Shiga toxin-producing *Escherichia coli* (STEC) other than the top-7: multiplex PCR assays development and validation for detection and identification of the predominant serogroups in cattle feces.

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by

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Abstract

Shiga toxin-producing *E. coli* (STEC) are major food borne pathogens that cause mild to severe hemorrhagic colitis in humans. Seven serogroups, which include O26, O45, O103, O111, O121, O145 and O157, considered the 'top-7 STEC', cause a majority of food borne STEC infections. Cattle are a major reservoir for the STEC. These organisms reside in the hindgut and are shed in the feces. Additionally, cattle harbor a number of other serogroups (n=113) of STEC. A few of the serogroups have been implicated to cause sporadic human infections. Traditionally, serogrouping of *E. coli* is done by agglutination reaction with serogroup-specific antisera. Multiplex PCR (mPCR) assays targeting STEC serogroups, other than the top-7, were designed, optimized, and validated. The assays targeted one or more serogroup-specific genes, *wzx, wzy, gnd, wbdA* of the O-antigen gene cluster. A total of 11 sets of mPCR assays targeting 113 serogroups of STEC were designed and validated with known strains of STEC. The assays were utilized to identify serogroups of *E. coli* strains (n=358), positive for *stx*1, *stx*2 (Shiga toxins 1 and 2), and or *eae* (intimin) genes, and negative for the top 7 serogroups, isolated from fecal samples collected from commercial; feedlots. Of the 358 strains from multiple feedlots located in two states, 298 (83.2%) strains belonged to 21 serogroups and 60 strains (16.8%) were unidentifiable. The twenty one sergogroups included O168 (29.9%), O109 (17.9%), O131 (8.1%), O2 (7.0%), O104 (4.7%), O171 (4.2%), O74 (3.6%), O8 (2.8%), O136 (0.84%), O178 (0.56%), O102 (0.56%), O175 (0.56%), O98 (0.28%), O118 (0.28%), O113 (0.28%), O96 (0.28%), O76 (0.28%),O35 (0.28%), O20 (0.28%), O160 (0.28%), and O169 (0.28%). The detection of the STEC strains indicated that serogroups, O2, O74, O109, O131, O168, and O171, were the most predominant. A six-plex PCR was designed and validated to detect the prevalence of the top six major non top-7 STEC in cattle feces from feedlot cattle. Among the six

serogroups of the major non-top 7 STEC, serogroups of O2 (48.7%), O109 (88.1%), O168 (67.9%), and O171 (80.4%) were the dominant serogroups in cattle feces collected from commercial feedlots. Fecal samples that contained the six serogroups were also positive for the three major virulence genes, *stx*1, *stx*2, and *eae*. In conclusion, cattle harbor and shed in the feces a number of serogroups of STEC other than the top 7, with O2, O109, O168, and O171 being the predominant STEC. The potential risk of these STEC to cause human infections is not known.

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Dedication

This work in dedicated to the Ludwig and Behrman Family. Their belief in higher education and continuous learning is just one example of how they allow me to stand on the shoulders of giants.

Chapter 1 - An overview of Shiga toxin-producing *Escherichia coli* (STEC) other than the top-7, in cattle.

Introduction

Escherichia coli, belonging to the *Enterobacteriacae* family, is a Gram negative, facultatively anaerobic, rod shaped organism. The species is present in various habitats, including gastrointestinal tract of animals and humans, soil and water. Most strains of *E. coli* are non-pathogenic, however, some have become pathogenic because of acquisition of virulence genes via mobile genetic elements. The pathogenic *E. coli* or pathotypes are broadly categorized into those that cause intestinal infections and extra-intestinal infections. The categorization of *E. coli* pathotypes is based on the virulence mechanisms, such as patterns and effects of bacterial attachment onto host cells, effects of attachment on host cells, production of toxins, and invasiveness. The pathotypes that cause intestinal infections, also called diarrheagenic, include at least five major categories: Shiga toxin-producing *Escherichia coli* (STEC) of which enterohemorrhagic *E. coli* (EHEC) is a subset, enteropathogenic *Ea coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAggEC) of the pathotypes, Shiga toxin-producing *E. coli* is the most common in terms of prevalence, particularly in the gastrointestinal tract habitat of cattle.

Shiga toxin-producing *E. coli* (STEC) are major zoonotic, foodborne pathogens that are associated with various disease outcomes in humans. Infections caused by STEC can range from mild enteritis to hemorrhagic colitis (HC) to complications of hemolytic uremic syndrome (HUS), particularly in children. The food borne infections are caused by consumption of contaminated beef, produce, or water. Cattle are generally considered major reservoirs for

STEC, however, other ruminant and domestic animal reservoirs have been linked to STEC infections (Gannon et al. 1993). The STEC pathotype possesses a variety of virulence factors that are critical to their adherence to host cells and cytotoxicity. Of the many, Shiga toxins are major virulence factors. There are two types of Shiga toxins, Shiga toxin1 and Shiga toxin-2, encoded by *stx1* and *stx2* genes, respectively. Additionally, there are a number of other virulent factors associated with STEC. The intimin, which is encoded by the *eae* gene, is responsible for adhesion to host cells and in the formation of attaching and effacing lesions in the intestinal epithelium. The possession of this gene distinguishes a subset of STEC called enterohemorrhagic *E. coli* (EHEC; Fratamico et al. 2011, Fagan et al. 1999).

Based on the antigenicity of cell surface structures, *E. coli* is classified into serogroups and serotypes. Serogrouping of *E. coli* is based on the O-antigen of the lipopolysaccharide, which is part of the outer membrane, and is composed of repeating units of one or more sugars or their derivatives. Serotyping includes identification based on the H-antigen, which is the flagellar protein. The importance of this classification system allows for distinguishing strains during outbreaks and for surveillance (DebRoy et al. 2016). The "gold standard" for *E. coli* serotyping based on agglutination reactions, carried out on slides, in tubes, or in 96-well microtiter plates (Debroy et al., 2011).

Seven serogroups of STEC, O26, O45, O103, O111, O121, O145, and O157 (Hara-Kudo et al. 2016), often called 'top-7', have been associated with a majority of human infections. The top 7 serogroups have been shown to colonize the hindgut of cattle and are shed in the feces, which is the source of contamination of food products (Tozzi et al., 2003). Among the top 7serogroups,

the most common serotype is O157:H7. In 1994, the USDA Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 as an adulterant in raw ground beef in response to a large outbreak associated with undercooked ground beef. The FSIS began verification testing of ground beef for this pathogen (Wasilenko et al. 2014). In addition to O157, the USDA-FSIS declared the remaining top six serogroups as adulterants in 2012. According to the Centers for Disease Control and Prevention, STEC serogroups O26, O45, O103, O111, O121, and O145 cause a majority of cases of illness due to non-O157 STEC in the US and some of them are major STEC serogroups in other countries (Fratamico et al. 2012). The morbidity and mortality associated with large outbreaks of STEC disease have highlighted the threat these organisms pose to public health.

There have been a total of 184 serogroups of *E. coli* identified, illustrating the vast diversity of *E. coli* (Bettelheim, 2007, Iguchi et al. 2015; DebRoy et al., 2016). A number of serogroups of STEC, other than the top-7, have been identified in cattle (n=108). These serogroups are also harbored in the hindgut and are shed in the feces. According to Ennis et al. (2012) and Monaghan et al. (2011) there have been reports of STEC other than the top-7 linked to human infections. Additionally, Aidar-Ugrinovich et al. (2007) and Gonzalez et al. (2016) have linked STEC serogroups not within the top-7 to causal contaminants of food products and human infections.

Shiga toxin-producing *Escherichia coli* other than the top 7 in cattle.

A total of 113 serogroups of STEC have been identified in feces (Bettellheim, 2007). O1, O2/50, O3, O4, O5, O6, O7, O8, O10, O11, O15, O16, O17/O44/O73/O77/O106, O18 (ab/ac), O19,

O20, O21, O22, O23, O25, O29, O35, O37, O38, O39, O40, O42, O43, O46/O134, O49, O51, O53, O55, O62/O68, O63, O69, O70, O74, O75, O76, O79, O80, O81, O82, O84, O85, O86, O87, O88, O90, O91, O92, O93, O96, O98, O101/O162, O102, O105, O107/O117, O108, O109, O110, O112, O113, O114, O115, O116, O118/O151, O119, O120, O124, O125 (ab/ac), O126, O128, O130, O132, O136, O139, O140, O141, O146, O148, O149, O150, O152, O153, O154, O156, O159, O160, O161, O163, O165, O168, O169, O170, O171, O172, O174, O175, O176, O177, O178, O179, O181, O182 (Table 1). These serogroups are believed to be colonized in the hindgut and are shed in the feces, therefore of concern for food safety(Iguchi et al., 2015, Bettelheim, 2007, Aidar-Ugrinovich et al., 2007, Gonzalez et al., 2016, Guth et al., 2003, Meichtri et al., 2004, Monaghan et al., 2011, Padola et al., 2004)..

Of the 113 STEC serogroups that have been identified in cattle, 85 serogroups have been associated with human illnesses. The serogroups include O1, O2/50, O3, O4, O5, O6, O7, O8, O11, O15, O16, O17/O44/O73/O77/O106, O18 (ab/ac), O20, O21, O22, O23, O25, O38, O39, O40, O43, O46/O134, O53, O55, O62/O68, O69, O70, O74, O75, O76, O79, O80, O81, O82, O84, O85, O86, O87, O88, O91, O92, O93, O96, O98, O101/O162, O102, O105, O107/O117, O109, O110, O112, O113, O114, , O115, O116, O118/O151, O119, O120, O125 (ab/ac), O126, O128, O132, O136, O146, O148, O150, O152, O153, O154, O156, O160, O161, O163, O165, O168, O169, O171, O172, O174, O175, O177, O178, O179, O181 (Iguchi et al., 2015, Bettelheim, 2007, Aidar-Ugrinovich et al., 2007, Gonzalez et al., 2016, Guth et al., 2003, Meichtri et al., 2004, Monaghan et al., 2011, Padola et al., 2004, Hornitzky et al.,2005, Fukushima et al., 2004, Beutin et al., 2007, Zweifel et al., 2005, Irino et al., 2005, Werber et al., 2008). Among the list are specific serogroups that are linked to a higher likelihood to cause

human infections. For example, according to Gonzalez et al., 2016, Meichtri et al., 2004, O8 and O165 have been connected to HUS in children. In addition to O8 and O165, O91, O113, O117, O118, O128 and O146 have a higher frequency of being linked to food contamination and health complications, such as gastroenteritis and hemorrhagic colitis (Blanco et al., 2004). Serogroup O178 has also emerged as a non top-7 STEC that is associated with human illnesses. The O178 serogroup has been frequently isolated in Germany (Beutin et al., 2007; Werber et al., 2008; Slanec et al., 2009) and South America (Cergole-Novella et al., 2007; Oliveira et al., 2008) and connected to sporadic cases of HUS in both regions.

Detection methods of STEC other than the top-7

Agglutination and antisera detection

To date, the traditional method of detection for STEC, other than the top-7, is based on serology. This method determines the O and H antigens by agglutination test carried out in microtiter plates and tubes. The O antisera is produced in a number of laboratories across the world and made available for use. Additionally, strains within a serotype can be isolated based on virulence factors that correspond to the serogroup identified (Hornitzky et al.,2005, Fukushima et al., 2004, Beutin et al., 2007, Zweifel et al., 2005, Irino et al., 2005, Werber et al., 2008, Iguchi et al., 2015, Bettelheim et al., 2007, Aidar-Ugrinovich et al., 2007, Gonzalez et al., 2016, Guth et al., 2003, Meichtri et al., 2004, Monaghan et al., 2011, Padola et al., 2004).

Culture based detection

Culture based detection of STEC, other than top-7, is generally with the use of non-selective media, such as MacConkey agar, and confirmation of the serogroup by agglutination and Shiga

toxin gene by PCR. Hornitzky et al. (2001), using the dual technique (culture and PCR) detected STEC in fecal samples and estimated a prevalence of 18.7%. Fecal samples are cultured onto blood agar supplemented with vancomycin-cefixime-cefsulodin, allowing for the detection of hemolytic colonies, then positive colonies were subjected to DNA extraction and PCR assays

PCR detection

Iguchi et al. (2015) have used 162 primary pairs for the identification of O groups. Of the 162 pairs, 147 pairs were used to identify 147 serogroups based on unique O genes and the other 15 pairs were used to identify 15 groups or strains. Each of these groups contained strains with identical or very similar o antigen genes and included a total of 35 O groups. The authors then used the 162 primer pairs to develop 20 multiplex PCR sets, with each set consisting of 6 to 9 primer pairs of different amplicon sizes. Primer pairs targeted unique sequences specific to O group biosynthesis genes, for example *wzx*, *wzy*, *wzm*, and *wzt*. Iguchi et al. (2015) concluded that PCR-based method is a promising tool for the serogrouping of *E. coli* strains.

Conclusion

Shiga toxin-producing *E. coli* is a major zoonotic foodborne pathogen. The diseases caused by STEC range from mild enteritis to hemorrhagic colitis (HC) and serious complications of hemolytic uremic syndrome (HUS). The complication of HUS is particularly seen in children and is fatal if it is not diagnosed early (Tarr et al., 2005). The top 7 STEC (O26, O45, O103, O111, O121, O145, and O157) are most commonly associated with human infections. Cattle harbor a number of other serogroups of STEC other than the top-7. Although there are several detection strategies for STEC that are the top-7, the detection of other STEC is by random

isolation and confirmation of serogroup by serology. A PCR-based method of detection has been described to identify 147 serogroups, which minclude STEC and non-STEC

Table 1. Shiga toxin-producing *E. coli*, other than the top-7 reported in cattle feces

Animal	Type of sample	Diet	Methodology	Serogroup (Prevalence)	Reference
Cattle	Fecal samples	Not reported	Serology (Agglutination with O-group specific antisera)	O5:H-, O91:H21, O113:H21	Hornitzky et al., 2001.
Beef Steers 14-16 months old	Stool samples and rectal swabs-taken at slaughter level	Not reported	Serology (Agglutination with O-group specific antisera)	O8(16 strains), O113(14 strains), O91(4), O171(3), O174(3), O25(2), O112(2), O2, O11, O128, O143, O146	Meichtri et al., 2004.
Cattle	Fecal samples	Not reported	Serology (Agglutination with O-group specific antisera)	O119, O146, O159, O177, O8	Hornitzky et al., 2005.
Calves, heifers and diarrheagenic animals	Fecal samples of calves, heifers and diarrheagenic animals	Not reported	Serology (Agglutination with O-group specific antisera)	O113, O178, O79 O77, O88, O98	Irino et al., 2005.

cattle, ovines	Isolates recovered from humans, cat-tle, ovines and food (ground beef)	Not reported	Serology (Agglutinatio n with O-group specific antisera)	O4, O8, O20, O26, O111, O118, O118, O171, O177	Mora et al., 2005.
Bovine,	Strains from human food, environment	Not reported	Serology (Agglutinatio n with O-group specific antisera)	O8, O26, O103, O111, O113 and O128.	Bettelheim et al., 2007.
Steers (80)	Rectal fecal samples	Not reported	Serology (Agglutinatio n with O-group specific antisera)	O2, O49, O110, O132 O156, O172	Diarra et al., 2009.
Cattle	Fecal samples	Not reported	Serology (Agglutinatio n with O-group specific antisera)	O2, O26, O63, O148, O149, O174	Scott et al., 2009.
Cattle	Fecal samples	Not reported	Serology (Agglutinatio n with O-group specific antisera)	O2, O113, O131	Monaghan et al., 2011.

