Shiga toxin-producing *Escherichia coli*: detection and isolation from swine feces and wheat grains by PCR and culture methods

by

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B.S., Kansas State University, 2017

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Veterinary Biomedical Sciences
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2020

Approved by:

Major Professor
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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are major foodborne pathogens that cause mild to hemorrhagic colitis, which could lead to a serious complication of renal failure, called hemolytic uremic syndrome, particularly in children. Seven serogroups of STEC, O26, O45, O103, O111, O121, O145, and O157, often called top-7, account for most of the STEC-associated illness in humans in the United States. Two Shiga toxins, Shiga toxin 1 and 2, encoded by *stx*1 and *stx*2, respectively, and intimin, a protein that mediates attachment of STEC onto enterocytes, encoded by *eae* gene, are major virulence factors involved in STEC infections. Cattle are a major reservoir of the 'top-7' serogroups, in which STEC colonize the hindgut and are shed in the feces. The feces serve as a major source of food, feed and water contaminations. In addition to cattle, other domestic animals and even wild animals harbor STEC and shed them in the feces. Sources of STEC foodborne illness outbreaks have been traced to food of animal and plant origin. Two studies were conducted to detect and isolate STEC, based on culture- and PCR methods, from swine feces and wheat grains.

Swine fecal samples (n=598), collected from ten swine farms with finisher pigs, located in eight states, were enriched with EC broth. The enriched samples were subjected to a three-plex quantitative PCR (qPCR) assay targeting three virulence genes, *stx*1, *stx*2, and *eae*. Samples positive for either of the two Shiga toxin genes were then tested by a multiplex PCR assay targeting top-7 serogroups (O26, O45, O103, O111, O121, O145, and O157) and the O104 serogroup. Also, *stx*-positive samples were subjected to an eight-plex PCR assay, designed and validated to detect 8 serogroups (O8, O20/O137, O59, O86, O91, O100, O120, and O174) considered to be the top-8 prevalent STEC in swine feces. Samples positive for the top-7 plus O104 serogroups were subjected to a serogroup-specific IMS culture method and plating on

selective media for detection and isolation of top-7 serogroups of STEC. Samples positive for *stx*1 or *stx*2 gene and negative for the top-7 serogroups were directly plated onto MacConkey and Eosin-Metheylene Blue agar. Putative colonies, up to ten per sample and medium, were picked, pooled and tested for *stx* genes. If pooled colonies were positive for *stx*1 or *stx*2 gene, then each colony in the pool was tested individually to identify *stx*1 and/or *stx*2-carrying *E. coli*.

Of the 598 fecal samples tested by qPCR for the three major virulence genes, 155 (25.9%), 389 (65.1%), and 398 (66.6%) samples were positive for *stx*1, *stx*2, and *eae* genes, respectively. Based on the mPCR assay for the top-7 plus O104 serogroups, the three predominant serogroups detected were O26 (10.7%; 64/598), O121 (17.6%; 105/598), and O157 (11.5%; 69/598). The 8-plex PCR assay designed to detect the top-8 serogroups of swine STEC indicated the prevalence of 88.6% of O8 (530/598), 35.5% of O86 (212/598), 24.1% of O174 (144/598), 20.2% of O100 (121/598), 15.6% of O91, 4.3% of O59 (26/598), 4.2% of O120 (25/598), and 3.2% of O20/O137 (19/598). The culture method identified STEC O121 as the dominant top-7 STEC serogroup (3.8%; 23/598). None of the O157 isolates (3.5%; 21/598) carried the s*tx* gene. Isolates that were non top-7 (or O104) were tested by 12 different PCR assays that can detect 130 serogroups to identify the serogroups of STEC. The most prevalent non top-7 STEC serogroups isolated were STEC O8 and STEC O86. In conclusion, our study indicated that the major top-7 STEC was O121, and among the non-top-7 STEC, serogroups of O8 and O86 were the dominant. Interestingly, the O157 serogroup implicated in outbreaks traced to pork products, was shed in the feces, but none of the isolates carried the *stx* gene. Data on the prevalence and virulence potential of STEC from swine will be useful information to have to evaluate management strategies and mitigate the effects on food borne illnesses in the United States.

