tested or 32.4% of the samples that were PCR positive were super-shedders (≥ 4 log CFU/g) for one or more of the six non-O157 serogroups. The difference is not surprising because quantitative PCR assays are more sensitive for detection or quantification than the culture method because quantitative PCR quantifies both viable and nonviable bacterial cells. Similarly, differences between PCR-based and culture-based methods have been reported for *E. coli* O157 and other bacteria (Jacob et al., 2014; Noll et al., 2015a; Sonawane and Tripathi, 2013).

The presence of super shedders of *E. coli* O157 in a pen or herd has been reported to influence the overall fecal prevalence and subsequent hide contamination and carcass transmission (Arthur et al., 2009; Fox et al., 2008; Jacob et al., 2010; Matthews et al., 2006; Stephens et al., 2009). Serotype O157:H7 is usually shed at concentrations of < 2 log CFU/g of feces (Chase-Topping et al., 2007). Omisakin et al. (Omisakin et al., 2003) reported that 9% of the cattle shedding *E. coli* O157:H7 were super-shedders (≥ 4 log CFU/g of feces). In the present study, 650 (82.6%) of 787 and 669 (67.6%) of 989 of the culture- and PCR-positive samples,

respectively, were not quantifiable, suggesting that a majority of non-O157 serogroups are also shed at concentrations below the detection limit (2 log CFU/g). Therefore, this finding supports the need for fecal samples to be enriched before being subjected to culture or PCR method for detection of non-O157 serogroups.

The majority of quantifiable samples detected by either spiral plating (135 of 137, 98.5%) or mqPCR (239 of 320, 74.7%) were shedding only one serogroup. *E. coli* O103 was the most common serogroup quantified in fecal samples by both spiral plating and mqPCR. The second most common serogroup was O26 followed by O45, O121, and O145. None of the quantifiable samples tested positive for O111 by the spiral plating method, but 4 of 12 fecal samples that were PCR positive were quantifiable by mqPCR. For the spiral plating method, testing of individual colonies by mPCR not only confirmed the serogroup, but also allowed us to identify whether they carry the genes for Shiga toxins and intimin. Of the total serogroup-confirmed colonies (n=333) tested, only one was positive for *stx*1 and *eae*, and 38 colonies were positive for *eae*. This indicates that almost all super shedders in this population of cattle were shedding *stx*-negative non-O157 *E. coli*. This is not surprising because only a small proportion (3.6%) of non-O157 serogroups isolated from cattle feces were *stx* positive (Noll et al., 2015a).

We analyzed the association between the presence of a non-O157 super shedder within a pen with the within-pen prevalence of serogroups and found a positive association with O26, O45, O121, and O145 serogroups. Previous studies have indicated that super shedders are responsible for contamination of the environment, leading to increased re-inoculation, colonization, and fecal shedding in cattle (Arthur et al., 2009). Although *E. coli* O103 was the most common serogroup detected and quantified by culture method and qPCR assays in our

study, the association between the presence of *E. coli* O103 super shedders within a pen with its pen-level prevalence was not statistically significant. The reason is not known.

In conclusion, the spiral plating method can be used to quantify non-O157 serogroups in cattle feces. This method allows identification of cattle shedding at concentrations $\geq 10^4$ CFU/g of feces, called super shedders. Another potential application of the spiral plating method would be to quantify serogroup concentrations on carcass surfaces to generate data on efficacy of intervention steps. In the present study, we identified a subset of cattle that were super-shedders of non-O157 *E. coli*, similar to *E. coli* O157. The number of super-shedders detected by the spiral plating method was lower compared with that of mqPCR assays. However, the advantage of the spiral plating method is that it can quantify STEC, as opposed to serogroup only by mqPCR assays.

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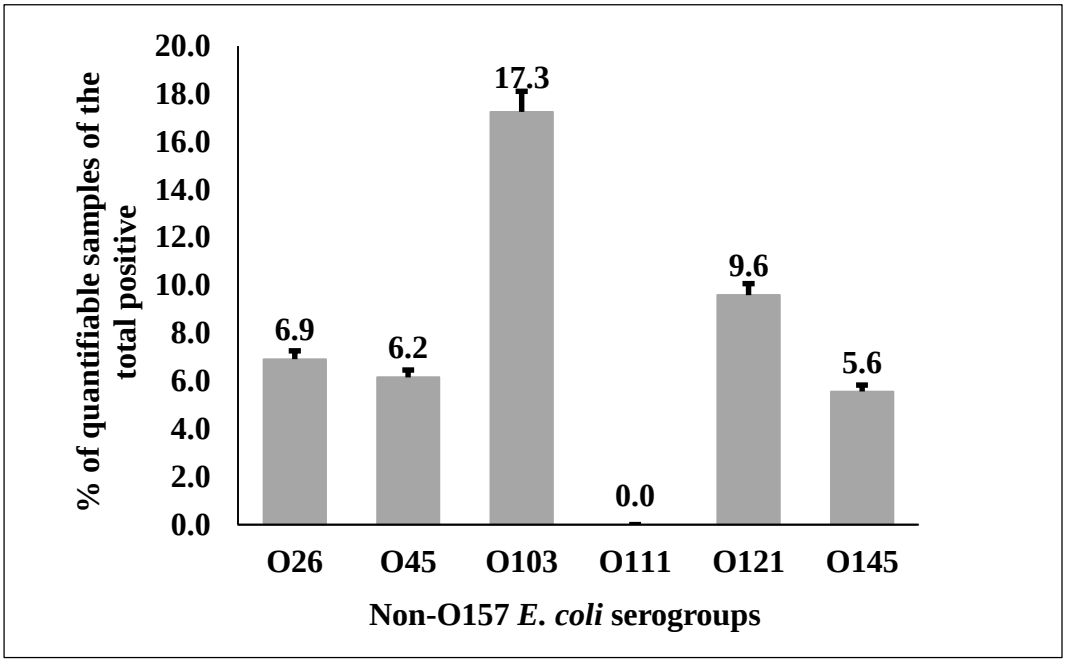
Figure 3-1. Percentage of samples positive by culture method and multiplex quantitative PCR (mqPCR) that were quantifiable by spiral plating method (A) and mqPCR (B) assays, respectively (n=1, 152)

A



B

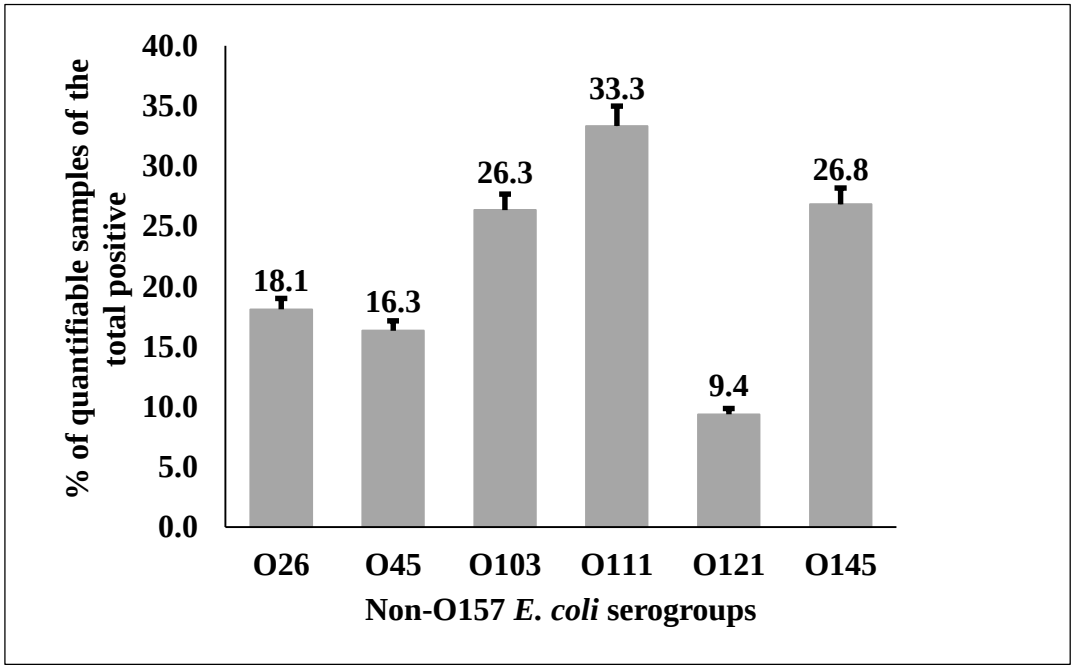


Table 3.1. Source and virulence gene profiles of strains used for validation of the spiral plating method

Serogroup	Strain	Source	Virulence gene[a]			
			*stx*1	*stx*2	*eae*	*ehxA*
O26	2013-3-6D	Bovine feces	+	-	+	+
O45	CDC-96-3285	Human	+	-	+	+
O103	2013-3-174C	Bovine feces	+	-	+	+
O111	2013-3-390E	Bovine feces	+	-	+	+
O121	KDHE 48	Human	-	+	+	-
O145	2013-3-86C	Bovine feces	+	-	+	+

[a] +, present; -, absent

Table 3.2. Quantification of pure cultures of non-O157 *E. coli* serogroups by the spiral plating method

Serogroups	Inoculum concentration (log CFU/ml)[a]							
	8.2	7.2	6.2	5.2	4.2	3.2	2.2	1.2
	Concentrations of non-O157 *E. coli* serogroups determined by spiral plating method, log CFU/ml							
O26	GPLC[b]	GPLC	6.5	5.7	4.7	3.3	0	0
O103	GPLC	GPLC	6.7	5.5	4.7	3.5	0	0
O111	GPLC	GPLC	6.7	5.6	4.7	3.0	0	0
O45	GPLC	GPLC	6.5	5.6	4.8	3.0	0	0
O121	GPLC	GPLC	6.6	5.6	4.7	3.3	0	0
O145	GPLC	GPLC	6.7	5.6	4.7	3.0	0	0

[a]Determined by the spread-plating method.

[b] GPLC: Greater than 500,000 estimated spiral plate count per ml.

Table 3.3. Quantification of non-O157 *E. coli* serogroups in pooled pure cultures by the spiral plating method

Serogroup	Inoculum concentrations of pooled pure cultures of O26, O103 and O111 serogroups (log CFU/ml)[a]							
	8.1	7.1	6.1	5.1	4.1	3.1	2.1	1.1
	Concentrations of non-O157 *E. coli* serogroups determined by spiral plating method (log CFU/ml)							
O26	GPLC[b]	GPLC	6.4	4.8	4.5	3.0	0	0
O103	GPLC	GPLC	6.1	5.1	4.4	3.5	0	0
O111[c]	GPLC	GPLC	5.7	5.1	3.8	0	0	0
Serogroup	Inoculum concentrations of pooled pure cultures of O45, O121 and O145 serogroups (log CFU/ml)[a]							
	8.2	7.2	6.2	5.2	4.2	3.2	2.2	1.2
	Concentrations of non-O157 *E. coli* serogroups determined by spiral plating method (log CFU/ml)							
O45[c]	GPLC	GPLC	6.1	5.1	4.0	0	0	0
O121	GPLC	GPLC	6.1	5.0	4.3	3.0	0	0
O145	GPLC	GPLC	6.2	5.4	4.3	3.3	0	0

[a] Determined by the spread-plating method.

[b] GPLC=Greater than 500,000 estimated spiral plate count per ml.

[c]Significant ($P < 0.05$) difference between the mean concentration of non-O157 *E. coli* serogroups determined by the spiral plating method and the inoculum concentrations used for spiral plating.

Table 3.4. Quantification of non-O157 *E. coli* serogroups in cattle fecal samples spiked with pooled pure cultures by the spiral plating method

Serogroups	Inoculum concentrations of pooled pure cultures of O26, O103 and O111 serogroups (log CFU/ml)[a]							
	8.1	7.1	6.1	5.1	4.1	3.1	2.1	1.1
	Concentrations of non-O157 *E. coli* serogroups determined by spiral plating method (log CFU/g)							
O26	GPLC[b]	GPLC	6.3	5.9	5.8	5.4	0	0
O103[c]	GPLC	GPLC	5.7	6.2	5.9	0	0	0
O111[c]	GPLC	GPLC	6.1	5.3	0	0	0	0
Serogroups	Inoculum concentrations of pooled pure cultures of O45, O121 and O145 serogroups (log CFU/ml)[a]							
	8.3	7.3	6.3	5.3	4.3	3.3	2.3	1.3
	Concentrations of non-O157 *E. coli* serogroups determined by spiral plating method, log CFU/g							
O45[c]	GPLC	GPLC	5.9	6.0	5.3	5.2	0	0
O121[c]	GPLC	GPLC	6.3	6.1	0	0	0	0
O145[c]	GPLC	GPLC	6.0	5.9	5.8	0	0	0

[a] Determined by spread-plating method.

[b] GPLC=Greater than 500,000 estimated spiral plate count per ml.

[c] Significant ($P < 0.05$) difference between the mean concentrations of non-O157 *E. coli* serogroups in spiked fecal samples determined by the spiral plating method and the inoculum concentrations used for spiral plating.

Table 3.5. Comparison of culture and multiplex quantitative PCR (mqPCR) methods for detection and quantification of non-O157 *E. coli* serogroups in cattle feces (n=1,152)

Serogroups	Culture method						mqPCR method				
	Total no.(%) of positive samples[a]	No. (%) of quantifiable samples[b]	No. of quantifiable samples (log CFU/g)[b]				Total no. (%) of positive samples[c]	No. (%) of quantifiable samples[d]	No. of quantifiable samples (log CFU/g)[d]		
			3- < 4	4 - < 5	5 - < 6	6 - < 7			4 - < 5	5 - < 6	6 - < 7
O26[e]	275 (23.9)	19 (1.6)	1	10	8	0	442 (38.4)	80 (6.9)	52	25	3
O45	195 (16.9)	12 (1.0)	1	9	2	0	398 (34.5)	65 (5.6)	49	15	1
O103[e]	574 (49.8)	99 (8.6)	13	34	49	2	797 (69.2)	210 (18.2)	71	128	11
O111	9 (0.8)	0	0	0	0	0	12 (1.0)	4 (0.3)	3	1	0
O121	73 (6.3)	7 (0.6)	0	4	2	1	405 (35.2)	38 (3.3)	27	11	0
O145	36 (3.1)	2 (0.2)	0	1	1	0	41 (3.6)	11 (1.0)	9	1	1
Total[f]	787 (68.3)	137 (11.9)					989 (85.9)	320 (27.8)			

[a]Detected by immunomagnetic separation-based culture method in post-enriched fecal samples.

[b]Quantified by the spiral plating method in pre-enriched fecal samples

[c]Detected by mqPCR assays in post-enriched fecal samples

[d]Quantified by mqPCR assays in pre-enriched fecal samples

[e]One sample was positive for two different genotypes by spiral plating method

[f]Positive for one or more of the six serogroups

Table 3.6. Quantification of non-O157 *E. coli* serogroups and associated virulence genes in naturally-shedding cattle fecal samples (n=1,152) by the spiral plating method

Serogroups	Total no. (%) of quantifiable samples[a]	Virulence genes (*stx*1, *eae*)	No. of samples positive in each concentration (log CFU/g)				
			3- < 4	4 - < 5	5 - < 6	6 - < 7	GPLC[b]
O26[c]	19 (1.6)	None	0	4	2	0	0
		eae	1	6	6	0	1
		eae + *stx*$_1$	0	0	0	0	0
O45	12 (1.0)	None	1	7	2	0	0
		eae	0	2	0	0	0
		eae + *stx*$_1$	0	0	0	0	0
O103[c]	99 (8.6)	None	9	23	42	2	1
		eae	4	11	7	0	0
		eae + *stx*$_1$	0	0	1	0	0
O111	0	None	0	0	0	0	0
		eae	0	0	0	0	0
		eae + *stx*$_1$	0	0	0	0	0
O121	7 (0.6)	None	0	4	2	1	0
		eae	0	0	0	0	0
		eae + *stx*$_1$	0	0	0	0	0
O145	2 (0.2)	None	0	1	1	0	0
		eae	0	0	0	0	0
		eae + *stx*$_1$	0	0	0	0	0

[a] Total number of quantifiable samples by the spiral plating method in pre-enriched fecal samples

[b] GPLC=Greater than 500,000 estimated spiral plate count per ml

[c] One sample was positive for two different genotypes

Table 3.7. Agreement between the spiral plating method and multiplex quantitative PCR (mqPCR) assays for pre-enriched fecal samples for quantification, and between the culture method and mqPCR assays for post-enriched fecal samples for detection of non-O157 *E. coli* serogroups

Serogroups	Kappa statistic (95% CI)[a]	
	mqPCR vs. spiral plating (n=1,152)	mqPCR vs. culture (n=1,152)
O26	0.33 (0.21 - 0.44)	0.60 (0.55-0.65)
O45	0.25 (0.12 - 0.37)	0.49 (0.44-0.54)
O103	0.25 (0.18 - 0.32)	0.54 (0.50-0.59)
O111	0	0.86 (0.70-1.00)
O121	0.08 (-0.03 - 0.19)	0.20 (0.15-0.24)
O145	-0.003 (-0.007 - 0.001)	0.83 (0.73-0.92)

Interpretation of the Kappa statistic was based on the scale proposed by (Landis and Koch, 19

Chapter 4 - Shiga Toxin Subtypes of Non-O157 *Escherichia coli* Serogroups Isolated from Cattle Feces

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Abstract

Shiga toxin producing *Escherichia coli* (STEC) are important foodborne pathogens responsible for human illnesses. Cattle are a major reservoir that harbor the organism in the hindgut and shed in the feces. Shiga toxins (Stx) are the primary virulence factors associated with STEC illnesses. The two antigenically distinct Stx types, Stx1 and Stx2, encoded by *stx*1 and *stx*2 genes, share approximately 56% amino acid sequence identity. Genetic variants exist within Stx1 and Stx2 based on differences in amino acid composition and in cytotoxicity. The objective of our study was to identify the *stx* subtypes in strains of STEC serogroups, other than O157, isolated from cattle feces. Shiga toxin gene carrying *E. coli* strains (n = 192), spanning 27 serogroups originating from cattle (n = 170) and human (n = 22) sources, were utilized in the study. Shiga toxin genes were amplified by PCR, sequenced, and nucleotide sequences were translated into amino acid sequences using CLC main workbench software. Shiga toxin subtypes were identified based on the amino acid motifs that define each subtype. Shiga toxin genotypes were also identified at the nucleotide level by *in silico* restriction fragment length polymorphism (RFLP). Of the total 192 STEC strains, 93 (48.4%) were positive for *stx*1 only, 43 (22.4%) for *stx*2 only, and 56 (29.2%) for both *stx*1 and *stx*2. Among the 149 strains positive for *stx*1, 132 (88.6%) were *stx*1a and 17 (11.4%) were *stx*1c. Shiga toxin 1a was the most

common subtype of *stx*1 among cattle (87.9%; 123/140) and human strains (100%; 9/9) of non-O157 serogroups. Of the total 99 strains positive for *stx*2, 79 were stx2a (79.8%), 11 (11.1%) were *stx*2c, 12 (12.1%) were *stx*2d. Of the 170 strains originating from cattle feces, 58 (34.1%) were stx2a subtype, 11 (6.5%) were *stx*2c subtype, and 11 were of subtype *stx*2d (6.5%). All but one of the human strains were positive for *stx*2a. Three strains of cattle origin were positive for both *stx*2a and *stx*2d. In conclusion, a number of non-O157 STEC serogroups harbored by cattle possess a wide variety of Shiga toxin subtypes, with *stx*1a and *stx*2a being the most predominant *stx* subtypes occurring individually or in combination. Cattle are a reservoir of a number of non-O157 STEC serogroups and information on the Shiga toxin subtypes is useful in assessing the potential risk as human pathogens.

Introduction

Shiga toxin producing *Escherichia coli* (STEC) are major foodborne pathogens responsible for human illnesses, characterized by non-bloody to bloody diarrhea, sometimes leading to complications of hemolytic uremic syndrome (HUS), particularly in children (Gyles, 2007). *Escherichia coli* O157:H7 is the major serotype responsible for many of the STEC illness outbreaks in humans. However, there is increasing incidence of outbreaks associated with non-O157 STEC in recent years, particularly O26, O45, O103, O111, O121, and O145, referred to as top six non-O157 STEC. According to FoodNet sites, incidence of top six non-O157 STEC infections increased from 0.12 per 100,000 population in 2,000 to 0.95 per 100,000 population in 2010 (Gould et al., 2013). Non-O157 STEC associated illnesses range from cases of sporadic to major outbreaks, and clinically, from mild watery diarrhea to life threatening complications of

HUS, similar to STEC O157 infections (Johnson et al., 2006). Cattle are a major reservoir of O157 and non-O157 STEC, which harbor the organisms in the hindgut and shed in the feces. Consumption of water, beef and fresh produce contaminated with cattle feces leads to human illnesses. In addition to O157 and the six top non-O157, cattle do harbor and shed in the feces a number of other serogroups of STEC (Bettelheim, 2007b; Hussein, 2007).

Shiga toxins (Stx) are the major virulence factors of STEC. Shiga toxins (Stx) belong to the AB5 family of protein toxins, with an enzymatically active A moiety and a B moiety involved in binding to the host cell receptor. The A subunit is responsible for the cleavage of N-glycosidic bond in the 28 s rRNA of 60 s ribosomal subunit, which leads to cytotoxicity (Endo et al., 1988; Fraser et al., 2004). The two antigenically distinct Stx types, Stx1 and Stx2, encoded by *stx*1 and *stx*2 genes, share approximately 56% amino acid sequence similarity (Strockbine et al., 1986; Weinstein et al., 1988). Although Stx1 and Stx2 are structurally similar, they differ in cellular distribution and cytotoxicity. Shiga toxin 1 is located in the periplasmic space of the bacterial cell, whereas Stx2 is in the extracellular fraction (Shimizu et al., 2009). Basu et al. (2015) have shown that A1 subunit of Stx2 has a higher affinity for ribosomes and a higher catalytic activity compared to A1 subunit of Stx1 (Basu et al., 2015), which makes Stx2 more cytotoxic than Stx1. Shiga toxin 2 is reported to be 400 times more toxic than Stx1 in a murine infection model (Tesh et al., 1993). Shiga toxin 2 is more commonly associated with complications of HUS than Stx1 (Brooks et al., 2005a; Ethelberg et al., 2004a). Variants exist within *stx*1 (*stx*1a, *stx*1c, and *stx*1d) and *stx*2 (*stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f, and *stx*2g) families based on differences in amino acid compositions and in the degree of cytotoxicity. The outcome of human illness associated with STEC strains has been shown to be influenced by Stx subtypes (Friedrich et al., 2002). Shiga toxin 2a and Stx2c are the major subtypes produced by *E.*

coli O157:H7 strains associated with HUS in humans (Persson et al., 2007). Therefore, identifying the subtypes of Stx is important to assess the potential risk for human illnesses associated with STEC infections. Subtyping method based on restriction fragment length polymorphism of PCR products (PCR-RFLP) has been developed to identify *stx*1 (*stx*1a, *stx*1c, and *stx*1d) and *stx*2 (*stx*2, *stx*2v-ha, *stx*2v-hb, *stx*2g, *stx*2-NV206, and *stx*2-EC1586) genotypes at the nucleotide level based on their unique restriction patterns (Beutin et al., 2007; Gobius et al., 2003). However, the demerits of this method are the lack of consistency in the nomenclature, and misinterpretation of *stx* subtypes due to single nucleotide changes (Scheutz et al., 2012). Scheutz et al. (2012) standardized the Stx nomenclature by designating stx1 subtypes as *stx*1a, *stx*1c and *stx*1d, and *stx*2 subtypes as *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f, and *stx*2g based on amino acid sequence similarity (Scheutz et al., 2012). There is a paucity of data on Shiga toxin subtypes carried by non-O157 *E. coli* serogroups isolated from cattle feces in the United States. The objective of our study was to determine the subtypes of *stx*1 and *stx*2 in non-O157 *E. coli* serogroups isolated from cattle feces.

Materials and Methods

Strains

Shiga toxin gene-positive *E. coli* strains (n=192) spanning 27 non-O157 *E. coli* serogroups isolated from cattle feces (n=170), and human clinical cases (n=22), available in our culture collection, were used in the study. A majority of strains belonged to the top six non-O157 *E. coli* serogroups: O26 (n=16), O45 (n=4), O103 (n=54), O111 (n=21), O121 (n=4), and O145 (n = 27). The other non-O157 *E. coli* serogroups included O6 (n=2), O8 (n=3), O15 (n=1), O22 (n=1), O38 (n=2), O39 (n=3), O74 (n=3), O88 (n=3), O91 (n=2), O96 (n=3), O104 (n=18), O113

(n=3), O116 (n=3), O117 (n=3), O130 (n=4), O141 (n=3), O146 (n=1), O153 (n=1), O163 (n=2), O171 (n=3), and O172 (n=2). Cattle strains were isolated from feces, primarily from commercial feedlots (Cull et al., 2017; Noll et al., 2015a; Renter et al., 2005; Shridhar et al., 2016b). A few human clinical strains, obtained from Michigan State University (MSU) and the Kansas Department of Health and Environment (KDHE), were also included in the study. The strains, stored at −80° C on cryo beads (CryoCareTM, Key Scientific Products, Round Rock, TX), were streaked onto blood agar plates (Remel, Lenexa, KS), colonies were suspended in 50 µl distilled water, boiled for 10 min, centrifuged at 9,300 × g for 5 min, and supernatant was used for PCR amplification and subsequent sequencing of the amplicons.

Primers Design

Nucleotide sequences of *stx*1 and *stx*2 of *E. coli* O157 and non-O157 *E. coli* were downloaded from NCBI Genbank. The sequences were aligned using CLC Main Workbench (CLC Bio, Cambridge, MA), and the conserved regions flanking the Shiga toxin genes were selected to design the oligonucleotide primers for amplification of *stx*1 and *stx*2 genes (Table 4.1). Sequences of *stx*2f subtype appeared to be more divergent from the other subtypes, therefore, a separate set of primers were designed for amplification (Table 4.1). Primers were obtained from Integrated DNA technologies (Coralville, Iowa).

PCR Assay Conditions

The *stx* genes were amplified by touchdown PCR method, where the annealing temperature of each cycle was lowered gradually to avoid amplification of non-specific sequences (Don et al., 1991). PCR was performed using Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

PCR amplification protocol for *stx*1 included an initial denaturation at 94° C for 5 min, 10 cycles of touch-down PCR (denature: 94° C for 30 s, annealing: 56–51° C (1-0.5° C) for 30 s; and extension: 72° C for 1 min 45 s) followed by 30 cycles of regular PCR (denature: 94° C for 30 s, annealing: 51° C for 30 s; and extension: 72° C for 1 min 45 s). PCR amplification protocol for *stx*2 included an initial denaturation at 94° C for 5 min, 10 cycles of touch-down PCR (denature: 94° C for 30 s, annealing: 47–44° C (Δ-0.3◦C) for 30 s; and extension: 72° C for 1 min 45 s) followed by 30 cycles of regular PCR (denature: 94◦C for 30 s, annealing: 44° C for 30 s; and extension: 72° C for 1 min 45 s). All PCR reagents were obtained from TaKaRa Bio USA, Inc. (CA).

Sequencing and Data Analyses

Amplified PCR products were visualized using a Qiaxcel capillary electrophoresis system (Qiagen, Valencia, CA) and purified using a QIAquick PCR purification kit (Qiagen). The purified PCR products were measured by a spectrophotometer (NanoDrop-Thermo Scientific, Wilmington, DE) to assess the DNA concentration and purity. PCR products and primers were shipped to Genewiz, Inc., (South Plainfield, NJ) for nucleotide sequencing. The chromatogram data were visualized using the CLC Main Workbench software for further analysis. All sequences were individually analyzed for conflicts, and secondary peaks, and consensus sequences were produced. Nucleotide sequences were translated to amino acid sequences after removing intergenic sequences. Shiga toxin subtypes were determined based on the amino acid motifs that define each *stx* subtype (Scheutz et al., 2012).

In silico Restriction Fragment Length Polymorphism (RFLP)

A subset of strains (n = 68) were subjected to *in silico* RFLP for identification of *stx*1 (*stx*1, *stx*1c, and *stx*1d) and *stx*2 (*stx*2, *stx*2vha, *stx*2v-hb, *stx*2-NV206, *stx*2g, and *stx*2-EC1586)

genotypes. For *stx*1, a 391-392 bp fragment starting with GAYTATCAT GGACAAGACTCYGTT and ending with TGACGATACYTTTACAGTTAAAGTGG was digested with *Rsa*I, *Fok*I, and *Nci*I enzymes using CLC Main Workbench software to identify the unique restriction sites for each *stx*1 genotype. For *stx*2, a 270 bp fragment starting with ATGAAGAAGATGTTTATG and ending with CAGTTTAATAATGACTGA was digested with *Hae*III, *Rsa*I, *Fok*I, and *Nci*I enzymes (Beutin et al., 2007) using CLC Main Workbench software. Restriction patterns of *stx*1 and *stx*2 sequences of the non-O157 STEC strains were compared to that of the reference sequences from the NCBI database to identify the *stx*1 and *stx*2 genotypes.

Cloning and Sequencing of PCR Products

Three strains belonging to serogroups O96, O113, and O130 that revealed double peaks in the chromatograms of *stx*2 sequences were subjected to cloning. PCR products were cloned using TOPO®TA Cloning kits and protocols by Invitrogen-Life Technologies (Grand Island, NY). Up to 12 transformants were selected and grown in Luria Bertani broth (LB; Becton, Dickinson Co., Sparks, MD) containing carbenicillin for 2 h. Clones obtained from the LB broth were subjected to sequencing using flanking M13 forward and reverse primers. The sequences were analyzed as mentioned above to identify the Shiga toxin subtypes.

Results

Of the total 192 non-O157 STEC strains (170 cattle and 22 human strains) belonging to 27 serogroups tested in the study, 93 (48.4%) were positive for *stx*1 only, 43 (22.4%) for *stx*2 only, and 56 (29.2%) for both *stx*1 and *stx*2. A total of 149 strains belonging to 23 serogroups were positive for *stx*1, and of those 132 (88.6%) were *stx*1a and 17 (11.4%) were *stx*1c (Table

4.2). Of the 140 *stx*1 positive strains originating from cattle feces, 123 (87.9%) were *stx*1a subtype, and 17 (12.1%) were *stx*1c. Shiga toxin 1a was the only *stx*1 subtype found in human STEC strains (9/9; 100%). The *stx*1a was also the most common subtype of *stx*1 identified in top six non-O157 *E. coli* serogroups (Table 4.2). None of the strains were positive for more than one *stx*1 subtype.

A total of 99 strains belonging to 25 serogroups were positive for *stx*2, and of those 79 were *stx*2a (79.8%), 11 (11.1%) were *stx*2c, 12 (12.1%) were *stx*2d. Three *stx*2 positive-strains belonging to O96, O113 and O130 serogroups of cattle origin revealed double peaks in the chromatogram of *stx*2 sequences, suggesting the presence of more than one *stx*2 subtype in the same strain. Subsequent cloning and sequencing revealed that all three strains carried a combination of *stx*2a and *stx*2d. All human clinical strains (n = 22), except one, were of subtype *stx*2a. Only one strain belonging to serogroup O145 carried *stx*2d. All strains positive for *stx*2c were of bovine origin (Table 4.2, Figure 4.1). Shiga toxin subtypes 2e, 2f, and 2g were not detected in any of the strains tested. A majority of cattle strains (n = 170) possessed subtype *stx*1a (123/170; 72.4%) followed by *stx*2a (58/170; 34.1%; Figure 4.1). However, a majority of human strains carried *stx*2a (21/22; 95.5%) followed by *stx*1a (9/22; 40.9%). Shiga toxin 1a was most commonly found in combination with *stx*2a in strains (n = 46) belonging to 18 serogroups. Six strains belonging to O8, O103, O117, and O130 carried a combination of *stx*1a and *stx*2d. Two strains of serogroup O74 carried a combination of *stx*1a and *stx*2c and a strain of serogroup O15 was positive for a combination of *stx*1c and *stx*2c. A strain of serogroup O130 was positive for three subtypes, *stx*1a, *stx*2a, and *stx*2d (Table 4.2).