Cattle	Fecal samples (from 12 beef farms, beef suckler and dairy herds)	Not reported	Serology (Agglutination with O-group specific antisera)	O2,O3,O26,O33, O69,O76,O88,O113,O 118,O136,O150, O153,O171	Ennis et al., 2012.
Cattle	Fecal samples collected from an abattoir and farms	Not reported	Serology (Agglutination with O-group specific antisera)	O1,O2,O8,O55,O84,O 91,O113 (8/39)	Mekata et al., 2014.

Chapter 2 - Development and validation of multiplex PCR assays to identify 'non-top-7' serogroups of Shiga toxin-producing *Escherichia coli* known to be present in cattle feces.

Introduction

Escherichia coli, a Gram-negative facultative anaerobic and rod-shaped organism, is a common inhabitant of the gut of animals and humans. The species is categorized into serogroups and serotypes based on O-antigen (somatic) and H-antigen (flagellar). The O-antigen, which is a polysaccharide, is part of the lipopolysaccharide portion of the outer membrane, and the H antigen is the flagellar protein (flagellin) of the bacteria. A total of (n=184) serogroups of *E. coli* have been recognized based on O antigens (Iguchi et al., 2014; Debroy et al., 2015). *E. coli* can be non-pathogenic or pathogenic. The pathogenic strains are categorized into several pathotypes based on the virulence mechanisms. Among the pathotypes, Shiga toxin producing *E. coli* (STEC) appears to be the major one based on the number of serogroups that have been shown to produce Shiga toxins or carry genes (*stx*) that code for Shiga toxins. Shiga toxin-producing *E. coli* are important foodborne pathogens that can cause a variety of diseases, ranging from mild to hemorrhagic enteritis that could lead to complications of hemolytic-uremic syndrome (HUS), particularly in children (Karmali et al., 2004). Specific serogroups and serotypes of STEC are associated with enteritis, hemorrhagic colitis, and hemolytic uremic syndrome. (Stenutz et al., 2006, Knirel et al., 2016). *Escherichia coli* O157 and six other serogroups, O26, O103, O111, O121, and O145, often referred to as the 'top-7', are considered as major STEC and have been declared as adulterants in ground beef and non-intact beef products (Fratamico et al., 2014). Cattle are a major reservoir of the top-7 STEC, in which the organisms colonize or reside in the hindgut and are shed in the feces (Hussein et al., 2005). Additionally, as many as 113

serogroups of STEC have been identified to be present feces of cattle (Battelheim, 2007; Aidar-Ugrinovich et al., 2007, Blanco et al., 2004, Gonzalez et al., 2016, Guth et al., 2003, Meichtri et al., 2004, Monaghan et al., 2011, Padola et al., 2004). Some of the non-top-7STEC have been shown to be associated with sporadic human illnesses (Bettelheim et al., 2007).

Traditionally, serogrouping of *E. coli* is done by agglutination reaction with serogroup-specific antisera, often carried out in a few reference laboratories. A broad serogrouping method based on restriction fragment length polymorphism pattern of amplified)-antigen biosynthesis gene cluster (O-AGC) has been described (Coimbra et al., 2000). In recent years, whole genome sequencing and DNA microarray have been used to identify serogroups of *E. coli* (Norman et al., 2015. Lacher et al., 2014; Liu and Fratamico et al., 2006, Joensen et al., 2015.). A PCR-based assay targeting serogroup-specific genes would be a simpler, low-cost alternative method, readily adaptable to any laboratory, to identify serogroups of *E. coli*. A number of PCR-based assays, end point or real time, have been developed and validated for one or multiple clinically relevant serogroups of *E. coli* (Gonzalez et al., 2016, Monaghan et al., 2011, Iguchi et al., 2014, Blanco et al., 2004; Bai et al., 2012;). Previously, we developed three sets mPCR assays to detect top-7 serogroups and three major virulence genes (Noll et al., 2015, Shridhar et al., 2016) Iguchi et al. (2015) have designed 162 primer pairs to identify 147 individual O-serogroups of *E. coli*, which were then used to create 20 multiplex PCR assays. Because the primary focus of our laboratory is STEC in cattle feces, we wanted to develop PCR based assays to detect 113 serogroups of STEC known to be present in cattle feces (Table 2). Therefore, our aim was to develop and validate several sets of multiplex PCR assays targeting serogroup-specific genes to detect 113 serogroups of STEC.

MATERIALS AND METHODS

Primer design

The nucleotide sequence of the targeted gene for each of the 113 *E. coli* serogroups were obtained from NCBI GenBank, and a search was performed to look for additional corresponding sequences using BLASTN on NCBI's Nucleotide collection (nr/nt) database. The *wzx* gene, which encodes for the O-antigen flippase (Liu et al., 2015) required for O-polysaccharide export, *wzy* gene (Liu et al., 2015), which encodes for the O-antigen polymerase required O antigen biosynthesis, and *wbd*A gene (Kido et al., 2000), which encodes for mannosyltransferase, were used to design primers for serogroup-specific identification. The sequences for each serogroup were aligned using ClustalX version 2.0 (Larkin et al., 2007). Primers were designed to amplify the targets with distinct amplicon sizes that can be differentiated on a capillary gel electrophoresis equipment. The primer sequences for the 113 serogroups are shown in Table 3.

PCR assay conditions and optimization

The PCR protocol for specific gene target included an initial denaturation at 94 °C for 5 min, followed by 25 or 30 cycles of denaturation at 94° C for 30 s, annealing for 30 s at 59-68°C, extension for 75 s at 68°C and a final step of extension at 68°C for 7 min. The number of PCR cycles and annealing temperatures varied based on optimization for each set (Table 4). All other conditions were same for all 11 sets of assays (Table 5).

Validation of PCR assays

Strains belonging to 113 serogroups of STEC, other than the top-7 (O26, O45, O103, O111, O121, O145, and O157), were used in the study (Table 3). The strains were obtained from our

culture collection (n=104), *E. coli* Reference Center at Pennsylvania State University (n=231), Michigan State University (n=5), University of Nebraska (n=5), and Food and Drug Administration (n=36). Strains, stored in protect beads at -80° C, were streaked onto blood agar plates (Remel, Lenexa, KS) and incubated at 37 C. Colonies were picked and suspended in 1 ml of distilled water, boiled for 10 min, centrifuged at 9,300 x g for 5 minutes and the supernatant was used for PCR assays. Each set of assays was validated by testing with known strains of STEC (n= 381) belonging to 113 serogroups (Table 5).

Results

A total of 11 mPCR assays, each targeting 8 to 11 serogroups, targeting a total of 113 serogroups of STEC were designed. Each set contained primer pair sequences that generated amplicons of different sizes that can be readily differentiated in a capillary electrophoretic system (Table 3); Figures 1 to 11). The running conditions ranged from 25 to 30 cycles and annealing temperatures varied from 59°C to 66°C (Table 4). Initial optimization and validation of the assay were done with one strain and subsequently validated with additional strains (Table 4).

Discussion

Traditionally, serogrouping and serotyping *E. coli* involved agglutination reactions with specific antisera raised against O and H antigens (DebRoy et al., 2011). This method is labor-intensive and in some instances, specificity is questionable because of cross-reactions with other serogroups (Ennis et al., 2012). The development of PCR assays would be a simpler and effective tool to identify serogroups. More importantly, the PCR assay is readily adaptable to any laboratory. Many PCR assays have been developed and validated, generally targeting 1 to 7

serogroups, often with major virulence genes (Shiga toxins 1 and 2, intimin, and enterohemolysin (Noll et al. 2015). The colonization and fecal prevalence of STEC is widespread among cattle, indicating the diverse possibility of contamination (Blanco et al., 2004). Additionally, the isolation and of non-top-7 STEC can be challenging because the lack of immunomagenetic beads and selective media differentiation (Hornitzky et al., 2001). STEC serogroups other than the top-7 have been previously identified in other studies. Serogroups that have been identified from cattle comprise of 108 serogroups. Of the 108 STEC serogroups that have been identified in cattle, 85 serogroups have been associated with human illness. (Iguchi et al., 2015, Bettelheim et al., 2007, Aidar-Ugrinovich et al., 2007, Gonzalez et al., 2016, Guth et al., 2003, Meichtri et al., 2004, Monaghan et al., 2011, Padola et al., 2004). Serogroups O8 and O165 have been found in association with HUS in other countries, including Brazil and Argentina (Gonzalez et al., 2016, Meichtri et al., 2004). Additionally, humans have a higher likelihood of becoming exposed to O91, O113, O117, O118, O128 and O146 because of their higher prevalence in animals and subsequent contamination of food and water. These serogoups are frequently associated with severe illnesses (Blanco et al., 2004). The design of multiplex PCR assays allows for a higher throughput and increased specificity compared to other methods such as agglutination reactions with antisera, or culture method approach. (Irino et al., 2005, Zweifel et al., 2005).

Figure 1. Multiplex PCR assay (Set 1) to detect serogroups O4, O86, O84, O156, O80, O168, O91, and O109 of Shiga toxin-producing *Escherichia coli.*

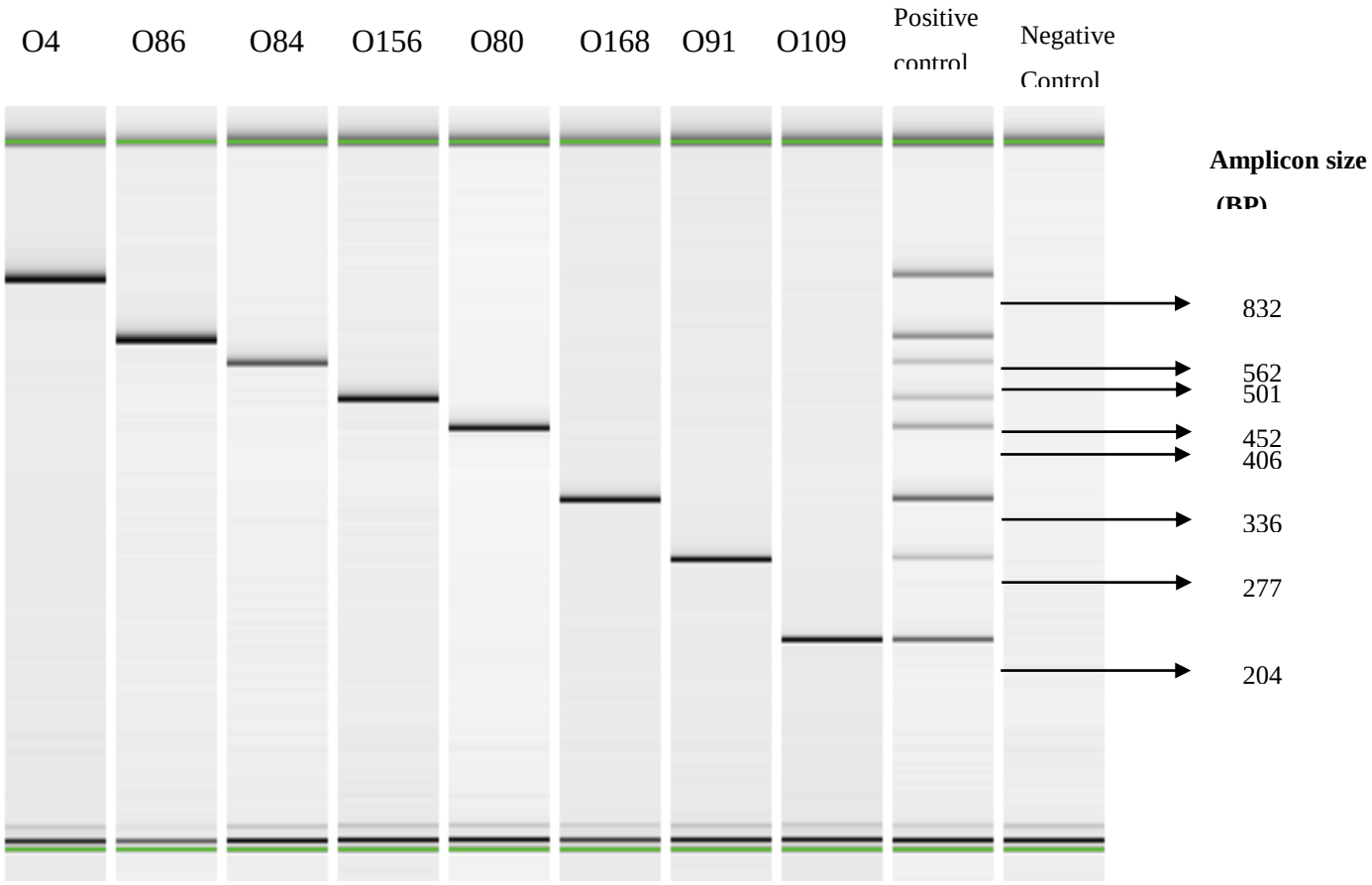

Figure 2. Multiplex PCR assay (Set 2) to detect serogroups O128, O138, O123, O120, O119, O13, O175, O171, O22, and O5 of Shiga toxin-producing *Escherichia coli*.