Wheat grain samples (n=626), collected from different regions of the country and transported to the laboratory, were enriched using two different media, modified Buffered Peptone Water with pyruvate (mBPWp) and *Escherichia coli* (EC) broth and subjected to a mPCR assay to detect the top-7 serogroups (O26, O45, O103, O111, O121, O145, and O157) and three major virulence genes (*stx*1, stx2, and *eae*). Samples positive by PCR for any of the top-7 serogroups and or *stx* genes were then cultured using serogroup-specific immunomagnetic separation (IMS) and plating on selective media for detection and isolation of top-7 STEC. Based on the mPCR assay, the prevalence of the top-7 serogroups in wheat grain samples, enriched by mBPWp, were 0.2% of O26 (1/626), 0.6% of O45 (4/626), 0.2% of O103 (1/626), and 0.6% of O157 (4/626), and the prevalence of virulence genes were 0.2% of *stx*1 (1/626), 0.2% of *stx*2 (1/626), and 0.8% of *eae* (5/626). The prevalence of top-7 serogroups, enriched in EC broth, were 0.2% of O26 (1/626), 1.9% of O45 (12/626), 0.5% of O103 (3/626), and 1.0% of O157 (6/626), and that of virulence genes were *stx*1 (0.3%; 2/626), *stx*2 (1.3%; 8/626), and *eae* (1.1%; 7/626). The number of wheat grain samples positive for STEC serogroups and or virulence genes, by PCR method (36/626; 5.8%), was higher in samples enriched by EC broth than mBPWp broth (15/626; 2.4%). Based on culture methods, the top-7 serogroups prevalent in wheat grain samples, enriched in mBPWp, were 0.2% of O26 (1/626), 0.5% of O45 (3/626), 0.2% of O103 (1/626), and 0.6% of O157 (4/626), and none of the isolates was positive for any of the three virulence genes. In wheat grain samples enriched in EC broth, the prevalence of the top-7 serogroups were 0.2% of O26 (1/626), 0.8% of O45 (5/626), 0.3% of O103 (2/626), and 0.8% of O157 (5/626), with no detection of virulence genes. The number of wheat grain samples positive for STEC serogroups and or virulence genes, by culture method, was higher in samples enriched by EC broth than mBPWp broth (5/36 and 0/15, respectively). None of the isolates of

the top-7 serogroups by either enrichment method was positive for the Shiga toxin genes. A total five isolates that carried the Shiga toxin 2 gene were isolated from wheat grains enriched in EC broth. The five isolates were confirmed as serogroups O8 (0.64%; 4/626) and O130 (0.16%; 1/626) by PCR. Our study shows that wheat grains were contaminated with the top-7 serogroups of *E. coli,* but none of the isolates carried the Shiga toxin genes. The two *stx*2-positive serogroups that were isolated, O8 and O130, are not major STEC pathogens and have only been implicated in sporadic diarrheal cases in animals and humans.

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Acknowledgements

I would like to acknowledge my major advisor, Raghavendra Amachawadi, for his continued support in my career in veterinary medicine in both my undergraduate and graduate career. I would like to also acknowledge TG Nagaraja, for his endless patience and guidance in my graduate curriculum. Further, I cannot forget to acknowledge the student workers and technicians at the Kansas State College of Veterinary Medicine Pre-Harvest Food Safety Lab. Particularly Leigh Ann George, Neil Wallace, and Xiaorong Shi, for being incredible and assisting me through every step of the research process. After working in these labs for over four years, everything I have gained is because of these individuals, and they could never be acknowledged enough.

Dedication

I owe so many dedications to so many people, as I have such an incredible support system. My parents, my brothers, the Bonavia family, Harley, Skyler, and Brendan are just a few who have supported me every step of the way in my education. These individuals make me the most blessed person in the world. Not only have they supported me through this graduate curriculum, they support me through every challenge in life and remind me to always chase my passion for veterinary medicine. As I move through the veterinary school curriculum, I hope to make each one of them as proud as possible. I dedicate this thesis, and every wonderful moment in my life, to these extraordinary people.