The sequences from a subset of strains (n = 68) were also analyzed by *in silico* RFLP. The sizes of fragments generated by restriction enzyme digestion are shown in Table 4.3. The

two *stx*1 genotypes (*stx*1 and *stx*1c) determined by *in silico* RFLP corresponded to the two subtypes (*stx*1a and *stx*1c) determined based on the amino acid motifs. For *stx*2, genotypes, such as *stx*2, *stx*2-NV206, and *stx*2v-ha determined based on the restriction patterns corresponded to *stx*2a, *stx*2d, and *stx*2c subtypes, respectively, determined based on amino acid sequence. However, some strains positive for *stx*2v-hb gene (based on restriction patterns) corresponded to *stx*2c and some to *stx*2d subtype (based on amino acid sequence motifs). Restriction patterns of *stx*1 and *stx*2 genotypes determined by *in silico* RFLP are shown in Figure 4.2.

Discussion

Shiga toxins are the major virulence factors of STEC, which are responsible for foodborne illnesses, including life threatening complications of HUS in humans. Shiga toxins exist as several subtypes, which vary in their cytotoxicity, and therefore in the extent of their involvement in human illness (Friedrich et al., 2002; Fuller et al., 2011; Persson et al., 2007). Fuller et al. (2011) demonstrated that *stx*2a and *stx*2d subtypes were more potent than *stx*2b, *stx*2c, and *stx*1 based on in vitro (using primary human renal proximal tubule epithelial cells and Vero cells) and in vivo (using mice) experiments (Fuller et al., 2011). Karve and Weiss (2014) demonstrated stronger binding of *stx*2a, *stx*2c, and *stx*2d to a mixture of Gb3 and glycolipids when compared to *stx*1 and *stx*2b (Karve and Weiss, 2014). Determining the Shiga toxin subtype carried by the STEC strains is important to estimate the risk of human illness associated with specific serotype or source of transmission. Studies have shown that STEC strains carrying *stx*2a and *stx*2c are most commonly associated with HUS in humans (Friedrich et al., 2002; Iyoda et al., 2014; Persson et al., 2007). Production of elastase activatable Stx2d subtype in STEC strains has been reported to be a predictor of severity of clinical illness (Bielaszewska et al., 2006). The

cleavage of C-terminal amino acids of A2 peptide of Stx2d by elastase has been reported to increase the cytotoxicity of this Shiga toxin subtype (Kokai-Kun et al., 2000; Melton-Celsa et al., 2002). The Stx2e subtype is most commonly associated with porcine STEC, however, it is also associated with STEC from asymptomatic humans and fresh produce (Beutin et al., 2008; Feng and Reddy, 2013; Friedrich et al., 2002). Although pigeons are a primary reservoir of *stx*2f carrying *E. coli* strains, this subtype has also been isolated from human diarrheic patients (Prager et al., 2009; Schmidt et al., 2000). There are studies reporting the distribution of Shiga toxin subtypes in STEC strains isolated from humans and fresh produce (Feng and Reddy, 2013; Friedrich et al., 2002). There are very limited studies on the distribution of Shiga toxin subtypes in cattle, particularly of non-O157 serogroups, in the United States.

In this study, we identified Shiga toxin subtypes associated with 27 serogroups of non-O157 STEC strains of cattle (n=170) and human (n=22) origin based on the amino acid sequences deduced from nucleotide sequences. Additionally, we also identified *stx* genotypes by *in silico* RFLP, based on the restriction patterns obtained after *in silico* digestion of nucleotide sequences with specific restriction enzymes. The most common subtype of *stx*1 carried by non-O157 STEC strains of cattle and human origin was *stx*1a. A similar finding was also reported in a study on cattle and human STEC strains in Canada (Chui et al., 2015a). Tostes et al. (2017) reported that *stx*1a and *stx*2a are the most common subtypes in both *E. coli* O157 and non-O157 *E. coli* strains of human and bovine origin in Alberta (Tostes et al., 2017). In the present study, a majority of the cattle STEC strains (21.8%; 37/170) and human STEC strains (40.9%; 9/22) carried a combination of *stx*1a and *stx*2a. A majority of *E. coli* O157:H7 strains isolated from outbreaks and sporadic cases in Canada carried a combination of *stx*1a and *stx*2a (Chui et al., 2015a). In our study, 17 (10%) cattle STEC strains carried *stx*1c, while none of the human STEC

strains carried this subtype. STEC strains carrying *stx*1c have been isolated from asymptomatic humans and patients with uncomplicated diarrhea (Friedrich et al., 2002). However, *stx*1c carrying *E. coli* O78 was isolated from a 2-week old boy suffering from bacteremia and HUS in Finland (Lienemann et al., 2012).

Shiga toxin subtype 2a was the most common *stx*2 subtype (41.1%; 79/192) found in cattle and human sources. It was the most common subtype detected in STEC strains isolated from patients suffering from HUS (Persson et al., 2007) and those isolated from fresh produce (Feng and Reddy, 2013). It was also the most common subtype of *stx*2 in STEC strains isolated from cattle in Australia (Brett et al., 2003) and France (Bertin et al., 2001). The second most frequent subtype of *stx*2 associated with STEC strains isolated from cattle was *stx*2c, however, none of the human STEC strains included in this study contained *stx*2c. The *stx*2c subtype has been reported in STEC strains isolated from human patients suffering from HUS (Persson et al., 2007). It was the third most common subtype (next to *stx*2d) associated with STEC strains isolated from fresh produce (Feng and Reddy, 2013). Tostes et al. (2017) have reported the distribution of *stx*1 and *stx*2 subtypes associated with *E. coli* O157 and non-O157 *E. coli* serogroups of bovine and human origin in Alberta, and found that the *stx*2c subtype was found only in *E. coli* O157 strains (Tostes et al., 2017). Shiga toxin 2c was the most common subtype associated with STEC O157 strains of cattle origin in Italy, however, none of the STEC O157 strains harbored *stx*2a (Bonardi et al., 2015). However, in our study, 6.5% of the non-O157 STEC strains isolated from cattle carried *stx*2c subtype. In the present study, only a small proportion (6.5%) of the bovine strains carried *stx*2d subtype, and only one human STEC strain carried *stx*2d. Tasara et al. (2008) have reported *stx*2d carrying STEC strains isolated from cattle and sheep (Tasara et al., 2008). Presence of *stx*2d carrying STEC strains in human patients was

reported to be significantly associated with HUS (Bielaszewska et al., 2006). In our study, three cattle strains carried two different *stx*2 subtypes (*stx*2a and *stx*2d). STEC strains carrying more than one *stx*2 subtypes have been reported in previous studies (Persson et al., 2007; Scheutz et al., 2012). The significance of multiple *stx*2 subtypes with regard to virulence of STEC has not been studied yet. The presence of multiple *stx* subtypes in STEC is assumed to be the result of recombination of *stx* phages (Ashton, 2015).

We also identified Shiga toxin genotypes by *in silico* RFLP. Some strains positive for *stx*2v-hb gene (based on restriction patterns; (Beutin et al., 2007)) corresponded to *stx*2c and some to *stx*2d subtype (based on amino acid sequence motifs). Single nucleotide changes within the restriction sites could lead to misinterpretation of *stx* subtypes based on the restriction pattern (Scheutz et al., 2012). In conclusion, a number of non-O157 STEC serogroups isolated from cattle possessed a wide variety of Shiga toxin subtypes, with *stx*1a and *stx*2a being the most predominant *stx* subtypes occurring individually or in combination. Cattle harbor a number of non-O157 STEC serogroups and identification of the Shiga toxin subtypes is useful in assessing the potential risk to cause human illnesses.

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Figure 4-1. Percentage of *stx*1 and *stx*2 subtypes in non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from cattle (n=170)

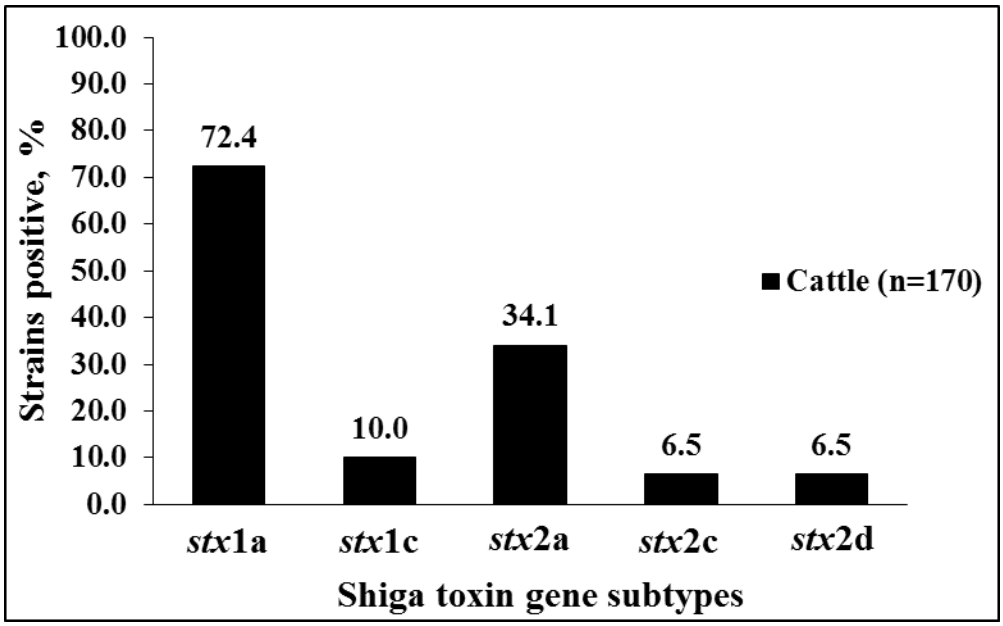

Figure 4-2. *In silico* restriction fragment length polymorphism (RFLP) of *stx*1 and *stx*2 genes of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains. (A) RFLP pattern of *stx*1a of a non-O157 STEC strain (O103 serogroup) isolated from cattle feces; (B) RFLP pattern of *stx*1c of a non-O157 STEC strain (O15 serogroup) isolated from cattle feces; (C) RFLP pattern of *stx*2 of a non-O157 STEC strain (O145 serogroup) isolated from cattle feces.

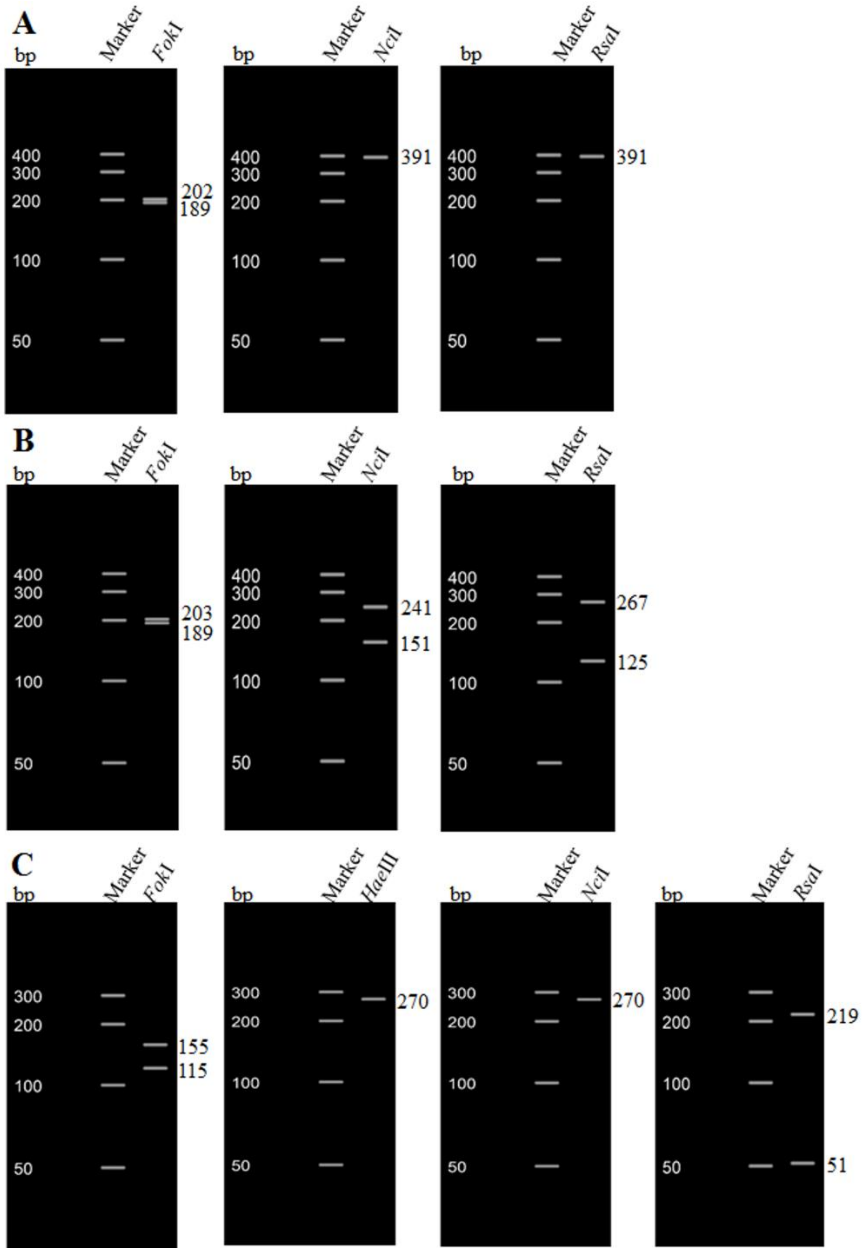

Table 4.1. Target genes, primers used and size of amplicons

Target gene	Primers	Sequence	Amplicon size (bp)	Reference
*stx*1	Forward	GCTCAAGGAGTATTGTGTAATATG	1,233	This study
	Reverse	TCGCTGAATCCCCYTC		
*stx*2	Forward	CGAATCCAGTACAACGC	1,390	This study
	Reverse	CCCACATACCACGAATC		
*stx*2f	Forward	CGTCATTCACTGGTTGG	766	This study
	Reverse	GCTGAGCACTTTGTAACA		

Table 4.2. Shiga toxin subtype distribution in non-O157 Shiga toxin-producing *E. coli* (STEC) serogroups of cattle and human origin (n = 192)

Serogroup	Cattle (n=170)					Human (n=22)				
	***stx*1a**	***stx*1c**	***stx*2a**	***stx*2c**	***stx*2d**	***stx*1a**	***stx*1c**	***stx*2a**	***stx*2c**	***stx*2d**
Top non-O157 STEC (n=126)										
O26 (n=16)	9		3			2		4 (2[a])		
O45 (n=4)	4									
O103 (n=54)	54				1 (1[b])					
O111 (n=21)	14		13 (13[a])			6		7 (6[a])		
O121 (n=4)								4		
O145 (n=27)	11		8 (1[a])	4	1	1		3 (1[a])		1
Total	92	0	24	4	2	9	0	18	0	1
Other non-O157 STEC (n=66)										
O6 (n=2)	1		1 (1[a])	1						
O8 (n=3)	3		1 (1[a])		2 (2[b])					
O15 (n=1)		1		1 (1[c])						
O22 (n=1)	1		1 (1[a])							
O38 (n=2)	1		2 (1[a])							
O39 (n=3)			3							
O74 (n=3)	3		1 (1[a])	2 (2[d])						
O88 (n=3)	3		3 (3[a])							

O91 (n=2)	1		2 (1[a])							
O96 (n=3)	2		3 (2[a])		1 (1[e])					
O104 (n=18)		16						2		
O113 (n=3)			2 (1[e])		2					
O116 (n=3)	3		3 (3[a])							
O117 (n=3)	2			1	2 (2[b])					
O130 (n=4)	3		2 (1[a])		2 (1[b],1[f])			1		
O141 (n=3)	3		3 (3[a])							
O146 (n=1)	1		1 (1[a])							
O153 (n=1)	1		1 (1[a])							
O163 (n=2)	2		2 (2[a])							
O171 (n=3)	1		1 (1[a])	2						
O172 (n=2)			2							
Total	31	17	34	7	9	0	0	3	0	0

[a]Contains strains positive for both *stx*1a and *stx*2a.

[b]Contains strains positive for both *stx*1a and *stx*2d.

[c]Contains strains positive for both *stx*1c and *stx*2c.

[d]Contains strains positive for both *stx*1a and *stx*2c.

[e]Contains strains positive for both *stx*2a and *stx*2d.

[f]Contains strains positive for *stx*1a, *stx*2a and *stx*2d.

Numbers in the parenthesis indicate the presence of multiple *stx* subtype

Table 4.3. Restriction fragments of *stx*1 and *stx*2 genotypes based on *in silico* RFLP

Shiga toxin subtypes	Restriction enzymes			
	***Hae*III**	***Rsa*I**	***Fok*I**	***Nci*I**
	Size(s) of restriction fragments (bp)			
*stx*1	-	391	202, 189	391
*stx*1c	-	267, 125	203, 189	241, 151
*stx*2	270	219, 51	155, 115	270
*stx*2v-ha	143, 127	139, 80, 51	270	270
*stx*2v-hb	143, 127	219, 51	270	141, 129
*stx*2-NV206	270	219, 51	270	141, 129

Chapter 5 - Genotypic characterization of top six non-O157 *Escherichia coli* serogroups isolated from feces of feedlot cattle using DNA Microarray

Abstract

Shiga toxin-producing *E. coli* (STEC) are major foodborne pathogens responsible for human illnesses. Cattle are a major reservoir and shed these organisms in the feces. Although *E. coli* O157:H7 is the major serotype responsible for most of the outbreaks, non-O157 STEC, particularly O26, O45, O103, O111, O121, and O145, are gaining more attention in recent years as they are responsible for more than 70% of human non-O157 STEC infections in North America. Shiga toxins are the major virulence factors responsible for the human illness, including life-threatening complications, such as hemolytic uremic syndrome. In addition, other virulence factors such as adhesins, toxins, receptor proteins, siderophores, and secretion systems also contribute to the pathogenesis. Identification of the genes that code for a variety of virulence factors is important to assess the virulence potential of non-O157 *E. coli*. The objective of this study was to determine the virulence potential of top six non-O157 *E. coli* serogroups isolated from cattle feces using DNA microarray. We subjected 48 non-O157 *E. coli* strains belonging to O26 (n=12), O45 (n=8), O103 (n=12), O111 (n=4), O121 (n=4) and O145 (n=8) serogroups isolated from cattle feces and five human strains (one strain each of O26, O45, O103, O111, and O145 serogroups) to FDA-ECID DNA microarray. Splitstree analysis was performed to determine the phylogenetic relationship among the non-O157 *E. coli* strains. The non-O157 *E. coli* strains harbored diverse flagellar (H) antigens with O26 strains positive for H9, H11 and

H25, O45 strains for H2, H4, H16, H19, and H31, O103 strains for H2 and H16, O111 strains for H8, O121 strains for H7 and H11, and O145 strains for H28 and H7. Shiga toxin 1 encoding gene was present in both bovine (four each of O26, O45, O103, O111 serogroups, and three strains of O145 serogroup) and human strains (one strain each of O26, O45, O103, O111, and O145 serogroups). All O111 strains of cattle and human origin, and one O145 strain of bovine origin were positive for *stx*2. The strains were also positive intimin (*eae*; 34/53) belonging to various subtypes (α, β, γ, ρ, and ϵ), and other adhesins such as IrgA homologue adhesin (*iha*; 21/53), long polar fimbriae (*lpfA*; 28/53), mannose specific adhesin (*fimH*; 50/53) and curli (*csgA*; 41/53). In addition to LEE encoded type III secretory system associated genes, such as *esp*D, *esc*D, *esc*C, *esc*R, *esc*T, and *sep*Q, non-LEE encoded effector genes (*nle*B, *nle*C, *nle*E, *nle*F, *nle*G5, and *nle*H) were also present in both human and cattle strains except O121 strains. The strains were also positive for other virulence genes such as *katP*, *toxB, irp2,* and *fyuA.* Phylogenetic analysis of non-O157 *E. coli* strains revealed that the strains clustered according to their flagellar type and pathotype. Bovine non-O157 *E. coli* strains possessed major virulence genes, such as those encoding Shiga toxins (1 and 2), adhesins, type III secretory system associated proteins, and plasmid encoded virulence factors, similar to human clinical strains. Because these virulence factors are involved in the pathogenesis of STEC, bovine strains have the potential to cause human illness.

Introduction

Foodborne illness outbreaks associated with *E. coli* serogroups, other than O157, is increasing in recent years. There are over 100 non-O157 Shiga toxin gene-carrying *E. coli* (STEC) serogroups, however, only a few of them have been implicated in human illnesses

(Bettelheim, 2007a). Six serogroups, O26, O45 O103, O111, O121, and O145, often referred to as 'top six non-O157' are responsible for a majority of the non-O157 STEC foodborne illness outbreaks in the USA (Gould et al., 2013; Scallan et al., 2011a). The six non-O157 STEC were declared as adulterants in ground beef and non-intact raw beef products by the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS, 2011). Cattle are a major carrier of the six non-O157 serogroups and their feces are a major source of contamination of food and water, which could lead to human illnesses. In cattle, these top six non-O157 *E. coli* serogroups can be enterohemorrhagic or Shiga toxigenic (EHEC/STEC), enteropathogenic (EPEC) or putative non-pathotype, based on the presence or absence of a few major genes that code for one or both Shiga toxins and intimin (Noll et al., 2015a).

Several PCR-based assays, end-point or real time, have been used for the characterization of non-O157 *E. coli* serogroups (Bai et al., 2012; Karama et al., 2008; Monaghan et al., 2011; Nielsen and Andersen, 2003a). However, such assays, besides being labor intensive and time-consuming, do not provide a comprehensive characterization of non-O157 *E. coli* serogroups. DNA microarray and whole genome sequence analysis provide a rapid identification of serotype and genome-wide characterization of *E. coli* strains (Chattaway et al., 2016; Ju et al., 2012b; Patel et al., 2016). A custom Affymetrix (Santa Clara, CA) DNA microarray developed by the US Food and Drug Administration with genetic signatures derived from a number of available *E. coli* and *Shigella* whole genome sequences, called Food and Drug Administration-*E. coli* Identification (FDA-ECID) DNA microarray, detects over 41,000 genes and over 9,000 single nucleotide polymorphisms to provide serotyping, strain identification and relatedness, and virulence gene profiles of *E. coli* isolates (Patel et al., 2016). The FDA-ECID microarray has been used to identify the O and H types, Shiga toxin (*stx*) and intimin (*eae*) subtypes and other

virulence genes to assess the health risks associated with *E. coli* isolated from humans and food sources (Feng et al., 2014; Lacher et al., 2014; Patel et al., 2016).

There are only a few studies on the comprehensive characterization of major non-O157 *E. coli* serogroups such as O26, O103, and O111 isolated from cattle feces based on whole genome sequencing and microarray (Garrido et al., 2006; Gonzalez-Escalona et al., 2016; Ju et al., 2012b; Krüger et al., 2015). Therefore, the objective of our study was to utilize FDA-ECID DNA microarray to determine the virulence potential of top six non-O157 *E. coli* serogroups isolated from cattle feces.

Materials and Methods

Bacterial strains

Forty-eight non-O157 *E. coli* strains belonging to six major serogroups, O26 (n=12), O45 (n=8), O103 (n=12), O111 (n=4), O121 (n=4), O145 (n=8), isolated from feedlot cattle feces (Cernicchiaro et al., 2013; Cull et al., 2016; Dewsbury et al., 2015), were used in the study. Seventeen strains were positive for *stx* and *eae* (EHEC; four each of O26, O103, O111, and O145 strains, and one O45 strain), three strains were *stx* positive and *eae* negative (STEC; all O45), 12 strains were *stx* negative and *eae* positive (EPEC; four O26, two O45, three O103, and three O145), and 16 were negative for both *stx* and *eae* (putative non-pathotype; four O26, two O45, five O103, four O121, one O145) based on end-point PCR (Bai et al., 2012). Additionally, five human strains positive for *stx* and *eae* (EHEC) obtained from the Kansas Department of Health and Environment (KDHE) were included in the study (one each of O26, O45, O103, O111, and O145). A list of strains used in the study is provided in Table 5.1.

Microarray analysis

The non-O157 *E. coli* strains of human and cattle origin were characterized using FDA-ECID DNA microarray according to the procedure described by (Patel et al., 2016). Briefly, the strains were grown overnight in Luria broth (Sigma-Aldrich Co., St. Louis, MO) at 37° C, and total DNA was extracted from overnight cultures using the DNeasy kit (Qiagen, Venlo, Netherlands). The DNA was fragmented by digesting with RQ1 RNase- Free DNase I, followed by labeling of the 3' end of DNA fragments with biotin. The labeled fragments were hybridized onto FDA-ECID gene chips and incubated at 45°C for 16 h, followed by washing, and scanning the arrays using the Affymetrix GeneAtlas system. Robust MultiArray Averaging (RMA) method and MAS5.0 algorithm were used to determine the presence/absence of each gene. Splitstree analysis was performed to determine the phylogenetic relationship among the strains.

Results

E. coli O26 serogroup

The bovine EHEC (n=4), EPEC (n=4), and human EHEC (n=1) strains carried *fliC*$_{H11}$, and bovine putative non-pathotype strains carried *fliC*$_{H9}$ (3/4) and *fliC*$_{H25}$ (1/4). Both bovine and human EHEC strains carried *stx*1, but were negative for *stx*2. All the bovine EHEC (n=4), EPEC (n=4), and human EHEC (n=1) strains carried *eae* subtype β. The O26 strains also carried a wide variety of other adhesins, such as *iha* (IrgA homolog adhesin), *lpfA* (long polar fimbriae), *fimH* (mannose specific adhesin), and *csgA* (major curlin subunit). Bovine (4/4) and human EHEC (1/1) strains carried *iha*, all the bovine and human O26 strains except one bovine EPEC strain carried *fimH*, all except non-pathotypic O26 strains carried *lpfA*, and all except one non-pathotypic O26 strain carried *csgA*. The strains were also positive for genes encoding proteins

associated with the type III secretory system, such as *escD* (4/4 bovine and 1/1 human EHEC strains), *escR* (4/4 bovine EHEC, 4/4 bovine EPEC, and 1/1 human EHEC strain), and *escT* (4/4 bovine EHEC, 4/4 bovine EPEC, and 1/1 human EHEC strain), and non-LEE encoded (*nle*) effector genes such as *nle*E (4/4 bovine EHEC, 4/4 bovine EPEC, and 1/1 human EHEC strains), *nle*F (3/4 bovine EHEC, 3/4 bovine EPEC, and 1/1 human EHEC strains), *nle*G5 and *nle*H (4/4 bovine EHEC, 4/4 bovine EPEC, and 1/1 human EHEC strains). Plasmid-encoded virulence genes, such as *kat*P (catalase peroxidase) and *toxB* (toxin B), were also present in bovine and human EHEC strains. Bovine EHEC, EPEC, and human EHEC strains were also positive for other virulence genes such as *irp*2 (yersiniabactin biosynthetic protein) and *fyuA* (Yersisniabactin receptor) (Table 5.2).

E. coli O45 serogroup

Escherichia coli O45 bovine strains belonged to five different serotypes: O45:H2 (1/1 bovine and 1/1 human EHEC strains), O45:H16 (3/3 bovine STEC strains), O45:H4 (2/2 bovine EPEC strains), O45:H19 (1/2 bovine non-pathotype strains), and O45:H31 (1/2 bovine non-pathotype strains). Bovine EHEC (1/1), STEC (3/3) and human EHEC (1/1) strains carried *stx*1, and none of them carried *stx*2. Bovine EHEC (1/1) strain carried *eae* subtype α, *eae* genes of two bovine EPEC strains were of subtype ρ, and one human EHEC strain carried *eae* subtype ϵ. They carried other adhesins such as *iha* (1/1 bovine EHEC strain), *fimH* (all O45 strains), *lpfA* (1/1 bovine EHEC, 3/3 bovine STEC, and 2/2 bovine non-pathotype strains), *csgA* (all O45 strains). They carried genes encoding proteins associated with the type three III secretory system such as *escD* (1/1 bovine and human EHEC, and 2/2 bovine EPEC strains), *escC* (1/1 bovine EHEC strain), *escR* (1/1 bovine and human EHEC strains), and *escT* (1/1 bovine and human EHEC strains) and *nle* genes such as *nle*B (1/2 bovine EPEC and 1/1 human EHEC strains), *nle*E (1/1

bovine and human EHEC strains), *nle*F (1/1 bovine and human EHEC strains), *nle*G5 (2/2 bovine EPEC and 1/1 human EHEC strains), and *nleH* (1/1 human EHEC strain). Bovine strains also carried other virulence genes such as *irp*2 and *fyuA* (1/1 bovine EHEC, 3/3 bovine STEC, and 1/2 bovine non-pathotype strains). Enterohemolysin (*ehxA*) was present in all O45 strains except bovine non-pathotype strains (Table 5.3).

E. coli O103 serogroup

All the bovine and human strains, except one strain, carried *fliC*$_{H2}$, and only one bovine non-pathotype strain carried *fliC*$_{H16}$. Bovine EHEC (4/4) and human EHEC (1/1) strains carried *stx*1, and none of them carried *stx*2. Bovine EHEC (4/4), EPEC (3/3) and human EHEC (1/1) strains carried *eae* of the subtype ϵ. They also carried other adhesins such as *fim*H (all O103 strains), *csgA* (4/4 bovine and 1/1 human EHEC, 3/3 bovine EPEC, and 2/5 bovine non-pathotype strains), *lpfA* (5/5 bovine non-pathotype strains), and *iha* (1/4 bovine EHEC strain). They also carried genes encoding proteins associated with the type III secretory system: *escD* (4/4 bovine EHEC, 3/3 bovine EPEC, and 1/1 human EHEC strains), *sepQ* (2/4 bovine EHEC, and 2/3 bovine EPEC strains), *escR* (all except bovine non-pathotype strains) and *escT* (all except bovine non-pathotype strains), and *nle* genes such as *nleB* (4/4 bovine EHEC, 2/3 bovine EPEC strains, and 1/1 human EHEC strains), *nleC* (3/4 bovine EHEC, 1/3 bovine EPEC, and 1/1 human EHEC strains), *nleE* (4/4 bovine EHEC, 1/3 bovine EPEC, and 1/1 human EHEC strains), *nleF* (4/4 bovine EHEC, 2/3 bovine EPEC, and 1/1 human EHEC strains), *nleG5* (4/4 bovine EHEC, 3/3 bovine EPEC, and 1/1 human EHEC strains), and *nleH* (4/4 bovine EHEC, and 1/1 human EHEC strains). Plasmid-encoded virulence gene, *katP* was present in bovine (3/4) and human EHEC (1/1) strains. Enterohemolysin (*ehxA*) was present in all bovine and human strains except bovine non-pathotype strains (Table 5.4).

E. coli O111 and O121 serogroups

All bovine and the human EHEC O111 strains possessed *fliC*$_{H8}$ and carried both *stx*1 and *stx*2. All bovine and human EHEC O111 strains carried adhesins such as *eae* (γ subtype), *iha*, *fimH, lpfA*, and *csgA*. They were also positive for genes encoding proteins associated with the type III secretory system (*escC*, *escD*, *escR*, and *escT*) and *nle* genes (*nleE*, *nleG5*, and *nleH*) (Table 5.5).

Two of the bovine non-pathotype O121 strains carried *fliC*$_{H7}$ and two of them carried *fliC*$_{H11}$. None of them carried *stx*1 or *stx*2. They were also negative for *eae*, however, they were positive for other adhesins such as *fimH* (2/4), *lpfA* (2/4), and *csgA* (4/4). They were negative for genes encoding proteins associated with the type three secretory system and *nle* genes (Table 5.5).

E. coli O145 serogroup

All bovine and human strains belonged to serotype O145:H28, and only one bovine non-pathotype strain belonged to serotype O145:H7. Three of the bovine and one human EHEC strains carried *stx*1, one of the bovine EHEC strains carried *stx*2. Intimin encoding gene (subtype γ) was present in all bovine and human strains except the bovine non-pathotype strain. Other adhesins, such as *iha* and *fimH*, were present in all O145 strains, and *lpfA* and *csgA* were present only in bovine non-pathotype strain. Genes encoding proteins associated with type III secretory system such as *espD*, *escC*, *escD*, *escR*, and *escT* were present in all strains except bovine non-pathotype strain. The strains also carried genes encoding non-LEE encoded effector proteins such as *nleB* (3/4 bovine and 1/1 human EHEC strains), *nleC* (3/4 bovine and 1/1 human EHEC strains), *nleE* (4/4 bovine EHEC, 3/3 bovine EPEC, and 1/1 human EHEC strains), *nleF* (3/4 bovine and 1/1 human EHEC strains), *nleG5* and *nleH* (present in all the strains). Plasmid

encoded virulence genes such as *katP*, *ehxA* and *toxB* were present in all except bovine non-pathotype strain (Table 5.6).