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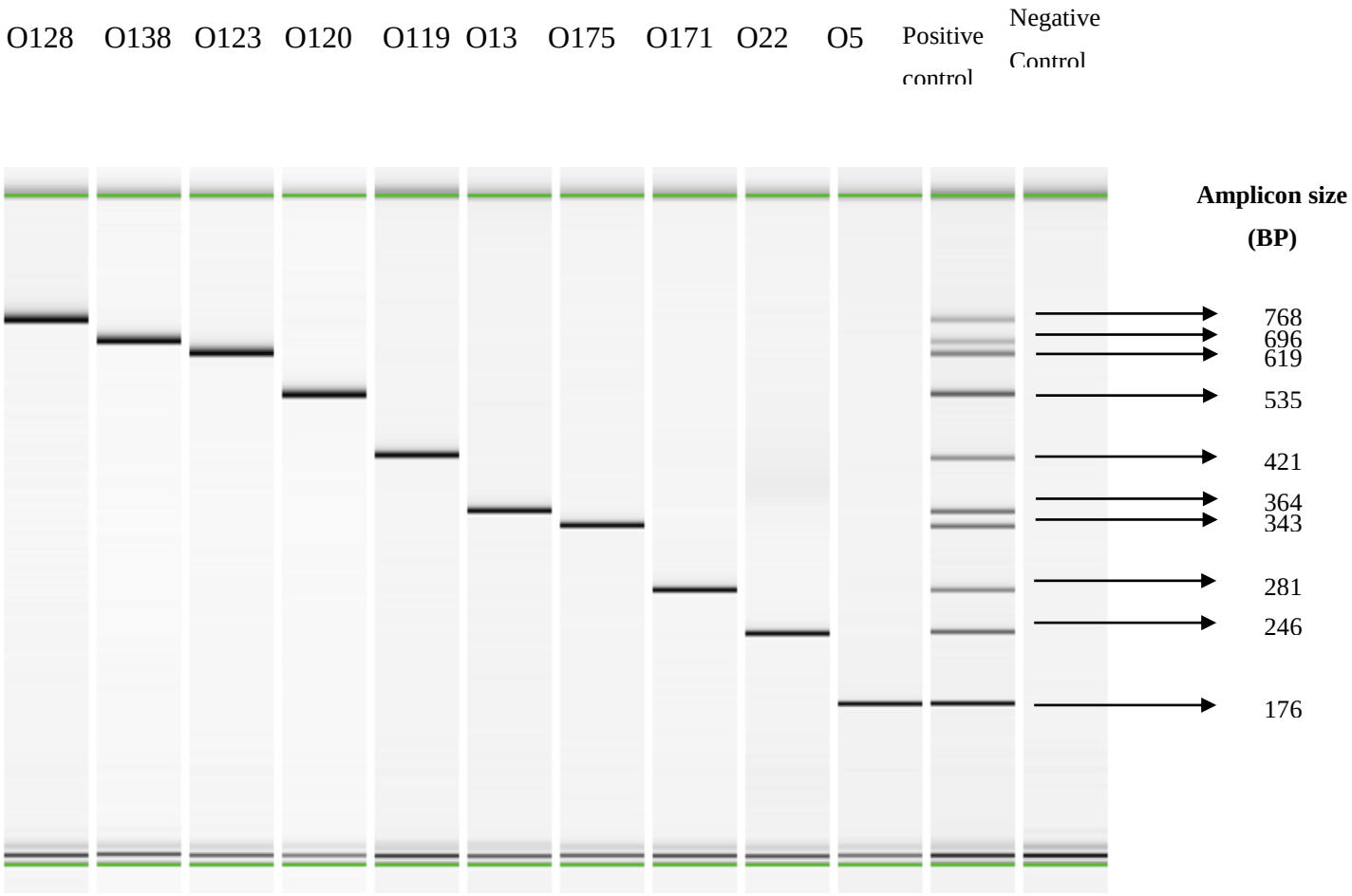

Figure 3. Multiplex PCR assay (Set 3) to detect serogroups O6, O98, O181, O75, O33, O116, O150, O79, and O25 of Shiga toxin-producing *Escherichia coli.*

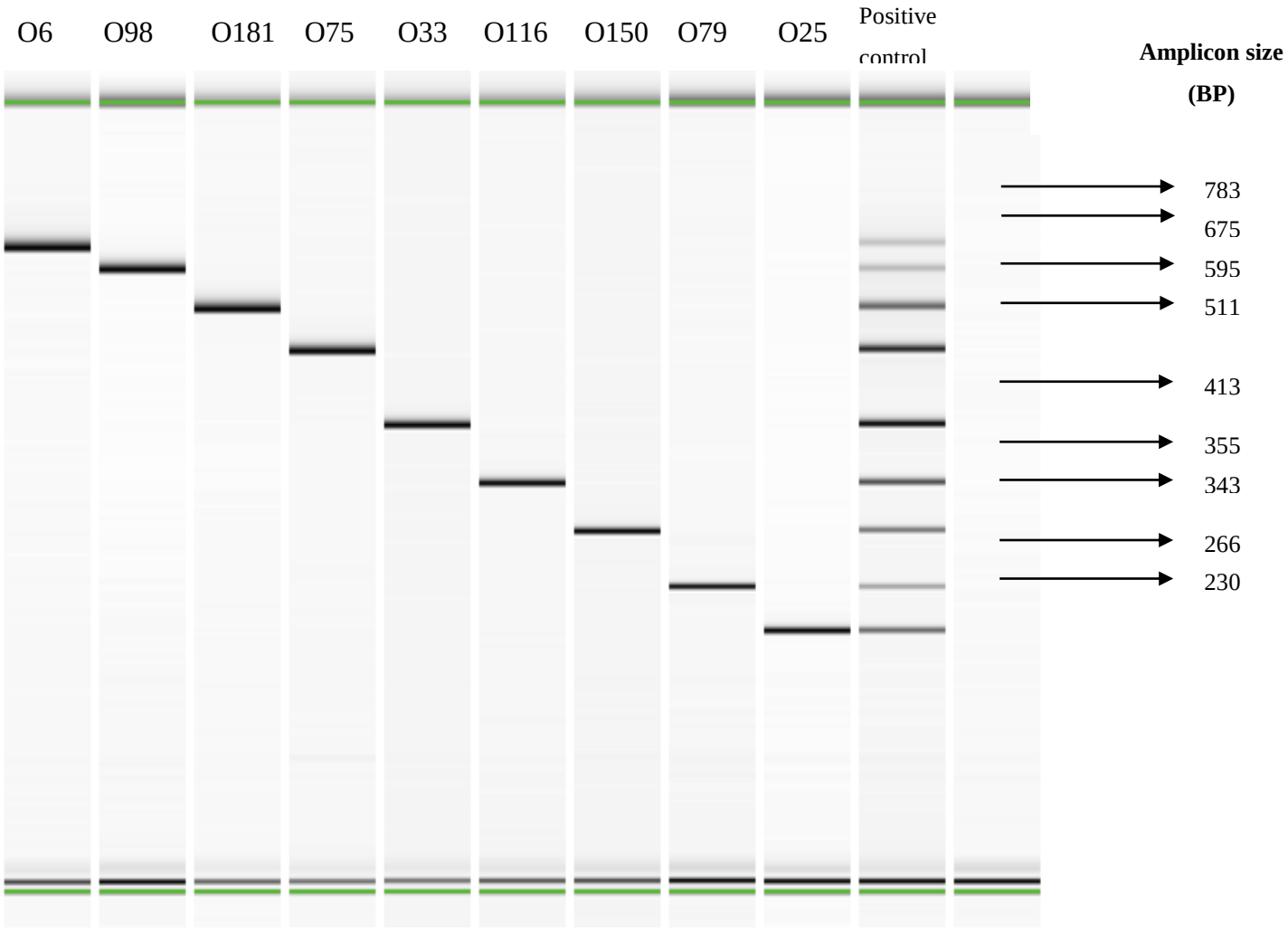

Figure 4. Multiplex PCR assay (Set 4) to detect serogroups O78, O2, O146, O76, O178, O126, O113, O118, O15, and O147 of Shiga toxin-producing *Escherichia coli*.

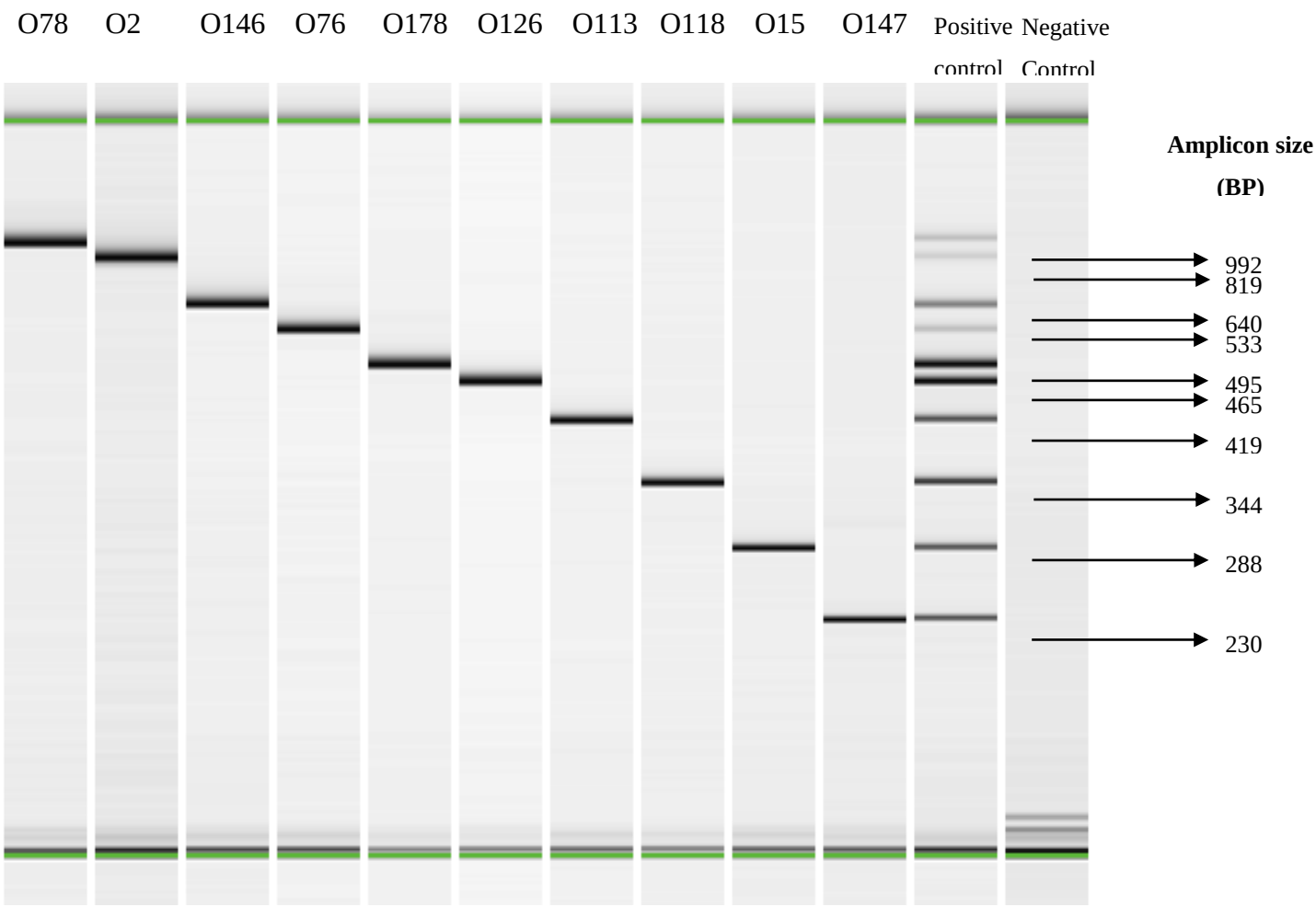

Figure 5. Multiplex PCR assay (Set 5) to detect serogroups O62/O68, O7, O163, O136, O8, O92, O87, O55, and O20 of Shiga toxin-producing *Escherichia coli.*

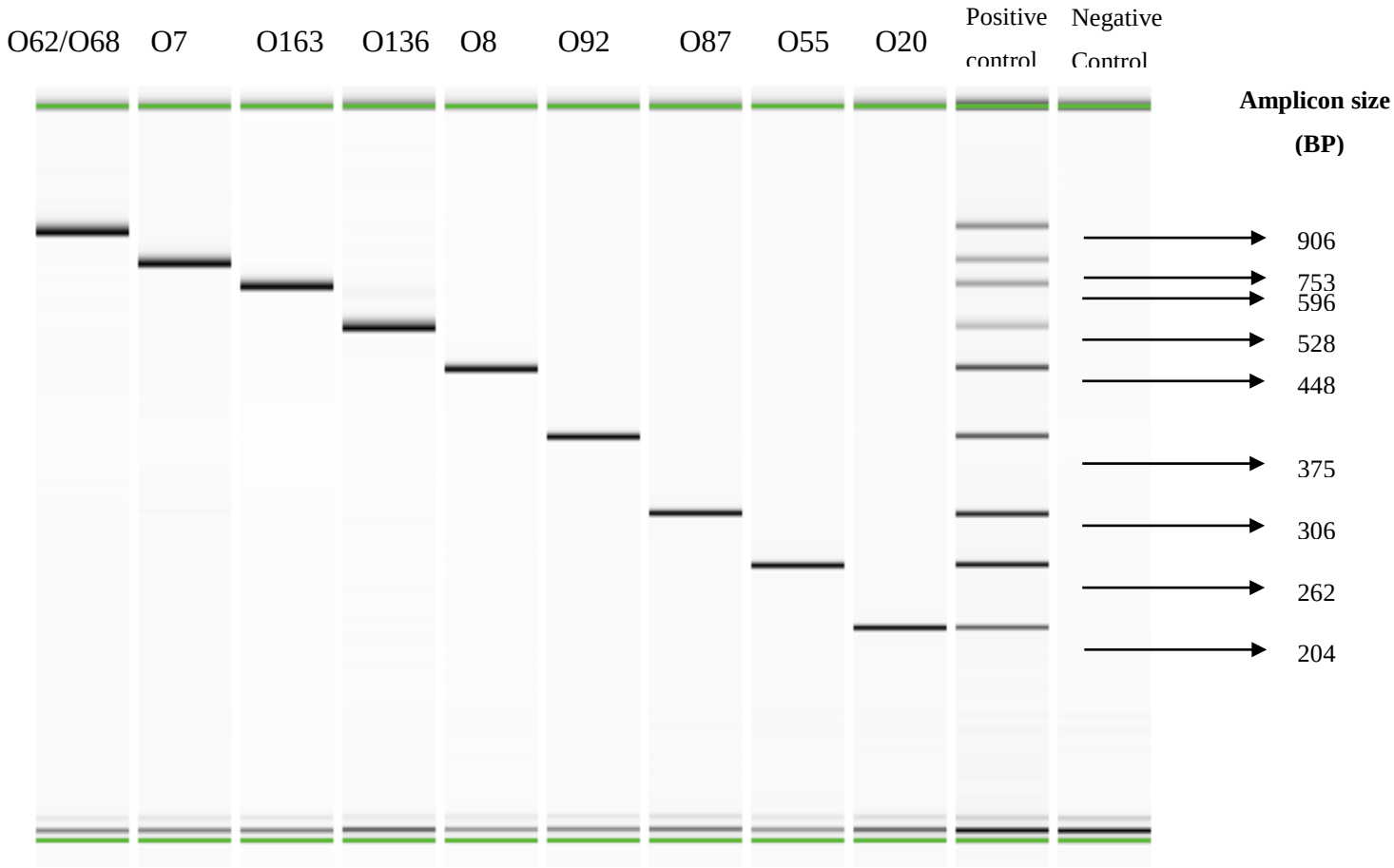

Figure 6. Multiplex PCR assay (Set 6) to detect serogroups O141, O153, O132, O130, O108, O96, O88, O107/O117, O74, O38, O39, and O115 of Shiga toxin-producing *Escherichia coli.*