Phylogenetic relationship

Non-O157 *E. coli* strains belonging to the same H-type generally clustered together. Within each serotype, strains belonging to the same pathotype clustered separately. However, two of the O26:H11 bovine EPEC strains clustered separately from two other O26:H11 EPEC strains (Fig 5.1). Interestingly, the human O45:H2 EHEC strain was more closely related to bovine and human O103:H2 EHEC strains compared to the bovine O45:H2 EHEC strain (Fig 5.1).

Discussion

Cattle are a major reservoir of a wide number of *E. coli* serogroups and serotypes, however, not all of them are pathogenic to humans. Therefore, assessment of virulence potential of the bovine strains is important to estimate the potential risk to cause human illness. Genome level characterization of *E. coli* strains can be performed using methods such as microarray and whole genome sequencing. Microarray-based characterization is less expensive and less time-consuming compared to whole genome sequencing. Custom DNA microarrays designed using available whole genome sequences contains several genes enabling rapid and comprehensive characterization of pathogens. The *E. coli* Identification microarray developed by the U.S. Food and Drug Administration (Food and Drug Administration-*E. coli* identification; FDA-ECID) is a custom Affymetrix DNA microarray which detects more than 40,000 gene targets of *E. coli* enabling rapid genome level characterization of *E. coli* strains (Patel et al., 2016).

The bovine strains used in the microarray analysis were isolated from feces of feedlot cattle. We also included a human clinical strain for each of the five serogroups to compare the virulence gene profiles. The serogroup O121 did not have a STEC strain. In all of the studies we have done with fecal samples collected from feedlot steers located in multiple feedlots in the Midwest region of the US, we have not found an O121 strain that carried a *stx* gene. The EHEC/STEC strains of bovine and human origin belonging to O26, O45, O103, O111, and O145 serogroups carried either or both *stx*1 and *stx*2. Shiga toxin 2 is more commonly associated with severe human illness and associated complications like hemolytic uremic syndrome compared to *stx*1 (Brooks et al., 2005b; Ethelberg et al., 2004b). The top six non-O157 *E. coli* serogroups (34 strains) belonging to EHEC/STEC pathotypes except O121 carried *eae*. Intimin is an adhesin encoded by *eae* within the locus of enterocyte and effacement (LEE) pathogenicity island. It is responsible for attachment and effacement lesion (A/E) in the host intestinal epithelial cells (Jerse et al., 1990). All *eae* carrying O26 strains were of subtype β, O45 strains carried α/ρ/ϵ subtypes, O103 strains carried the ϵ subtype, and O111 and O145 carried the γ subtype. Intimin subtypes have been reported to be associated with particular serogroups and flagellar types. Intimin subtypes γ, β, and θ are commonly carried by STEC serotypes isolated from outbreak cases of HUS and hemorrhagic colitis (Beutin et al., 2004; Ramachandran et al., 2003; Shen et al., 2015; Tostes et al., 2017). In our study, of the 48 non-O157 *E. coli* strains isolated from cattle feces, 17 were EHEC, 3 were STEC, 12 were EPEC, and 16 were putative non-pathotypes. All the human strains were EHEC, thereby carrying either or both *stx*1 and *stx*2 and *eae*. Shiga toxin 2 subtypes could not be identified by microarray because of high sequence similarity between *stx*2 subtypes (Patel et al., 2016). All of the bovine EPEC strains were atypical since none of them carried *bfp* gene (encoding bundle forming pili), an adhesin encoded by EPEC

adherence factor (EAF) plasmid (Giron et al., 1991; Nataro and Kaper, 1998). Bovine and human EHEC/EPEC non-O157 strains (n=34) were also positive for genes encoding proteins associated with the type III secretory system. The products of type three secretory system encoded by LEE are critical in the formation of A/E lesion in the host epithelial cells (McDaniel et al., 1995b; McDaniel and Kaper, 1997). They also carried non-LEE effector proteins encoding genes (*nleB, nleC, nleE, nleF, nleG5, nleH*). Non-LEE-encoded effector proteins have been reported to be significantly associated with severe human illness and its associated complications such as HUS (Bugarel et al., 2010; Karmali et al., 2003a). Non-LEE-encoded effector proteins such as NleH1 and NleH2 have been reported to inhibit NF- κB, a pro-inflammatory transcription factor, leading to improved colonization of enteropathogenic *E. coli* (Royan et al., 2010).

Apart from *eae,* non-O157 *E. coli* serogroups also carried other adhesins such as *lpfA*, *iha*, *fimH*, and *csgA*. Long polar fimbriae (*lpf*) has been shown to be involved in initial adherence of *E. coli* O104:H4 to host intestinal epithelial cells (Ross et al., 2015). Long polar fimbriae (*lpf*) and IrgA homolog adhesin (*iha*) are the most prevalent adhesins among different seropathotypes of STEC strains isolated from food, animals and human sources (Toma et al., 2004).

Plasmid encoded virulence genes such as *katP* and *toxB* were also present in human and bovine non-O157 STEC/EHEC/EPEC strains. The adherence of EHEC to host epithelial cells has been reported to be promoted through the production of type III secretory system proteins by ToxB, encoded by the *toxB* gene carried by plasmid O157 (Tatsuno et al., 2001). The human pathogenic STEC strains most commonly carry a combination of *stx*2, *eae* and *katP* genes (Pradel et al., 2008).

Phylogenetic analysis revealed that non-O157 *E. coli* serogroups carrying the same flagellar type clustered together. This is in agreement with the previous studies (Ferdous et al., 2016; Ju et al., 2012b). Shiga toxin-producing *E. coli* carrying the same flagellar types have been hypothesized to share a common ancestor (Ju et al., 2012b).

In conclusion, the non-O157 *E. coli* strains isolated from cattle feces characterized in this study belonged to EHEC, STEC or atypical EPEC pathotypes, and few were putative non-pathotypes. Comprehensive characterization of *E. coli* serogroups shed in cattle feces is essential to determine their pathotypes and to estimate the risk of human illness associated with cattle feces. The flagellar types and virulence gene profiles of some of the cattle fecal non-O157 *E. coli* strains characterized in this study were similar to those of human clinical strains, suggesting the potential of cattle fecal strains belonging to the top six non-O157 *E. coli* serogroups to cause human illness.

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Figure 5-1. Phylogenetic analysis of non-O157 *E. coli* strains (n=53) based on the FDA-ECID DNA microarray gene difference data from 41,000 probe sets using SplitsTree4. The tree was developed using the Neighbor net algorithm using Neighbor joining method. The scale bar represents 0.01 base substitutions per site

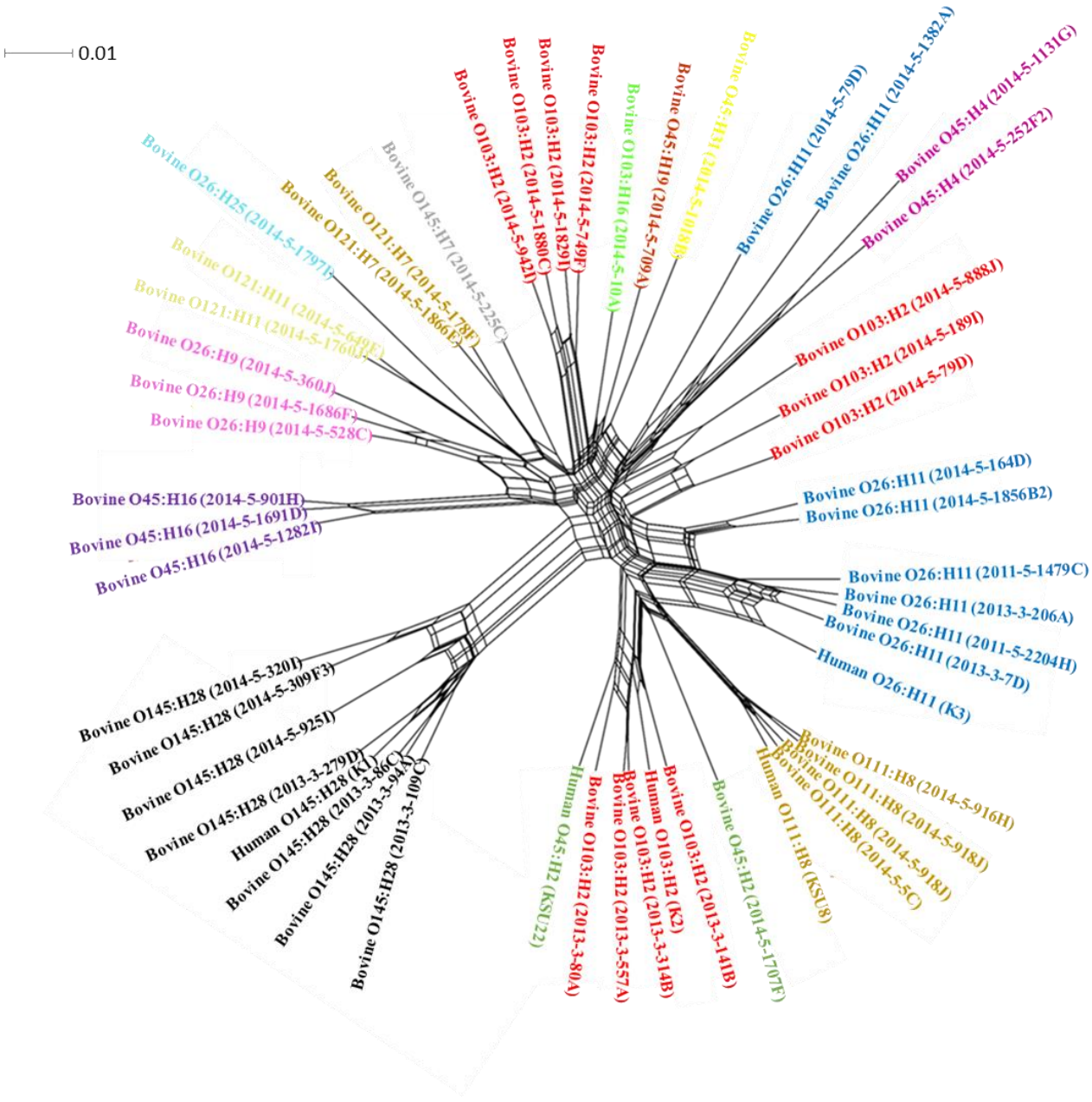

Table 5.1. Pathotypes of *E. coli* strains belonging to top six non-O157 *E. coli* serogroups used in the study (n=53) determined based on the presence/absence of *stx* and *eae* genes using end-point PCR

Serogroup	Host	EHEC (*stx*$^+$, *eae*$^+$)	STEC (*stx*$^+$, *eae*$^-$)	EPEC (*stx*$^-$, *eae*$^+$)	Non-pathotype (*stx*$^-$, *eae*$^-$)
O26	Bovine (n=12)	4	-	4	4
(n=13)	Human (n=1)	1	-	-	-
O45	Bovine (n=8)	1	3	2	2
(n=9)	Human (n=1)	1	-	-	-
O103	Bovine (n=12)	4	-	3	5
(n=13)	Human (n=1)	1	-	-	-
O111	Bovine (n=4)	4	-	-	-
(n=5)	Human (n=1)	1	-	-	-
O121 (n=4)	Bovine (n=4)	-	-	-	4
O145	Bovine (n=8)	4	-	3	1
(n=9)	Human (n=1)	1	-	-	-

Table 5.2. Distribution of virulence genes in enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype *Escherichia coli* O26 serogroup isolated from cattle feces and a human clinical case based on microarray data

Virulence genes	Product	Bovine EHEC (n=4)	Bovine EPEC (n=4)	Bovine Non-pathotype (n=4)	Human EHEC (n=1)
Flagellar types					
fliC$_{H9}$	Flagellar antigen H9			3/4	
fliC$_{H11}$	Flagellar antigen H11	4/4	4/4		1/1
fliC$_{H25}$	Flagellar antigen H25			1/4	
Toxins					
*stx*1	Shiga toxin 1	4/4			1/1
*stx*2	Shiga toxin 2				
ehxA	Enterohemolysin	4/4	3/4		1/1
Intimin subtypes					
eae (β)	Intimin subtype β	4/4	4/4		1/1
Adhesins					
Iha	IrgA homolog adhesin	4/4			1/1
fimH	Mannose specific adhesin	4/4	3/4	4/4	1/1
lpfA	Long polar fimbriae	4/4	4/4		1/1
csgA	Major curli subunit	4/4	4/4	3/4	1/1
Type III secretory system proteins					
espD	*E. coli* secreted protein D				
escD	*E. coli* secreted component protein D	4/4			1/1
escC	*E. coli* secreted component protein C				
sepQ	Secretion of *E. coli* protein Q				

escR	*E. coli* secreted component protein R	4/4	4/4		1/1
escT	*E. coli* secreted component protein T	4/4	4/4		1/1
	Non-LEE encoded proteins				
nleB	Non-LEE encoded effector protein B				
nleC	Non-LEE encoded effector protein C				
nleE	Non-LEE encoded effector protein E	4/4	4/4		1/1
nleF	Non-LEE encoded effector protein F	3/4	3/4		1/1
nleG5	Non-LEE encoded effector protein G5	4/4	4/4		1/1
nleH	Non-LEE encoded effector protein G5	4/4	4/4		1/1
	Others				
fyuA	Pesticin/yersiniabactin receptor protein	4/4	4/4		1/1
gadAB	Glutamate decarboxylase	4/4	3/4	4/4	1/1
Irp2	Yersiniabactin biosynthetic protein	4/4	4/4		1/1
katP	Catalase peroxidase	3/4			1/1
toxB	Toxin B	4/4			1/1

Table 5.3. Distribution of virulence genes in enterohemorrhagic (EHEC), Shigatoxigenic (STEC), enteropathogenic (EPEC) and putative non-pathotype *Escherichia coli* O45 serogroup isolated from cattle feces and a human clinical case based on microarray data

Virulence genes	Product	Bovine EHEC (n=1)	Bovine STEC (n=3)	Bovine EPEC (n=2)	Bovine Non-pathotype (n=2)	Human EHEC (n=1)
Flagellar types						
fliC$_{H2}$	Flagellar antigen H2	1/1				1/1
fliC$_{H4}$	Flagellar antigen H4			2/2		
fliC$_{H16}$	Flagellar antigen H16		3/3			
fliC$_{H19}$	Flagellar antigen H19				1/2	
fliC$_{H31}$	Flagellar antigen H31				1/2	
Toxins						
*stx*1	Shiga toxin 1	1/1	3/3			1/1
*stx*2	Shiga toxin 2					
ehxA	Enterohemolysin	1/1	3/3	2/2		1/1
Intimin subtypes						
eae (ϵ)	Intimin subtype ϵ					1/1
eae (α)	Intimin subtype α	1/1				
eae (ρ)	Intimin subtype ρ			2/2		
Adhesins						
Iha	IrgA homolog adhesin	1/1				
fimH	Mannose specific adhesin	1/1	3/3	2/2	2/2	1/1
lpfA	Long polar fimbriae	1/1	3/3		2/2	
csgA	Major curlin subunit	1/1	3/3	2/2	2/2	1/1
Type III secretory system proteins						
espD	*E. coli* secreted protein D					
escD	*E. coli* secreted component protein D	1/1		2/2		1/1

escC	*E. coli* secreted component protein C	1/1				
sepQ	Secretion of *E. coli* protein Q					
escR	*E. coli* secreted component protein R	1/1				1/1
escT	*E. coli* secreted component protein T	1/1				1/1
	Non-LEE encoded proteins					
nleB	Non-LEE encoded effector protein B			1/2		1/1
nleC	Non-LEE encoded effector protein C					
nleE	Non-LEE encoded effector protein E	1/1				1/1
nleF	Non-LEE encoded effector protein F	1/1				1/1
nleG5	Non-LEE encoded effector protein G5			2/2		1/1
nleH	Non-LEE encoded effector protein G5					1/1
	Others					
fyuA	Pesticin/yersiniabactin receptor protein	1/1	3/3		1/2	
gadAB	Glutamate decarboxylase	1/1	3/3	2/2	2/2	1/1
Irp2	Yersiniabactin biosynthetic protein	1/1	3/3		1/2	
katP	Catalase peroxidase					
toxB	Toxin B					

Table 5.4. Distribution of virulence genes in enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype *Escherichia coli* O103 serogroup isolated from cattle feces and a human clinical case based on microarray data

Virulence genes	Product	Bovine EHEC (n=4)	Bovine EPEC (n=3)	Bovine Non-pathotype (n=5)	Human EHEC (n=1)
Flagellar types					
fliC$_{H2}$	Flagellar antigen H2	4/4	3/3	4/5	1/1
fliC$_{H16}$	Flagellar antigen H16			1/5	
Toxins					
*stx*1	Shiga toxin 1	4/4			1/1
*stx*2	Shiga toxin 2				
ehxA	Enterohemolysin	4/4	3/3		1/1
Intimin subtypes					
eae (ϵ)	Intimin subtype ϵ	4/4	3/3		1/1
Adhesins					
Iha	IrgA homolog adhesin	1/4			
fimH	Mannose specific adhesin	4/4	3/3	5/5	1/1
lpfA	Long polar fimbriae			5/5	
csgA	Major curlin subunit	4/4	3/3	2/5	1/1
Type III secretory system proteins					
espD	*E. coli* secreted protein D				
escD	*E. coli* secreted component protein D	4/4	3/3		1/1
escC	*E. coli* secreted component protein C				
sepQ	Secretion of *E. coli* protein Q	2/4	2/3		

escR	*E. coli* secreted component protein R	4/4	3/3		1/1
escT	*E. coli* secreted component protein T	4/4	3/3		1/1
	Non-LEE encoded proteins				
nleB	Non-LEE encoded effector protein B	4/4	2/3		1/1
nleC	Non-LEE encoded effector protein C	3/4	1/3		1/1
nleE	Non-LEE encoded effector protein E	4/4	1/3		1/1
nleF	Non-LEE encoded effector protein F	4/4	2/3		1/1
nleG5	Non-LEE encoded effector protein G5	4/4	3/3		1/1
nleH	Non-LEE encoded effector protein G5	4/4			1/1
	Others				
fyuA	Pesticin/yersiniabactin receptor protein				
gadAB	Glutamate decarboxylase	4/4	3/3	5/5	1/1
Irp2	Yersiniabactin biosynthetic protein				
katP	Catalase peroxidase	3/4			1/1
toxB	Toxin B				

Table 5.5. Distribution of virulence genes in enterohemorrhagic (EHEC) *Escherichia coli* O111 isolated from cattle feces and a human clinical case, and putative non-pathotype *Escherichia coli* O121 serogroup isolated from cattle feces based on microarray data

Virulence genes	Product	Bovine O111	Human O111	Bovine O121
		EHEC (n=4)	EHEC (n=1)	Non-pathotype (n=4)
Flagellar types				
fliC$_{H7}$	Flagellar antigen H7			2/4
fliC$_{H8}$	Flagellar antigen H8	4/4	1/1	
fliC$_{H11}$	Flagellar antigen H11			2/4
Toxins				
*stx*1	Shiga toxin 1	4/4	1/1	
*stx*2	Shiga toxin 2	4/4	1/1	
ehxA	Enterohemolysin	4/4	1/1	
Intimin subtypes				
eae (γ)	Intimin subtype γ	4/4	1/1	
Adhesins				
iha	IrgA homolog adhesin	4/4	1/1	
fimH	Mannose specific adhesin	4/4	1/1	2/4
lpfA	Long polar fimbriae	4/4	1/1	2/4
csgA	Major curlin subunit	4/4	1/1	4/4
Type III secretory system proteins				
espD	*E. coli* secreted protein D			
escD	*E. coli* secreted component protein D	4/4	1/1	
escC	*E. coli* secreted component protein C	4/4	1/1	

sepQ	Secretion of *E. coli* protein Q			
escR	*E. coli* secreted component protein R	4/4	1/1	
escT	*E. coli* secreted component protein T	4/4	1/1	
	Non-LEE encoded proteins			
nleB	Non-LEE encoded effector protein B			
nleC	Non-LEE encoded effector protein C			
nleE	Non-LEE encoded effector protein E	4/4	1/1	
nleF	Non-LEE encoded effector protein F			
nleG5	Non-LEE encoded effector protein G5	4/4	1/1	
nleH	Non-LEE encoded effector protein G5	4/4	1/1	
	Others			
fyuA	Pesticin/yersiniabactin receptor protein			
gadAB	Glutamate decarboxylase	4/4	1/1	4/4
Irp2	Yersiniabactin biosynthetic protein			
katP	Catalase peroxidase		1/1	
toxB	Toxin B			

Table 5.6. Distribution of virulence genes in enterohemorrhagic (EHEC), enteropathogenic (EPEC) and putative non-pathotype *Escherichia coli* O145 serogroup isolated from cattle feces and a human clinical case based on microarray data

Virulence genes	Product	Bovine EHEC (n=4)	Bovine EPEC (n=3)	Bovine Non-pathotype (n=1)	Human EHEC (n=1)
Flagellar types					
fliC$_{H7}$	Flagellar antigen H7			1/1	
fliC$_{H28}$	Flagellar antigen H28	4/4	3/3		1/1
Toxins					
*stx*1	Shiga toxin 1	3/4			1/1
*stx*2	Shiga toxin 2	1/4			
ehxA	Enterohemolysin	4/4	3/3		1/1
Intimin subtypes					
eae (γ)	Intimin subtype γ	4/4	3/3		1/1
Adhesins					
iha	IrgA homologue adhesin	4/4	3/3	1/1	1/1
fimH	Mannose specific adhesin	4/4	3/3	1/1	1/1
lpfA	Long polar fimbriae			1/1	
csgA	Major curlin subunit			1/1	
Type III secretory system proteins					
espD	*E. coli* secreted protein D	4/4	3/3		1/1
escD	*E. coli* secreted component protein D	4/4	3/3		1/1
escC	*E. coli* secreted component protein C	4/4	3/3		1/1
sepQ	Secretion of *E. coli* protein Q				
escR	*E. coli* secreted component protein R	4/4	2/3		1/1
escT	*E. coli* secreted component protein T	4/4	2/3		1/1
Non-LEE encoded proteins					

nleB	Non-LEE encoded effector protein B	3/4			1/1
nleC	Non-LEE encoded effector protein C	3/4			1/1
nleE	Non-LEE encoded effector protein E	4/4	3/3		1/1
nleF	Non-LEE encoded effector protein F	3/4			1/1
nleG5	Non-LEE encoded effector protein G5	4/4	3/3	1/1	1/1
nleH	Non-LEE encoded effector protein G5	4/4	3/3	1/1	1/1
	Others				
fyuA	Iron acquisition outer membrane yersiniabactin receptor				
gadAB	Glutamate decarboxylase	4/4	3/3	1/1	1/1
Irp2	Yersiniabactin biosynthetic protein				
katP	Catalase peroxidase	4/4	3/3		1/1
toxB	Toxin B	4/4	3/3		1/1

Chapter 6 - Analysis of Virulence Potential of *Escherichia coli* O145 Isolated from Cattle Feces and Hide Samples Based on Whole Genome Sequencing

Abstract

Escherichia coli O145 serogroup is one of the top-7 Shiga toxin producing *E. coli* (STEC) that causes foodborne infections in the United States. The O145 serogroup is responsible for several outbreaks in the United States and other countries. Cattle are a major reservoir of STEC, harboring them in the hindgut and shedding them in feces. Cattle feces is the main source of hide and carcass contamination during harvest, potentially leading to foodborne illnesses in humans. The objective of our study was to determine the virulence potential and phylogenetic relationship of STEC O145 strains isolated from cattle feces and hide samples. A total of 87 STEC O145 strains from cattle feces (n=18), hide (n=67), and human clinical samples (n=2) were used in the study. The strains were subjected to whole genome sequencing using the Illumina MiSeq platform. All strains were confirmed to be of the O145 serogroup, and *fliC*$_{H28}$ was the only flagellar type found among all the strains. The average genome sizes of the fecal, hide, and human clinical strains were 5.41, 5.28, and 5.29 Mb, respectively. The average number of genes associated with virulence, disease and defense in cattle fecal, hide and human clinical strains were similar. The average number of genes associated with mobile genetic elements (phages, prophages, transposable elements, and plasmids) was 260, 259, and 236 in strains from cattle feces, cattle hide, and human clinical cases, respectively. Shiga toxin 1a was the most common Shiga toxin gene subtype among all the strains, followed by *stx*2a (only in bovine fecal

and human strains) and *stx*2c (only in bovine strains). All the strains were positive for *eae* subtype γ1. The strains also contained genes encoding for type III secretory system proteins, *nle* genes, and plasmid-encoded virulence genes. Phylogenetic analysis of the strains revealed clustering of all the strains isolated from cattle feces separately from those isolated from hide samples, and the human clinical strains were more closely related to fecal strains than the hide strains. A majority of the strains belonged to sequence type (ST)-32, although seven cattle fecal and two hide strains belonged to ST-436. This suggests that most of the STEC O145 strains of cattle and human origin belonged to the same clonal complex. The virulence gene profile of STEC O145 strains isolated from cattle sources was similar to that of human clinical strains, and were phylogenetically more closely related to human clinical strains. The genetic analysis suggests that STEC O145 strains from cattle sources have the potential to cause human illnesses.

Introduction

Escherichia coli O157:H7 is the most extensively studied serotype among Shiga toxin-producing *E. coli* (STEC) because of its increased association with human foodborne illnesses. In recent years, there has been an increased incidence of non-O157 STEC-associated human illnesses. Six *E. coli* serogroups (O26, O45, O103, O111, O121, and O145) are responsible for more than 70% of non-O157 STEC-associated human illnesses in the United States (Brooks et al., 2005b; Scallan et al., 2011b). In 2011, the U. S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) declared these six non-O157 STEC to be adulterants in ground beef and non-intact raw beef products (USDA-FSIS, 2011). The serogroup O145 is responsible for several outbreaks in the US and other countries, including Germany (Beutin et al., 1998), Argentina (Rivero et al., 2010) and Belgium (De Schrijver et al., 2008). In the US, two cases of

E. coli O145 associated infections were reported in a day care in Minnesota in 1999 (Luna and Mody, 2010). The serogroup was also responsible for a waterborne human illness in Oregon in 2005 (Yoder et al., 2008), and in 2010, a multistate outbreak was associated with the consumption of romaine lettuce, leading to 45% hospitalization, with 10% of the patients developing hemolytic uremic syndrome (Taylor et al., 2013).

Cooper et al. (2014) have analyzed the whole genome sequences (WGS) of two strains of *E. coli* O145:H28 that were associated with the romaine lettuce outbreak in the US and ice cream outbreak in Belgium, and compared them to genome sequences of *E. coli* and *Shigella*. They reported that *E. coli* O145 and O157 strains evolved from a common lineage, and the core genome profile of *E. coli* O145 strains was more similar to that of *E. coli* O157 than to other *E. coli* strains (Cooper et al., 2014). Carter et al. (2016) have studied the genetic diversity, population structure, virulence potential, and antimicrobial resistance profiles of environmental *E. coli* O145 strains (cattle feces, feral pigs, wildlife, sediment, water and human clinical cases). They reported a great deal of genetic diversity among the strains, and antimicrobial resistance appeared to be widespread in environmental strains with over half of the cattle strains resistant to at least one of the 14 antibiotics tested (Carter et al., 2016).

Cattle are major reservoirs of STEC, harboring them in the hindgut and shedding them in feces. Cattle feces are the main source of hide and carcass contamination during harvest, potentially leading to foodborne illnesses in humans. Several studies have shown the association between STEC prevalence in cattle feces and subsequent hide and carcass contamination (Elder et al., 2000; Jacob et al., 2010). However, it is important to determine the virulence potential of the strains in order to estimate the risk associated with each source of contamination, and to design intervention strategies to prevent foodborne illness in humans. The objective of our study

was to assess the virulence potential and phylogenetic relationship of STEC O145 strains isolated from cattle feces and hide samples based on the WGS-based analysis.

Materials and Methods

Escherichia coli O145 strains

A total of 85 STEC O145 strains isolated from cattle feces and hide samples were used for the study. The strains isolated from cattle feces (n=18) and hide swab samples (n=67) were collected from feedlot cattle and at abattoirs, respectively (Cull et al., 2017; Noll et al., 2015a). Two human clinical strains obtained from the Kansas Department of Health and Environment were included in the study. The strains were positive for *stx*1 (n=76) only, *stx*2 only (n=9), both *stx*1 and *stx*2 (n=1), and *eae* (n=87) by PCR (Bai et al., 2012). The strains were cultured onto Tryptone soy agar (TSA; BD Difco, Sparks, MD) slants and shipped overnight on ice to the University of Maryland for whole genome sequencing.

DNA extraction and whole genome sequencing

Escherichia coli O145 strains on the TSA slants were restreaked onto blood agar and then subcultured in TSB. The genomic DNA was extracted from the broth culture using DNeasy Blood and Tissue Kit with the QIAcube robotic workstation (Qiagen, Germantown, MD). The genomic libraries were constructed using Nextera XT DNA Library Preparation Kit and MiSeq Reagent Kits v2 (500 Cycles) (Illumina, Inc.). Whole genome sequencing was performed using an Illumina MiSeq platform (Illumina, San Diego, CA). *De novo* genome assembly was performed using SPAdes 3.6.0 (Bankevich et al., 2012) using default settings. Average genome coverage of the *E. coli* O145 strains isolated from cattle feces, hide and human clinical strains (n=87) was 45x. Subsequent analysis was performed on the assembled draft genomes.

Sequence analysis

The initial annotation of draft genomes of STEC O145 strains was performed using RAST (Rapid Annotation using Subsystem Technology (Aziz et al., 2008)). The O and H-types were identified using SerotypeFinder 1.1 and BLAST tools, respectively. The number of genes categorized as associated with virulence, disease and defense, mobile elements (phages, prophages, transposable elements, and plasmids), membrane transport, iron acquisition and metabolism, and stress response in each strain was determined using RAST. A single factor analysis of variance test was performed to determine whether genome size and number of genes associated with different functional categories were significantly different between cattle fecal, hide and human clinical strains. Tukey adjustment for multiple comparisons was performed, using SAS 9.4 with Proc Glimmix, to test each pairwise comparison for significant differences ($P < 0.01$), if the means were significantly different ($P < 0.01$). The virulence gene profile and antimicrobial resistance genes were determined using VirulenceFinder 1.4 (Joensen et al., 2014) and ResFinder 2.1 (Zankari et al., 2012), web-based tools developed by the Center for Genomic Epidemiology (CGE) at the Danish Technical University (Lyngby, Denmark) (http://www.genomicepidemiology.org/). Plasmid and phage sequences were identified using PlasmidFinder v1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and Phage Search Tool Enhanced Release (PHASTER; http://phaster.ca/), respectively. The sequence types of each strain were determined using the *in silico* MLST tool, MLST v1.8 (Jaureguy et al., 2008; Wirth et al., 2006), a web-based tool developed by CGE. The phylogenetic relationship among the STEC O145 strains of cattle and human origin was determined using Parsnp v1.2 (http://harvest.readthedocs.io/en/latest/content/parsnp.html) (Treangen et al., 2014), which

performs core genome alignment followed by construction of a maximum likelihood tree. The tree was visualized using FigTree 1.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

All strains were confirmed to be of the O145 serogroup by SerotypeFinder 1.1 using default parameters (select threshold for % ID = 85%, and select minimum length=60%) for 71 strains, with 40% select minimum length for 13 strains and 20% select minimum length for three strains. All of the STEC O145 strains carried *fliC*$_{H28}$. The flagellar genes of the strains showed ≥ 99% identity to the *fliC*$_{H28}$ reference sequences (GenBank accession no.LN555740, LN555741, LN649615).

RAST subsystem summary

Based on the RAST subsystem annotation, the average genome size of STEC O145 strains isolated from cattle feces and hide were 5.41 (5.25-5.63) Mb and 5.28 (5.21-5.46) Mb, respectively. The average genome size of fecal strains was significantly larger ($P < 0.01$) than the hide and human clinical strains. The average number of genes associated with mobile genetic elements (phages, prophages, transposable elements, and plasmids) was significantly higher ($P < 0.01$) in strains isolated from cattle feces (260 [224-291]) compared to hide strains (236 [203-268]). There was no significant difference in the average number of genes associated with membrane transport, iron acquisition and metabolism, and stress response categories between cattle fecal, hide and human clinical strains. The number of genes associated with the major subsystem categories in all of the bovine and human strains is provided in Table 6.1.