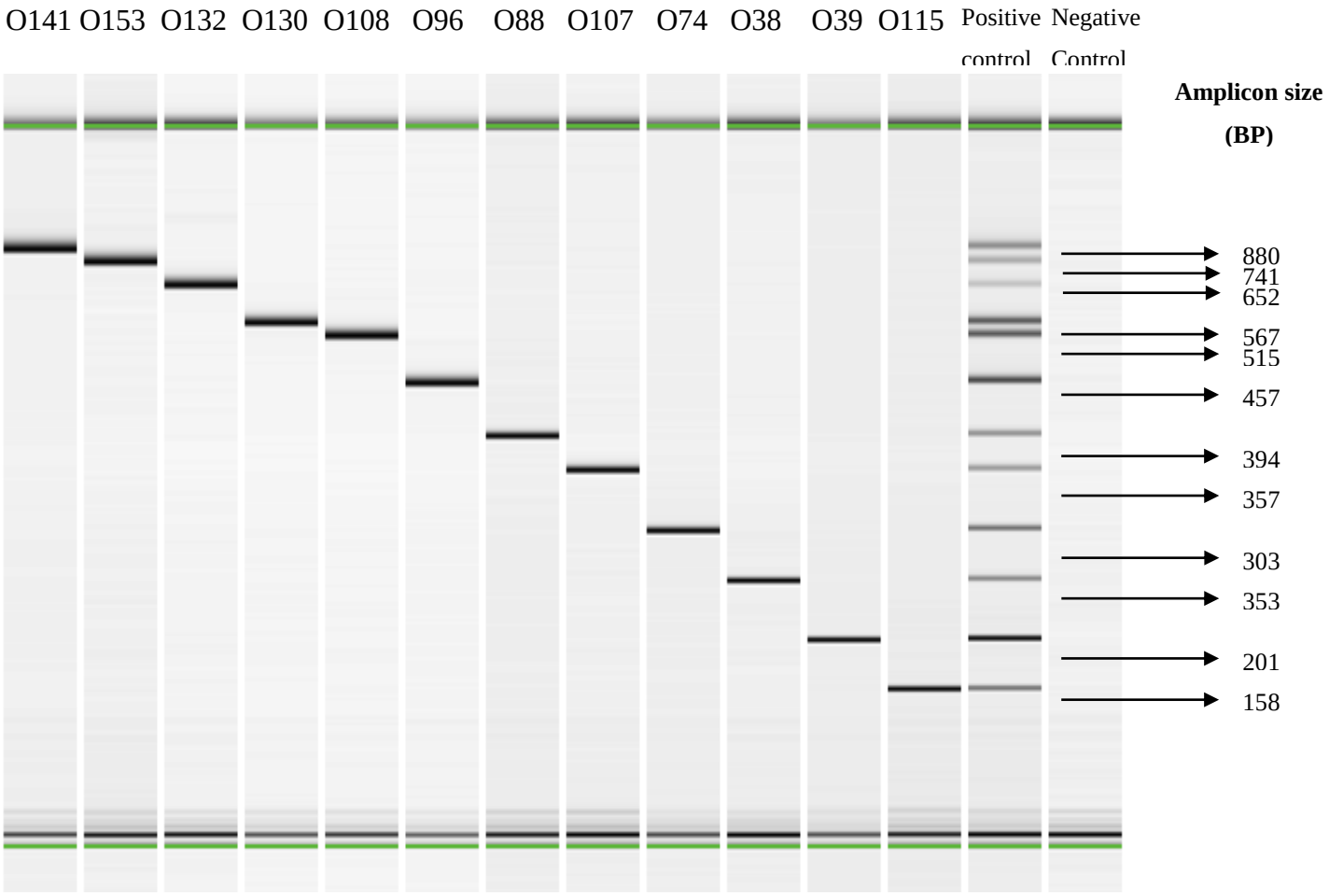

Figure 7. Multiplex PCR assay (Set 7) to detect serogroups O70, O53, O69, O51, O17/O44/O73/O77/O106, O43, O40, O37, O35, O28/O42 (O20ac), O18 (ab/ac), and O1 of Shiga toxin-producing *Escherichia coli.*

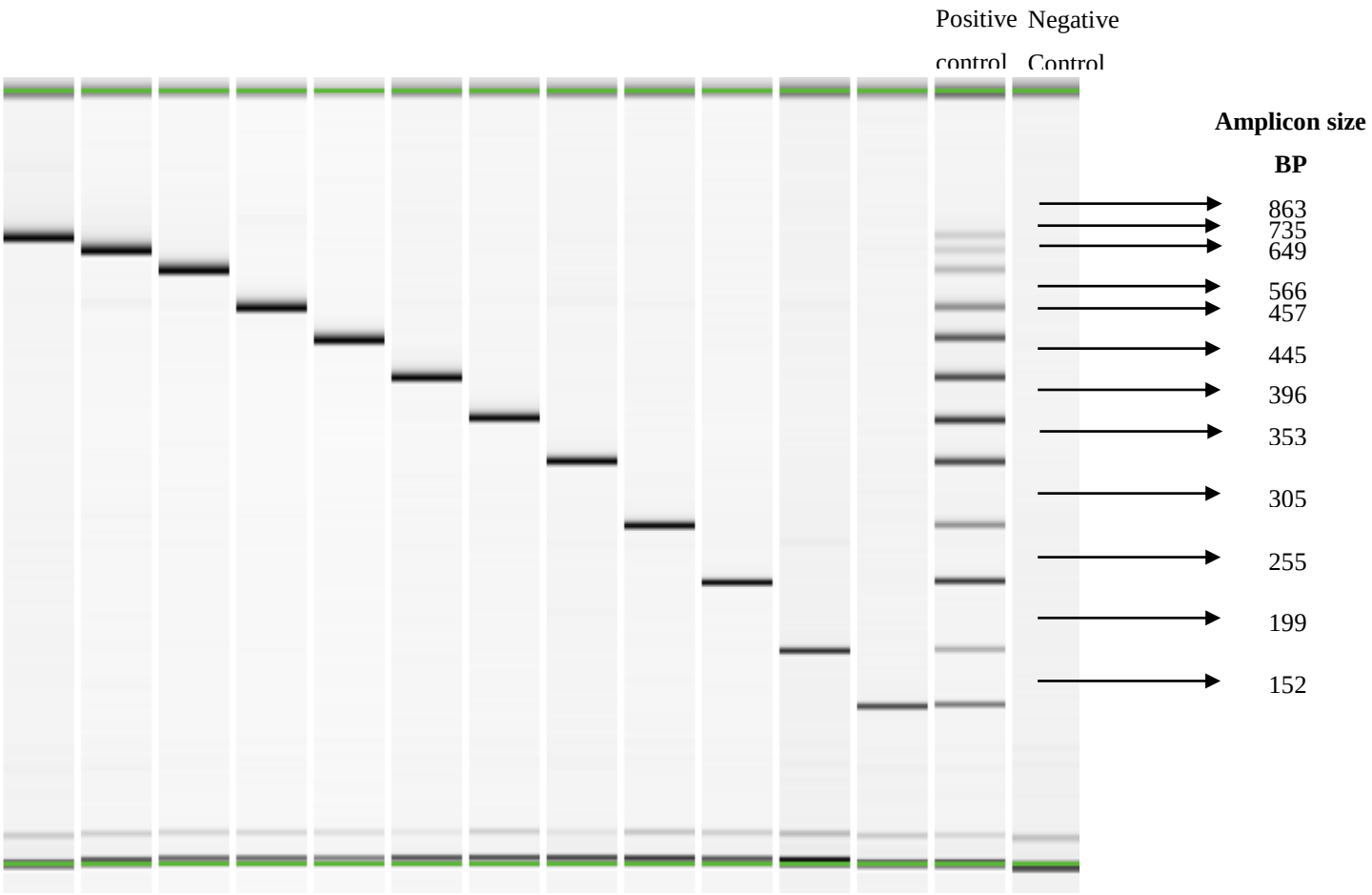

Figure 8. Multiplex PCR assay (Set 8) to detect serogroups O139, O125(ab/ac), O124/O164, O90/O127, O102, O105, O85, O82, O81, O148and O140 of Shiga toxin-producing *Escherichia coli.*

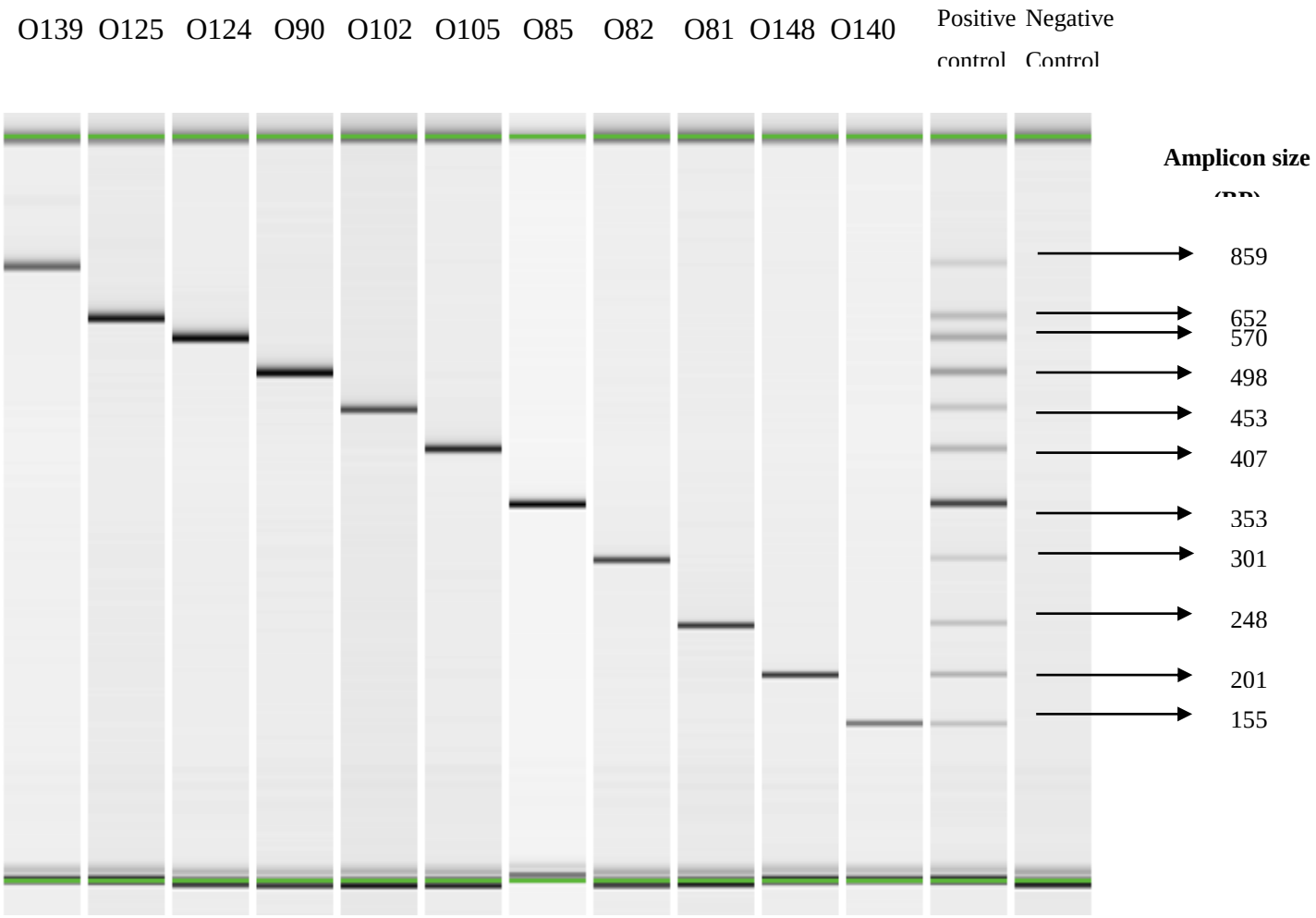

Figure 9. Multiplex PCR assay (Set 9) to detect serogroups O169, O161, O154, O114, O110, O93, O149, O49, and O21 of Shiga toxin-producing *Escherichia coli.*

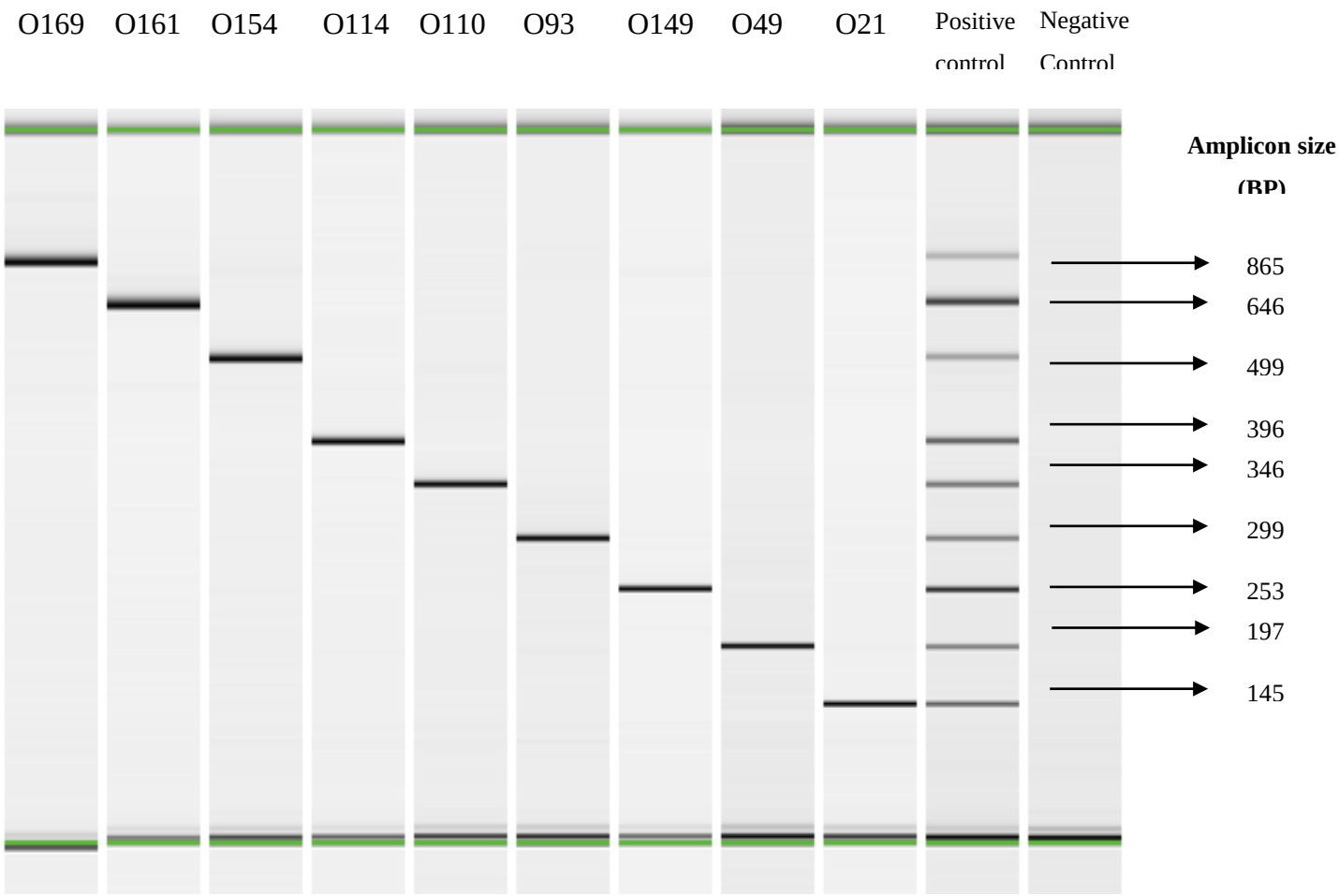

Figure 10. Multiplex PCR assay (Set 10) to detect serogroups O165, O160, O182, O179, O46/O134, O177, O176, O174, O172, O170, O159, and O152 of Shiga toxin-producing *Escherichia coli*.