Virulence genes

Of the 87 strains, 76 strains were positive for *stx*1 only (10 fecal, 65 hide and one human strains), nine strains for *stx*2 only (8 fecal and one hide strains) and one human strain was positive for both *stx*1 and *stx*2. Shiga toxin 1a was the most common subtype found in bovine fecal (10/18; 55.6%), hide (65/67; 97%) and human clinical strains (2/2; 100%). Shiga toxin 2a was present only in bovine fecal (6/18; 33.3%) and human clinical strains (1/2; 50%), but absent in cattle hide strains. Similarly, *stx*2c was present only in bovine fecal (2/18; 11.1%) and hide strains (1/67; 1.5%), but absent in human clinical strains. All of the STEC O145 strains of bovine fecal, hide, and human origin carried intimin (*eae*) subtype γ1. They also carried LEE-encoded type three secretory system proteins such as *tir, espA, espB,* and *espF*. All strains carried *tir*, *espA*, and *espB,* whereas *espF* was present at a frequency of 88.9%, 86.6%, and 100% in cattle fecal, hide and human clinical strains, respectively. Apart from intimin, they also carried other adhesins such as *iha* (IrgA homolog adhesin). The non-LEE encoded effector protein encoding genes such as *nleA*, *nleB,* and *nleC* were present in all strains. Additionally, they also carried phage-encoded type III secretory system protein encoding genes such as *espI, espJ, cif,* and *tccp*. All of the strains carried *espJ* (except one human strain) and *cif* (except one human and one cattle hide strain), and *tccp* was present at a frequency of 88.9%, 80.6%, and 100% in cattle fecal, hide and human clinical strains, respectively. Plasmid-encoded virulence genes such as *ehxA* (88.9% of cattle fecal, 67.2% of cattle hide, and 50% of human clinical strains), *katP* (61.1% of cattle fecal, 77.6% of cattle hide, and 50% of human clinical strains), and *espP* (100% of cattle fecal, 89.6% of cattle hide, and 50% of human clinical strains) were also present in STEC O145 strains. The gene encoding EAST-1 heat-stable toxin (*astA*) was present in all of the

strains. The virulence gene content of cattle fecal, hide and human strains is provided in Table 6.2.

Antimicrobial resistance genes

Antimicrobial resistance genes to aminoglycosides, tetracyclines, sulfonamides, phenicols, β-lactams were found in three fecal and two hide strains. Aminoglycoside resistance genes (*strA* and *strB*) were found in two fecal and two hide strains. Tetracyclin resistance genes, *tetA* was carried by three strains (two fecal and one hide strain) and *tetB* was carried by one fecal and one hide strain. Sulfonamide resistance gene (*sul2*) was present in three fecal and one hide strain. Phenicol resistance gene (*floR*) was carried by two fecal and one hide strain. Beta-lactamase resistance gene ($bla_{\text{CMY-2}}$) was carried by one fecal and one hide strains (Table 6.2).

Plasmid and prophage sequences

The most common plasmid sequences found in STEC O145 strains were IncFIB (18 cattle fecal, 61 cattle hide, and one human strains) and IncB/O/K/Z (18 cattle fecal, 65 cattle hide, and one human strains). Other plasmid sequences found were IncI2 (two cattle fecal), IncI2A (one cattle fecal), IncA/C2 (one cattle fecal and one cattle hide), pO111 (one cattle fecal), and IncR (one cattle fecal) (Table 6.3). The average number of phage sequences were 17.2 (11-23) and 15.3 (9-22) in cattle fecal and hide strains, respectively. The average number of intact, incomplete and questionable phage sequences based on PHASTER scores in cattle and human strains is provided in Table 6.4.

Phylogenetic relationship and sequence types

Phylogenetic analysis of the strains revealed clustering of all the strains isolated from cattle feces separately from those isolated from hide samples, and human clinical strains were more closely related to fecal strains than the hide strains. Only four hide strains clustered with

fecal strains (Fig 6.1). Most of the cattle fecal, hide and human strains belonged to ST-32, except a few cattle fecal (n=7) and hide strains (n=2) which belonged to ST-436.

Discussion

Understanding the virulence potential of STEC O145 strains isolated from cattle feces and hide samples is useful in estimating the risk associated with different sources of human illnesses. The whole genome sequences of STEC O145 strains isolated from cattle feces, hide and human clinical strains were analyzed to determine their virulence potential and phylogenetic relationship. All strains carried flagellar type H28, which is the most common flagellar type carried by the human outbreak and environmental O145 strains (Carter et al., 2016; Cooper et al., 2014). The average genome size of cattle fecal strains was larger than cattle hide and human clinical strains, which appears to be because of the higher average number of genes associated with mobile genetic elements in cattle fecal strains than cattle hide and human clinical strains. This suggests that the size of the genomes were proportional to the number of mobile genetic elements.

The virulence gene profile of STEC O145 strains isolated from cattle feces, hide, and human clinical strains were similar. They carried Shiga toxins, LEE-encoded type three secretory system proteins, and plasmid-encoded virulence genes. The environmental and outbreak *E. coli* O145 strains were also found to carry core EHEC virulence determinants (Carter et al., 2016). Shiga toxin 1a was the only subtype of *stx*1 found in all strains. Shiga toxin 2a (*stx*2a) and *stx*2c were the subtypes of *stx*2 found in *E. coli* O145 strains of cattle and human origin. Similar findings were also reported in environmental *E. coli* O145 strains by (Carter et al., 2016). Shiga toxin 2a was also carried by *E. coli* O145 strains isolated from an ice-cream associated outbreak

in Belgium and lettuce associated outbreak in the United States (Cooper et al., 2014). In our study, a majority of the fecal strains carried *stx*2a (44.4%), however, it was absent in hide strains. One strain isolated from cattle hide was negative for *stx*, although, it tested positive for *stx*2 by end-point PCR, which may likely be due to the loss of *stx*-encoding phage. *Escherichia coli* O145 strains from all three sources carried genes associated with LEE and phage-encoded type III secretory system and *nle* genes. Locus of enterocyte effacement-encoded type three secretory system proteins are involved in the formation of attachment and effacement lesions in host epithelial cells (McDaniel et al., 1995a; McDaniel and Kaper, 1997). Genes encoding non-LEE encoded effectors have been reported to be associated with complications of STEC infections such as HUS (Bugarel et al., 2010; Karmali et al., 2003b). Subtype γ1 was the only subtype of intimin found among all the strains. Intimin gamma 1 subtype was found to be frequently associated with *E. coli* O145, O55, and O157 strains isolated from cattle and human sources (Oswald et al., 2000). Strains from all three sources were also positive for plasmid-encoded (pO157) virulence genes such as *katP*, *ehxA*, and *espP*. Whole genome sequence-based analysis of *E. coli* O145 outbreak strains revealed that the virulence genes carried by pO145 were similar to those carried by pO157, although they lacked *katP* (Cooper et al., 2014). Plasmid-encoded virulence genes have been reported to be involved in the pathogenesis of STEC (Brunder et al., 1997a; Schmidt and Karch, 1996; Zhang et al., 2012). In our study, the pO157 plasmid sequence was not found in O145 strains, although they carried virulence genes encoded by pO157, which could be due to sequencing error. *Escherichia coli* heat-stable enterotoxin 1 (EAST1), encoded by *astA*, was present in *E. coli* O145 strains isolated from all three sources. This gene was reported to be frequently associated with diarrhea caused by typical and atypical enteropathogenic *E. coli* (Silva et al., 2014).

IncFIB was the most frequently found plasmid sequence in cattle fecal, hide and human strains. IncF plasmid has been most frequently found in *E. coli* strains carrying antibiotic resistance genes such as *tet*(A), $bla_{\text{TEM-1}}$, and $bla_{\text{CTX-M-15}}$ (Lyimo et al., 2016; Mshana et al., 2009). A majority of the virulence associated plasmids in *E. coli* belong to the IncF incompatibility family (Johnson and Nolan, 2009). Another most commonly identified plasmid sequence among STEC O145 strains was IncB/O/K/Z. The genes encoding penicillin resistance (bla_{TEM}) carried by IncB/O/K/Z plasmid was found in *Shigella* strains associated with outbreaks (Kozyreva et al., 2016). IncA/C2 plasmid sequence was present in one each of cattle fecal and hide strains. IncA/C2 plasmid carrying antibiotic resistance genes were found in *E. coli* O145 strains associated with a multistate outbreak in the US in 2010 (Folster et al., 2011).

The antimicrobial resistance genes were present only in a few (5/87; three cattle fecal and two hide strains) of the STEC O145 strains. They carried genes encoding resistance for tetracycline (*tetA* and *tetB*), aminoglycoside (*strA* and *strB*), sulphonamide (*sul2*), phenicol (*floR*) and beta-lactam ($bla_{\text{CMY-2}}$). Antimicrobial resistance genes, such as *floR, strA, strB, sul2,* and *tetA,* were found in *E. coli* O145 strains associated with the previously mentioned multistate outbreak in the US in 2010 (Folster et al., 2011).

All O145 strains except a few fecal (n=7) and hide (n=2) strains belonged to ST-32. *Escherichia coli* O145 strains isolated from wildlife belonging to ST-32 was previously reported (Carter et al., 2016). Additionally, human clinical strains were phylogenetically more closely related to cattle fecal strains and belonged to the same sequence type (ST-32).

In conclusion, STEC O145 strains isolated from cattle feces and hide samples carried the same flagellar type (H28) and their virulence gene profiles were similar. Most of the strains also belonged to the same sequence type (ST-32). Additionally, all of the cattle fecal strains clustered

separately from hide strains, and human clinical strains clustered closely with cattle fecal strains. Separate clustering of fecal and hide strains could be attributed to core genome diversity between fecal and hide strains. Our study demonstrated the presence of potentially pathogenic STEC O145 strains in cattle feces and hides, which could cause foodborne illness in humans.

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Figure 6-1. Phylogenetic tree of *E. coli* O145 strains isolated from cattle feces, hide and human clinical strains constructed using Parsnp v1.2 and visualized using FigTree 1.4.3

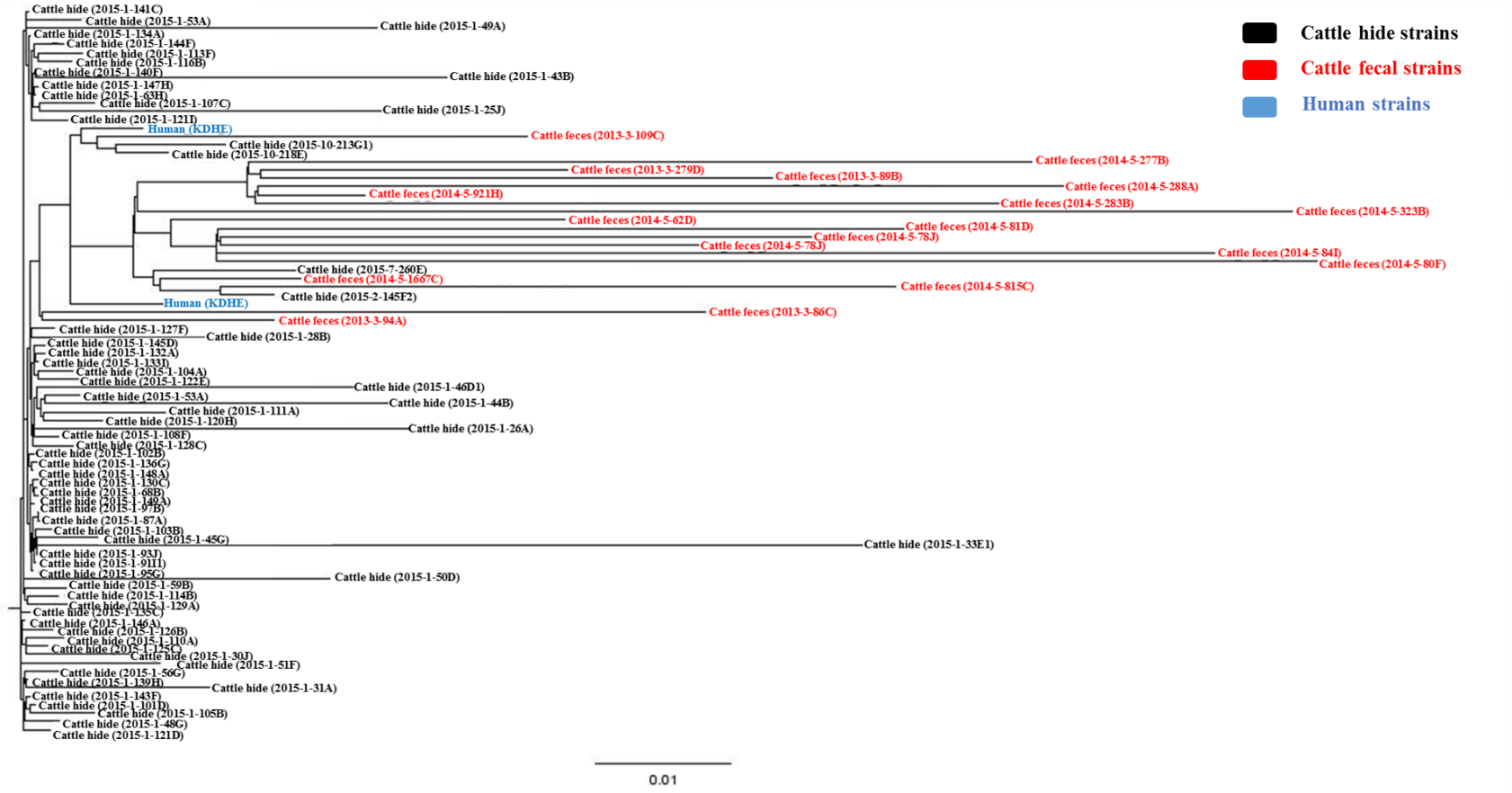

Table 6.1. Average genome size and average number of different categories of genes in STEC O145 strains (n=87) isolated from human and cattle sources based on RAST subsystem annotation

Source	Genome size (Mb)	Functional categories of genes, Mean (Range)				
		Virulence, disease, and defense	Phages, prophages, transposable elements and plasmids	Membrane transport	Iron acquisition and metabolism	Stress response
Cattle feces (n=18)	5.41 (5.25-5.63)	111 (110-118)	260 (224-291)	173 (154-199)	75 (74-75)	189 (185-196)
Cattle hide (n=67)	5.28 (5.21-5.46)	111 (110-115)	236 (203-268)	176 (146-188)	75 (74-75)	190 (183-192)
Human clinical (n=2)	5.29 (5.24-5.33)	110 (110-111)	259 (252-266)	170 (160-179)	75	190 (187-192)

Table 6.2. Distribution of virulence genes in STEC O145 strains from cattle and human sources (n=87) determined using Virulence Finder 1.4

Virulence genes	Product	Source of *E. coli* O145 strains		
		Cattle feces (n=18)	Cattle hide (n=67)	Human (n=2)
Shiga toxins		**No. of strains positive (%)**		
*stx*1a	Shiga toxin 1 subtype a	10 (55.6)	65 (97.0)	2 (100)
*stx*2a	Shiga toxin 2 subtype a	6 (33.3)	0	1 (50)
*stx*2c	Shiga toxin 2 subtype c	2 (11.1)	1 (1.5)	0
Adhesins				
eae	Intimin	18 (100)	67 (100)	2 (100)
iha	IrgA homologue adhesin	18 (100)	67 (100)	2 (100)
LEE encoded Type III secretory system proteins				
tir	Translocated intimin receptor	18 (100)	67 (100)	2 (100)
espA	EPEC secreted protein A	18 (100)	67 (100)	2 (100)
espB	EPEC secreted protein B	18 (100)	67 (100)	2 (100)
espF	EPEC secreted protein F	16 (88.9)	58 (86.6)	2 (100)
Non-LEE encoded effector proteins				
nleA	Non-LEE encoded effector protein A	18 (100)	67 (100)	2 (100)
nleB	Non-LEE encoded effector protein B	18 (100)	67 (100)	2 (100)
nleC	Non-LEE encoded effector protein C	18 (100)	67 (100)	2 (100)
Phage encoded type III secretory system proteins				
espJ	*E. coli*-secreted protein J	18 (100)	67 (100)	1 (50)
cif	Cell-cycle inhibiting factor	18 (100)	66 (98.5)	1 (50)
tccp	Tir-cytoskeleton coupling protein	16 (88.9)	54 (80.6)	2 (100)
Plasmid encoded virulence factors				
ehxA	Enterohemolysin	16 (88.9)	45 (67.2)	1 (50)
katP	Catalase peroxidase	11 (61.1)	52 (77.6)	1 (50)
espP	Extracellular serine protease	18 (100)	60 (89.6)	1 (50)

Antimicrobial resistance genes				
tetA	Tetracycline resistance	2 (11.1)	1 (1.5)	0
tetB	Tetracycline resistance	1 (5.6)	1 (1.5)	0
strA	Aminoglycoside resistance	2 (11.1)	2 (3.0)	0
strB	Aminoglycoside resistance	2 (11.1)	2 (3.0)	0
sul2	Sulphonamide resistance	3 (16.7)	1 (1.5)	0
floR	Phenicol resistance	2 (11.1)	1 (1.5)	0
bla_{CMY2}	Beta-lactam resistance	1 (5.6)	1 (1.5)	0
Other				
gad	Glutamate decarboxylase	18 (100)	67 (100)	2 (100)
iss	Increased serum survival	18 (100)	67 (100)	2 (100)
cba	Colicin B	0	1 (1.5)	0
astA	EAST-1 heat-stable toxin	18 (100)	67 (100)	2 (100)

Table 6.3. Plasmid sequences in STEC O145 strains (n=87) isolated from human and cattle sources identified by PlasmidFinder 1.3

Source	Type of plasmid sequences[a],						
	IncFIB	IncB/O/ K/Z	IncI2	IncI2A	IncA/C2	pO111	IncR
	no. of strains positive						
Cattle feces (n=18)	18	18	2	1	1	1	1
Cattle hide (n=67)	61	65	0	0	1	0	0
Human clinical (n=2)	1	1	0	0	0	0	0

[a]Plasmid types were identified using Plasmid Finder1.3 (Carattoli et al., 2014)

Table 6.4.Total number of phage sequences in STEC O145 strains (n=87) isolated from human and cattle sources based on PHASTER

Source	Type of phage sequences, Mean (Range)[a]			
	Total	Intact	Incomplete	Questionable
Cattle feces (n=18)	17.2 (11-23)	8.5 (6-13)	6.5 (0-14)	2.2 (0-4)
Cattle hide (n=67)	15.3 (9-22)	7.8 (4-13)	6.2 (2-9)	1.3 (0-7)
Human clinical (n=2)	15.5 (15-16)	7 (6-8)	7 (6-8)	1.5 (1-2)

[a]Phage sequences were classified as intact, questionable and incomplete based on the PHASTER scores >90, 70-90, <70 (based on the proportion of phage genes in the identified region), respectively (Arndt et al., 2016; Zhou et al., 2011)

Chapter 7 - *Escherichia coli* O104 in Feedlot Cattle Feces: Prevalence, Isolation and Characterization

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Abstract

Escherichia coli O104:H4, a hybrid pathotype of Shiga toxigenic and enteroaggregative *E. coli*, involved in a major foodborne outbreak in Germany in 2011, has not been detected in cattle feces. Serogroup O104 with H type other than H4 has been reported to cause human illnesses, but their prevalence and characteristics in cattle have not been reported. Our objectives were to determine the prevalence of *E. coli* O104 in feces of feedlot cattle, by culture and PCR detection methods, and characterize the isolated strains. Rectal fecal samples from a total of 757 cattle originating from 29 feedlots were collected at a Midwest commercial slaughter plant. Fecal samples, enriched in *E. coli* broth, were subjected to culture and PCR methods of detection. The culture method involved immunomagnetic separation with O104-specific beads and plating on a selective chromogenic medium, followed by serogroup confirmation of pooled colonies by PCR. If pooled colonies were positive for the wzx_{O104} gene, then colonies were tested individually to identify wzx_{O104}-positive serogroup and associated genes of the hybrid strains. Extracted DNA from feces were also tested by a multiplex PCR to detect wzx_{O104}-positive serogroup and associated major genes of the O104 hybrid pathotype. Because wzx_{O104} has been shown to be present in *E. coli* O8/O9/O9a, wzx_{O104}-positive isolates and extracted DNA from fecal samples were also tested by a PCR targeting $wbdD_{O8/O9/O9a}$, a gene specific for *E. coli* O8/O9/O9a serogroups. Model-adjusted prevalence estimates of *E. coli* O104 (positive for wzx_{O104} and

negative for *wbdD*$_{O8/O9/O9a}$) at the feedlot level were 5.7% and 21.2%, and at the sample level were 0.5% and 25.9% by culture and PCR, respectively. The McNemar's test indicated that there was a significant difference ($P < 0.01$) between the proportions of samples that tested positive for *wzx*$_{O104}$ and samples that were positive for *wzx*$_{O104}$, but negative for *wbdD*$_{O8/O9/O9a}$ by PCR and culture methods. A total of 143 isolates, positive for the *wzx*$_{O104}$, were obtained in pure culture from 146 positive fecal samples. Ninety-two of the 143 isolates (64.3%) also tested positive for the *wbdD*$_{O8/O9/O9a}$, indicating that only 51 (35.7%) isolates truly belonged to the O104 serogroup (positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$). All 51 isolates tested negative for *eae*, and 16 tested positive for *stx*1 gene of the subtype 1c. Thirteen of the 16 *stx*1-positive O104 isolates were from one feedlot. The predominant serotype was O104:H7. Pulsed-field gel electrophoresis analysis indicated that *stx*1-positive O104:H7 isolates had 62.4% homology to the German outbreak strain and 67.9% to 77.5% homology to human diarrheagenic O104:H7 strains. The 13 isolates obtained from the same feedlot were of the same PFGE subtype with 100% Dice similarity. Although cattle do not harbor the O104:H4 pathotype, they do harbor and shed Shiga toxigenic O104 in the feces and the predominant serotype was O104:H7.

Introduction

In the summer of 2011, Germany and other European countries experienced a large outbreak of foodborne illness affecting nearly 4,000 people, with about 900 developing hemolytic uremic syndrome, leading to 54 deaths (Karch et al., 2012). The causative agent was identified as *Escherichia coli* O104: H4, a hybrid serotype possessing characteristics of two pathotypes of *E. coli*, Shiga toxin-producing *E. coli* (STEC) and enteroaggregative *E. coli* (EAEC). The outbreak strain carried Shiga toxin 2 gene (*stx*2) and genes characteristic of EAEC,

such as *aatA* (pAA virulence plasmid marker gene), *aggA* (pilin subunit of aggregative adherence fimbriae I), *aggR* (aggregative adherence fimbriae I transcriptional regulator), but was negative for other enterohemorrhagic *E. coli* (EHEC) genes, such as *stx*1 (Shiga toxin 1), *eae* (intimin) and *ehxA* (enterohemolysin) (Bielaszewska et al., 2011). The serogroup O104 has serotypes other than O104:H4 and at least two of them have been implicated in human illnesses. A *stx*2-carrying O104:H21 serotype that was also negative for *eae* (similar to the O104:H4 German strain) was implicated in an outbreak of hemorrhagic colitis associated with consumption of raw milk in Helena, Montana in 1994 (Feng et al., 2001). Sporadic cases of diarrhea caused by O104:H7, carrying *stx*1 or *stx*2, also negative for *eae*, have been reported (Hussein, 2007; Miko et al., 2013). Non-Shiga toxigenic strains of *E. coli* O104 that were either EAEC or enteropathogenic (EPEC) pathotype have been reported in human patients with diarrhea in South Africa (Tau et al., 2012).

Because cattle are a primary reservoir of STEC, studies have been conducted to determine whether cattle harbor the O104:H4 serotype. Wieler et al. (Wieler et al., 2011) tested 100 cattle fecal samples from 34 different farms in the outbreak region of Germany and found that none of the fecal samples were positive for *E. coli* strains carrying genes characteristic of O104:H4. Auvray et al. (Auvray et al., 2012) analyzed 1,468 cattle fecal samples collected from several slaughter facilities in France by real-time and conventional PCR assays that targeted *stx*2, wzx_{O104}, $fliC_{H4}$ (H4 flagellar gene) and *aggR* and reported that none of the fecal samples were positive for all four genes. We conducted a study to detect *E. coli* O104:H4 in feedlot cattle fecal samples (n = 248) using a multiplex PCR that targeted O104 (wzx_{O104}), H4 ($fliC_{H4}$), aggregative adherence fimbriae 1 (*aggA*), Shiga toxins 1 and 2 (*stx*1 and *stx*2), intimin (*eae*), tellurite resistance (*terD*), and enterohemolysin (*ehxA*), characteristic of the outbreak serotype and

reported that cattle feces were positive for the O104 serogroup, but negative for the hybrid pathotype (Paddock et al., 2013). In that study, fecal samples positive for *wzx*$_{O104}$ were plated onto several selective and differential media, and only a small number of PCR-positive samples yielded pure cultures of serogroup O104 and none of the isolates carried Shiga toxin genes. The likely reason for the poor recovery of O104 from PCR-positive fecal samples was that the culture method did not have an immunomagnetic separation (IMS) step because O104-specific IMS beads were not available at the time the study was conducted. Therefore, our objectives were to determine the prevalence of *E. coli* O104 in feedlot cattle feces utilizing a culture method involving an IMS step and a PCR-based method of detection, and to characterize the isolated strains. The culture method utilized in the study involved an enrichment step, followed by IMS with O104-specific IMS beads, plating on a chromogenic selective medium and confirming the O104 serogroup and major virulence genes by a multiplex PCR. The *wzx*$_{O104}$ gene that was targeted in the PCR assay to detect O104 has also been reported in O8, O9 and O9a serogroups of *E. coli* (Whitfield and Roberts, 1999). Therefore, pre- and postenriched fecal suspensions and putative *E. coli* O104 isolates were also subjected to a PCR assay with primers that targeted *wbdD* (which codes for methyl and kinase transferase), specific for *E. coli* O8, O9 and O9a serogroups (Wang et al., 2001).

Materials and Methods

Sample collection

The study was approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC # 3172). Rectal content samples were collected from feedlot cattle immediately after slaughter at a Midwest slaughter plant during two visits, one week apart, in July 2013. The permission to collect samples was given under an agreement that the name and

location of the abattoir will not be disclosed. Rectums were incised and contents were scooped with a plastic spoon. The spoon with the contents (approximately 10 to 20 g) were placed in a Whirl-Pak bag (Nasco, Ft. Atkinson, WI), transported on ice in a cooler to the Preharvest Food Safety Laboratory at Kansas State University and processed within 24 h. Rectal content samples were collected from a total of 757 cattle (both heifers and steers) originating from 29 feedlots located in six Midwestern States (IA, IL, MN, MO, NE and SD). Sixteen to 38 samples were collected per lot of cattle from a total of 35 lots, with each lot consisting of 36 to 227 animals. Cattle from one feedlot (No. 2) were sampled in both weeks.

Culture method of detection

Approximately two grams of fecal samples were suspended in 18 ml of *E. coli* broth (EC; Difco™, Becton, Dickinson Co., Sparks, MD) and incubated at 40° C for 6 h (Paddock et al., 2013). Post-enrichment fecal samples were subjected to a culture-based procedure, which involved immunomagnetic separation with O104 serogroup-specific beads (Abraxis1, Warminster, PA), and plating onto a selective chromogenic Possé medium (Possé et al., 2008) modified to include novobiocin at 5 mg/l and potassium tellurite at 0.5 mg/l (MP) (Noll et al., 2015a). After 20–24 h of incubation at 37° C, six chromogenic colonies (mauve, pink, or purple) were picked and streaked onto blood agar and incubated at 37° C for 24 h. The six colonies were pooled, boiled and the lysate was subjected to an eight-plex PCR targeting O-antigen genes of O104 (wzx_{O104}) and the seven major serogroups of STEC (O26, O45, O103, O111, O121, O145 and O157). If pooled colonies were positive for wzx_{O104}, then the six colonies were tested individually by a nine-plex PCR to identify pure culture of putative O104 serogroup (wzx_{O104}) and associated major genes of the STEC and EAEC pathotypes: *stx*1 (Shiga toxin 1), *stx*2 (Shiga toxin 2), *eae* (intimin), *ehxA* (enterohemolysin), *terD* (tellurite resistance), *aggA* (pilin subunit of

aggregative adherence fimbriae 1), *bfpA* (bundle-forming pilus) and *fliC*$_{H4}$ (H4-specific flagella). The primer pairs used for the eight-plex and nine-plex PCR assays are listed in Table 7.1. PCR amplification protocol for both assays included an initial denaturation at 94° C for 5 min followed by 25 cycles (pure culture) or 35 cycles (fecal suspension in broth) of 94° C for 30 s, 65° C for 30 s, 68° C for 75 s, and the final extension was 68° C for 7 min (Paddock et al., 2013). Isolates confirmed as positive for the *wzx*$_{O104}$ gene were stored on cryogenic beads (CryoCare™, Key Scientific Products, Round Rock, TX).

PCR method of detection

One ml of pre- and post-enriched fecal suspensions were removed, boiled for 10 min and centrifuged at 9,300 x g for 5 min. The DNA in the supernatant was purified using a GeneClean® Turbo Kit (MP Biomedicals LLC., Solon, OH). DNA extracted from pre- and post-enrichment samples were tested by a nine-plex PCR to detect O antigen of O104 serogroup (*wzx*$_{O104}$) and associated major genes of the STEC and EAEC (*stx*1, *stx*2, *eae*, *ehxA*, *terD*, *aggA*, *bfpA* and *fliC*$_{H4}$) and a single-plex PCR assay to detect *wbdD*$_{O8/O9/O9a}$, a gene specific for serogroups O8, O9 and O9a (Wang et al., 2001).

Characterization of the *E. coli* O104 isolates

All putative O104 isolates (positive for *wzx*$_{O104}$) were tested by a PCR assay targeting O8/O9/O9a (*wbdD*$_{O8/O9/O9a}$), and isolates positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$ were considered as truly O104, and were further characterized. The flagellar types of the O104 isolates were identified by a multiplex PCR targeting five flagellar types (H2, H4, H7, H11 and H21) (Table 1) using the PCR running conditions as described before for the nine- or eight-plex PCR. The subtype of Shiga toxin genes was identified by nucleotide sequencing. Shiga toxin genes of the *wzx*$_{O104}$-positive isolates were amplified (F-GCTCAAGGAGTATTGTGTAATATG and R-

TCGCTGAATCCCCYTC) by a touchdown PCR method where the annealing temperature of each cycle was lowered gradually to avoid amplification of non-specific sequences (Don et al., 1991). PCR amplification protocol included an initial denaturation at 94°C for 5 min, 10 cycles of touchdown PCR (denature: 94°C for 30 s, annealing: 56–51°C (Δ-0.5°C) for 30 s; extension: 72°C for 1 min 45 s) followed by 30 cycles of regular PCR (denaturation: 94°C for 30 s, annealing: 51°C for 30 s; extension: 72°C for 1 min 45 s). Amplicons (1,233 bp) were purified using QIAquick1 PCR Purification Kit. The purity and concentration of purified PCR products were determined using a spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE). The products were then shipped to Genewiz Inc., (South Plainfield, NJ) for sequencing. The sequence data were aligned using the CLC Main Workbench 6.8.4 software for further analysis. Shiga toxin subtypes were determined according to the procedure described by Scheutz et al., (Scheutz et al., 2012). Briefly, nucleotide sequences were translated to amino acid sequences after removing intergenic regions. The subtype of Shiga toxins was determined based on the amino acid motifs that define each Shiga toxin subtype (Scheutz et al., 2012). Additionally, the subtyping of Shiga toxin genes was confirmed by PCR-RFLP (restriction fragment length polymorphism) (Beutin et al., 2007). Briefly, the B subunit of *stx*1 genes was amplified yielding a 283 bp amplicon. PCR amplification protocol included an initial denaturation at 94.0°C for 5 min, followed by 35 cycles of 94.0°C for 30 s, 51.3°C for 60 s, 72.0°C for 40 s. PCR products were purified using QIAquick® PCR purification Kit, digested separately with *BstE*II, *Hae*II and *Pflm*I enzymes. Restriction fragments were visualized by microcapillary electrophoresis using Qiaxcel (Qiagen, Valencia, CA). Additionally, subtyping of *stx*1was conducted by *in silico* RFLP. A 283 bp fragment starting with CAGTTGAGGGGGGTAAAATG (forward primer used for amplification of *stx*1 in PCR-RFLP) and ending with GATTCAGCGAAGTTATTTTCCG

(reverse complement of reverse primer used for amplification of *stx*1 in PCR-RFLP) was digested by *BstE*II, *Hae*II and *Pflm*I using CLC Main Workbench 6.8.4 software. Restriction patterns of O104 STEC isolates were compared to reference sequences of three *stx*1 subtypes from the NCBI database (*stx*1a: Accession no. M16625.1; *stx*1c: Accession no. DQ449666.1; *stx*1d: Accession no. AY170851.1).