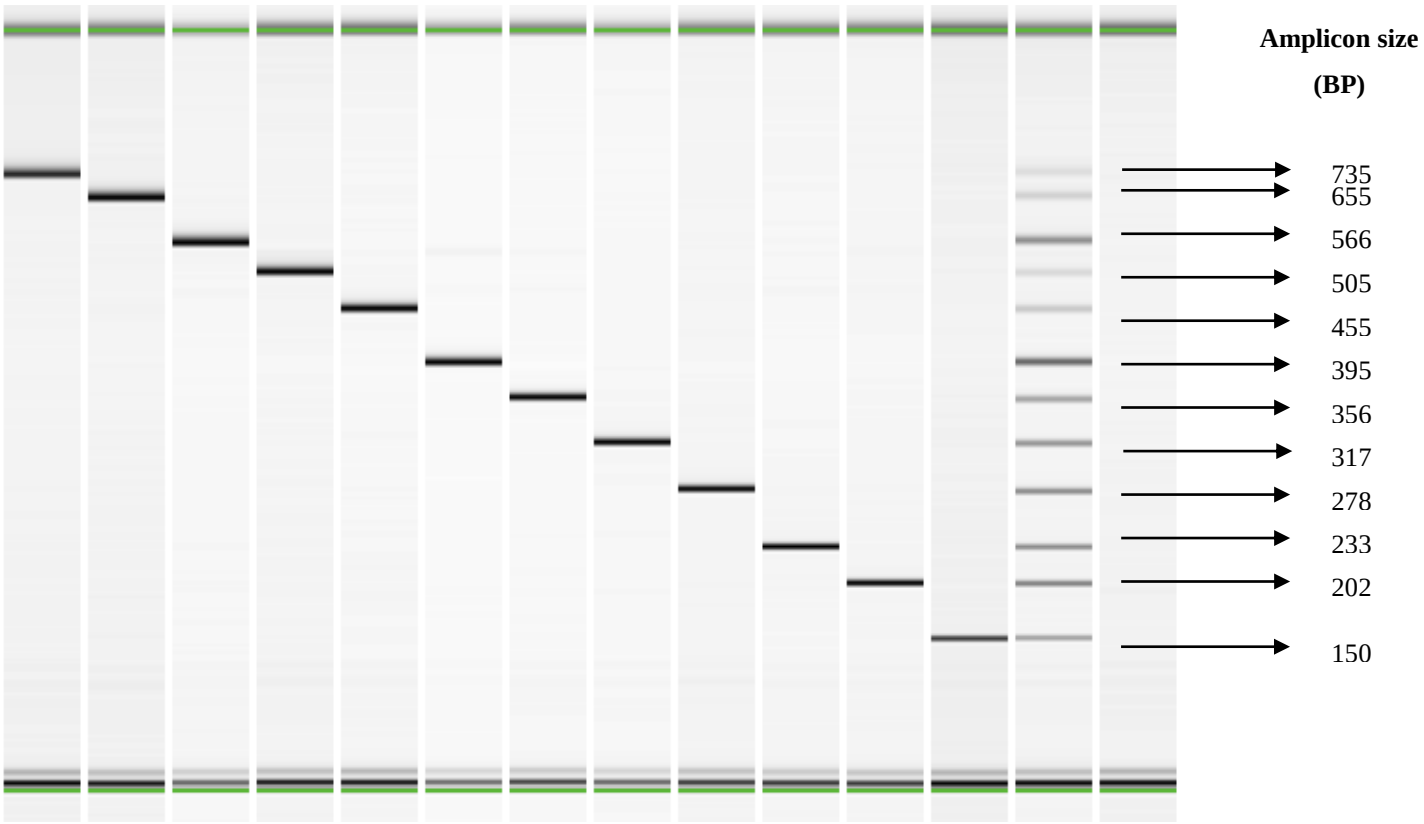

Figure 11. Multiplex PCR assay (Set 11) to detect serogroups O131, O19, O16, O63, O23, O29, O101/O162, O112(ab), O11, O10, and O3 of Shiga toxin-producing *Escherichia coli*.

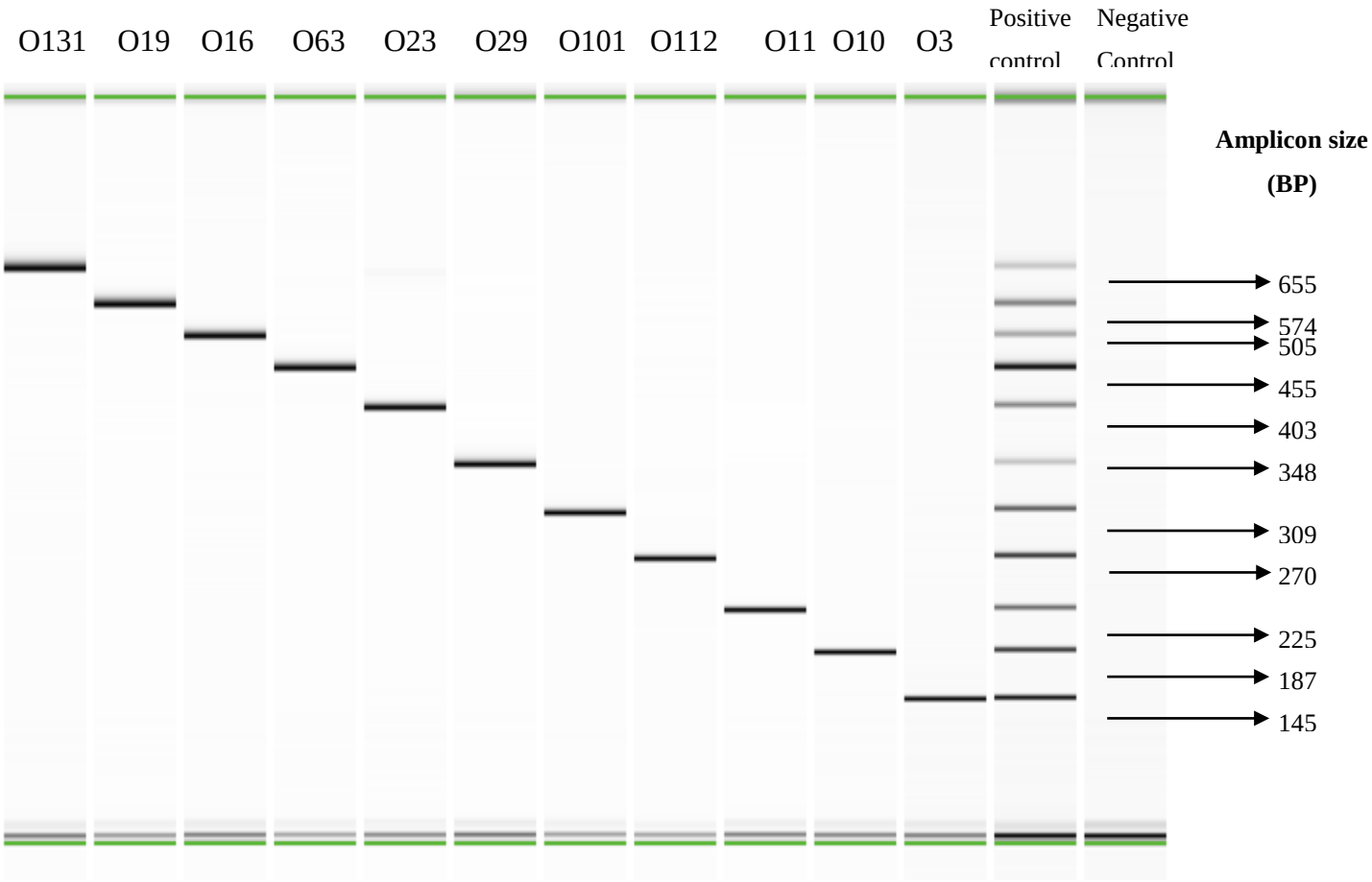

Table 2. Shiga toxin-producing *Escherichia coli* serogroups identified in cattle feces.

O1	O2/50	O3	O4	O5	O6	O7	O8	O10	O11
O15	O16	O17/O44/ O73/O77/ O106	O18	O19	O20	O21	O22	O23	O25
O29	O35	O37	O38	O39	O40	O42	O43	O46	O49
O51	O53	O55	O62	O63	O69	O70	O74	O75	O76
O79	O80	O81	O82	O84	O85	O86	O87	O88	O90
O91	O92	O93	O96	O98	O101	O102	O105	O107	O108
O109	O110	O112	O113	O114	O115	O116	O118	O119	O120
O124	O125	O126	O128	O130	O131	O132	O136	O139	O140
O141	O146	O147	O148	O149	O150	O152	O153	O154	O156
O159	O160	O161	O163	O165	O168	O169	O170	O171	O172
O174	O175	O176	O177	O178	O179	O181	O182		

Table 3. Multiplex PCR (mPCR) assay sets, serogroups and serogroup-specific genes targeted, and size of amplicons

mPCR set	Serogroup	Genes targetd	Primers sequence	Amplicon size (bp)
1	O4	*wzx*	F: TGAAACAGCAGTGCGTGCATTCTC	832
			R: GATTGCCGTGCCAATTATGCCGAA	
	O80	*wzx*	F: GAAGCATGGCTTCTAGGGGG	406
			F: TGACAAAGGTAGCCACGGAA	
	O84	*wzx*	F: TCAGCGTTCCAAGAAGCACT	501
			R: TGGTGTGCACTTATACATCCGA	
	O86	*wzy*	F: ATTTGAGGCTGACGCGTATGGACT	562
			R: AGCAACACTTCCAATGATCCACCC	
	O91	*wzy*	F: CGCATTTAAGGACTGGCTGT	277
			R: GTAGCAGATATGCCGACCGT	
	O109	*wzx*	F: TCTCTCTCGACATACCCGCGCTT	204
			R: ACCGTAGCCCAAAGAGCCACA	
	O156	*wzx*	F: TGCTCATGCGTTTAAAATGG	452
			R: CAACAAGAACAGGCATCAGG	
	O168	*wzx*	F: TGTCGACTTTGGGAAATGTGG	336
			R: CTGCAGAGGCCAATTCAGGT	
2	O5	*wzy*	F: AGCCCAAGAGGCCGCAGTATTT	176
			R: TCCAACCACCATTCTCCGCCAT	
	O13/O129/ O135	*wzx*	F: CGGGAGAGCAGTGTTTCCAA	364
			R: TATTGCGGCACCCAGTAACC	
	O22	*wzy*	F: TTTACTGGCTGCTGCTAGTGCT	246
			R: TCATCTCCACCACGAGTCGAAAGT	
	O119	*wzx*	F: CTGGGGCAATCTGCTTTCCT	421
			R: CCCAAGGTATTGTTGCCCCT	

	O120	*wzx*	F: CTGGTTTTGTTGTTGCATTGCT	535
			R: CCAGTTGGTGCCAACCAAAG	
	O123/O186	*wzx*	F: ACAATTAGGGCCTGGTGCAT	619
			R: TGTGCTAGCGCTAAAGGACT	
	O128	*wzx*	F: GCCATTACGACGTTGATGACT	768
			R: TGCAACCCCAATAGCAAAAGC	
	O138	*wzx*	F: GCAGCAATGCCTGCTGTTTT	696
			R: AGCGTATGCAACCCCAATGA	
	O171	*wzx*	F: TGCTCAAGTGGCATGCAGAT	281
			R: TGCAACCTGATATCCAGCAGT	
	O175	*wzx*	F: TGTTTAATTGTTCCTCCGCTCA	343
			R: GCAGCCCAACCAAACTTAGC	
3	O6	*wzy*	F: GGATGACGATGTGATTTTGGCTAAC	783
			R: TCTGGGTTTGCTGTGTATGAGGC	
	O25	*wzy*	F: AGAGATCCGTCTTTTATTTGTTCGC	230
			R: GTTCTGGATACCTAACGCAATACCC	
	O33	*wzx*	F: GGACCTCTGTTTGTGGCTGT	413
			R: GGTGTGCCTATCGCATAACC	
	O75	*wzy*	F: GAGATATACATGGGGAGGTAGGCT	511
			R: ACCCGATAATCATATTCTTCCCAAC	
	O79	*wzx*	F: GCTATAGTGCACCAGGATTGT	266
			R: GATTGCCGTCTGCCCTAAT	
	O98	*wzx*	F: GGGCGTATTGAGGTTCTTGT	675
			R: CAACTGAAATGGAGCAGCAAAT	
	O116	*wzx*	F: CTTTTGCGTTGTGCCTCGAA	355
			R: ATACTGCCCTACGTTTGCGG	
	O150	*wzx*	F: TGCGTTCACTTGCTGGTTTG	313
			R: ATGAGTGCAGGCACTTGAA	
	O181	*wzx*	F: TGGAGTAACGAAATACACCGCT	595
			R: AGATTGCCAATAACCAGAAGCA	
4	O2/O50	*wzx*	F: TGGCCTTGTTCGATATACTGCGGA	819

			R: TCACGAGCTGAGCGAAACTGTTCA	
	O15	*wzx*	F: CCAAATTGGTTTTTGCAAGG	288
			R: TGCACTAGTCGCAAGTGTTGA	
	O76	*wzx*	F: CATATGCAGATTGAAGGTAG	533
			R: GAAAGCCATAAAGTGCC	
	O78	*wzx*	F: GGTATGGGTTTGGTGGTA	992
			R: AGAATCACAACTCTCGGCA	
	O113	*wzy*	F: GCATGTATGATGCATAGCTTCGCC	419
			R: TGATATCGTTCGCTAACCACCCA	
	O118/O151	*wzx*	F: GTGGGAGTCTGAATCAAGTTGCGA	344
			R: AGCAACCTTACCCAATCCTAAGGG	
	O126	*wzy*	F: CGCATTAAATGGACCTGATAAAGCATCG	465
			R: ACTAGCGCACATATCGTTAGCACG	
	O146	*wzx*	F: AGGGTGACCATCAACACACTTGGA	640
			R: AGTTCAATACTGTCGCAGCTCCTC	
	O147	*wzx*	F: GCTTGATTGGCGGTAGTGTAT	230
			R: TGGTAATCCAGCCACAAAAGAA	
	O178	*wzx*	F: CTGTCCGTAGTGAGGTTGGC	495
			R: ACCTCCAGATCGGTCCTTAATC	
5	O7	*wzx*	F: GCCAGGCAAGGATTATTATGG	753
			R: TGTCATTGCAGGTACGCTAGA	
	O8	*Orf469*	F: CCAGAGGCATAATCAGAAATAACAG	448
			R: GCAGAGTTAGTCAACAAAAGGTCAG	
	O20	*wzx*	F: GAGCAGCGGAATACTTTCCA	204
			R: TCCAAGAGTCCTGATGCAAAT	
	O55	*wzx*	F: TGCCAATACGTAATACCCAAAA	262
			R: GTGGCCACAGGCAATCTTAT	
	O62/O68	*wzx*	F: TGAAATGTTTGAGCGTGAATTT	906
			R: CATATCGCCGAATGAGTAACC	
	O87	*wzx*	F: TGGGTTTTTGTCAAGCATCA	306
			R: TCGCTTTCATTTTCTCCATTC	