Pulsed-field gel electrophoresis (PFGE)

The clonal relationships between O104 STEC strains isolated from cattle feces were assessed by PFGE typing. The human clinical strains, German (O104:H4; BAA-2326) and Montana outbreak (O104:H21; BAA-178) and O104:H7 strains (06–3637, 07–3598, 08–4061, 2011C-3665,2012C-3400; provided by Nancy A. Strockbine, Centers for Disease Control and Prevention, Atlanta, GA) were also included for comparison. The PFGE was performed according to the Centers for Disease Control and Prevention's PulseNet protocol. Briefly, agarose embedded DNA of the O104 isolates were digested with *Xba*I followed by separation of restriction fragments by electrophoresis. *Salmonella enterica* serotype Braenderup (strain H9812) DNA digested with *Xba*I was used as DNA marker. Gel images were captured with a Gel Doc 2000 system (Bio-Rad). PFGE patterns were analyzed using the Bionumerics software (Applied Maths, Inc., Austin, TX). Band-based Dice similarity coefficients and unweighted pair-group method for clustering with a position tolerance setting of 1.5% were used for optimization and band comparison. Isolates with 100% homology were grouped as subtypes and those isolates which were > 96% but less than 100% homologous were grouped as types based on the Dice coefficients.

Statistical analysis

Generalized linear mixed models (GLMM) were fitted using a binary or binomial (events/trials) distribution, logit link, Laplace estimation, and Newton-Raphson and Ridging optimization, to estimate fecal prevalence of *wzx*$_{O104}$ and *wzx*$_{O104}$/*wbdD*$_{O8/O9/O9a}$ in Proc Glimmix (SAS 9.3, SAS Institute Inc., Cary, NC). Sample-, lot- and feedlot-level prevalence were calculated as the proportion of samples, samples within lots or samples within feedlots testing positive for *wzx*$_{O104}$ (serogroup O104 and or O8/O9/O9a) and positive for *wzx*$_{O104}$ but negative for *wbdD*$_{O8/O9/O9a}$ (serogroup O104 only) by PCR or culture methods, and divided by the total number of samples tested per lot or per feedlot, respectively. Random effects were used to account for the hierarchical structure of the data (samples within lots, lots within feedlots and feedlots within states). Prevalence estimates (and 95% confidence intervals) were obtained from model intercepts using the formula $p = e^{\beta 0} / (1 + e^{\beta 0})$, where β^0 is the coefficient of the model intercept. The Cohen's Kappa statistic was used to compute the agreement beyond that due to chance between PCR- and culture-based methods for detection of serogroup O104 and or O8/O9/O9a (positive for *wzx*$_{O104}$) and serogroup O104 only (*wzx*$_{O104}$ positive but negative for *wbdD*$_{O8/O9/O9a}$) in fecal samples. Interpretation of the Kappa statistic was based on the scale proposed by Landis and Koch (Landis and Koch, 1977). The McNemar's Chi-square test was used to compare the proportion of positive samples detected by both methods (McNemar, 1947). When the *P*-value of the McNemar's test is not significant ($P > 0.01$), there is little evidence to conclude that the proportion of positives are different, whereas when the *P*-value is significant ($P < 0.01$), there is a significant disagreement between tests, indicating little value in assessing agreement. In the latter case, Cohen's Kappa statistics are provided for reference only.

Results

Culture method of detection

Of the total 757 fecal samples tested, 146 samples (19.3%) tested positive for serogroup O104 (and/or O8/O9/O9a), based on PCR testing (for the *wzx*$_{O104}$ gene) of the six-pooled colonies picked from the O104 IMS beads-inoculated MP plates. Fecal samples from 21 of 29 feedlots (72.4%) and 27 of 35 lots (77.1%) tested positive for the serogroup O104 (and/or O8/O9/O9a) (Table 7.2). At the sample level, the crude prevalence of serogroup O104 (and or O8/O9/O9a) within feedlots ranged from 0 to 93.8%. Because six chromogenic colonies were picked from O104 beads-plated medium, pooled, and tested by a multiplex PCR targeting a total of eight serogroups (O26, O45, O103, O104, O111, O121, O145, and O157), other *E. coli* serogroups were also detected occasionally. Pooled colonies from fecal samples (n = 757) also tested positive for O26 (3.7%), O45 (0.9%), O103 (5.8%), O145 (0.5%) and O157 (4.9%). Serogroups O111 and O121 were not detected in any of these samples. The testing of individual colonies was designed to identify *wzx*$_{O104}$-positive serogroup and associated virulence genes only; therefore, individual isolates of the other seven serogroups were not identified. A total of 143 isolates, positive for the *wzx*$_{O104}$, were obtained in pure culture from 146 positive (based on pooled colonies) fecal samples. Ninety-two of the 143 isolates (64.3%) also tested positive for the *wbdD*$_{O8/O9/O9a}$ (Table 7.2), indicating that only 51 (35.7%) isolates truly belonged to the O104 serogroup (positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$). After excluding the samples that yielded isolates positive for O8/O9/O9a, only 14 of the 29 (48.3%) feedlots, 20 of the 35 lots (57.1%), and 49 of the 757 (6.5%) samples were considered truly positive for the serogroup O104.

PCR method of detection

Of the nine genes included in the multiplex PCR assay, two genes, *aggA* and *bfpA*, which code for aggregative adherence fimbriae 1 and bundle forming pili, respectively, were not detected in any of the fecal samples, either before or after enrichment in EC broth (Table 7.3). The overall prevalence of *wzx*$_{O104}$ gene in samples before and after enrichment was 5% (38/757) and 46.1% (349/757), respectively. Based on the single-plex assay of samples targeting *wbdD*$_{O8/O9/O9a}$, 13 (1.7%) and 238 (31.4%) of the 757 samples tested were positive-before and after enrichment, respectively. Thirty-four (4.5%) and 194 (25.6%) fecal samples were positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$, suggesting that those fecal samples truly contained *E. coli* O104 (Table 3). A higher proportion of fecal samples tested positive for the *stx*2 than *stx*1 gene (66.8 vs. 27.5%) in enriched samples. Among the genes tested, *ehxA* that codes for enterohemolysin was the most prevalent in both pre- and post-enriched samples, followed by *flic*H4, *terD*, *eae*, *stx*2 and *stx*1 genes (Table 7.3). Based on the PCR assay of post-enriched fecal suspensions, 26 of 29 feedlots (89.7%) and 32 of 35 lots (91.4%) contained one or more fecal samples that tested positive for *wzx*$_{O104}$ (serogroup O104 and or O8/O9/O9a). At the sample level, the crude prevalence of *wzx*$_{O104}$ within feedlots ranged from 0 to 95%. Of the three feedlots that were negative for *wzx*$_{O104}$, two had samples that tested positive for *wbdD*$_{O8/O9/O9a}$. Twenty-three of the 29 feedlots (79.3%) and 29 of the 35 lots (82.9%) were positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$, indicating that cattle feces from these feedlots can be considered truly positive for serogroup O104. Five of the 29 feedlots were negative for *wbdD*O8/O9/O9a and of those four were positive for *wzx*$_{O104}$ (Table 7.4). Model-adjusted prevalence estimates of *wzx*$_{O104}$ -positive fecal samples (serogroups O104 and/or O8/O9/O9a) and *wzx*$_{O104}$ -positive fecal samples that were negative for *wbdD*$_{O8/O9/O9a}$ (serogroup O104 only) at the sample-, lot- and feedlot-

levels detected by culture and PCR methods are presented in Table 7.5. The McNemar's test indicated that there was a significant difference ($P < 0.01$) between the proportions of samples that tested positive for *wzx*$_{O104}$ and samples that were positive for *wzx*$_{O104}$, but negative for *wbdD*$_{O8/O9/O9a}$ by PCR and culture methods, hence the Kappa statistics (κ = 0.27; κ 95% CI = 0.21-0.32 for samples that tested positive for *wzx*$_{O104}$ and κ = 0.10; κ 95% CI = 0.04–0.17 for samples that were positive for *wzx*$_{O104}$, but negative for *wbdD*$_{O8/O9/O9a}$) are provided for reference only.

Characteristics of the *E. coli* O104 isolates

Of the 51 O104 isolates (positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$), 16 isolates (31.4%) carried *stx*1 gene and all 16 also tested positive for *ehxA* and *terD* genes (Table 7.6). None of the O104 isolates carried *stx*2, *fliC*$_{H4}$, *eae*, *bfpA* and *aggA* genes (Table 7.6). Thirteen of the 16 *stx*1-positive O104 isolates were from one feedlot. Based on PCR assays targeting flagellar genes, 37 isolates tested positive for H7, four for H2, one each for H11 and H21, eight were unidentified, and none of the isolates tested positive for H4. The 16 *stx*1-positive O104 isolates possessed the H7 flagellar type. Amino acid sequences deduced from nucleotide sequences of *stx*1 amplicons indicated that the Shiga toxin genes of all O104 isolates (n = 16) were of subtype 1c. Digestion of *stx*1 amplicon (283 bp) with *BstE*II yielded two fragments of 224 and 59 bp whereas a single undigested fragment of 283 bp was produced with *Hae*II and *PflM*I enzymes. *In silico* RFLP analysis indicated that restriction patterns of Shiga toxin genes of O104 isolates were similar to that of *stx*1c of the reference strain (*E. coli* strain BCN26-Accession no. DQ449666.1; Fig 7.1) and matched the PCR-RFLP results (data not shown).

Pulsed-field gel electrophoresis (PFGE)

The 16 *stx*1-positive *E. coli* O104 isolates obtained in the present study, German (O104:H4) and Montana outbreak (O104:H21) strains and the five human O104:H7 strains formed seven separate PFGE clusters (Fig 7.2). Bovine O104 strains were 62.4% similar to the German and Montana outbreak strains and 67.9% to 77.5% similar to human O104:H7 strains (Fig 7.2). The Dice similarity between the German outbreak and the Montana strains was 73%. The *stx*1-positive O104 strains (n = 13) obtained from the same feedlot were of the same PFGE subtype with 100% similarity, and the remaining three STEC O104 strains from different feedlots were of the same PFGE type (96–100% similarity).

Discussion

The prevalence of *wzx*$_{O104}$-positive *E. coli* was determined in feedlot cattle feces by culture and PCR methods of detection. Our primary goal was to detect the prevalence of serogroup O104. However, the target gene, *wzx*$_{O104}$, used in the culture and PCR methods of detection is also present in *E. coli* O8, O9, and O9a serogroups (Wang et al., 2001). In fact, the O antigen gene cluster of O104 serogroup has the same genes (O antigen polymerase gene, O antigen flippase gene [*wzx*$_{O104}$], three CMP-sialic acid synthesis genes, and three glycosyl transferase genes) in the same order as that of the gene cluster that codes for the K9 capsular antigen of O8, O9 and O9a serogroups (Wang et al., 2001; Whitfield and Roberts, 1999). Therefore, a *wzx*$_{O104}$-positive fecal sample could contain *E. coli* O104 and or O8/O9/O9a. In order to distinguish O104 from O8/O9/O9a, we assayed all fecal samples and pure cultures of *wzx*$_{O104}$-positive isolates by PCR with primers targeting *wbdD*$_{O8/O9/O9a}$, a gene that is specific for

O8/O9/O9a (Wang et al., 2001). A fecal sample or an O104 isolate that was positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$ was considered as truly positive for serogroup O104.

The fecal samples collected in our study to estimate prevalence of *wzx*$_{O104}$-positive *E. coli* were representative of a large population of cattle originating from 29 feedlots located in six Midwestern states. The prevalence of *wzx*$_{O104}$-positive fecal sample, determined by PCR, reported in our study (46.1%) was higher than that reported (20.6%) by Paddock et al.(Paddock et al., 2013), possibly because samples collected were from multiple feedlots (29 vs 8). Based on the culture method, 19.3% of fecal sample were positive for *E. coli* containing *wzx*$_{O104}$ compared to 2.8% reported by Paddock et al (Paddock et al., 2013). In addition, the use of the O104-specific IMS beads likely increased the sensitivity of detection. None of the previous studies has utilized IMS beads because O104-serogroup specific beads had been commercially available only recently (Baranzoni et al., 2014).

In the culture method, fecal samples that tested positive (pooled colonies) for *E. coli* possessing the *wzx*$_{O104}$ gene indicated the sample was positive for O104 and/or O8.O9/O9a. However, isolates positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$, which could be considered as truly *E. coli* O104, were obtained from 6.5% fecal samples. Ninety-two of the 143 *wzx*$_{O104}$-positive isolates (64.3%) were also positive for *wbdD*$_{O8/O9/O9a}$ by PCR, which means the isolates were not O104, but could be *E. coli* O8, O9, or O9a. Isolates positive for *wzx*$_{O104}$ and *wbdD*$_{O8/O9/O9a}$ have been reported in previous studies (Delannoy et al., 2012a; Paddock et al., 2013). Fecal prevalence of *E. coli* O8/O9/O9a has been previously reported in cattle (Amézquita-López et al., 2012; Manna et al., 2010). Manna et al. (2010) tested cattle feces collected at a slaughter plant in India for *E. coli* O8 and reported a prevalence of 2% (Manna et al., 2010). Some of the chromogenic colonies picked from O104 beads-plated medium tested positive for

other *E. coli* serogroups, such as O26, O45, O103, O145 and O157, which indicates some cross-reactivity of O104 beads with other serogroups. Unfortunately, the multiplex PCR targeting eight serogroup-specific genes (O104, O157 and 6 non-O157) that was used to test pooled colonies did not include the *wbdD*$_{O8/O9/O9a}$ gene. Therefore, we could not ascertain the prevalence of serogroups of O8/O9/O9a in the pooled colonies. The detection of O157 and six non-O157 serogroups was low suggesting that non-specificity of the O104 beads does not appear to be an issue compared to IMS beads for other serogroups, particularly O103 (Noll et al., 2015a). We picked colonies with a range of color because chromogenic colonies of pure cultures of O104 serogroup on MP medium were indistinguishable from other serogroups of STEC.

Our study showed that feedlot cattle harbor *E. coli* O104 serogroup (positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$) in the gut and shed these organisms in their feces, however only a small proportion of the O104 isolates obtained carried the Shiga toxin gene and none exhibited the enteroaggregative genes of the pathotype of the German outbreak strain. Unlike other predominant STEC serogroups (O157 and non-O157 STEC) causing human illnesses, O104:H4 serotype has never been reported in animals. Previous studies that aimed at detecting the O104:H4 serotype carrying genes characteristic of the German outbreak strain in cattle feces reported that cattle do not harbor the combination of genes (*wzx*$_{O104}$, *stx*1, *stx*2, *fliC*$_{H4}$, *aggA* or *aggR*) that are unique to this pathotype (Auvray et al., 2012; Paddock et al., 2013; Wieler et al., 2011). The present study confirms the absence of the unique pathotype in cattle feces in this population of feedlot cattle, based on both PCR and culture-based detection methods. *E. coli* O104 strains with H antigen, other than H4, have been isolated from animals (Blanco et al., 2004; Blanco et al., 2003; European Centre for Disease Prevention and Control, 2013). Blanco et al. (Blanco et al., 2003) have reported that O104:H7, positive for *stx*1 and

negative for *stx*2 and *eae*, was one of the eight non-O157 serotypes more frequently detected among STEC strains in sheep in Spain, and interestingly, in the same study, none of the non-O157 STEC strains isolated from cattle included O104. Serotype O104:H21, positive for *stx*1 and *stx*2, but negative for *eae*, has been isolated from feces of healthy and diarrheic cattle in Spain (Blanco et al., 2004). None of the O104 isolates in our study tested positive for *stx*2 and the one isolate of H21 serotype obtained was negative for *stx*. To our knowledge, this is the first report of Shiga toxin carrying O104 serogroup in feces of cattle in the US.

Of the 51 O104 isolates (positive wzx_{O104} and negative for $wbdD_{O8/O9/O9a}$), 16 (31.4%) carried a combination of *stx*1, *terD* and *ehxA* genes. Because the modified MP medium contained potassium tellurite, it is possible that there was a selection pressure exerted for *terD*-positive isolates. The *st*x1 of O104 isolates were of subtype *stx*1c based on nucleotide sequencing. Shiga toxin subtyping based on amino acid sequences were further confirmed by *in silico* RFLP, which matched results obtained from PCR-RFLP. Our study shows that *in silico* RFLP, a simple and rapid method, is a reliable alternative to PCR-RFLP for subtyping of *stx*. None of the O104 isolates obtained in the present study were positive for *eae*, indicating that serogroup O104 in our study population could be Shiga toxigenic, but not enterohemorrhagic *E. coli*. The absence of *eae* appears to be a feature of the serogroup O104 because previously reported serotypes such as O104:H4 (German outbreak strain; (Bielaszewska et al., 2011)), O104:H21 (Montana outbreak strain; (Feng et al., 2001)), and O104:H7 (CDC strains from sporadic diarrheal cases (Delannoy et al., 2012a; Miko et al., 2013) were all negative for *eae*. Based on PFGE typing, the O104:H7 strains of cattle origin were only 67.9% to 77.5% similar to human O104:H7 strains. Intimin-negative STEC isolates of serogroups O5, O76, O78, O113, O128, O146, O174, O178, and O181 carrying *stx*1c have been isolated from stools of asymptomatic carriers and individuals with

diarrhea (Friedrich et al., 2003). The O104 strains were also negative for *aggA* and *bfpA*, which are responsible for adherence to host cells in enteroaggregative *E. coli* (Nataro et al., 1993) and enteropathogenic *E. coli* (Cleary et al., 2004), respectively. All 16 O104 STEC strains isolated in our study carried H7 flagellar type and possessed the same profile of virulence genes tested (*ehxA* and *terD*). Miko et al. (Miko et al., 2013) have reported that STEC strains carrying same flagellar type generally harbor similar virulence genes. Thirteen of the 16 *stx*1-positive isolates were from the same feedlot and all 13 were of the same PFGE type, suggesting spread of a single clone within a feedlot.

Escherichia coli is a continuously evolving organism with the capacity to acquire virulence genes from other pathogenic organisms and become virulent (Moriel et al., 2012). Sialic acid which has been reported to be an important component of *E. coli* O104 antigen and other organisms such as *E. coli* O24, O56, *Campylobacter jejuni*, *Salmonella enterica*, and *Citrobacter freundii* (Gamian and Kenne, 1993; Kedzierska, 1978), is also an important component of animal tissues. This trait of bacterial antigens may contribute to evasion of immune system by mimicking the host tissue component (Wang et al., 2001). Therefore, STEC O104:H7 serotype has the potential to be a human pathogen. Because the prevalence of O104 is low in cattle and only a small proportion of O104 is STEC, cattle are not likely to be a major reservoir for *E. coli* O104. *Escherichia coli* O104:H4 involved in the German outbreak in 2011 is a classic example of the emergence of a highly virulent pathogen by acquisition of prophage encoding Shiga toxin 2 through horizontal gene transfer (Hao et al., 2012). Similarly, *E. coli* O104 with H types other than H4 has the potential to emerge as a virulent pathogen by acquiring Shiga toxins 1 and or 2 via phage-mediated transfer.

Conclusions

Cattle harbor and shed *eae*-negative serogroup O104 in feces, however, none of the isolated strains in this study carried genes characteristic of the hybrid serotype reported in Germany (*stx*2, *aggA* and *fliC*$_{H4}$) and only a small proportion of O104 strains carried the *stx*1 gene. The predominant STEC serotype detected in cattle feces was O104:H7, which has been previously isolated from sporadic cases of diarrhea in humans. Based on our results, cattle are not a reservoir of O104:H4 serotype, however, they do harbor other O104 serotypes, such as O104:H2, O104:H7, O104:H11 and O104:H21.

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Figure 7-1. *In silico* restriction fragment length polymorphism (RFLP) subtyping of Shiga toxin genes of O104 isolates. (A) RFLP pattern of Shiga toxin of an O104 isolate; (B) RFLP pattern of *stx*1c of a reference sequence (Accession no. DQ449666.1); (C) RFLP pattern of *stx*1a of a reference sequence (Accession no. M16625.1); (D) RFLP pattern of *stx*1d of a reference sequence (Accession no. AY170851.1)

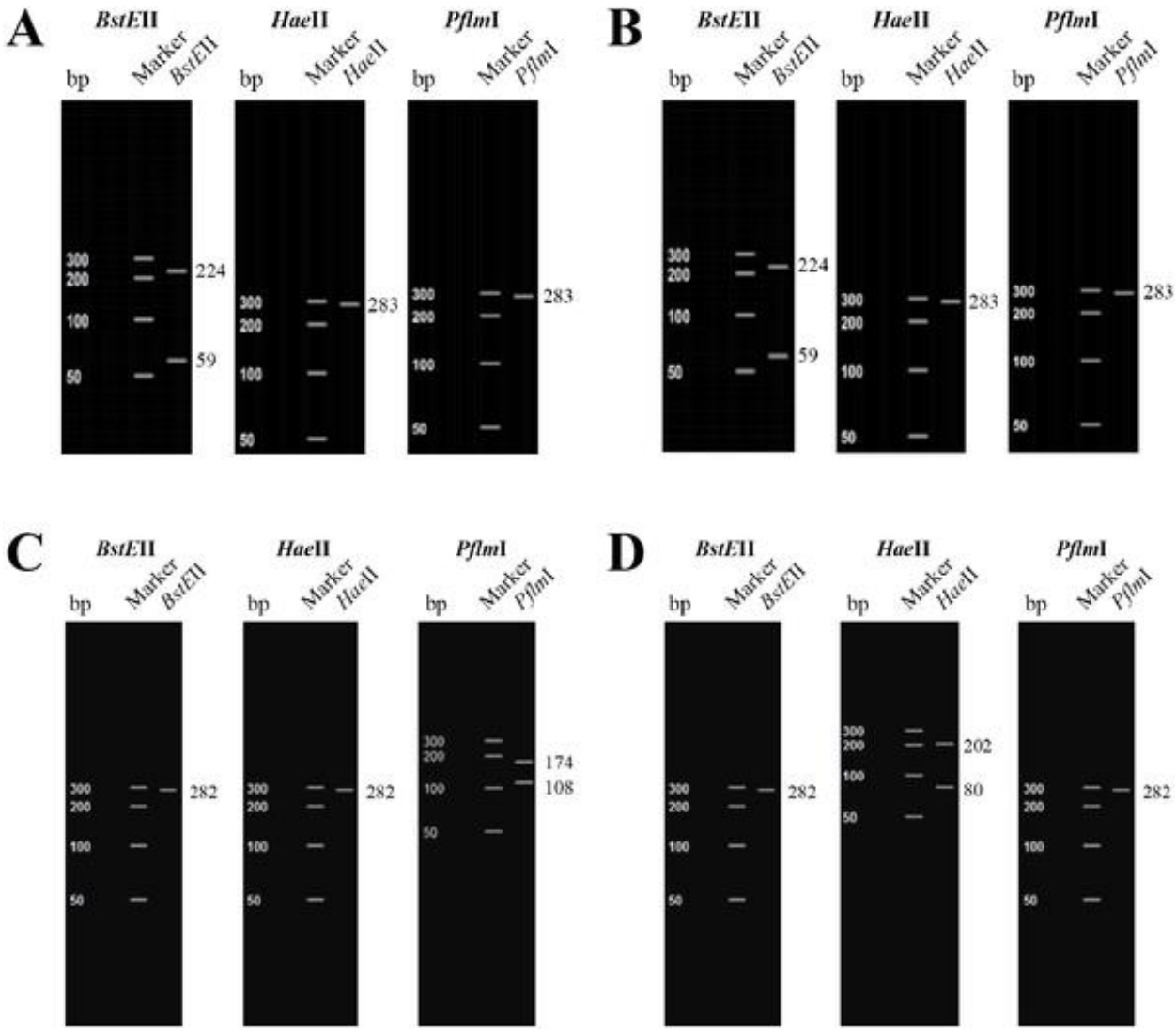
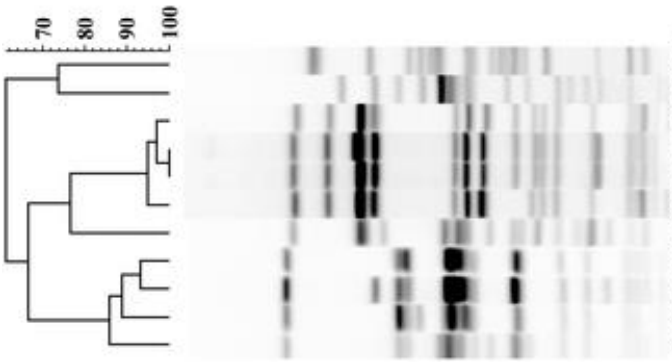


Figure 7-2. Pulsed-field gel electrophoresis-based clustering of *Escherichia coli* O104 strains from cattle feces and human clinical strains (O104:H4; O104:H21; and O104:H7)



Serotype	Strain	Source	Virulence genes		
			*stx*1	*stx*2	*eae*
O104:H4	BAA-2326	Human	-	+	-
O104:H21	BAA-178	Human	-	+	-
O104:H7	2013-6-669B	Cattle	+	-	-
O104:H7	2013-6-48C	Cattle	+	-	-
O104:H7	2013-6-122E	Cattle	+	-	-
O104:H7	2013-6-148B	Cattle	+	-	-
O104:H7	2011C-3665	Human	+	-	-
O104:H7	07-3598	Human	-	-	-
O104:H7	08-4061	Human	-	+	-
O104:H7	06-3637	Human	-	+	-
O104:H7	2012C-3400	Human	-	+	-

Table 7.1. Target genes, primer sequences used and size of amplicons in PCR assays

Target gene	Primer sequences	Amplicon size (bp)	Reference
***wzx*$_{O26}$**	F:AGGGTGCGAATGCCATATT R:GACATAATGACATACCACGAGCA	417	Bai et al., 2012
***wzx*$_{O45}$**	F: GGGCTGTCCAGACAGTTCAT R: TGTACTGCACCAATGCACCT	890	Bai et al., 2012
***wzx*$_{O103}$**	F: GCAGAAAATCAAGGTGATTACG R: GGTTAAAGCCATGCTCAACG	740	Bai et al., 2012
***wzx*$_{O104}$**	F: GGTTTTATTGTCGCGCAAAG R: TATGCTCTTTTTCCCCATCG	337	Paddock et al., 2013
***wzx*$_{O111}$**	F: ACAAGAGTGCTCTGGGCTTC R: AAACTAAGTGAGACGCCACCA	230	Noll et al., 2015
***wbq*E + *wbq*F$_{O121}$**	F: TCAGCAGAGTGGAACTAATTTTGT R: TGAGCACTAGATGAAAAGTATGGCT	587	Bai et al., 2012
***wzx*$_{O145}$**	F: TCAAGTGTTGGATTAAGAGGGATT R: CACTCGCGGACACAGTACC	523	Bai et al., 2012
***rfb*E$_{O157}$**	F: CAGGTGAAGGTGGAATGGTTGTC R: TTAGAATTGAGACCATCCAATAAG	296	Bai et al., 2010
***wbdD*$_{O8/O9/O9a}$**	F: GGCATCGGTCGGTATTCC R: TGCGCTAATCGCGTCTAC	1000	Wang et al, 2001
***stx*1**	F: TGTCGCATAGTGGAACCTCA R: TGCGCACTGAGAAGAAGAGA	655	Bai et al., 2010
***stx*2**	F: CCATGACAACGGACAGCAGTT R: TGTCGCCAGTTATCTGACATTC	477	Bai et al., 2010
eae	F: CATTATGGAACGGCAGAGGT R: ACGGATATCGAAGCCATTTG	375	Bai et al., 2010
ehxA	F: GCGAGCTAAGCAGCTTGAAT R: CTGGAGGCTGCACTAACTCC	199	Bai et al., 2010
terD	F: AGTAAAGCAGCTCCGTCAAT	434	

	R: CCGAACAGCATGGCAGTCT		Bielaszewska et al., 2011
aggA	F: CGTTACAAATGATTGTCCTGTTACTAT R: ACCTGTTCCCCATAACCAGAC	151	Paddock et al., 2013
bfpA	F: CAGAAGTAATGAGCGCAACG R: CGTAGCCTTTCGCTGAAGTA	285	This study
***fliC*$_{H4}$**	F: ACGGCTGCTGATGGTACAG R: CGGCATCCAGTGCTTTTAAC	244	Paddock et al., 2013
***fliC*$_{H2}$**	F: GCAACGGCTGAAACAACCTA R: TGCAGTTACAACTTCGGTTTTG	585	This study
***fliC*$_{H4}$**	F: ACGGCTGCTGATGGTACAG R: CGGCATCCAGTGCTTTTAAC	244	Paddock et al., 2013
***fliC*$_{H7}$**	F: AGCTGCAACGGTAAGTGATTT R: GGCAGCAAGCGGGTTGGTC	949	Bai et al., 2010
***fliC*$_{H11}$**	F: TCTGACACAAACATAGCTGGTACA R: TGTCTCACTCGTAATCAAAGAAGC	228	This study
***fliC*$_{H21}$**	F: TCGATGGCGCGCAGAAAGCA R: GGCTGTCGTAGGGGCAACGG	419	Sekse et al., 2011

Table 7.2. Number of fecal samples from feedlot cattle positive for *wzx*$_{O104}$ possessing *E. coli* (serogroups O104 and/or O8/O9/O9a) based on the culture method and *wzx*$_{O104}$-positive isolates that tested positive for *wbdD*$_{O8/O9/O9a}$ (serogroups O8/O9/O9a) or negative for *wbdD*$_{O8/O9/O9a}$ (serogroup O104)

Week	Feedlot no.	Lot No.	No. of samples collected	No. of samples positive for *wzx*$_{O104}$ (%)[a]	No. of *wzx*$_{O104}$-positive isolates	
					Positive for *wbdD*$_{O8/O9/O9a}$ (%)[b]	Negative for *wbdD*$_{O8/O9/O9a}$ (%)[b]
1	1	1	38	18 (47.4)	20 (52.6)	0
	2	2	38	5 (13.2)	4 (10.5)	2 (5.3)
	2	3	19	3 (15.8)	0	1 (5.3)
	3	4	38	0	0	0
	4	5	19	6 (31.6)	4 (21.1)	2 (10.5)
	4	6	19	9 (47.4)	4 (21.1)	5 (26.3)
	5	7	38	1 (2.6)	0	1 (2.6)
	6	8	19	3 (15.8)	0	3 (15.8)
	7	9	19	11 (57.9)	10 (52.6)	1 (5.3)
	8	10	19	0	0	0
	9	11	19	0	0	0
	10	12	19	0	0	0
	11	13	19	4 (21.1)	0	3 (15.8)
	12	14	19	1 (5.3)	0	0
	13	15	19	8 (42.1)	2 (10.5)	5 (26.3)
	14	16	16	15 (93.8)	16 (100.0)	2 (12.5)
2	2	17	20	2 (10.0)	0	2 (10.0)
	2	18	20	5 (25.0)	3 (15.0)	1 (5.0)
	15	19	20	1 (5.0)	0	1 (5.0)
	16	20	20	2 (10.0)	2 (10.0)	0
	17	21	20	1 (5.0)	0	1 (5.0)

	18	22	20	2 (10.0)	0	0
	19	23	20	0	0	0
	20	24	20	2 (10.0)	2 (10.0)	0
	21	25	20	10 (50.0)	9 (45.0)	0
	22	26	20	5 (25.0)	2 (10.0)	3 (15.0)
	22	27	20	5 (25.0)	2 (10.0)	3 (15.0)
	23	28	20	11 (55.0)	11(55.0)	0
	24	29	20	1 (5.0)	0	1 (5.0)
	25	30	20	0	0	0
	26	31	20	9 (45.0)	0	9 (45.0)
	26	32	20	5 (25.0)	1 (5.0)	4 (20.0)
	27	33	20	1 (5.0)	0	1 (5.0)
	28	34	20	0	0	0
	29	35	20	0	0	0
Total	29	35	757	146 (19.3)	92 (12.2)	51 (6.7)

[a]Samples positive by PCR of pooled colonies

[b]The percentages in parentheses are number of *wzx*$_{O104}$ isolates that were positive or negative for *wbdD*$_{O8/O9/O9a}$ from the total number of samples in each lot.

Table 7.3. Number of fecal samples from feedlot cattle positive for *wzx*$_{O104}$ and or *wbdD*$_{O8/O9/O9a}$ and associated major genes of the O104 hybrid pathotype (O104:H4) in cattle feces based on the PCR method

Genes	Protein or Function	No. of samples (n = 757) positive	
		Before enrichment, n (%)	After enrichment[a], n (%)
***wzx*$_{O104}$**	O104 antigen flippase	38 (5.0)	349 (46.1)
***wbdD*$_{O8/O9/O9a}$**	Kinase and methyl transferase	13 (1.7)	238 (31.4)
Only[b] *wzx*$_{O104}$		34 (4.5)	194 (25.6)
***stx*1**	Shiga toxin 1	29 (3.8)	208 (27.5)
***stx*2**	Shiga toxin 2	156 (20.6)	506 (66.8)
eae	Intimin	112 (14.8)	549 (72.5)
ehxA	Enterohemolysin	370 (48.9)	710 (93.8)
terD	Tellurite resistance	339 (44.8)	624 (82.4)
aggA	Aggregative adherence fimbriae 1	0 (0)	0 (0)
bfpA	Bundle forming pili	0 (0)	0 (0)
***fliC*$_{H4}$**	H4 flagellar antigen	200 (26.4)	659 (87.1)

[a]Feces were enriched in *Escherichia coli* broth at 40° C for 6 h

[b]Positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$

Table 7.4. Number of fecal samples from feedlot cattle positive for *E. coli* O104 and O8/O9/O9a based on PCR assays of *wzx*$_{O104}$ and *wbdD*$_{O8/O9/O9a}$

Week	Feedlot no.	Lot No.	No. of samples collected	Positive for:		
				wzx$_{O104}$	*wbdD*$_{O8/O9/O9a}$	*wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$
1	1	1	38	31 (81.6)	12 (31.6)	21 (55.3)
	2	2	38	10 (26.3)	9 (23.7)	6 (15.8)
	2	3	19	9 (47.4)	7 (36.8)	5 (26.3)
	3	4	38	3 (7.9)	5 (13.2)	2 (5.3)
	4	5	19	14 (73.7)	16 (84.2)	0
	4	6	19	18 (94.7)	7 (36.8)	11 (57.9)
	5	7	38	26 (68.4)	19 (50.0)	12 (31.6)
	6	8	19	7 (36.8)	2 (10.5)	5 (26.3)
	7	9	19	12 (63.2)	5 (26.3)	8 (42.1)
	8	10	19	0	1 (5.3)	0
	9	11	19	0	0	0
	10	12	19	2 (10.5)	0	2 (10.5)
	11	13	19	8 (42.1)	0	8 (42.1)
	12	14	19	5 (26.3)	1 (5.3)	4 (21.1)
	13	15	19	10 (52.6)	1 (5.3)	10 (52.6)
	14	16	16	10 (62.5)	3 (18.8)	8 (50.0)
2	2	17	20	5 (25.0)	3 (15.0)	4 (20.0)
	2	18	20	11 (55.0)	3 (15.0)	10 (50.0)
	15	19	20	5 (25.0)	0	5 (25.0)
	16	20	20	14 (70.0)	5 (25.0)	11 (55.0)
	17	21	20	4 (20.0)	1 (5.0)	4 (20.0)
	18	22	20	19 (95.0)	18 (90.0)	1 (5.0)
	19	23	20	13 (65.0)	20 (100.0)	0
	20	24	20	6 (30.0)	5 (25.0)	4 (20.0)

	21	25	20	16 (80.0)	7 (35.0)	9 (45.0)
	22	26	20	18 (90.0)	9 (45.0)	9 (45.0)
	22	27	20	15 (75.0)	12 (60.0)	5 (25.0)
	23	28	20	17 (85.0)	14 (70.0)	3 (15.0)
	24	29	20	1 (5.0)	5 (25.0)	0
	25	30	20	2 (10.0)	15 (75.0)	1 (5.0)
	26	31	20	9 (45.0)	9 (45.0)	4 (20.0)
	26	32	20	9 (45.0)	10 (50.0)	5 (25.0)
	27	33	20	4 (20.0)	13 (65.0)	1 (5.0)
	28	34	20	0	1 (5.0)	0
	29	35	20	16 (80.0)	0	16 (80.0)
Total	29	35	757	349 (46.1)	238 (31.4)	194 (25.6)

Table 7.5. Model-adjusted prevalence estimates of fecal samples from feedlot cattle positive for *wzx*$_{O104}$/*wbdD*$_{O8/O9/O9a}$ and *wzx*$_{O104}$ at the feedlot-, lot- and sample- levels

Target genes, serogroups, and detection method	Level of prevalence estimation	Mean Prevalence, % (95% confidence interval)
Positive for *wzx*$_{O104}$ (Positive for O104 and/or O8/O9/O9a) [a, b]		
Culture method	Feedlot	17.6 (6.3-40.3)
	Lot	13.5 (4.8-32.9)
	Sample	11.8 (6.3-21.0)
PCR method	Feedlot	49.5 (29.3-69.9)
	Lot	42.5 (22.6-65.1)
	Sample	41.7 (27.1-57.8)
Positive for *wzx*$_{O104}$ and negative for *wbdD*$_{O8/O9/O9a}$ (Positive for O104 only) [a, c]		
Culture method	Feedlot	5.7 (2.9-10.7)
	Lot	2.8 (1.1-7.2)
	Sample	0.50 (0.2-1.2)
PCR method	Feedlot	21.2 (14.7-29.5)
	Lot	20.1 (13.2-29.2)
	Sample	25.9 (17.5-36.6)

[a]The proportions of samples that tested positive by culture and PCR methods were significantly different by McNemar's Chi square test ($P < 0.01$).

[b]Kappa statistics: κ=0.27; κ 95% CI = 0.21 – 0.32

[c]Kappa statistics: κ=0.10; κ 95% CI = 0.04 – 0.17

Table 7.6. Virulence gene profiles of strains of *Escherichia coli* O104 isolated from feedlot cattle feces

Week of sample collection	Feedlot no.	No. of samples collected	Total no. of O104 isolates[a]	Genes				
				*stx*1	*stx*2	*eae*	*ehxA*	*terD*
1	2	38	2	1, 0	0	0	1, 0	1, 0
	2	19	1	1	0	0	1	1
	4	19	2	1, 0	0	0	1, 0	2
	4	19	5	0	0	0	0	0
	5	38	1	0	0	0	0	1
	6	19	3	0	0	0	0	0
	7	19	1	0	0	0	0	1
	11	19	3	0	0	0	0	1, 0, 0
	13	19	5	0	0	0	0	3, 0, 0
	14	16	2	0	0	0	0	1, 0
2	2	20	2	0	0	0	0	0
	2	20	1	0	0	0	0	0
	15	20	1	0	0	0	0	1
	17	20	1	0	0	0	0	0
	22	20	3	0	0	0	0	1, 0, 0
	22	20	3	0	0	0	0	0
	24	20	1	0	0	0	0	1
	26	20	9	9	0	0	9	9
	26	20	4	4	0	0	4	4
	27	20	1	0	0	0	0	0
	Total	**425**	**51**	**16**	**0**	**0**	**16**	**27**

[a]Isolates positive for *wzx*$_{O104}$ gene and negative for O8/O9/O9a

All isolates were negative for *bfpA*, *aggA* and *fliC*$_{H4}$ genes

Chapter 8 - DNA microarray-based assessment of virulence potential of Shiga toxigenic *Escherichia coli* O104:H7 isolated from feedlot cattle feces

Abstract

Escherichia coli O104:H4, a hybrid pathotype of Shiga toxin-producing *E. coli* (STEC) and enteroaggregative *E. coli* reported in a large 2011 foodborne outbreak in Germany, has not been detected in cattle feces. However, cattle harbor and shed in the feces other O104 serotypes, particularly O104:H7, which has been associated with sporadic cases of diarrhea in humans. The objective of our study was to assess the virulence potential of STEC O104:H7 isolated from feces of feedlot cattle using DNA microarray. Six strains of STEC O104:H7 isolated from cattle feces were analyzed using FDA-ECID Affymetrix DNA microarray to assess their virulence potential and compare to the virulence gene profiles of human strains of STEC O104:H4 (German outbreak), O104:H21 (milk-associated Montana outbreak strain) and five O104:H7 (clinical) strains. Microarray scatter plots were generated to visualize the gene-level differences between bovine and human O104 strains, and Pearson correlation coefficients (r) were determined. Splitstree analysis was performed to determine the phylogenetic relationship among the strains. Bovine *E. coli* O104 strains were negative for *eae* similar to human O104 strains. The bovine strains were positive for genes encoding Shiga toxin 1 subtype c (*stx*1c), enterohemolysin (*ehxA*), tellurite resistance gene (*terD*), IrgA homolog protein (*iha*), type 1 fimbriae (*fimH*), and were negative for those encoding intimin (*eae*) and effector proteins of type III secretory system. The six cattle derived O104 strains were closely related (r=0.86-0.98) to each other, except for a

few differences in phage related and non-annotated genes. One of the human clinical (O104:H7; 2011C-3665) strains was more closely related to bovine O104:H7 strains (r=0.81-0.85). None of the bovine *E. coli* O104 strains carried genes characteristic of the *E. coli* O104:H4 German outbreak strain, and unlike other human strains, were also negative for Shiga toxin 2. Because the cattle *E. coli* O104:H7 strains characterized in our study possess *stx*1c and genes that code for enterohemolysin and a variety of adhesins, the serotype has the potential to be a diarrheagenic foodborne pathogen in humans.

Introduction

Escherichia coli O104:H4, a hybrid pathotype possessing genes characteristic of enteroaggregative *E. coli* (EAEC) and Shiga toxin producing *E. coli* (STEC), was responsible for a large foodborne outbreak of hemorrhagic colitis and hemolytic uremic syndrome in Europe, mainly Germany, in 2011. Based on whole genome sequence analysis, the hybrid pathotype is hypothesized to have emerged by sequential loss and gain of chromosome- and plasmid-associated virulence genes (Mellmann et al., 2011). *Escherichia coli* O104:H4 serotype has not been detected in cattle, unlike other major STEC serotypes that cause human illnesses (Auvray et al., 2012; Paddock et al., 2013; Wieler et al., 2011). However, cattle harbor O104 serotypes other than H4 in the gut and shed them in the feces (Paddock et al., 2013; Shridhar et al., 2016b). A *stx*2-carrying O104:H21 serotype, possibly of cattle origin, was implicated in an outbreak of hemorrhagic colitis associated with consumption of raw milk in Helena, Montana in 1994 (Feng et al., 2001). We have conducted a study with cattle feces collected from a number of feedlots (n=29) to determine the prevalence of O104 serogroup and reported that serotype O104:H7 was the most commonly isolated *stx*-carrying O104 serogroup (Shridhar et al., 2016). The O104:H7

serotype has been reported to be associated with sporadic diarrheal cases in humans (Delannoy et al., 2012a; Miko et al., 2013), however, there has been no evidence to suggest that cattle were the source of human infections. Not much is known about the virulence potential of the strains of *E. coli* O104:H7 of cattle origin, particularly in relation to human strains of O104:H7. Therefore, the objectives of our study were to analyze the gene content to assess the virulence potential of cattle O104:H7 strains using FDA-ECID (Food and Drug Administration-*E. coli* Identification) DNA microarray and to compare their virulence gene profiles with that of human STEC O104:H7 (clinical strains), O104:H21 (milk-associated Montana outbreak strain) and O104:H4 (German outbreak) strains.

Materials and Methods

E. coli O104 strains

Six strains of STEC O104:H7 (2013-6-659A, 2013-6-672E, 2013-6-685A, 2013-6-48C, 2013-6-122E, and 2013-6-148B), isolated from feedlot cattle fecal samples collected at a Midwest slaughter plant (Shridhar et al., 2016), were utilized in this study. Additionally, human STEC clinical strains of O104:H4 (BAA-2326; German outbreak), O104:H21 (BAA-178; Montana outbreak) and five strains of O104:H7 (06-3637, 07-3598, 08-4061, 2011C-3665, and 2012C-3400; provided by Nancy A. Strockbine, Centers for Disease Control and Prevention, Atlanta, GA) were included in the study.

Microarray assay and data analysis

The strains were subjected to a custom Affymetrix DNA microarray developed by the FDA for identification and characterization of *E. coli* (Patel et al., 2016). The array was designed using 368 *E. coli* and *Shigella* sequence sets to identify 55,918 annotated open reading

frames. The array incorporates 41,932 probe sets, which includes 54 closed chromosomes, 47 closed plasmids, and 267 whole genome shotgun sequences. The microarray assay was performed according to the protocol described by Patel et al. 2016. Briefly, each strain was grown overnight in Luria broth (Sigma-Aldrich Co., St. Louis, MO) at 37° C. Total DNA was extracted from 1 ml of the culture and fragmented by a brief digestion with RQ1 RNase- Free DNase I. The 3' end of the digested DNA was labelled with biotin, hybridized to FDA-ECID gene chip and incubated for 16 h at 45° C. The presence or absence of each gene was determined using Robust MultiArray Averaging (RMA) method and MAS5.0 algorithm. Splitstree analysis was performed to determine the phylogenetic relationship among the bovine and human *E. coli* O104 strains. Pearson correlation coefficients (r) were determined to compare the strains. In addition, pair wise scatter plots were generated to visualize the gene level differences between the strains.

Results

The gene contents of six O104:H7 strains, isolated from cattle feces, were compared to human O104:H7 strains associated with sporadic diarrhea, and the German outbreak strain of O104:H4 (BAA-2326) and the Montana outbreak strain of O104:H21 (BAA-178). All O104 strains, cattle or human, were negative for *eae* (intimin) and for genes associated with the type III secretory system (*esc*R, *esp*A, *esp*D, *esc*D, *esc*R, *esp*D, *esp*B, *ler*, *esc*T, *sep*L, *sep*Q, and *tir*).

Cattle O104:H7

All bovine O104:H7 strains were positive for the following virulence genes characteristic of STEC: *stx*1 (subtype C), *terD* (tellurite resistance protein), and *iha* (IrgA homolog adhesin). Enterohemolysin (*ehxA*) was present in four of the six bovine strains. The strains were also

positive for other adhesins, *lpfA* (long polar fimbriae), *fim*H (mannose specific adhesin), and *sfm*A (fimbriae like adhesin); and antibiotic resistance genes, such as *ampH* (penicillin binding protein), *marC* (multiple antibiotic resistance protein), and *pmrD* (polymyxin resistance protein) (Table 8.1). Bovine strains were also positive for invasion protein encoded by *ibe*B, and glutamate decarboxylase gene (*gadAB*), which is responsible for acid resistance. The six cattle O104 strains were closely related (r=0.86-0.98; Table 8.2) to each other, except for a few differences in phage related and non-annotated genes (data not shown). Scatter plot data of the strain pairs revealed that three bovine O104:H7 strains, 2013-6-659A, 2013-6-672E, and 2013-6-685A, isolated from the same feedlot, were almost indistinguishable (Fig 8.1A).

Cattle O104:H7 vs. human O104:H7

Of the five human O104:H7 strains characterized, three were positive for *stx*2, one was positive for *stx*1 (subtype C) and another was negative for both. Virulence genes such as *ehxA*, *ibeB*, *ampH*, *lpfA*, *fimH*, *sfmA*, *pmrD*, *marC*, *gadAB*, and *cfaC* (CFA/I fimbrial subunit C) were present in both human and bovine O104:H7 strains (Table 8.1). The human strains contained the following virulence genes that were absent in the bovine strains: *sub* (4/5 strains; encodes for subtilase cytotoxin), *esp*P (4/5 strains; encodes for extracellular serine protease) *saa* (4/5 strains; encodes for STEC auto agglutinating adhesin, and *acf*D (4/5 strains; which encodes for accessory colonization factor). Some of the virulence genes present in bovine O104:H7 strains such as *ter*D and *iha* were absent in human O104:H7 strains. One human clinical (O104:H7; 2011C-3665) strain was more closely related to bovine O104:H7 strains (r=0.81-0.85; Table 8.2; Fig 8.1B) than the other four human strains (Fig 8.1C; Fig 8.2). The human strain, 2011C-3665 had the lowest number of probe sets (3,344 to 4,024) different from any of the bovine strains.

Comparison of bovine O104:H7 strains with the other four human clinical (O104:H7) strains revealed probe set differences ranging from 3,481 to 5,412.

Cattle O104:H7 vs. human O104:H4 and O104:H21

Characteristic virulence genes of enteroaggregative *E. coli* (*pet*- Per-activated serine protease autotransporter enterotoxin, *aatA*- EAEC virulence plasmid (*pAA*), *aggR*- Transcriptional regulator, *pic*-Protein involved in intestinal colonization, and *aatP*- permease) were present in the German outbreak (O104:H4) strain (Table 8.3), but absent in bovine O104:H7 and O104:H21 strains. Extracellular serine protease (*esp*P) was present only in the Montana outbreak strain, but absent in the German outbreak and bovine O104:H7 strains. The bovine O104:H7 strains and the German O104:H4 strain were positive for *terD*. Enterohemolysin gene (*ehxA*), absent in the O104:H4 strain, was present in four bovine O104:H7 strains and in the O104:H21 strain. Some of the virulence genes (*ibeB, ampH, pmrD, marC, sfmA, gadAB, lpfA,* and *cfaC*) detected in bovine O104:H7 strains and human clinical O104:H7 strains were also present in O104:H4 and O104:H21 strains (Tables 8.1 and 8.3). The complete list of genes detected in bovine *E. coli* O104:H7 and human O104:H4 and O104:H21 outbreak strains is shown in Table 8.3.

Phylogenetic relationship

Phylogenetic analysis revealed that all bovine O104:H7 strains clustered together, and 4 of 5 human clinical O104:H7 strains clustered together (Fig 8.3). The human O104:H7 strain, 2011C-3665, clustered with the bovine O104:H7 strains. However, none of the bovine or human O104:H7 strains clustered with the two human outbreak strains (O104:H4 and O104:H21) (Fig 8.3).

Discussion

Several molecular methods are available for the genetic characterization of foodborne pathogens, including Shiga toxin-producing *E. coli*. PCR-based methods to detect genes, besides being labor-intensive, are limited by the number of targets that can be simultaneously detected. Molecular techniques, such as DNA microarray and whole genome sequencing (WGS), allow genome-wide characterization of the organisms. The limitation of the microarray is that it allows the detection of only specific gene targets, unlike WGS, which enables the detection of novel genes in the target organisms. However, WGS is time-consuming and laborious compared to microarray (Joensen et al., 2014; Patel et al., 2016). Custom DNA microarrays detecting a multitude of genes, designed using published whole genome sequences of *E. coli* and *Shigella*, enable rapid genome-scale analysis of pathogenic *E. coli* (Baranzoni et al., 2016; Lacher et al., 2014; Lacher et al., 2016; Patel et al., 2016).

All of the bovine O104:H7 strains were negative for *stx*2, which is more frequently associated with human illnesses, particularly hemolytic uremic syndrome, than *stx*1 (Siegler et al., 2003). The bovine O104:H7 strains and one of the five human O104:H7 strain were positive for *stx*1 of subtype 1c. Other STEC serogroups carrying *stx*1c have been associated with diarrhea and in a few cases serious complications in human patients (Zhang et al., 2002). For example, STEC O78:H- carrying *stx*1c was reported to be the causative agent of HUS in a 2-week old boy in Finland (Lienemann et al., 2012). All O104:H7 strains, both bovine and human, similar to O104:H4 and O104:H21 outbreak strains (Feng et al., 2001; Shridhar et al., 2016b), were negative for intimin (*eae*), an adhesin involved in the attachment of *E. coli* to the host cells (Jerse et al., 1990). However, O104:H7 carried other adhesins, such as IrgA homologue adhesin (*Iha*), mannose-specific adhesin (*fim*H), long polar fimbriae (*lpfA*), and fimbriae like adhesin

(*sfmA*), which are involved in adhesion of bacteria to the host epithelial cells (Ross et al., 2015; Tarr et al., 2000; Wold et al., 1988). These adhesins may be involved in the attachment of the bacteria to the host cells in the absence of intimin. Long polar fimbriae (*lpfA*), which was also present in the German outbreak strain (O104:H4), has been reported to be involved in initial attachment and colonization of the intestine (Ross et al., 2015). Enterohemolysin (*ehxA*) was present in four of the bovine strains and in all human O104:H7 strains, except one, and in the Montana outbreak strain (O104:H21). Previous studies have reported the association of *ehxA* with diarrhea and hemolytic uremic syndrome (Schmidt and Karch, 1996). Cheng et al. (2015) reported that hemolysin encoded by *ehxA* in *E. coli* O157:H7 is involved in cytotoxicity of macrophages and release of IL-1β in humans (Cheng et al., 2015). All bovine O104:H7 strains and the German outbreak strain were positive for *ter*D, which codes for tellurite resistance protein. Shiga toxin-producing *E. coli* serogroups such as O26, O45, O111, and O157 have been reported to carry the tellurite resistance gene (Orth et al., 2007). The tellurite resistance gene has been reported to be involved in the survival of bacteria by conferring protection against host defense (Valková et al., 2007).

Based on the scatter plots, phylogenetic analysis and Pearson correlation coefficients, all the bovine O104:H7 strains were closely related to each other, and one of the five human clinical strains (O104:H7; 2011C-3665) included in the study was more closely related to the bovine strains. This suggests that cattle feces could potentially be a source of O104:H7 strains associated with diarrhea in humans.

Although there was some similarity in the virulence gene profile of human and bovine O104 strains, the key virulence genes, such as *stx*2 responsible for hemolytic uremic syndrome, was absent in bovine strains, but present in human clinical and outbreak strains. The *E. coli*

O104:H4 responsible for the large food-borne illness outbreak in Germany has been reported to be evolved by the uptake of *stx*2 encoding bacteriophages found in cattle (Beutin et al., 2013). Similarly, bovine O104:H7 strains could evolve into highly pathogenic strains by acquiring other virulence genes responsible for human illnesses by gain and loss of genomic islands, prophages, and plasmids.

In conclusion, microarray-based analysis of genes of bovine O104:H7 strains isolated from cattle feces characterized in our study suggest the potential of the serotype to be a diarrheagenic human pathogen. Because cattle harbor these strains and shed them in feces, O104:H7 strains also have the potential to be foodborne pathogens to humans.

Acknowledgments

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Figure 8-1. Microarray scatter plots of pairwise comparisons demonstrating gene-level differences between *Escherichia coli* O104:H7 strains of bovine and human origin, where each spot represents the robust multiarray average (RMA)-summarized probe set intensity. (A) Comparison of two bovine O104:H7 strains; (B) and (C) Comparison of bovine (Y-axis) and human (X-axis) O104:H7 strains. Dots clustered on the diagonal line represent genes present in both genomes; scattered dots indicate genome-specific probe sets: upper-left for the genes represented by the Y-axes, and lower-right for the genes represented by the X-axes

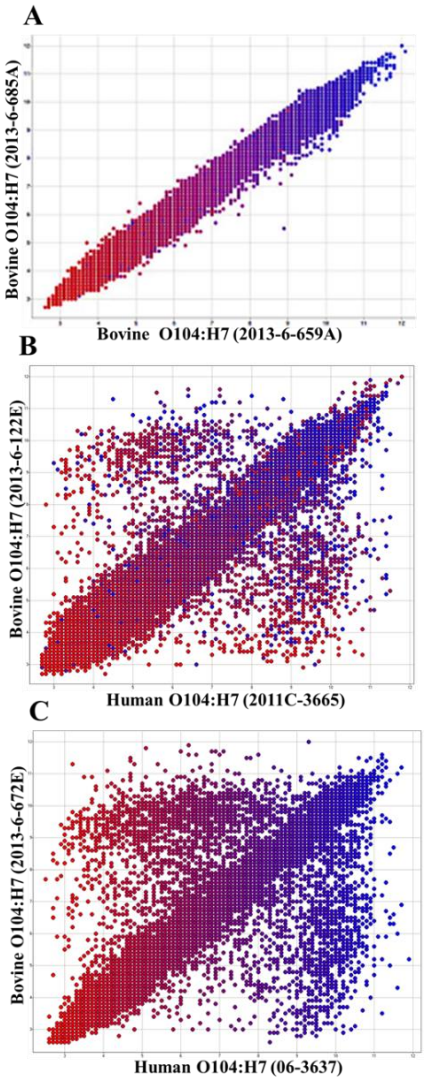
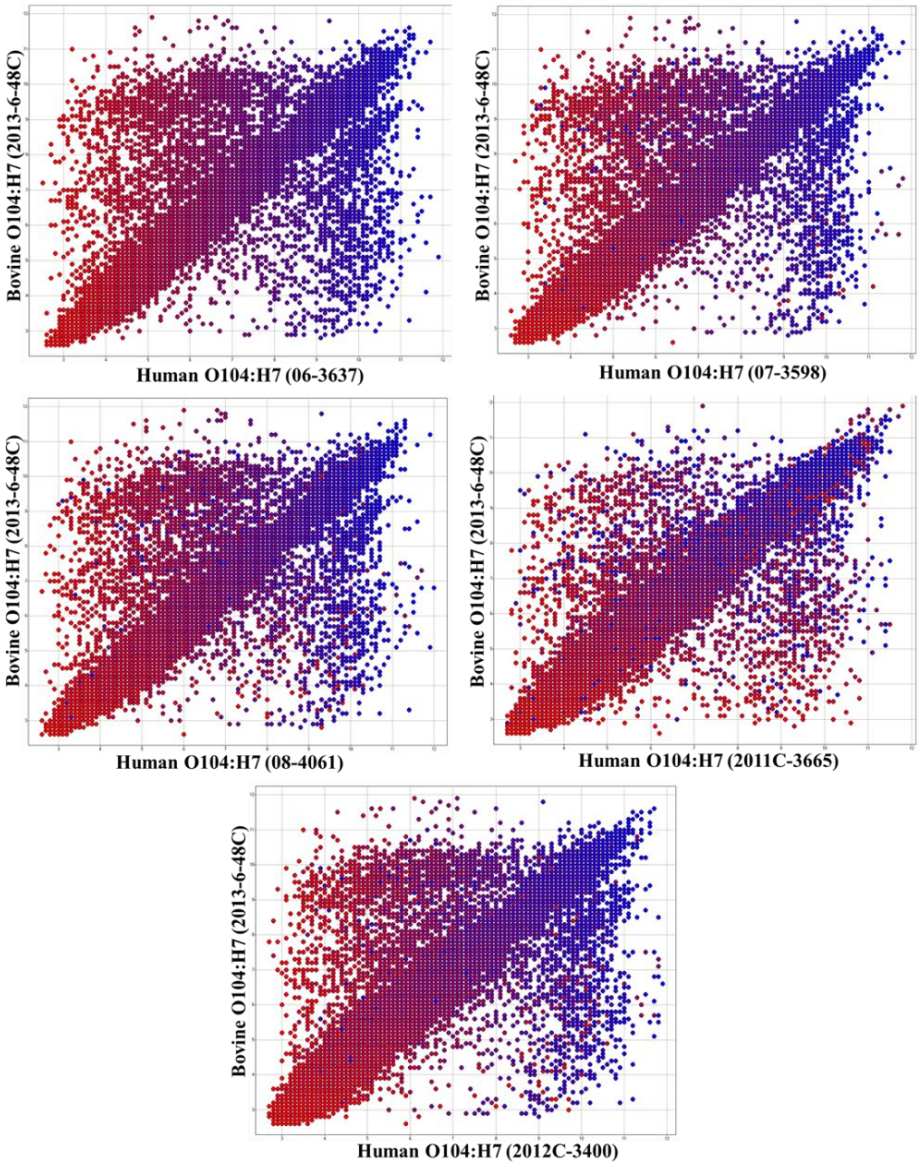
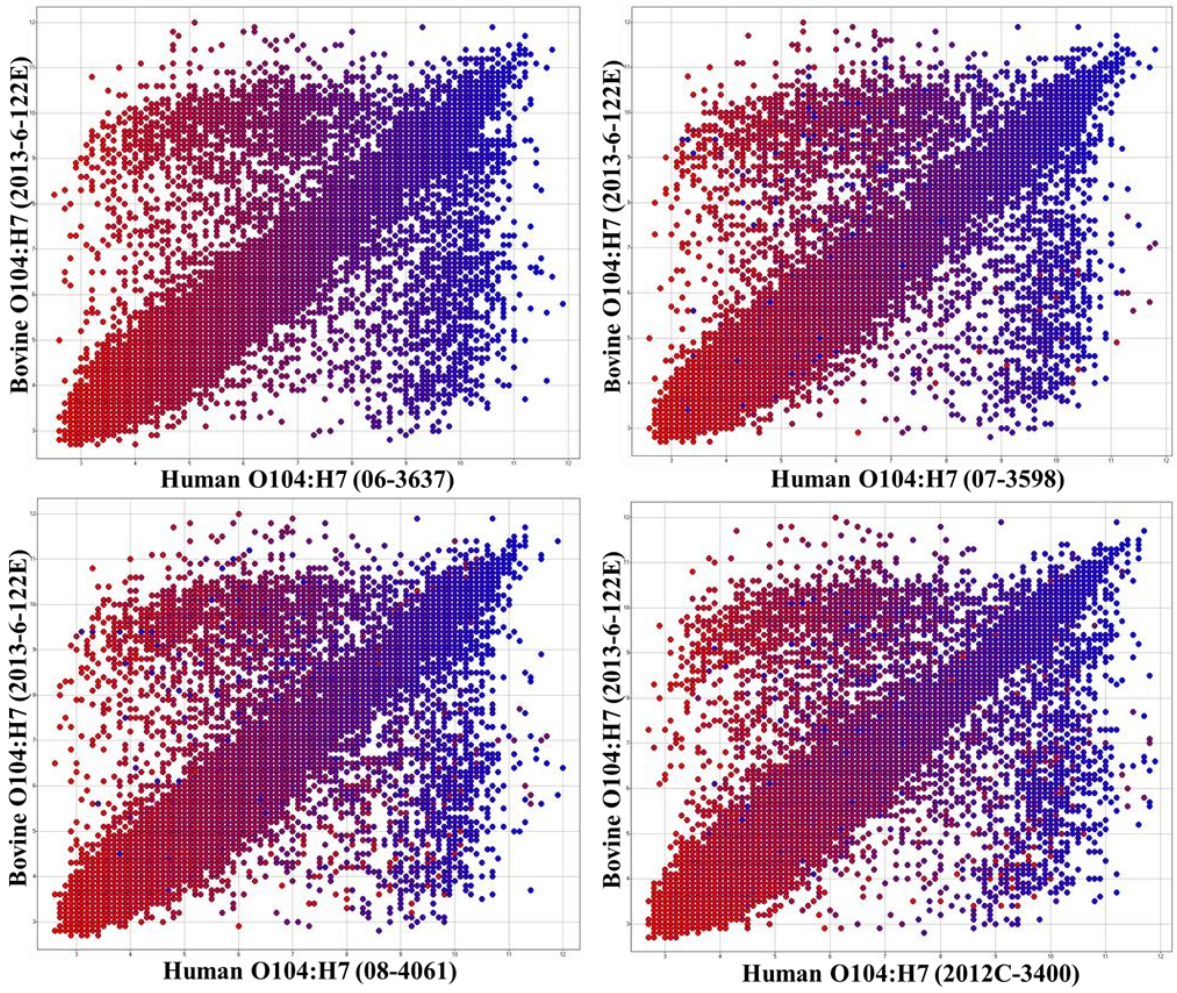


Figure 8-2. Microarray scatter plots of pairwise comparisons demonstrating gene-level differences between *Escherichia coli* O104:H7 strains of bovine (Y-axis) and human origin (X-axis), where each spot represents the robust multiarray average (RMA)-summarized probe set intensity. Dots clustered on the diagonal line represent genes present in both genomes; scattered dots indicate genome-specific probe sets: upper-left for the genes represented by the Y-axes, and lower-right for the genes represented by the X-axes