	O92	*gnd*	F: TGCGAAGTGGTCTGAATCTG	375
			R: TTCCCAAGTACAATGCACGA	
	O136	*wzx*	F: GCCACGTACAAAAATTTAGCC	528
			R: CCCCTTCTTGATCCATTTGA	
	O163	*wzy*	F: TTGCTGATGATGAGGTTGTGA	596
			R: ATGTTCCACTCCCTGCAAGT	
6	O38	*wzx*	F: AAGAGGCTGGGGCATTTAGT	253
			R: TGCGCCGATAAATCCATATT	
	O39	*wzx*	F: CACAAGCATTTCAGCCAAAG	201
			R: TGAGAAAATCACCAGCCTGC	
	O74	*wzx*	F: CTGGTCAATGGCAAGCTGTA	303
			R: ATGCAAAAATCCAAGCCAAT	
	O88	*wzx*	F: TTCTCTCCCTTCGTTGGCTA	394
			R: TTCCCACACCAGCATTAACA	
	O96	*wzy*	F: GGGCTGTTTACGAGAGCATT	457
			R: GCACTACTAGGATCATCCGCA	
	O107/O117	*wzx*	F: TTTGATGTCGTTGCTCCGTA	357
			R: TGCACCAATTGTCGGTGA	
	O108	*wzx*	F: GGTGGTCGTGAGCAACTAGG	515
			R: TTATACGCGAGATGCTTTGC	
	O115	*wzx*	F: GTGAATTTGCTCGCCTCTCT	158
			R: TGCTACCAACCATTTATGCG	
	O130	*wzx*	F: GAGGGCTAATTGCATCCGTA	567
			R: GCAACAAATGAACGCATGAC	
	O132	*wzx*	F: TGGCAATCCCTCTGATCTTC	652
			R: ATCAAACATAACCCGCCTGA	
	O141	*wzx*	F: GTGTATTTAACATTTGCTCAAGCC	880
			R: ACGCTGAAGGAATCGTCAAC	
	O153	*wzx*	F: TTCGTCCTATGCGCAGTATTC	741
			R: ATTGCCTGAACACGGAAGAT	
7	O1	*wzx*	F: CTTTTAGTGCAAGTCGTGCG	152

			R: AGCTTGCACAGGCAAAAGAT	
	O18	*wzx*	F: CGAGAAGTCGCAATTGAAAA	199
			R: TGGATGCTGCGAGAATTTTA	
	O28	*wzx*	F: CTCAAGTCATTGGCGCATTA	255
			R: GGCTGACTGGGGTCGTTAT	
	O35	*wzx*	F: TGGCATAGCGGTGTTCTATG	305
			R: CCCAAGAAAGTGGAAAAACAA	
	O37	*wzx*	F: TAGCCATATGCCTTGTCGTG	353
			R: TGAATCGCTGGTAACGAACA	
	O40	*wzx*	F: CCTGAAGAGGCTGGGAGAAT	396
			R: AAGAAAACTCATTGGCACACC	
	O43	*wzx*	F: TTGAATCGAAGGCTTTTTGC	445
			R: AGGTATCACACCATGCACGA	
	O44/17/73/77/106	*wzx*	F: GGAGAAGATGTAGGCGGAGA	500
			R: AGCACATCAAAAGCTGACGTT	
	O51	*wzy*	F: TTGGTCGCCTTATTTATTGCTT	566
			R: AACGTAATGAAAAACCAGGAACA	
	O53	*wzx*	F: CGACCTCCTTTATTGGTTGC	735
			R: TCTTTCCCATAATTCAATCCAA	
	O69	*wzx*	F: ATCATTTGCAGGGCTTATGG	649
			R: CTTCCTTTCATCCCGAGAAT	
	O70	*wzx*	F: ATACCGGTCCATCAGGAATG	863
			R: AACATCCCGCGAAAAATAAA	
8	O81	*wzx*	F: AGATCGCAGGCTTCGTTG	248
			R: GGGCAGGACCTTCTTAAACA	
	O82	*wzx*	F: TCGGGATATCTGGATTTGGA	301
			R: AGCCAATTCATTTGGAATGTTT	
	O85	*wzx*	F: TGGATACTGCAGGTGAGTGG	353
			R: AATGCTAAGTACAGCGCAACC	
	O90	*wzx*	F: CAGTTCGGCTGGTTGGTT	498

			R: CCCAGTTGTGCAACAATAACA	
	O102	*wzx*	F: GCTCGTGAATTGGGAGCA	453
			R: TGCACATGTCAGGGTTGG	
	O105	*wzx*	F: TCAAAGATGAGAGTGGGAATAGC	407
			R: CGGCTCTTCATCATTGCAT	
	O124/O164	*wzx*	F: CCGCGATGAATGATTCTGTA	570
			R: TCACTCCCTGAACTCTGCAC	
	O125	*wzx*	F: GAGAGGAGCATTGATTGCTGT	652
			R: TTGAGCATTTTCGACGTAGC	
	O139	*wzx*	F: GGGATTAGGCGGCTTTTTAAT	859
			R: TACTTGCCCCAAAAGAGACG	
	O140	*wzx*	F: AAACGCAATAAGTGGGTTGG	155
			R: GCAATTGGACCAGAAACAAA	
	O148	*wzx*	F: TCGCTAAACTGACAGGTGTGAT	201
			R: ACGGCCACGCTTCTTTTA	
9	O21	*wzx*	F: GCTCAATATGAGTGAGGCTGGT	145
			R: AAAAACGCCTCGTGCTTTTA	
	O49	*wzx*	F: TTTACCGCGAATAACGGTTC	197
			R: ACAGGGGCAATACGTTCATC	
	O93	*wzx*	F: GGCAGGAGAATATGCAAGGA	299
			R: TAAATGCCTCCCAAACACCA	
	O110	*wzx*	F: TGTTGCAAGCATGAAACTTGA	346
			R: TAAGCCATTTTCGCCAAATC	
	O114	*wzx*	F: TGCTACAAGTGGTGGATTGC	396
			R: CCCATACAACCATCGCAAAT	
	O149	*wzx*	F: AGCGGTGCAAAGTTAATTCC	253
			R: CACCAAACACATTCTGCGTAA	
	O154	*wzx*	F: TTGGTTTTTCCAAGGGATACA	499
			R: CAAAGCAAATACCACCATCG	
	O161	*wzx*	F: GGATATCTGGTTGGCGACAT	646
			R: AGCTTAAAACATGAGGGAGCTG	

	O169	*wzx*	F: GAAGGAATGGGATTTGAAAAGA	865
			R: CGCTTTAACAATTGCTTTCG	
10	O46/O134	*wzx*	F: TCAGGTGCGCCATTATTTTT	455
			R: TCACCCCCATAATAACCATCTT	
	O152	*wzx*	F: TCTGTCGCTATTGTCTTTTCACA	150
			R: TGCCGTTAACGTACCAAAAA	
	O159	*wzx*	F: AAATCCATTGGTGGAGTAGGAA	202
			R: CTTCCGCAAATAAGGTCCAA	
	O160	*wzx*	F: TGGCGTTCTAGGTCTTGCTAT	655
			R: TGCAGCCCCTCTGATTCTAT	
	O165	*wzx*	F: TGCGATTGCTTTATTTGAGC	735
			R: TCACGCTTTAACGCATACAGA	
	O170	*wzx*	F: AATAGAATCGCCTGGGGATAG	233
			R: CAGTGCTTAAAGGATGGTTGG	
	O172	*wzx*	F: CGGGCATGTTTGTTTTTGAT	278
			R: AGCATCCCACCTCTCACAAT	
	O174	*wzx*	F: GGAGATAAAGCTGCAGGTGAG	317
			R: CCAGTAAGCGGGCCTAAAAG	
	O176	*wzx*	F: TTTTTAGATTTGTTGATCCATTGGT	356
			R: CATAAGCGGCACTGCAATTA	
	O177	*wzx*	F: GGGGTATACACGCTCGCTAT	395
			R: CTTACAGCAGGCAACGCAAC	
	O179	*wzx*	F: ATGCCATTGCCGATGATATG	505
			R: ACCTCCACCCCAGCATAACT	
	O182	*wzx*	F: TTAGTTAATGCAGCCGTCACA	566
			R: GCCGATACTCCCATAAAAAGC	
11	O3	*wzx*	F: CGCTTAATTCGACCCAAATC	145
			R: AGTCGTTCTTGAGCCATCTGTA	
	O10	*wzx*	F: AGGAAAACATTTGCCGGAAT	187
			R: TATGGAGCGGTGATGAAACA	
	O11	*wzx*	F: CCATTGGTCACACTGCCTTA	225

			R: CAAACCAAGAGATGCTGCAA	
	O16	*wzx*	F: TTTTGGGCGGTGATATTTTC	505
			R: CCGCATTAAAATTCCCTACG	
	O19	*wzx*	F: GCGGAATATCTCGTGCAGTT	574
			R: GCGAAATGTGAGACAGCAAA	
	O23	*wzx*	F: TTTATCTTGGGCGGCTTAAA	403
			R: CCCACCCAAGCATTTACTTT	
	O29	*wzx*	F: TTATTGGGTTGTCGCCATTT	348
			R: CGGTCAACTTGTGAAAGCAA	
	O63	*wzx*	F: GGGCAGATGCAAGGATTTAC	455
			R: AAGACTCCTCCCCCAATCAG	
	O101/O162	*wzm*	F: TGGACCACATTAGGTTGGTTAGAG	309
			R: AAGCATCATAAAATTTCGCCATAG	
	O112	*wzx*	F: ACAACGTGAAATTTTTGTGTGG	270
			R: GCACGTATGCATTCTCAAGC	
	O131	*wzx*	F: GTGATTTCTGGGGCAACATT	655
			R: AAGCCTGCCCTAAACAAAGC	

Table 4. Multiplex PCR assays running conditions for the detection Shiga toxin-producing *Escherichia coli* (STEC) serogroups, other than the top-7 STEC.

Assays	Number of O groups	PCR cycles	Annealing temperature	O groups
Set-1	8	25	65°C	O4, O80, O84, O86, O91, O109, O156, O168
Set-2	10	30	65°C	O5, O13/O129/O135, O22, O119, O120, O123/O186, O128, O138, O171, O175
Set-3	9	30	64°C	O6, O25, O33, O75, O79, O98, O116, O150, O181
Set-4	10	30	63°C	O2/O50, O15, O76, O78, O113, O118/O151, O126, O146, O147, O178
Set-5	9	30	61°C	O7, O8, O20, O55, O62/O68, O87, O92, O136, O163
Set-6	12	30	66°C	O38, O39, O74, O88, O96, O107/O117, O108, O115, O130, O132, O141, O153
Set-7	12	30	63°C	O1,O17/O44/O73/O77/ O106, O18 (O18ab/O18ac), O28(O28ac/O42), O35, O37, O40, O43, O51, O53, O69, O70
Set-8	11	25	68°C	O81, O82, O85, O90/O127, O102, O105, O124/O164, O125 (O125ab/O125ac), O139, O140, O148
Set-9	9	25	63°C	O21, O49, O93, O110, O114, O149, O154, O161, O169
Set-10	12	25	59°C	O46/O134, O152, O159, O160, O165, O170, O172, O174, O176, O177, O179, O182

Set-11	11	30	62°C	O3, O10, O11, O16, O19 O23, O29, O63, O101/O162, O112, O131
Total	113			

Table 5. Validation of multiplex PCR assays to detect 'non-top-7' Shiga toxin producing *Escherichia coli*

mPCR set	Serogroup	Source of strains					No. of strains positive/No. of strains tested
		Kansas State University	Pennsylva-nia State University	Michig-an State University	Univers-ity of Nebraska	Food and Drug Administ ration	
1	O4	6282	O4 std., 14.0484, 14.0486, 14.1232				5/5
	O80		O80 std., 13.0406, 12.3395, 9.1802, 9.1803				5/5
	O84	10340-1, 12824-1					2/2
	O86		O86 std., 14.0607, 14.0615, 14.0747, 14.0748				5/5
	O91	4162, sPRH-561					2/2
	O109	12662-2, 15150-1, 15166-1	13.0166		E10F-6		5/5
	O156		O156 std., 15.0353,				4/4

			15.0354, 15.0355	
	O168		O168 std., 15.0133, 15.0181, 15.0185, 14.0047	5/5
2	O5		14.0590, 14.1660	2/2
	O13/O129/ O135		13.0102, 12.2598	2/2
	O22	1245, 4590, 4570, 1027-4	13.0145	5/5
	O119		15.1553	1/1
	O120		O120 std., 13.0021, 13.0022, 13.0023, 13.0024	5/5
	O123/O186		O123 std., 8.0269, 7.0571, 5.0769, 15.0862	5/5
	O128		O128 std., 13.0062, 13.0027, 13.1024, 14.0696	5/5
	O138		O138 std., 13.1113	2/2

	O171	1044-1, 12772		2/2
	O175		O175 std., 9.1703, 10.0047, 10.0048, 10.0472	5/5
3	O6	5750-1, 16857, 8276-1		3/3
	O25		O25 std, 3.1421, 14.1138, 14.1119, 14.0200, 14.0052	6/6
	O33		O33 std., 13.0050, 13.1199, 15.1254, 16.0504	5/5
	O75		O75 std., 11.1836, 12.0940, 12.0944, 12.1624	5/5
	O79		O79 std., 5.2457	2/2
	O98	sPRH-571	13.1267, 15.0710	3/3
	O116	1238-1, 1582-1, 99-3708, 3702-1,		7/7

		7712-1, 7716, 7752-2			
	O150		12.3283, 12.3280, 12.3284, 3.4896, 3.4897		5/5
	O181		O181 std., 11.0228, 11.0392, 12.0697		4/4
4	O2/O50	99-18866, 99-18858	14.1652		3/3
	O15	2794, 18925-1, S918, s915, N5789, N15018	O13 std	TW1681	8/8
	O76		O76 std., 14.0706, 14.0760, 14.0940, 14.0986		5/5
	O78	ATCC 35401, ATCC 43896	O78 std., 10.0182		4/4
	O113	1598-2, 1862-1, 3517			3/3
	O118/O151	KDHE 14, KDHE 52	13.1018		3/3

	O126	99-17409	16.0457, 16.0511, 13.0069		4/4
	O146		7.2907, 7.2663, 7.2803, 7.3159, 14.0976		5/5
	O147	13466-1		TW7464	2/2
	O178		O178 std., 14.0800, 14.0828, 14.0829, 14.0908		5/5
5	O7		16.0405, 14.0598, 14.0551		3/3
	O8	2089-2, 4202, 4950-2, sPRH-559, 5702-1, 6102-2, 6504-1, 6846-2, 6849-1, 15484-1,4544-1, 4964-1, 18370-1, 4542-1, 6412-1, 6416-1,	86.1006		19/19