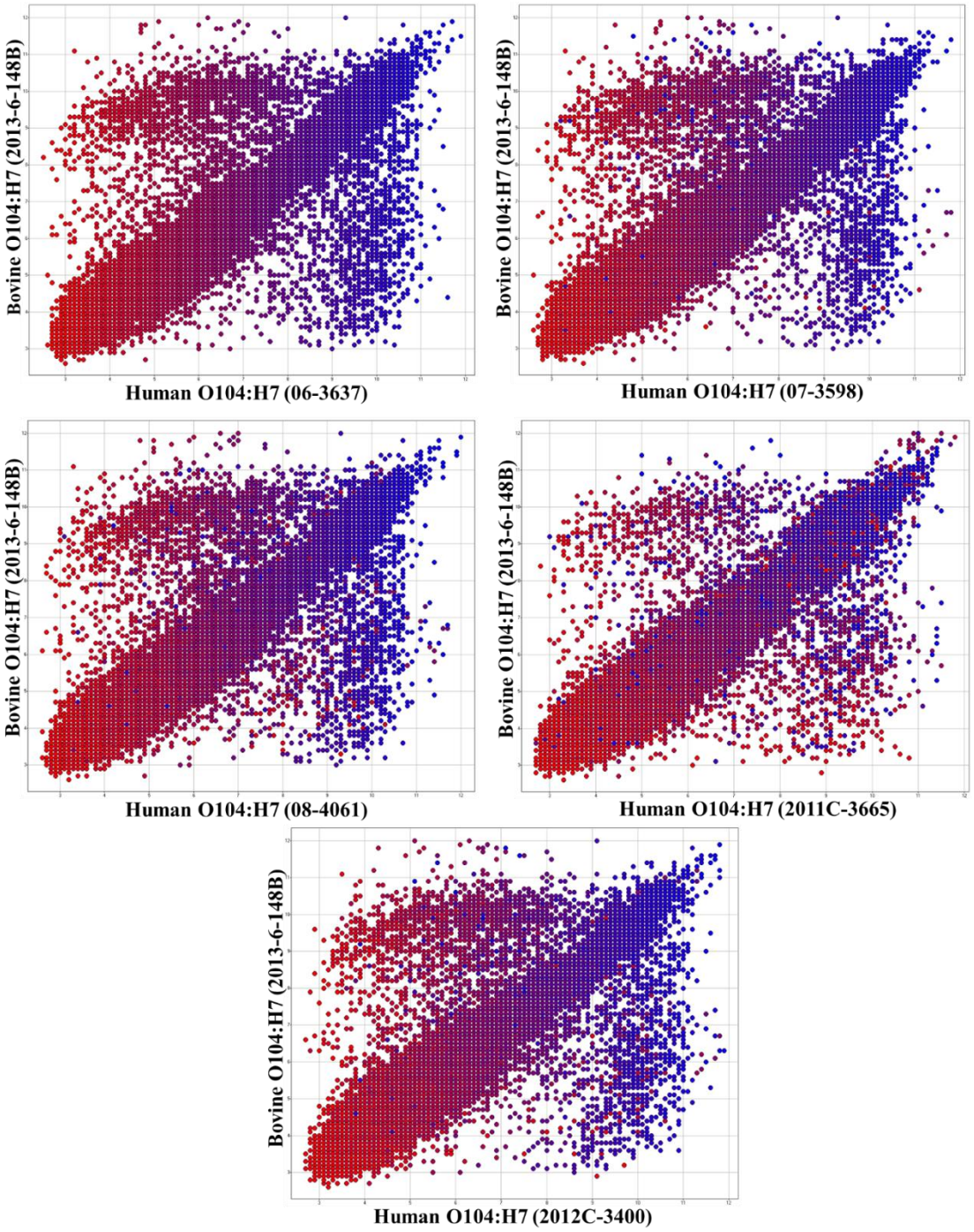
(A) Comparison of bovine O104:H7 strain (2013-6-48C) with human O104:H7 strains



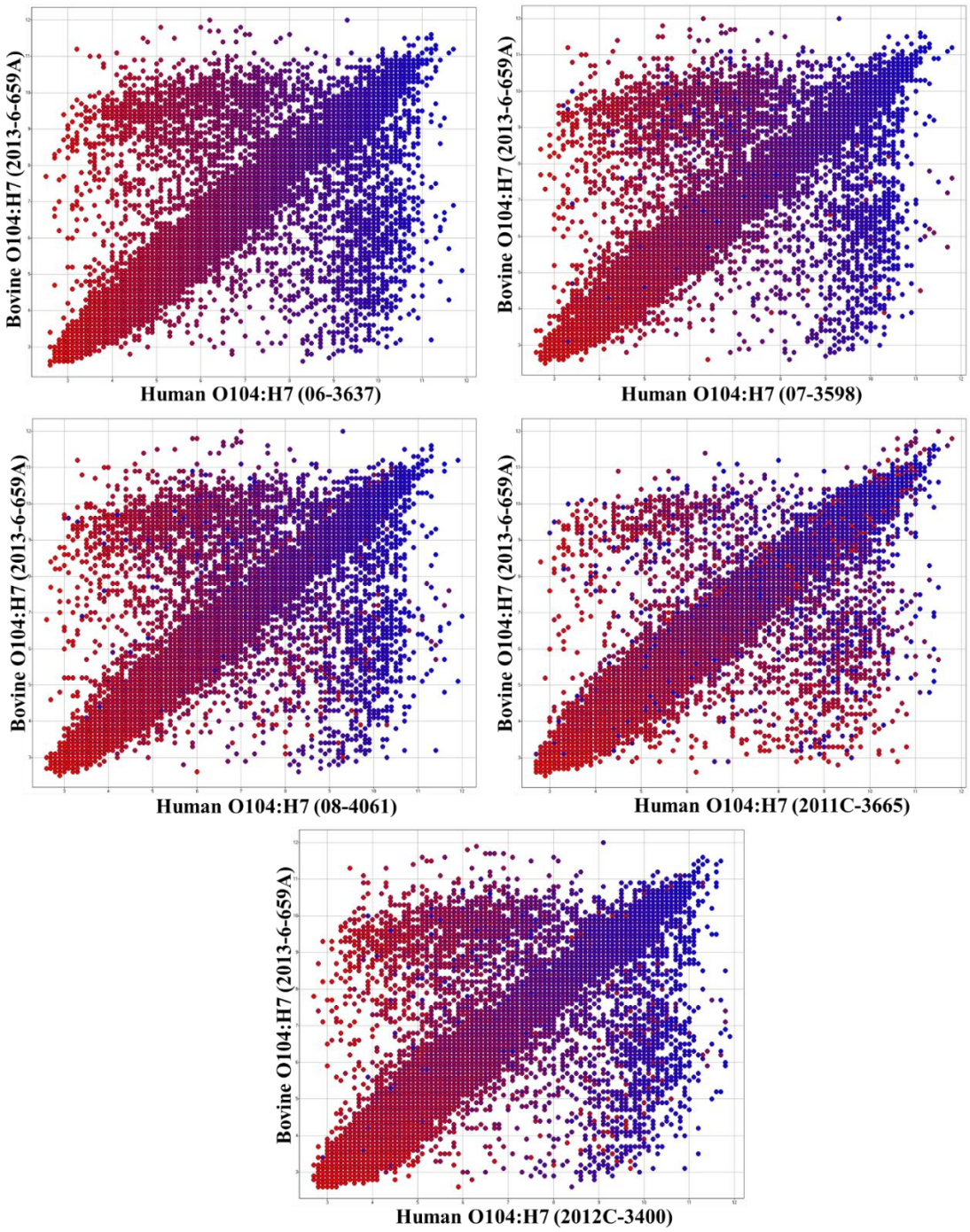
B) Comparison of bovine O104:H7 strain (2013-6-122E) with human O104:H7 strains



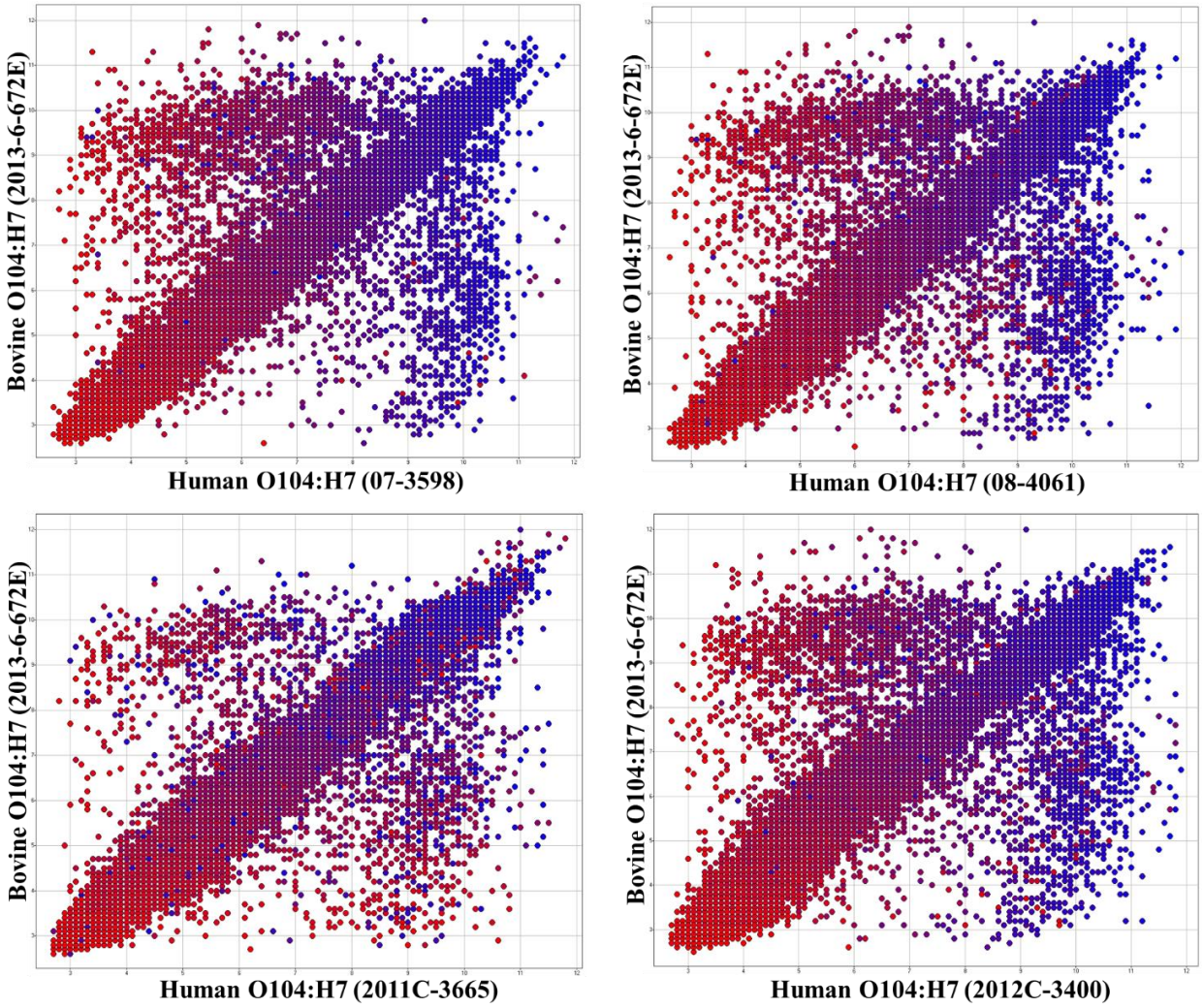
C) Comparison of bovine O104:H7 strain (2013-6-148B) with human O104:H7 strains



D) Comparison of bovine O104:H7 strain (2013-6-659A) with human O104:H7 strains



E) Comparison of bovine O104:H7 strain (2013-6-672E) with human O104:H7 strains



F) Comparison of bovine O104:H7 strain (2013-6-685A) with human O104:H7 strains

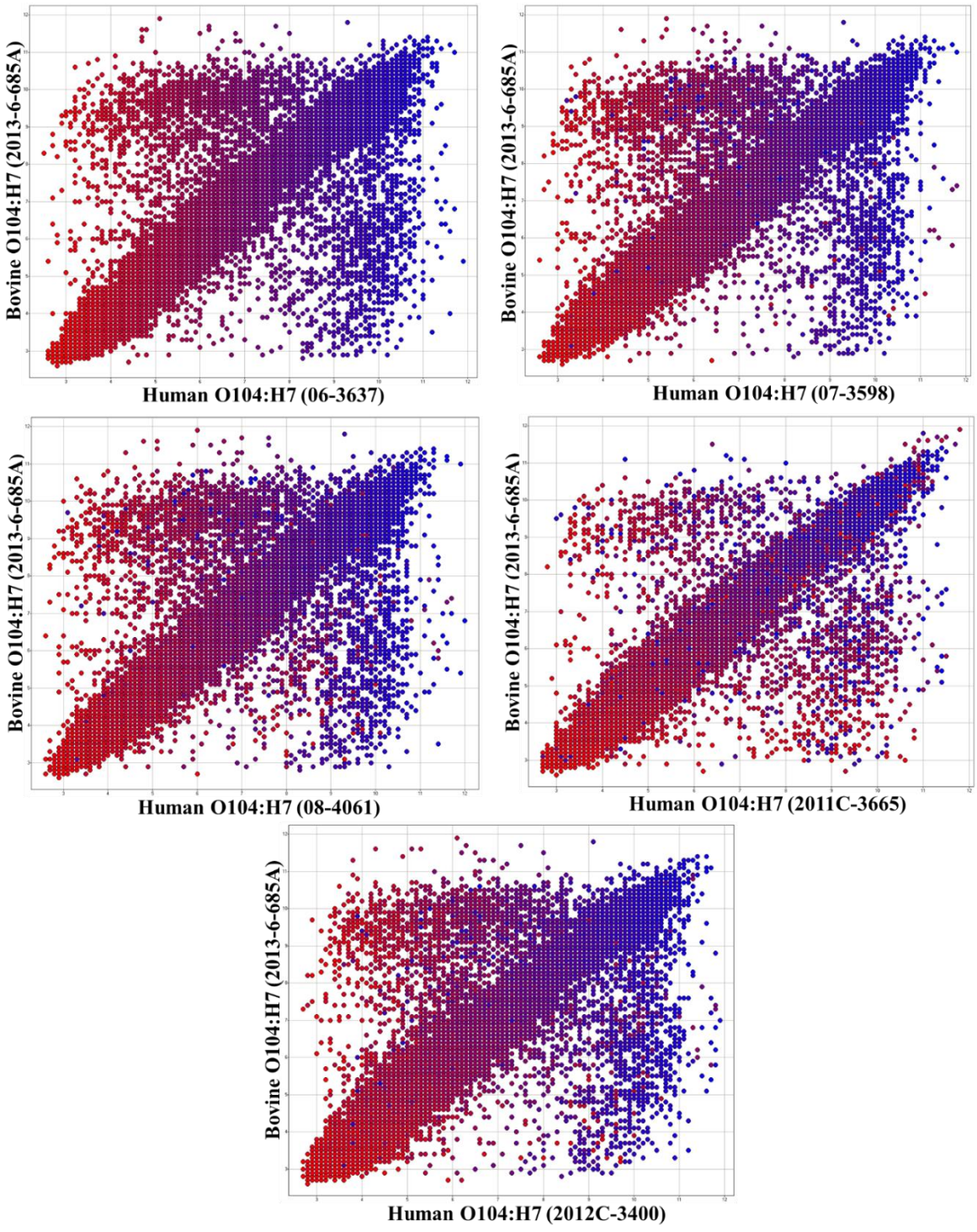


Figure 8-3. Phylogenetic analysis of bovine and human *E. coli* O104 strains based on the FDA-ECID DNA microarray gene difference data from 41,000 probe sets using SplitsTree4. The tree was developed using the Neighbor net algorithm using Neighbor joining method. The scale bar represents 0.01 base substitutions per site

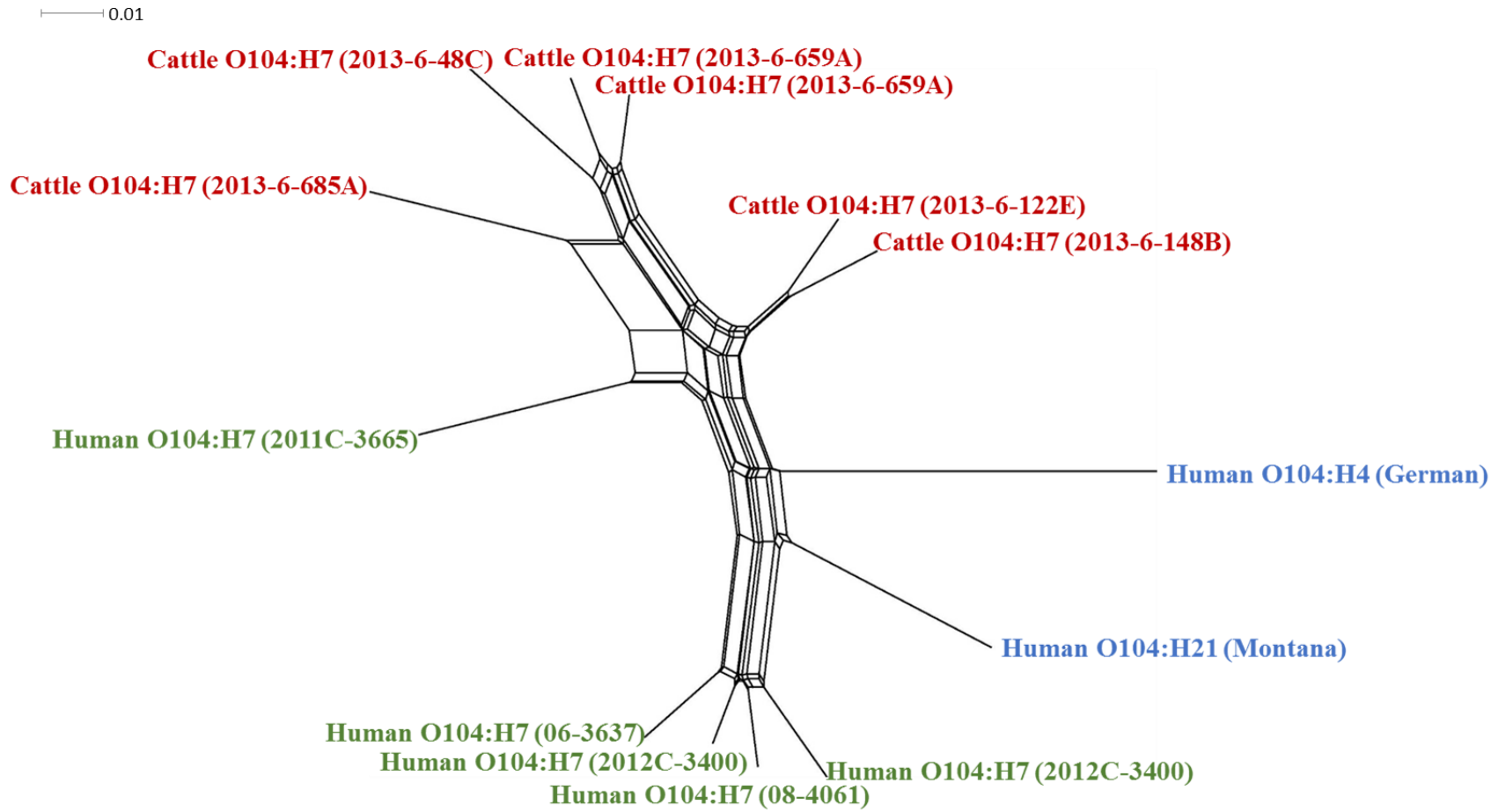


Table 8.1. Comparison of bovine *Escherichia coli* O104:H7 with human O104:H7 strains by microarray

Genes	Product	Bovine O104:H7 strains						Human O104:H7 strains				
		2013-6-659A	2013-6-672E	2013-6-685A	2013-6-48C	2013-6-122E	2013-6-148B	06-3637	07-3598	08-4061	2011C-3665	2012C-3400
Enterohemorrhagic *E. coli* (EHEC)												
*stx*1	Shiga toxin 1	+	+	+	+	+	+				+	
*stx*2	Shiga toxin 2							+		+		+
terD	Tellurite resistance protein	+	+	+	+	+	+					
ehxA	Enterohemolysin	+	+			+	+	+	+	+		+
sub	Subtilase cytotoxin							+	+	+		+
Iha	IrgA homologue adhesin	+	+	+	+	+	+					
saa	Shiga toxin-producing *E. coli* autoagglutinating adhesin							+	+	+		+
Other genes												
ampH	Penicillin binding protein	+	+	+	+	+	+	+	+	+	+	+
lpfA	Long polar fimbriae	+	+	+	+	+	+	+	+	+	+	+
fimH	Mannose specific adhesin	+	+	+	+	+	+	+	+	+	+	+

sfmA	Fimbriae like adhesin	+	+	+	+	+	+	+	+	+	+	+
pmrD	Polymyxin resistance protein	+	+	+	+	+	+	+	+	+	+	+
marC	Multiple antibiotic resistance protein	+	+	+	+	+	+	+	+	+	+	+
acfD	Accessory colonization factor							+	+	+		+
gadAB	Glutamate decarboxylase isozyme	+	+	+	+	+	+	+	+	+	+	+
*esp*P	Extracellular serine protease							+	+	+		+
ibeB	Invasion protein	+	+	+	+	+	+	+	+	+	+	+
*cfa*C	CFA/I fimbrial subunit C	+	+	+	+	+	+	+	+	+	+	+

Table 8.2. Pearson correlation analysis of bovine O104:H7 and human O104:H7 strains

Strains	Bovine O104:H7						Human O104:H7				
	2013-6-659A	2013-6-672E	2013-6-685A)	2013-6-48C	2013-6-122E	2013-6-148B	06-3637	07-3598	08-4061	2011C-3665	2012C-3400
Bovine O104:H7											
2013-6-659A	1	0.98	0.94	0.94	0.91	0.9	0.78	0.78	0.77	0.83	0.76
2013-6-672E	0.98	1	0.95	0.94	0.92	0.9	0.77	0.78	0.77	0.83	0.76
2013-6-685A	0.94	0.95	1	0.91	0.91	0.91	0.77	0.77	0.77	0.85	0.76
2013-6-48C	0.94	0.94	0.91	1	0.89	0.86	0.77	0.77	0.76	0.81	0.75
2013-6-122E	0.91	0.92	0.91	0.89	1	0.93	0.78	0.79	0.78	0.85	0.78
2013-6-148B	0.90	0.90	0.91	0.86	0.93	1	0.77	0.78	0.77	0.84	0.77
Human O104:H7											
06-3637	0.78	0.77	0.77	0.77	0.78	0.77	1	0.94	0.93	0.78	0.91
07-3598	0.78	0.78	0.77	0.77	0.79	0.78	0.94	1	0.95	0.78	0.93
08-4061	0.77	0.77	0.77	0.76	0.78	0.77	0.93	0.95	1	0.78	0.93
2011C-3665	0.83	0.83	0.85	0.81	0.85	0.84	0.78	0.78	0.78	1	0.77
2012C-3400	0.76	0.76	0.76	0.75	0.78	0.77	0.91	0.93	0.93	0.77	1

Table 8.3. Comparison of bovine *E. coli* O104:H7 strains with human O104:H4 and O104:H21 outbreak strains by microarray

Genes	Product	Human strains		Bovine O104:H7 strains					
		O104:H4 (German)	O104:H21 (Montana)	2013-6-659A	2013-6-672E	2013-6-685A	2013-6-48C	2013-6-122E	2013-6-148B
Enterohemorrhagic *E. coli* (EHEC)									
*stx*1	Shiga toxin 1			+	+	+	+	+	+
*stx*2	Shiga toxin 2	+	+						
terD	Tellurite resistance protein	+		+	+	+	+	+	+
ehxA	Enterohemolysin		+	+	+			+	+
sub	Subtilase cytotoxin		+						
iha	IrgA homolog adhesin	+	+	+	+	+	+	+	+
saa	Shiga toxin producing *E. coli* autoagglutinating adhesin		+						
Enteroaggregative *E. coli* (EAEC)									
pet	Per-activated serine protease autotransporter enterotoxin EspC	+							
aatA	EAEC virulence plasmid (pAA)	+							
aggR	Transcriptional regulator	+							

pic	Protein involved in intestinal colonisation	+							
aatP	AatP permease	+							
				Other genes					
ampH	Penicillin binding protein	+	+	+	+	+	+	+	+
lpfA	Long polar fimbriae	+	+	+	+	+	+	+	+
sfmA	Fimbriae like adhesin	+	+	+	+	+	+	+	+
pmrD	Polymyxin resistance protein	+	+	+	+	+	+	+	+
marC	Multiple antibiotic resistance protein	+	+	+	+	+	+	+	+
acfD	Accessory colonization factor	+							
gadAB	Glutamate decarboxylase isozyme	+	+	+	+	+	+	+	+
fimH	FimH protein precursor (mannose-specific adhesin)		+	+	+	+	+	+	+
ibeB	Invasion protein	+	+	+	+	+	+	+	+
*esp*P	Extracellular serine protease		+						
*cfa*C	CFA/I fimbrial subunit C	+	+	+	+	+	+	+	+
mchC	MchC Protein	+							

fyuA	Iron acquisition outer membrane yersiniabactin receptor	+

Table 8.4. Pearson correlation analysis of the bovine *E. coli* O104:H7 and human O104:H4 and O104:H21 strains

Strains	Pearson correlation coefficients							
	O104:H4 (BAA-2326)	O104:H21 (BAA-178)	Bovine O104:H7 strains					
			2013-6-659A	2013-6-672E	2013-6-685A	2013-6-48C	2013-6-122E	2013-6-148B
Human O104:H4 (BAA-2326)	1	0.74	0.72	0.72	0.73	0.72	0.75	0.75
Human O104:H21 (BAA-178)	0.74	1	0.79	0.79	0.78	0.78	0.77	0.76
Cattle O104:H7 (2013-6-659A)	0.72	0.79	1	0.98	0.95	0.94	0.91	0.90
Cattle O104:H7 (2013-6-672E)	0.72	0.79	0.98	1	0.95	0.94	0.92	0.90
Cattle O104:H7 (2013-6-685A)	0.73	0.78	0.95	0.95	1	0.91	0.91	0.91
Cattle O104:H7 (2013-6-48C)	0.72	0.78	0.94	0.94	0.91	1	0.89	0.86
Cattle O104:H7 (2013-6-122E)	0.75	0.77	0.91	0.92	0.91	0.89	1	0.93
Cattle O104:H7 (2013-6- 148B)	0.75	0.76	0.90	0.90	0.91	0.86	0.93	1

Chapter 9 - Whole Genome Sequence-Based Analysis of Virulence Potential of *Escherichia coli* O104 Serotypes Isolated from Cattle Feces

Abstract

Escherichia coli O104:H4, a hybrid pathotype of Shiga toxin producing *E. coli* (STEC) and enteroaggregative *E. coli*, was responsible for a large outbreak of foodborne illness in Europe. The serotype has not been detected in cattle; however, serotypes other than H4 have been isolated from cattle feces. The dominant serotype prevalent in cattle feces is H7. The objectives of this study were to assess the virulence potential of *E. coli* O104 strains isolated from cattle feces and O104:H7 strains from human clinical cases by whole genome sequence (WGS)-based analysis. Ten bovine *E. coli* O104 strains (six O104:H7, one O104:H8, one O104:H12, and two O104:H23) and five O104:H7 human clinical strains were subjected to WGS using the Illumina Miseq platform. All bovine O104:H7 strains and one of the human strains carried *stx*1c. Three of the human O104 strains carried *stx*2; two were of subtype 2a and one was 2d. All bovine and human strains were negative for intimin (*eae*) and other genes associated with the type III secretory system, and non-LEE encoded effectors. Bovine and human strains carried other adhesins, such as irgA homolog adhesin (*iha*) and long polar fimbriae (*lpfA*). Plasmid-encoded virulence genes (*ehxA*, *epeA*, *espP*, *katP*) were also present in bovine and human strains. One of the human O104:H7 strains was phylogenetically closely related and belonged to the same sequence type (ST-1817) as that of bovine O104:H7 STEC strains. Because bovine O104:H7 strains characterized in our study carried virulence genes similar to human strains

associated with human illnesses and phylogenetically related to one of the O104:H7 human clinical strains, the serotype appears to have the potential to be a diarrheagenic foodborne pathogen in humans.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157 and six serogroups of non-O157 STEC, O26, O45, O103, O111, O121, and O145, are responsible for foodborne illnesses that range from mild to bloody diarrhea to serious complications of hemolytic uremic syndrome (HUS) in humans (Gould et al., 2013). The 'top 7' STEC have been declared as adulterants in ground beef and non-intact raw beef products by the U.S. Department of Agriculture, Food Safety and Inspection Service (Taylor MR, 1994; USDA-FSIS, 2011). In 2011, a novel pathotype, *E. coli* O104:H4, was responsible for a large outbreak of hemorrhagic colitis and HUS in humans in Germany. The serotype was a hybrid pathotype carrying genes characteristic of enteroaggregative (EAEC) and STEC (Bielaszewska et al., 2011). Another serotype, *E. coli* O104:H21 was responsible for a small outbreak of hemorrhagic colitis in Helena, Montana in 1994 due to consumption of contaminated milk (CDC, 1995). In addition, there are reports of sporadic cases of diarrhea associated with *E. coli* O104:H2, O104:H7, and O104:H12 (Delannoy et al., 2012a; Miko et al., 2013).

Cattle are a major reservoir of O157 and non-O157 STEC serogroups, harboring them in the hindgut and shedding them in feces. The German outbreak strain of *E. coli* O104:H4 has not been detected in cattle feces (Auvray et al., 2012; Paddock et al., 2013; Shridhar et al., 2016b; Wieler et al., 2011). However, cattle do harbor and shed other serotypes of O104, including O104:H2, O104:H7, O104:H11, O104:H12, O104:H21 and O104:H27 (Miko et al., 2013; Rump

et al., 2012; Shridhar et al., 2016b). Of all the serotypes, O104:H7 was the dominant serotype and the only serotype that carried Shiga toxin gene (*stx*1) (Shridhar et al., 2016b).

Shiga toxins (Stx) are the major virulence factors of STEC, which are responsible for life-threatening complications such as HUS associated with STEC infections (Karmali et al., 1985). Intimin, encoded by *eae*, a key adhesin expressed by enterohemorrhagic (EHEC), a subset of STEC, and enteropathogenic *E. coli* (EPEC), is responsible for the intimate attachment of bacteria to the host epithelial cells leading to attachment and effacement lesion (DeVinney et al., 1999). In addition, a number of other virulence factors, encoded by genes located on the chromosome or mobile genetic elements, are also involved in the pathogenesis of *E. coli* infections (Kaper et al., 1999). Therefore, understanding the virulence gene profiles of *E. coli* O104 serotypes isolated from cattle is required to assess their potential to cause infections. Whole genome sequence analysis provides a greater insight into the virulence potential and genetic diversity of strains within a serotype. The objectives of this study were to determine the virulence genes repertoire of *E. coli* O104 strains isolated from cattle by whole genome sequence analysis and to determine their phylogenetic relationship. Human clinical strains of *E. coli* O104:H7 were included for comparison.

Materials and Methods

E. coli O104 strains

Ten *E. coli* O104 strains isolated from rectal contents of feedlot cattle collected at slaughter (Shridhar et al., 2016b) and five *E. coli* O104 strains isolated from sporadic cases of diarrhea from human patients (Centers for Disease Control and Prevention, Atlanta, GA) were included in the study. Of the 10 cattle strains, six were identified to contain H7 flagellar antigen

(Shridhar et al., 2016b) and the other four were of unknown serotypes. All five human strains were of H7 flagellar type. The strains were cultured onto Tryptone soy agar (TSA; BD Difco, Sparks, MD) slants and shipped on ice overnight to the Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD for whole genome sequencing.

DNA extraction and whole genome sequencing

Escherichia coli O104 strains were streaked onto blood agar (Remel, Lenexa, KS). A single colony of each strain was inoculated into Luria Bertani (LB) broth and incubated on a shaker at 37°C. Genomic DNA was isolated from 1 ml overnight culture using the Qiagen DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA). The purity of the DNA was assessed spectrophotometrically using the Nanodrop (Thermo Scientific, Willmington, USA). Genomic libraries of the strains were constructed using Nextera XT DNA Library Preparation kit and whole genome sequencing was performed on an Illumina MiSeq benchtop sequencer (Illumina, Inc., San Diego, CA) using the MiSeq version 2 reagent kit with 2 × 250 cycles. CLC Genomics Workbench version 8.5.1 was used for *de novo* assembly of quality controlled trimmed sequenced reads using kmer size of 55. Average genome coverage of bovine and human *E. coli* O104 strains was 52x. Subsequent analysis was performed on the assembled draft genomes.

Sequence analysis

Draft genomes of *E. coli* O104 strains were initially annotated using the RAST (Rapid Annotation using Subsystem Technology) server. The RAST server also provides data on genes associated with various subsystems and the distribution of genes in various categories. A single factor analysis of variance (ANOVA) test was performed to determine whether genome size, and number genes associated with different functional categories were significantly different between bovine STEC, non-STEC and human strains. If the means were significantly different ($P < 0.01$),

then Tukey adjustment for multiple comparisons was performed, using SAS 9.4 with Proc Glimmix, to test each pairwise comparison. Serotypes, virulence genes and antibiotic resistance genes of all the strains were determined using serotype finder 1.1, virulence finder 1.5, and ResFinder 2.1, respectively, web-based tools developed by the Center for Genomic Epidemiology (CGE) at the Danish Technical University (Lyngby, Denmark) (http://www.genomicepidemiology.org/). The plasmids were identified by PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) tool. The total number of phage sequences were determined using Phage Search Tool Enhanced Release (PHASTER; http://phaster.ca/). It identifies intact, questionable, and incomplete phage sequences by scores of >90, 70-90, <70, respectively (Arndt et al., 2016; Zhou et al., 2011). The sequence types of the strains were determined *in silico* using MLST 1.8 (https://cge.cbs.dtu.dk/services/MLST/). The Harvest Suite, a software package which includes tools such as Parsnp and Gingr was used to determine the phylogenetic relationship among the *E. coli* O104 strains (Treangen et al., 2014). Parsnp v1.2 (http://harvest.readthedocs.io/en/latest/content/parsnp.html) was used to align the core genomes of human and bovine *E. coli* O104 strains, followed by the construction of maximum likelihood tree. *Escherichia coli* O104:H21, a human outbreak strain (Montana; GenBank accession no. CP009106.2) was also included in the phylogenetic tree to determine the genetic relatedness to the bovine O104 strains. The phylogenetic tree was subsequently imported to FigTree 1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/) for better visualization, and bootstrap values were reported for each branch.

Results

The serotypes of bovine (n=6) and human O104:H7 (n=5) strains were confirmed by Serotype finder 1.1, and the other four unknown serotypes from cattle were identified as O104:H8, O104:H12, and 104:H23 (n=2).

RAST annotation

The genome size ranged from 5.2 to 5.3 Mb and 4.7 to 5.0 Mb for STEC and non-STEC bovine strains, respectively, based on RAST subsystem annotations. The genome size of human *E. coli* O104 strains ranged from 4.9 to 5.4 Mb. The average genome size of bovine STEC strains (5.3 Mb) was significantly ($P < 0.01$) larger than bovine non-STEC strains (4.9 Mb). The functional categorization of genes revealed that the number of genes associated with virulence, disease, and defense ranged from 109 to 116 in bovine strains, and 110 to122 in human O104 strains. The number of genes carried on mobile elements such as phages, prophages, transposable elements and plasmids ranged from 60 to 201 in bovine O104 strains, and 80 to 201 in human O104 strains. The average number of genes associated with mobile genetic elements was significantly ($P < 0.01$) higher in bovine STEC strains (189) compared to non-STEC strains (71). There was also significant ($P < 0.01$) difference in the average number of genes associated with mobile genetic elements between human and bovine STEC O104:H7 strains. There was no significant difference in the average number of genes associated with the other subsystem categories (membrane transport, iron acquisition and metabolism, and stress response) between bovine and human strains (Table 9.1).

Virulence genes

Four bovine and one human STEC O104:H7 strains carried *stx*1c gene. Three of the human O104 strains carried *stx*2, two were of subtype 2a, and one was 2d (Table 9.2). The *ast*A

gene that codes for *E. coli* heat stable enterotoxin 1 was carried by one bovine strain (2013-6-173D that belonged to O104:H23 serotype). Another gene, *sub*A (subtilase toxin subunit) was present in 4 of 5 human strains of O104:H7, but absent in bovine strains. All of the bovine and human O104 strains were negative for intimin (*eae*) and other genes associated with the LEE pathogenicity island, and non-LEE encoded effectors. However, both bovine and human *E. coli* O104 strains carried other adhesins, such as *irg*A homolog adhesin (*iha*) and long polar fimbriae (*lpfA*).

Plasmid-encoded virulence genes (*ehx*A, *epe*A, *esp*P, *kat*P) were present in bovine and human strains. Four of the bovine strains, and three of the human strains carried *ehx*A (enterohemolysin). Enterohemorrhagic *E. coli* plasmid-encoded autotransporter (*epe*A) was present in one of the bovine strains (O104:H7; 2013-6-685A), and one human strain (O104:H7; 2011C-3665). Extracellular serine protease (*esp*P; 4/5) and catalase peroxidase (*kat*P; 1/5) were present only in human strains.

All bovine and human strains carried *gad* (Glutamate decarboxylase). Four bovine O104:H7 strains (2013-6-48C, 2013-6-122E, 2013-6-148B, and 2013-6-685A) and one human O104:H7 strain (2011C-3665) were positive for *aai*C (Type VI secretory system protein). The gene encoding increased serum survival (*iss*) was present in two bovine (2013-6-685A and 2013-6-210A) and four human O104:H7 strains (06-3637, 07-3598, 08-3046, and 2012C-3400). One of the bovine strains (O104:H7 2013-6-193B) carried *pic* (protein involved in intestinal colonization). Different types of colicin encoding genes were also present in bovine and human *E. coli* O104 strains. The colicin B gene (*cba*) was present in one of the bovine strains (2013-6-148B) and one of the human strains (08-4061), *celb* (endonuclease colicin E2) was present in three human strains (06-3637, 07-3598, and 2011C-3665), *cma* (Colicin M) was present only in

one human strain (2011C-3665). Virulence gene profiles of bovine and human strains are provided in table 9.2.

Antimicrobial resistance genes

Antimicrobial resistance genes such as *tet* (tetracycline resistance), *aad*A1 (aminoglycoside resistance), and *sul*1 (sulphonamide resistance) were present only in one of the human strains (2011C-3665). All the other bovine and human strains were negative for antimicrobial resistance genes (Table 9.2)

Plasmid and prophage sequences

All the bovine STEC and one (2013-6-289D) of the non-STEC O104:H7 strains carried IncFIB and IncFII plasmid sequences (Table 9.3). IncH12 and IncH12A were carried by all except one (O104:H7; 2013-6-193B) bovine non-STEC strains. IncY was present in one (O104:H8; 2013-6-380B) of the bovine non-STEC strains and one human STEC strain (08-4061). IncB/O/K/Z and IncFIB were present in all human strains except one strain (O104:H7; 2011C-3665). Col156 was present in two human STEC and one non-STEC strains. A complete list of plasmid sequences found in bovine and human *E. coli* O104 strains is provided in Table 9.3.

Total number of intact phages in bovine STEC O104:H7 strains was similar (n=8), whereas bovine non-STEC strains carried only 1 to 5 intact phages. The number of intact phages in human STEC O104:H7 ranged from 2-10, with 2011C-3665 strain carrying highest number (n=10) of intact phage sequences. The number of incomplete phage sequences ranged from 9-12, 1-5, and 4-7 for bovine STEC, bovine non-STEC, and human STEC strains, respectively. Total number of questionable phages ranged from 1-4, 0-1, and 1-4 for bovine STEC, bovine non-

STEC, and human STEC strains, respectively. One of the human non-STEC strains carried four intact, six incomplete and zero questionable phages (Table 9.4).

Phylogenetic relationship and sequence types

Bovine *E. coli* O104 strains carrying the same flagellar antigen clustered together (Fig 9.1). Among bovine O104:H7 strains, STEC strains clustered separately from non-STEC strains. Additionally, the O104:H8 strain (2013-6-380B) was more closely related to non-STEC O104:H7 strains (Fig 9.1). *Escherichia coli* O104:H12 strain (2013-6-210A) was distantly related to other O104 serotypes. All human O104:H7 strains, except one, clustered together. One of the human strains (2011C-3665) clustered with the bovine O104 STEC strains. The human Montana outbreak strain of O104:H21 (Montana outbreak) clustered more closely with one of the human (2011C-3665) and bovine O104:H7 STEC strains (Fig 9.1).

All the bovine O104 STEC strains and one of the human STEC strains (2011C-3665) belonged to ST-1817. The other 4 human O104:H7 strains belonged to ST-2773. One non-STEC bovine O104:H7 strain (2013-6-289D) belonged to ST-5014. Remaining bovine non-STEC O104 strains belonged to ST-678, ST-10, ST-6494, ST-129 and ST-939.

Discussion

Shiga toxin–producing *E. coli* O104 serotypes, other than O104:H4, have been reported to be associated with sporadic cases of diarrhea in humans (Hussein, 2007; Miko et al., 2013). Cattle are a major reservoir of enterohemorrhagic *E. coli*, harboring them in the hindgut and shedding them in feces. *Escherichia coli* O104:H4 (German) outbreak strain has not been detected in cattle feces (Paddock et al., 2013; Shridhar et al., 2016b). However, other O104 serotypes have been isolated from cattle feces (Shridhar et al., 2016b). Several studies have

characterized the strains belonging to various O104 serotypes by PCR-based detection of specific virulence targets (Miko et al., 2013; Shridhar et al., 2016b). However, there are very few studies on the genome-scale analysis of *E. coli* O104 serotypes other than the German outbreak strain. Yan et al. (2015) sequenced the genomes of *E. coli* O104:H21 (Human; Montana outbreak) and an O104:H7 STEC strain isolated from cattle and compared them to *E. coli* O104:H4 (German and Central Africa outbreak strains) strains. They reported that the O104:H7 STEC strain was more similar to the Montana strain (O104:H21) than to the German strain (O104:H4). To our knowledge, this is the first study to analyze the whole genome sequences of human O104:H7 strains and compare the virulence gene profiles of bovine O104:H7 strains.

Genomes of *stx* carrying bovine O104:H7 strains were significantly larger than the *stx*-negative strains of O104:H7 or other serotypes. However, the genomes of human O104 strains were smaller than bovine strains regardless whether they contained *stx* or not, except for one of the STEC O104:H7 strains (2011-3665C) which had the largest genome size. The genome sizes were proportional to the number of genes carried on the mobile genetic elements (phages, prophages, transposable elements and plasmids). Yan et al. (2015) have attributed the variation in the genomes of O104 strains to gain or loss of mobile genetic elements (Yan et al., 2015). IncF plasmids were most commonly found in bovine O104 STEC strains. Antimicrobial resistance genes, such as $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, and *aac(6')-Ib-cr*, are carried on IncF plasmids (Carattoli, 2009; Cergole-Novella et al., 2010). IncH12 plasmid which was present in bovine non-STEC O104 strains, has shown to be involved in the dissemination of antimicrobial resistance genes (Fernández et al., 2007).

Of all the bovine O104 serotypes, only *E. coli* O104:H7 serotype carried Shiga toxins. Two human O104 strains carried *stx*2 subtype a, and one of the strain carried *stx*2 subtype d.

Shiga toxin 2a and 2d have been reported to be most commonly associated with severe human illness and complications such as HUS (Bielaszewska et al., 2006; Iyoda et al., 2014; Persson et al., 2007). Four of the bovine and one of the human O104:H7 strains carried *stx*1 subtype c. Friedrich et al (2003) reported that *stx*1c carrying STEC strains were isolated from asymptomatic patients and patients with uncomplicated diarrhea (Friedrich et al., 2003). However, *stx*1c carrying O78:H- strain was isolated from 2-week old boy suffering from bacteremia and HUS, and the asymptomatic family members (Lienemann et al., 2012).

All the Bovine and human strains included in our study were negative for intimin and other LEE-encoded virulence genes, and non-LEE encoded effectors. A significant association between *stx*1c and LEE-negative strains has been reported (Haugum et al., 2014). Shiga toxin-producing *E. coli* strains negative for LEE-encoded virulence genes have been isolated from humans with HUS (Bonnet et al., 1998; Paton et al., 1999). Both the human and cattle *E. coli* O104 strains carried other adhesins such as *iha* and *lpf*A. Long polar fimbriae (*lpf*) and *iha* were the most prevalent adhesins identified in LEE-negative *E. coli* strains isolated from cattle and human sources (Galli et al., 2010). Additionally, none of the bovine and human strains investigated in our study were positive for *efa*1 (enterohemorrhagic *E. coli* factor for adherence). Previous studies have suggested a strong association between *eae* and *efa*1 genes (Galli et al., 2010). Human O104:H7 strains were positive for other toxins such as subtilase cytotoxin. Subtilase cytotoxin has been reported to be involved in causing HUS by damaging human microvascular endothelial cells (Amaral et al., 2013). It has also been detected in *eae* negative *E. coli* O103:H21 strains associated with sporadic cases of HUS (Paton et al., 1999). One of the *stx* negative bovine O104 strain was positive for *ast*A gene. Typical and atypical enteropathogenic

E. coli strains carrying *astA* gene have been reported to be frequently associated with diarrhea (Silva et al., 2014).

Both bovine and human *E. coli* O104 strains included in the present study were positive for plasmid encoded virulence genes such as *ehx*A, *esp*P, *kat*P (encoded by pO157), and *epe*A (encoded by pO113). Enterohemolysin, encoded by *ehxA*, has been reported to be involved in increased production of IL-1β and cytotoxicity (Zhang et al., 2012). It has also been reported to be frequently associated with *E. coli* O111 STEC strains isolated from patients with HUS (Schmidt and Karch, 1996). Extracellular serine protease (*esp*P) is responsible for mucosal hemorrhage observed in patients suffering from hemorrhagic colitis, due to cleavage of pepsin A and human coagulation factor V (Brunder et al., 1997b). An autotransporter protease, *epe*A has been reported to be frequently found in association with LEE-negative STEC strains, it is involved in adherence and colonization of small intestine due to its mucinolytic activity (Leyton et al., 2003).

One of the bovine O104:H7 (non-STEC) strain carried *pic* (protein involved in intestinal colonization), a protease secreted by *Shigella flexneri* and enteroaggregative *E. coli* involved in intestinal colonization (Henderson et al., 1999). It was also carried by *E. coli* O104:H4 (German outbreak strain) (Bielaszewska et al., 2011). One of the human O104:H7 strains was positive for antimicrobial resistance genes such as *aadA*1 (aminoglycoside resistance), *tet* (tetracycline resistance), and *sul*1 (sulphonamide resistance). Tetracycline resistance was the most common resistance in *E. coli* O157 strains isolated from bovine and human sources (Wilkerson et al., 2004). Non-O157 Shiga toxin producing *E. coli* serogroups isolated from human, bovine and ovine sources were found to be most commonly resistant to streptomycin, sulfisoxazole, and tetracycline (Wang et al., 2016).

Analysis of the phylogenetic relationship of O104 strains revealed that the O104:H21 strain was more closely related to Shiga toxin-carrying O104:H7 strains of bovine and human origin. This is in agreement with the previous study (Yan et al., 2015). In our study, O104 strains with the same H-type clustered together, however, STEC strains clustered separately from non-STEC strains. Miko et al. (2013) reported that the O104 STEC strain carrying same H-types had similar virulence gene profile and PFGE patterns (Miko et al., 2013). The genetic diversity of different O104 serotypes could be attributed to gain and loss of mobile genetic elements (Yan et al., 2015). Additionally, bovine O104 STEC strains and one of the human STEC strains (2011C-3665) which clustered together belonged to the same sequence type (ST-1817). This suggests that the bovine feces could be the source of human illness caused by *E. coli* O104 serogroup. One of the non-STEC O104 strain belonged to ST-678, similar to *E. coli* O104:H4 German outbreak strain (Mora et al., 2011).

Conclusions

Whole genome analyses of *E. coli* O104 serotypes provide a comprehensive insight into their pathogenic potential. The predominant serotype in cattle, O104:H7 could be of public health concern, because they carry several genes encoding virulence factors responsible for human illness. Also, one of the human O104:H7 strain was phylogenetically more closely related and belonged to the same sequence type (ST-1817) as that of bovine O104:H7 STEC strains. This study suggests the potential of bovine O104:H7 STEC strains to cause human illnesses.

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Figure 9-1. Phylogenetic tree of bovine and human *E. coli* O104 strains constructed using Parsnp v1.2 and visualized using FigTree 1.4.3. Numbers on the branches correspond to bootstrap values

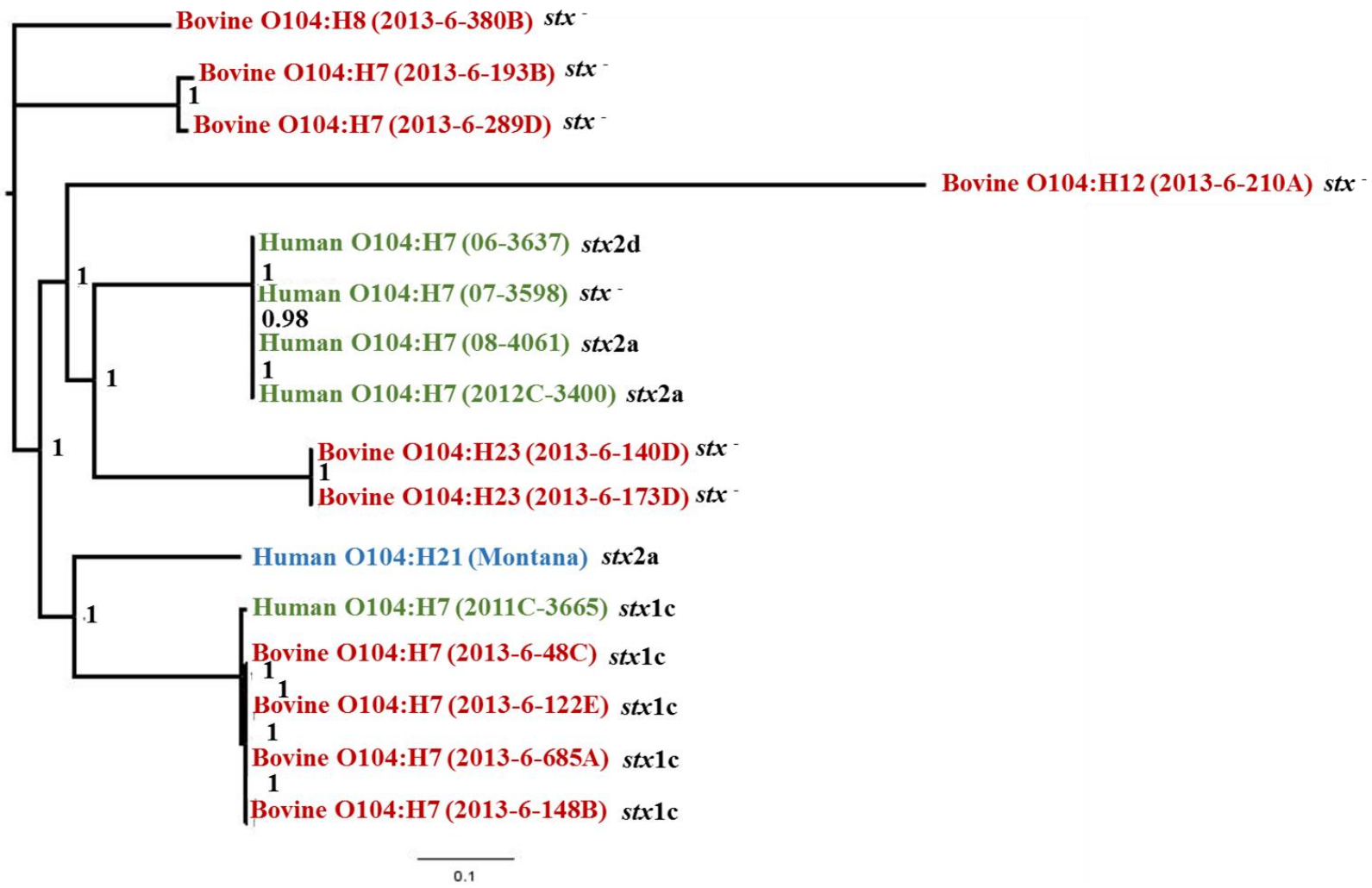

Table 9.1. Genome size and different categories of genes in *E. coli* O104 strains isolated from human and cattle sources based on RAST subsystem annotation

Strains	Serotype	Genome size (Mb)	*stx*	Virulence, disease, and defense	Phages, prophages, transposable elements and plasmids	Membrane transport	Iron acquisition and metabolism	Stress response
Cattle								
2013-6-685A	O104:H7	5.2	+	109	188	221	23	187
2013-6-48C	O104:H7	5.3	+	109	201	223	24	184
2013-6-122E	O104:H7	5.3	+	109	184	223	24	186
2013-6-148B	O104:H7	5.3	+	109	186	222	23	186
2013-6-193B	O104:H7	4.7	-	109	70	195	22	183
2013-6-289D	O104:H7	5.0	-	109	65	294	22	187
2013-6-380B	O104:H8	5.0	-	116	64	216	24	186
2013-6-210A	O104:H12	4.8	-	113	60	204	26	193
2013-6-140D	O104:H23	5.0	-	111	82	237	22	184
2013-6-173D	O104:H23	5.0	-	111	85	238	22	184
Human								
06-3637	O104:H7	5.0	+	112	95	328	22	185
08-4061	O104:H7	5.0	+	110	108	220	22	184

2011C-3665	O104:H7	5.4	+	122	201	316	22	187
2012C-3400	O104:H7	5.0	+	110	120	222	22	185
07-3598	O104:H7	4.9	-	110	80	254	22	184

Table 9.2. Virulence genes present in *E. coli* O104 strains isolated from cattle feces (n=10) and human sources (n=5)[a]

Genes	Product	Bovine						Human					Bovine			
		O104:H7 (2013-6-685A)	O104:H7 (2013-6-48C)	O104:H7 (2013-6-122E)	O104:H7 (2013-6-148B)	O104:H7 (2013-6-193B)	O104:H7 (2013-6-289D)	O104:H7 (06-3637)	O104:H7 (08-4061)	O104:H7 (2011C-3665)	O104:H7 (2012C-3400)	O104:H7 (07-3598)	O104:H8 (2013-6-380B)	O104:H12 (2013-6-210A)	O104:H23 (2013-6-140D)	O104:H23 (2013-6-173D)
Toxins																
*stx*1c	Shiga toxin 1 subtype c	+	+	+	+					+						
*stx*2a	Shiga toxin 2 subtype a								+		+					
*stx*2d	Shiga toxin 2 subtype d							+								
subA	Subtilase toxin subunit							+	+		+	+				
ehxA	Enterohemolysin	+	+	+	+			+	+			+				
astA	EAST-1 heat-stable toxin															+
Adhesins																
iha	*Irg*A homologue adhesin	+	+	+	+			+	+		+					
lpfA	Long polar fimbriae	+	+	+	+	+	+	+	+	+	+	+	+		+	+
Others																

aaiC	Type VI secretion protein	+	+	+	+					+									
gad	Glutamate decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
iss	Increased serum survival	+						+	+		+	+		+					
epeA	Enterohemorrhagic *E. coli* plasmid-encoded autotransporter	+								+									
cba	Colicin B				+				+										
celb	Endonuclease colicin E2							+		+		+							
espP	Extracellular serine protease plasmid-encoded							+	+		+	+							
cma	Colicin M									+									
katP	Plasmid-encoded catalase peroxidase									+									
pic	Serine protease autotransporters of Enterobacteriaceae (SPATE)					+													
Antimicrobial resistance genes																			
tet	Tetracycline resistance									+									
aadA1	Aminoglycoside resistance									+									
sul1	Sulphonamide resistance									+									

[a]Virulence gene profile was determined using VirulenceFinder 1.4 (Joensen et al., 2014)

Table 9.3. Plasmid types in *E. coli* O104 strains of bovine and human origin identified using PlasmidFinder 1.3[a]

Serotypes	Strains	*stx*	Plasmid types, strains positive						
			IncFIB	IncFII	IncB/O/K/Z	Col156	IncY	IncH12	IncH12A
Bovine									
O104:H7	2013-6-685A	1c	+	+					
O104:H7	2013-6-48C	1c	+	+					
O104:H7	2013-6-122E	1c	+	+					
O104:H7	2013-6-148B	1c	+	+					
O104:H7	2013-6-193B	-							
O104:H7	2013-6-289D	-	+	+				+	+
Human									
O104:H7	06-3637	2d	+		+	+			
O104:H7	08-4061	2a	+		+		+		
O104:H7	2011C-3665	1c		+		+			
O104:H7	2012C-3400	2a	+		+				
O104:H7	07-3598	-	+		+	+			
Bovine									
O104:H8	2013-6-380B	-					+	+	+
O104:H12	2013-6-210A	-						+	+

O104:H23	2013-6-140D	-	+	+
O104:H23	2013-6-173D	-	+	+

[a]Plasmid sequence types were identified by PlasmidFinder 1.3 (Carattoli et al., 2014)

Table 9.4. Total number of prophage sequences in bovine and human *E. coli* O104 strains identified using PHASTER

Serotypes	Strains	*stx*	Type of phage sequences[a]		
			Intact	Questionable	Incomplete
Bovine					
O104:H7	2013-6-685A	1c	8	1	9
O104:H7	2013-6-48C	1c	8	4	12
O104:H7	2013-6-122E	1c	8	3	10
O104:H7	2013-6-148B	1c	8	4	11
O104:H7	2013-6-193B	-	3	1	1
O104:H7	2013-6-289D	-	5	0	3
Human					
O104:H7	06-3637	2d	3	4	4
O104:H7	08-4061	2a	2	3	7
O104:H7	2011C-3665	1c	10	1	7
O104:H7	2012C-3400	2a	5	1	5
O104:H7	07-3598	-	4	0	6
Bovine					
O104:H8	2013-6-380B	-	5	0	5
O104:H12	2013-6-210A	-	1	0	3
O104:H23	2013-6-140D	-	3	0	3
O104:H23	2013-6-173D	-	4	0	1

[a]Phage sequences were classified as intact, questionable and incomplete based on the PHASTER scores >90, 70-90, <70 (based on the proportion of phage genes in the identified region), respectively (Arndt et al., 2016; Zhou et al., 2011)

Chapter 10 - Overall Conclusions and Future Directions

Shiga toxin-producing *E. coli* (STEC) are major foodborne pathogens that cause mild to bloody diarrhea, which can lead to hemolytic uremic syndrome (HUS) and even death. Serogroup O157 is the major STEC, however, non-O157 STEC, particularly O26, O45, O103, O111, O121 and O145, are gaining more attention in recent years as they are responsible for majority of the non-O157 STEC associated infections in humans. Cattle are major reservoirs of STEC, harboring them in the hindgut and shedding them in feces. Rapid and sensitive diagnostic methods are required to estimate the prevalence and concentration of non-O157 STEC serogroups in cattle feces. Two multiplex real-time PCR assays (assay 1: O26, O103, O111; assay 2: O45, O121, O145) were developed to detect and quantify six non-O157 *E. coli* serogroups in cattle feces. The assays were specific to all the target serogroups. The detection limit of the assays was 3 log CFU/ml, 4 log CFU/g and 2 log CFU/g for pure cultures, culture-spiked cattle feces before and after enrichment, respectively. The applicability of the assays to detect six non-O157 *E. coli* serogroups in cattle fecal samples collected from a commercial feedlot were compared to end-point PCR and culture-based method. The mqPCR assays detected a higher proportion of samples positive for non-O157 *E. coli* serogroups compared to end-point PCR and culture-based method. A spiral plating method was validated to quantify six major non-O157 *E. coli* serogroups in cattle feces. The quantification limit of the spiral plating method was 3, 3 - 4, 3 - 5 log CFU/ml or g for individual, pooled pure culture, and pooled pure culture-spiked cattle feces, respectively. The spiral plating method was utilized to quantify six major non-O157 *E. coli* serogroups in cattle feces collected from commercial feedlots and was compared to mqPCR assays. The mqPCR assays quantified higher proportion of samples positive for non-O157 *E. coli* serogroups compared to spiral plating method. Both methods

revealed that a subset of cattle shed non-O157 *E. coli* at a high concentration, and are termed as super shedders, similar to *E. coli* O157.

Cattle shed non-O157 *E. coli* serogroups belonging to different pathotypes (STEC, enterohemorrhagic, enteropathogenic, and putative non-pathotype), classified based on the major virulence genes. The virulence potential of *E. coli* strains shed in the cattle feces was determined by genetic characterization. Shiga toxins (Stx1 and Stx2) are the major virulence factors responsible for human STEC infections, and *stx*2 is more commonly associated with severe illness and life-threatening complications, such as HUS compared to *stx*1. Subtypes of *stx*2 have also been reported to influence the severity of clinical illness. The distribution of *stx*1 and *stx*2 subtypes in non-O157 STEC serogroups of cattle and human origin was determined. Non-O157 STEC serogroups isolated from cattle possessed a wide variety of Shiga toxin subtypes, with *stx*1a and *stx*2a being the most predominant subtypes occurring individually or in combination. The gene content of non-O157 *E. coli* cattle fecal and human clinical strains was analyzed by FDA-ECID DNA microarray. Bovine non-O157 *E. coli* strains possessed major virulence genes, such as those encoding Shiga toxins (1 and 2), adhesins, type III secretory system associated proteins, and plasmid-encoded virulence factors. The virulence gene profiles and flagellar types of the bovine strains were similar to that of human clinical strains, suggesting the potential of cattle fecal strains to cause human infections. The virulence potential of STEC O145 strains isolated from cattle feces and hide samples and human clinical strains were analyzed by whole genomes sequencing (WGS). Shiga toxin 1a was the most common subtype, followed by *stx*2a and *stx*2c (only in bovine strains). All O145 strains carried flagellar type H28 and *eae* subtype γ1. The strains also carried genes encoding type III secretory system proteins, *nle* genes, and plasmid-encoded virulence genes. The similarity in virulence gene profiles in strains isolated

from cattle feces, hide and human clinical cases suggest the potential of cattle STEC O145 strains to cause human illnesses.

Escherichia coli O104:H4 is a serotype responsible for a large outbreak of HUS in Germany in 2011. It is a hybrid pathotype carrying genes characteristic of enteroaggregative *E. coli* and STEC. The prevalence of *E. coli* O104 serogroup in cattle feces by PCR and culture methods and virulence potential of the isolated *E. coli* O104 strains were determined. Model adjusted prevalence estimates of *E. coli* O104 in cattle fecal samples collected from a Midwest slaughter plant were 0.5% and 25.9% by culture and PCR methods, respectively. Cattle do not appear to harbor *E. coli* O104:H4 hybrid pathotype, however, they do harbor and shed other O104 serotypes (O104:H2, O104:H7, O104:H11, O104:H21). *E. coli* O104:H7 was the predominant serotype and a small proportion of the isolated strains carried *stx*1c. Based on DNA microarray and WGS, none of the *E. coli* O104 strains carried genes encoded by LEE pathogenicity island. Cattle fecal O104:H7 strains characterized in our studies suggest the potential of the serotype to be a diarrheagenic human pathogen, since they possess *stx*1c, gene that code for enterohemolysin, and genes that encode a variety of adhesins.

In conclusion, qPCR and culture-based methods were developed to detect and quantify six major non-O157 *E. coli* serogroups in cattle feces. This is the first report of identification of a subset of cattle that are super-shedders of non-O157 *E. coli* serogroups. Further improvement in the selective media to phenotypically distinguish the STEC serogroups and minimize the growth of background flora could improve the detection and quantification by culture method. The virulence assessment of six major non-O157 *E. coli* serogroups and *E. coli* O104 by DNA microarray and WGS suggests the potential of cattle strains to cause human infections. However, confirmation of the virulence potential requires transcriptomic studies and possibly suitable

animal model studies. It is also important to determine the factors affecting the functional expression of virulence genes to help understand the molecular mechanisms involved in the non-O157 STEC pathogenesis.