		5344-1, 5799			
	O20	5481			1/1
	O55	5906, ATCC 12014		TW04062, TW08260	4/4
	O62/O68		O68 standard, 1.2557, 4.0175, 4.2378		4/4
	O87		15.0108, 14.0979, 12.3233		3/3
	O92		13.0124		1/1
	O136	4709-1, 10314-1, 10320-1	14.0889		4/4
	O163	9388-1, 13802-1, 18917-1			3/3
6	O38	748-1, 13950-1, 2072-3			3/3
	O39		13.1182, 15.1383, 16.1097		3/3
	O74	4558-1, 8608, 13472-1, 1229-1			4/4
	O88	1235-1, 2076-2, 8652-1,			5/5

		4560-1, 1240-2			
	O96	497, 3712-1, 18862-1, 3714-1			4/4
	O107/O117	3536-3, 9966-1, 3534-1			3/3
	O108	9924-1			1/1
	O115		13.1261		1/1
	O130	492-1, 1038-2, 1239-2, 3270-1			4/4
	O132	2067-1, sPRH-568			2/2
	O141		13.1660, 14.0312, 14.0314		3/3
	O153	1932, 9916-1			2/2
7	O1		14.0223	6227	2/2
	O17/O44/O73/O77/O106		8.2525, 8.2526, 14.0994, 14.0989, 14.0398	3829	6/6
	O18		12.0213, 13.0442, 13.1139	6853	4/4
	O28		4.0349, 5.0190	6404-2	3/3
	O35		1.2208, 6.089	6709	3/3

	O37	5.0628, 7.3209		6594	3/3
	O40	12.0572, 13.0486			2/2
	O43	12.0257, 15.1752, 16.0015		6861	4/4
	O51	11.1861, 12.2022, 15.0773		3724	4/4
	O53	12.0583		6503	2/2
	O69	14.1063, 14.1064		3560	3/3
	O70	95.1266, 96.1774		6388	3/3
8	O81	14.1380, 14.1397		6754	3/3
	O82	14.0846, 15.0429		5541	3/3
	O85	15.1549, 16.0447		3620	3/3
	O90/O127	12.3598	TW4549		2/2
	O102	12.3542, 12.3624, 14.0030		3558	4/4
	O105	9.0695, 9.0698		6810	3/3
	O124/O164	15.0880, 15.0790			2/2
	O125	13.0499, 13.1058		6517	3/3
	O139	13.0785, 14.0081		6672	3/3
	O140	12.066		5737	2/2

	O148				12.3461, 13.1357	2/2
9	O21		9.0069, 12.3518		6508	3/3
	O49		13.0005		5598	2/2
	O93		87.1672		6613	2/2
	O110		12.2044		3744	2/2
	O114		12.0637, 13.1247		6583	3/3
	O149		12.2184, 13.1187		6495	3/3
	O154		13.0130, 14.0265		6742	3/3
	O161				6341	1/1
	O169		12.0689		6992	2/2
10	O46/O134	1223-4	12.1020, 15.0242, 16.0631		3606	5/5
	O152		4.2414, 7.0560			2/2
	O159	1554-1	12.1465			2/2
	O160		15.1575, 14.0525			2/2
	O165	7050-4	13.0107, 14.1095		6757	4/4
	O170		13.0772, 14.1230		6833	3/3
	O172	9042-1, 11154-1				2/2
	O174		14.153		6129	2/2
	O176		14.0193		6996	2/2
	O177		14.1088, 14.1094	E28F-5, 89H-4, 58H-1		5/5

	O179		12.0472, 12.0696, 11.1855, 11.1606			4/4
	O182	sPRH-569	13.151, 14.1296		5745	4/4
11	O3		14.1045, 14.1048		6291	3/3
	O10		7.1714		6888	2/2
	O11		13.0841, 13.0861		6812	3/3
	O16				6734	1/1
	O19		14.0988, 15.0093, 16.054			3/3
	O23		1.2315, 3.2841		6556	3/3
	O29	10720-1	1.1116		3608	3/3
	O63		3.2810, 3. 4555		6441	3/3
	O101/O162			E907F-2		1/1
	O112		14.1406, 14.1409		5127	3/3
	O131		12.3205, 13.0803		6901	3/3

Chapter 3 - Detection of Serogroups, Other than the Top-7, of Shiga Toxin-Gene Carrying *Escherichia coli* (STEC) and Prevalence of Serogroups O2, O74, O109, O131, O168, and O171 in Cattle Feces.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are food borne pathogens that induce mild to severe bloody diarrhea (hemorrhagic colitis), which could lead to complications of hemolytic uremic syndrome (HUS), particularly in children. Cattle are major reservoirs of STEC, which reside in the hindgut and are shed in feces. Shiga toxins (Stx) are primary virulence factors associated with STEC induced illness in humans. Individual differences in amino acid sequences of Stx proteins, Stx1 and Stx2, or nucleotide sequences in *stx* genes, *stx*1 and *stx*2, as well as differences in cytotoxicity make it possible to identify Stx (stx) subtypes (Strockbine et al., 1986; Weinstein et al., 1988; Basu et al., 2015). Subtypes of *stx*1 and *stx*2 genes include *stx*1a, *stx*1c, and *stx*1d, and *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f, and *stx*2g, respectively. Outcomes of human illness are influenced by subtype. Shiga toxin 2 is more commonly associated with HUS than Stx1 (Ethelberg et al., 20014) and of the Shiga toxin subtypes, *stx*2a, and *stx*2c are more commonly associated with HUS (Persson et al., 2007). Intimin, encoded by *eae,* characteristic of enterohemorrhagic *E. coli* (EHEC), is responsible for adhesion of organisms onto intestinal epithelial cells causing attaching and effacing lesions, which is commonly seen in cases of severe human illnesses. The presence of *eae* and *stx*2 together result in higher risk for severe infections. However, Stx2 can cause severe illness without the the intimin gene as was seen in the O104:H4 outbreak in Germany in 2011 (Karch et al., 2012). Additionally, *E. coli* hemolysin, also called α-hemolysin (Cavalieri et al., 1984), EhxA, is also an important virulence factor that belongs to

the repeats-in-toxin (RTX) family and lyses red blood cells in the host, which could contribute to severity of illness induced by STEC or EHEC (Welch et al., 2005).

Escherichia coli O157 and six other serogroups, O26, O103, O111, O121, and O145, often referred to as the 'top-7', are considered as major STEC and have been declared as adulterants in ground beef and non-intact beef products. Additionally, as many as 108 serogroups of STEC have been identified to be present feces of cattle (Battelheim, 2007; Aidar-Ugrinovich et al., 2007, Blanco et al., 2004; Gonzalez et al., 2016; Guth et al., 2003; Meichtri et al., 2004; Monaghan et al., 2011; Padola et al., 2004). Some of the non-top-7 STEC have been shown to be associated with sporadic human illnesses (Bettelheim et al., 2007). Not much is known about the prevalence of the non-top-7 STEC in cattle feces. In our previous studies (Dewsbury et al., 2015; Cull et al., 2017) that were designed to study the prevalence of the top-7 STEC, a number of *E. coli* strains that were positive for Shiga toxin gene but negative to the top-7 serogroups were obtained. Therefore, our primary objective was to use previously developed multiplex PCR (mPCR) assays to identify the 'non-top-7 STEC' strains isolated from feedlot cattle feces and to determine subtypes of *stx* genes.

Because the detection of the STEC strains from the two feedlot studies indicated that serogroups, O2, O74, O109, O131, O168, and O171, were the most predominant, we wanted to determine the prevalence of the serogroups in cattle feces. Therefore, our secondary objectives were to, develop, validate, and use a mPCR assay to detect six predominant serogroups of non-top-7 STEC (O2, O74, O109, O131, O168 and O171) and utilize the assay to determine the prevalence of the predominant serogroups in feedlot cattle feces.

Materials and Methods

Non-top-7 STEC strains: A total of 359 STEC strains that were negative for the top-7 serogroups (O26, O45, O103, O11, O121, O145, and O157), obtained from two feedlot studies conducted in 2013 and 2014, were used. One-hundred-eighteen strains were isolated from twenty-four pen-floor fecal samples (n=576) collected weekly from two pens with 120-300 cattle each at a commercial feedlot in Nebraska during a 12-week period from June thru August of 2013 (Dewsbury et al., 2017). The remaining strains (n=241) were isolated from pen-floor fecal samples that were collected weekly from four-to-six pens with 30-450 cattle each at one commercial feedlot in Nebraska and one commercial feedlot in Texas during a 12-week period from June thru August of 2014 (n=1,886).

Both studies were designed to detect and estimate the prevalence of the top-7 STEC in cattle feces. For the detection and isolation of the top-7 STEC, serogroup-specific immunomagnetic beads were utilized to capture the top-7 STEC from the feces and then plated on selective media. Putative colonies from the selective media were subjected to a 10-plex PCR assay (Bai et al., 2012) that targeted seven serogroups and three major virulence genes (stx1, stx2, and eae). Isolates that were negative for the seven serogroups, but were positive one or both stx genes, were stored in Cryogenic beads (CryoCare™ , Key Scientific Products, Round Rock, TX).

Detection of serogroups: Eleven sets of mPCR assays, previously developed and validated (Ludwig et al., 2017) to detect serogroups of STEC, other than the top-7, were utilized to detect the serogroups of the 359 strains. Strains frozen in cryogenic beads were streaked onto blood agar plates (Remel, Lenexa, KS) and incubated overnight at 37 C. Single colony of each strain

was picked, suspended in 50 µl distilled water, boiled for 10 min, centrifuged at 9,300 x g for 5 min, and the supernatant was used in mPCR assays.

Development and Validation of mPCR targeting O2, O74, O109, O131, O168, and O171

Primer design

Serogroup-specific wzx gene, which encodes for the transmembrane lipid transporter enzyme, or flippase, required for O-polysaccharide export, was targeted in this assay. The nucleotide sequence of the targeted gene for each of the six serogroups were obtained from NCBI GenBank, and a search was performed for additional sequences using BLASTN on NCBI's Nucleotide collection (nr/nt) database. Distinct amplicon sizes were used as targets for amplification by the primers. Primers were designed to match available O-serogroup sequences. The sequences for each serogroup were aligned using ClustalX version 2.0 (Larkin et al., 2007) and primers were designed to amplify the targets with distinct amplicon sizes that can be differentiated by capillary gel electrophoresis (Table 8).

PCR running conditions

Primer stocks were prepared in 1 x TE buffer (10mM Tris, brought to pH 8.0 with HCL with 1mM EDTA; Integrated DNA Technologies, Inc., Coralville, IA) at concentrations of 0.1 nM/ µl (stock solution). Concentrations and volumes of six-primer pairs were mixed, equally. The PCR running conditions consisted of 94°C initial denaturation for 5 min followed by 30 cycles of 94°C denaturation for 30 s, 72°C annealing for 30 s, 68°C extension for 75 s, and a final step of 68°C for 7 min. The final reaction mixture consisted of 20 µl and included 10 µl of IQ Multiplex Powermix (2x), 1µl of six pairs of primer mix (after mix, 8.3 pM/ul for each primer), 2 µl of

template, and 7 µl of water. The primer sequences and amplicon sizes for serogroups, O2, O74, O109, O131, O168, and O171 are shown in Table 3.

Validation of the mPCR assay with pure cultures and feces spiked with pure cultures

The applicability of the assay to detect serogroups in cattle feces was validated with fecal samples spiked with pure cultures of the six serogroups. Initially, five feedlot steer's fecal samples, obtained from pen-floor fecal pats, from the university Beef Cattle Research Center, were enriched (1 g in 9 ml, incubated at 40°C for 6 h) in *Escherichia coli* (EC) broth (Difco™, Becton, Dickinson Co., Sparks, MD). One milliliter of enriched fecal sample was boiled for 10 min and centrifuged at 9,300 x g for 5 mins. The DNA in the supernatant was purified with a GeneClean® Turbo Kit (MP Biomedicals, LLC, Solon, Ohio) and subjected to the 6-plex PCR assay. A fecal sample confirmed as negative for the six serogroups was used to spike with pure cultures in the validation assay. Six STEC strains (14.1652, 4558-1, 12662-2, 12.3205, 15.013, 1044-1 of O2, O74, O109, O131, O168, and O171, respectively), previously isolated from cattle feces, were used to spike fecal samples. The strains were grown in Luria-Bertani (LB) broth, and cell concentration (CFU/ml) was determined by spread-plate method. Serial ten-fold dilutions of each strain (10-0 to 10-8) were prepared in LB broth and 100 µl of 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread-plated onto blood agar plates (four plates per dilution) to determine initial cell concentrations (CFU/ml). Approximately 50 g of PCR-negative feces was suspended in 450 ml of EC broth and dispensed into sterile tubes (9.4 ml/tube). Serial dilutions (10^{-0} to 10^{-8}) of pure cultures of each of the six serogroups were inoculated (100 µl) into 9.4 ml of EC broth. Spiked fecal samples were enriched (40°C for 6 h) in EC broth. Deoxyribonucleic acid was extracted from the enriched sample with a GeneClean® Turbo Kit, and subjected to the 6-plex PCR assay.

The experiment was repeated with a different fecal sample. The detection limit of the assay for *E. coli* serogroups O2, O74, O109, O131, O168, and O171 with DNA extracted directly from spiked cattle feces was ~ 10^6 CFU/g and ~ 10^2 CFU/g before and after enrichment in EC broth, respectively (Table 9).

Subtyping of *stx* genes

The subtyping of the *stx* genes of the 359 strains of non-top-7 STEC was according to Shridhar et al., 2017. A touchdown PCR method was used to amplify *stx* genes. The *stx*1 amplification protocol 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 (Δ-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). The *stx*2 amplification protocol 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). Sequencing analysis was preformed using methods from Shridhar et al., 2017, and Scheutz et al., 2012. Products of PCR were visualized using a Qiaxcel capillary electrophoresis system (Qiagen, Valencia, CA) and purified using a QIAquick PCR purification kit (Qiagen). DNA concentration and purity were analyzed using a spectrophotometer (NanoDrop-Thermo Scientific, Wilmington, DE). Nucleotide sequencing was performed on PCR products and primers by Genewiz, Inc., (South Plainfield, NJ). Chromatogram data was visualized using the CLC Main Workbench software. Individual analysis of conflicts and secondary peaks were conducted for all sequences. Consensus sequences were produced. Intergenic sequences were removed, resulting in the translation of nucleotide sequences to amino acid sequences. Shiga

toxin subtypes were determined based on the amino acid motifs that define each *stx* subtype (Scheutz et al., 2012).

Results

In the first feedlot study, of the 118 strains tested, 95 (80.5%) belonged to 11 serogroups and 23 (19.5%) were unidentifiable (Table 1). The 11 identified serogroups included the following: O168 (29.7%), O109 (20.3%), O171 (9.3%), O74 (6.8%), O104 (4.2%), O2 (4.2%), O8 (2.5 %), O175 (.8%), O131 (0.8%), O160 (0.8%), and O169 (0.8%). In the second feedlot study, 203 strains (84.2%) of the 241 belonged to 19 serogroups and 38 (15.8%) were unidentified. The 19 serogroups included O168, O109, O131, O2, O104, O8, O74, O171, O136, O178, O102, O98, O175, O118, O113, O96, O76, O35, and O20. (Table 1). The seven strains positive for the *st*x1 and *eae* genes were O118 (n=1), O98 (n=1), and O74 (n=5). The 36 strains positive for the *stx*2 and *eae* genes were O175 (n=1) and O109 (n=35). A total of 16 strains belonging to O76 (n=1), O104 (n=12), and O109 (n=3) were positive for stx1. Out of the 241 strains, 162 strains belonging to O20 (n=1), O35 (n=1), O96 (n=1), O102 (n=2), O136 (n=3), O171 (n=4), O8 (n=1), O2 (n=11), O131 (n=28), O169 (n=72) and unknown (n=38) were positive for *stx*2. The 15 strains positive for both *stx*1 and *stx*2 were O113 (n=1), O178 (n=2), O8 (n=4), O2 (n=7), and O109 (n=1). (Table 7)

Detection of STEC strains from the two feedlot studies indicated that serogroups O2, O74, O109, O131, O168, and O171were the predominant serogroups (Tables 6 and 7). In order to detect the prevalence of the six serogroups ofnon-top-7, a six-plex PCR assay was designed. The assay was validated with pure cultures and withy feces spiked with pure cultures. The viable cell concentrations of strains of serogroups O2, O74, O109, O131, O168, and O171, cultured individually in LB broth, were 3.2×10^8, 5.6×10^8, 3.1×10^8, 4.1×10^8, 4.5×10^8, and 6.5×10^8,

respectively. The detection limit of the assay for *E. coli* serogroups O2, O74, O109, O131, O168, and O171 with DNA extracted directly from spiked cattle feces was ~ 10^6 CFU/g and ~ 10^2 CFU/g before and after enrichment in EC broth, respectively (Table 9). A total of 911 fecal samples collected from commercial feedlots were subjected the six-plex PCR assay to determine the prevalence of the six predominant serogroups of non-top-7 STEC. Among the six serogroups of the major non-top 7 STEC, serogroups of O2 (59.4; 38.0), O109 (91.3; 84.9), O168 (78.4; 57.3), and O171 (86.8; 73.9) were the dominant serogroups in cattle feces collected from commercial feedlots from 2013 and 2014 studies, respectively (Tables 10 and 11). Only 29 (3.2%) fecal samples were negative for any of the six serogroups (Table 7). A majority of the fecal samples from the 2013 study (67.8%) were positive for 3 or 4 serogroups, and a majority from the 2014 study (51.5%) were positive for 2 or 3 serogroups (Table 12). Fecal samples that contained the six serogroups were also positive for the three major virulence genes, *stx*1, *stx*2, and *eae* (Tables 10 and 11).

Discussion

There are several pathotypes of intestinal pathogenic *E. coli:* Shiga toxin-producing *E. coli* (STEC)/ enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). Both STEC and EHEC cause mild to severe hemorrhagic colitis with complications of HUS, particularly in children. Serotype O157:H7 is the most common STEC involved in foodborne infections, although other serogroups can also cause hemorrhagic colitis, including HUS. According to the National Enteric Disease Surveillance of STEC's 2014 annual report (updated as of Feb. 8th, 2017), incidence of

infection due to non-O157 serogroups continued to rise to 0.79 cases per 100,000 people whereas O157 incidence of infection continued to decrease to 0.60 cases per 100,000 people, in 2014.. Additionally, infections in infants (< 1 year of age) were mostly due to non-O157 serogroups (1.19 vs. 1.97 cases per 100,000 population for girls and 0.69 vs. 2.18 cases per 100,000 population for boys).

Shiga toxins are major virulence factors of STEC. While presence of serotype serves as an initial risk indicator, determining the Shiga toxin subtype of STEC strains provides a more reliable estimate of risk of human illness. Studies show that *stx*2a and *stx*2c subtypes represent most HUS cases in humans (Friedrich et al., 2002; Persson et al., 2007). Shiga toxin 2 subtypes, d, e, and f, have each been recognized as a severity indicator for illness in humans (Bielaszewska et al., 2006).

Escherichia coli hemolysin, also called α-hemolysin (Cavalieri et al., 1984), encoded by *ehx*A, is a major virulence factor that belongs to the repeats-in-toxin (RTX) family and lyses red blood cells in the host. The *hly*A gene, also known as *ehx*A, encodes the toxin and is associated with *hly*C and *hly*B/D genes which encode for posttranslational modification, and components of the extracellular secretion system, respectively (Welch et al., 2005). Although much is unknown about the pathogenesis of Ehx/Hly, it is thought that iron from hemoglobin, released by mechanisms attributed to Hly, could stimulate the growth of STEC in the intestinal tract (Law et al., 1995).

In this study, prevalence of the major non top-7 STEC (O2, O74, O109, O131, O168, and O171) in commercial feedlot cattle was determined by developing and validating an end-point mPCR assay. Additionally, Shiga toxin subtypes associated with the major non top-7 STEC and others were identified based on restriction patterns after digestion of nucleotide sequences.

Among the six serogroups of non-top 7 STEC, serogroups of O2 (59.4; 38.0), O109 (91.3; 84.9), O168 (78.4; 57.3), and O171 (86.8; 73.9) were dominant in 2013 (%), and 2014 (%) studies, respectively. The most common subtypes carried by the dominant non top-7 STEC in this study were *stx*2a and *stx*2c for the 2013 and 2014 study which, as mentioned above, represent most HUS cases in humans; We found dominant serogroups O2, O109, O168, and O171 to be positive for *stx*2a at 72.0%, 56.3%, 87.9%, and 73.3%, respectively. Friedrich et al., (2002) found that one third of non-O157 serotypes positive for *stx*2c were also positive for *eae*, that 35.7% of HUS patients were positive for *stx*2c, and that the risk of developing HUS, post-infection, from the *stx2c* genotype was lower than the *stx*2 genotype in STEC.

Isolating STEC from fecal samples in an efficient and effective manner is an important step in the overall process of evaluating risk of illness in humans, especially regarding HUS. Screening fecal samples by PCR for *stx*, followed by rapid subtyping of *stx*2 genes in the presence or absence of *eae* provides physicians with trustworthy predictive values to assess the risk of their patients developing HUS from illness induced by STEC. This also provides a method of evaluation for those concerned about STEC shedding in cattle feces, which is a source of food and water contamination.

Table 6. Serogroup and virulence gene identification of enterohemorrhagic *Escherichia coli* and Shiga toxin-producing *Escherichia coli* strains isolated from commercial feedlot cattle feces (2013 study) by end-point PCR.

Serogroup	Total positive (%)	Enterohemorrhagic *E. coli*				Shiga toxin-producing *E. coli*		
		***stx*1**	***stx*2**	***eae***	***ehx*A**	***stx*1**	***stx*2**	***ehx*A**
O131	1 (0.8)	-	-	-	-	-	1 *stx*2d	1
O160	1 (0.8)	-	-	-	-	-	1 *stx*2a	-
O169	1 (0.8)	-	-	-	-	-	1 *stx*2a	1
O175	1 (0.8)	-	-	-	-	-	1 *stx*2d	1
O8	3 (2.5)	-	-	-	1	1 *stx*1a	2 *stx*2a; 1 *stx*2d	1
O2	5 (4.2)	-	-	-	-	1 *stx*1a	4 *stx*2a; 1 *stx*2c	5
O104	5 (4.2)	-	-	-	-	5 *stx*1c	-	5
O74	8 (6.8)	8 *stx*1a	-	8	8	-	-	-
O171	11 (9.3)	1 *stx*1a	-	1	-	-	9 *stx*2a; 1 *stx*2c	2
O109	24 (20.3)	-	12 *stx*2a; 4 *stx*2c	16	16	8 *stx*1a	-	-
O168	35 (29.7)	-	-	-	-	1 *stx*1a	31 *stx*2a; 1 *stx*2c; 2 *stx*2d	20
Unknown	23 (19.5)	-	-	-	1	1 *stx*1a	16 *stx*2a; 2 *stx*2c; 4 *stx*2d	22

Table 7. Serogroup and virulence gene identification of enterohemorrhagic *Escherichia coli* and Shiga toxin-producing *Escherichia coli* strains isolated from commercial feedlot cattle feces (2014 study) by end-point PCR.

Serogroup	Total positive (%)	Enterohemorrhagic *E. coli*				Shiga toxin-producing *E. coli*		
		***stx*1**	***stx*2**	***eae***	***ehx*A**	***stx*1**	***stx*2**	***ehx*A**
O20	1 (.4)	-	-	-	-	-	1 *stx*2a	1
O35	1 (.4)	-	-	-	-	-	1 *stx*2a	-
O76	1 (.4)	-	-	-	-	1 *stx*1a	-	-
O96	1 (.4)	-	-	-	-	-	1 *stx*2a	1
O113	1 (.4)	-	-	-	-	1 *stx*1a	1 *stx*2a	1
O118	1 (.4)	1 *stx*1a	-	1	1	-	-	-
O175	1 (.4)	-	1 *stx*2a	1	1	-	-	-
O98	1 (.4)	1 *stx*1a	-	1	1	-	-	-
O102	2 (.8)	-	-	-	-	-	2 *stx*2a	2
O178	2 (.8)	-	-	-	-	2 *stx*1a	2 *stx*2a	2
O136	3 (1.3)	-	-	-	-	-	2 *stx*2d; 1 *stx*2a	3
O171	4 (1.7)	-	-	-	-	-	2 *stx*2a; 1 *stx*2c; 1 *stx*2d	-
O74	5 (2.1)	5 *stx*1a	-	5	5	-	-	-
O8	7 (2.9)	-	-	-	-	4 *stx*1a	4 *stx*2a; 1 *stx*2d	5
O104	12 (5.0)	-	-	-	-	11 *stx*1c; 1 *stx*1a	-	12
O2	20 (8.3)	-	-	-	-	7 *stx*1a	14 *stx*2a; 1 *stx*2c; 3 *stx*2d	16
O131	28 (11.6)	-	-	-	-	-	19 *stx*2a; 9 *stx*2c	1

O109	40 (16.6)	-	24 *stx*2a; 5 *stx*2c; 7 *stx*2d	36	35	3 *stx*1a	1 *stx*2a	-
O168	72 (29.9)	-	-	-	-	-	63 *stx*2a; 6 *stx*2d; 3 *stx*2c	53
Unknown	37 (15.8)	-	-			-	27 *stx*2a; 4 *stx*2c; 6 *stx*2d	18

Table 8. Target genes, primers sequences, and amplicon sizes to detect six major non top-7 Shiga toxin-producing *Escherichia coli*.

Genes	Primer	Primers sequence	Amplicon size
*wzx*O2/O50	O2-F	TGGCCTTGTTCGATATACTGCGGA	819
	O2-R	TCACGAGCTGAGCGAAACTGTTCA	
wzxO131	O131-F	GTGATTTCTGGGGCAACATT	655
	O131-R	AAGCCTGCCCTAAACAAAGC	
wzxO168	O168-F	TGTCGACTTTGGGAAATGTGG	336
	O168-R	CTGCAGAGGCCAATTCAGGT	
wzxO74	O74-F	CTGGTCAATGGCAAGCTGTA	303
	O74-R	ATGCAAAAATCCAAGCCAAT	
*wzx*O171	O171-F	TGCTCAAGTGGCATGCAGAT	281
	O171-R	TGCAACCTGATATCCAGCAGT	
wzxO109	O109-F	TCTCTCTCGACATACCCGCGCTT	204
	O109-R	ACCGTAGCCCAAAGAGCCACA	

Table 9. Fecal spike validation of six major non top-7 Shiga toxin-producing *Escherichia coli* from cattle feces by end-point PCR method of detection.

Concentrations	Serogroups					
Pre-Enrichment	**O2**	**O74**	**O109**	**O131**	**O168**	**O171**
10^6	+	+	+	+	+	+
10^5	+	-	+	+	+	+
10^4	-	-	+	+	+	+
10^3	-	-	+	-	+	+
10^2	-	-	+	-	-	-
10^1	-	-	+	-	-	-
10^0	-	-	-	-	-	-
Post-Enrichment						
10^6	+	+	+	+	+	+
10^5	+	+	+	+	+	+
10^4	+	+	+	+	+	+
10^3	+	-	+	+	+	+
10^2	+	+	+	+	+	+
10^1	-	-	+	-	+	+
10^0	-	-	+	-	+	+

Table 10. Detection of six major non top-7 Shiga toxin-producing *Escherichia coli* and virulence genes, *stx*1, *stx*2, and *eae*, from cattle feces (2013 study) in a commercial feedlot setting by end-point PCR.

Serogroup	Total positive (%)	*stx*1	*stx*2	*eae*
O2	341 (59.4)	245 (71.8)	326 (95.6)	331 (97.6)
O74	113 (19.7)	83 (73.5)	107 (94.7)	111 (98.2)
O109	524 (91.3)	346 (66.0)	501 (95.6)	512 (97.7)
O131	10 (1.7)	5 (50.0)	10 (100.0)	10 (100.0)
O168	450(78.4)	310 (68.9)	437 (97.1)	442 (98.2)
O171	498 (86.8)	332 (66.7)	478 (96.0)	488 (98.0)

Table 11. Prevalence of six major non top-7 Shiga toxin-producing *Escherichia coli* and virulence genes, *stx*1, *stx*2, and *eae*, from cattle feces (2014 study) in a commercial feedlot setting by end-point PCR.

Serogroup	Total Positive (%)	*stx*1	*stx*2	*eae*
O2	128 (38.0)	85 (66.4)	108 (84.4)	126 (98.4)
O74	56 (16.6)	38 (67.9)	41 (73.2)	51 (91.1)
O109	286 (84.9)	157 (54.9)	220 (76.9)	263 (92.0)
O131	8 (2.4)	7 (87.5)	8 (100.0)	8 (100.0)
O168	193 (57.3)	120 (62.2)	167 (86.5)	181 (93.8)
O171	249 (73.9)	146 (58.6)	199 (79.9)	240 (96.4)

Table 12. Number and percentage of samples testing positive to multiple major non top-7 Shiga toxin-producing *Escherichia coli* serogroups ((O2, O74, 0109, 0131, 0168, 0171) by end-point PCR.

Study (no. samples tested)	Total positive (%)	Number of serogroups (%)						
		0	**1**	**2**	**3**	**4**	**5**	**6**
2013 (n=574)	560 (97.6)	14 (2.4)	33 (5.7)	63 (11)	156 (27.2)	233 (40.6)	73 (12.7)	2 (0.3)
2014 (n=337)	322 (95.5)	15 (4.5)	46 (13.6)	87 (25.8)	87 (25.8)	73 (21.7)	27 (8.0)	2 (0.6)

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