Chapter 1 - Shiga toxin producing *Escherichia coli* in swine: A literature review

Introduction

Escherichia coli are a diverse species of Gram-negative bacteria that include nonpathogenic and pathogenic strains. One of the pathotypes of *E. coli* that produces Shiga toxins (STEC) is an important food borne pathogen, which causes mild (colitis) to bloody diarrhea (hemorrhagic colitis), and in children, the infection could lead to hemolytic uremic syndrome because of renal failure, and even death. The number of STEC illnesses in the United States each year is estimated to be about 265,000 cases, with 3,600 of them resulting in hospitalizations and thirty of them resulting in deaths (Scallan et al., 2011). The serious complication of hemolytic uremic syndrome (HUS), principally in children, leads to destruction of red blood cells causing renal failure (Griffin et al., 1991; Karmali et al., 2010). Shiga toxins (Stx) are proteins secreted by *E. coli* and are cytotoxic because of inhibition of protein synthesis (Tesh et al., 1991). There are two types of Shiga toxins, Stx1 and Stx2, encoded by *stx*1 and *stx*2 genes, respectively, which are carried on a prophage (McDonough et al., 1999). Although the two Shiga toxins share similarity in some amino acid sequences and have the same mode of action, they are antigenically distinct and differ in the degree of cytotoxicity. Stx2 is more cytotoxic than Stx1, and within each, there are several subtypes. There are 3 subtypes of *stx*1: *stx*1a, *stx*1c, and *stx*1d (Zhang et al., 2002), and 7 subtypes of *stx*2: *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f, and *stx*2g (Scheutz et al., 2009). Certain subtypes of *stx*2, particularly *stx*2a, *stx*2c, and *stx*2d, are more often associated with hemorrhagic colitis and HUS (Melton-Celsa et al., 2002). Another important virulence protein is intimin, encoded by a gene called *eae*, which mediates the attachment of *E. coli* to intestinal epithelial cells is produced by enterohemorrhagic

E. coli (EHEC), a subset of STEC (CDC, 2019). Shiga toxin-producing *E. coli* (STEC) are found in animal gastrointestinal tracts, particularly food animals, and their environments. In animals, STEC are commensals and do not cause infections, except in swine in which a few serogroups carrying a subtype of Shiga toxin, called 2e, cause edema disease.

There are as many as 150 serogroups and more than 400 serotypes of STEC. Seven serogroups, O26, O45, O103, O111, O121, O145, and O157, referred to as top-7 STEC, are responsible for the majority of STEC infections in the US (CDC, 2019). The majority of the knowledge about STEC is derived from *E. coli* O157, which was first identified as a human pathogen in 1982 (Riley *et al.,* 1983). Infections caused by STEC are of public health concern because food animals that shed STEC in their feces can contaminate the food products. Although cattle are a major reservoir, different serogroups of STEC are capable of colonizing the hindgut of swine, and are shed in the feces, which serve as a source of food and water contaminations. This literature review addresses reports of foodborne outbreaks originating from swine and pork products, serogroups of STEC isolated from the feces of swine, and the pathogenesis of Stx2e in edema disease in swine to give foundational knowledge of STEC shedding and the overall impact on finisher swine.

Foodborne outbreaks of STEC traced to swine and pork products

Swine are capable of being a vehicle of STEC transmission, both by directly contaminating the pork products and the indirect contamination of other food products with their feces. An outbreak of *E. coli* O157:H7 was linked to the consumption of baby spinach, a product that is commonly not cooked before consumption (CDC, 2006). It was discovered that feral free-roaming swine were observed near the fields of spinach cultivation. This outbreak resulted in

205 cases of illnesses and three deaths. Of the forty feral swine fecal samples collected from the area, 5% of the samples were positive for the same PFGE type of STEC O157:H7, making them a possible source of contamination of the spinach (Jay et al., 2007).

Some of the foodborne outbreaks are related to pork products that are sold mixed with other animal meat products. An outbreak of twenty laboratory-confirmed cases of O157:H7 infection from Washington and California states that originated from a salami product made of a beef and pork mixture was reported over the span of two months. A total of eleven of these patients reported eating the dry-cured salami. Out of the twenty patients diagnosed, three required hospitalization and one developed hemolytic uremic syndrome (CDC, 1995). Further, two other outbreaks of O157:H7 resulting from the contamination of a salami product made of a beef and pork mixture in Canada have been reported. In 1998, Ontario had an outbreak of O157:H7 that originated from a Genoa salami product made of a beef and pork mixture. A total of thirty-nine cases had been reported to be linked to this product, and thirty-six of the product samples that were provided to the case report were positive for *E. coli* O157 with the same pulsed-field gel electrophoresis pattern and phage type. (Williams et al., 2000). A year later, British Columbia, Canada had a total of 143 cases of O157:H7 infection that originated from an unspecified salami product made of a beef and pork mixture. The outbreak strain of O157:H7 was isolated from 135 fecal samples over 12 weeks and had the same pulsed-field gel electrophoresis pattern as that of the strain from the contaminated salami product, and STEC with unidentified serotype was isolated from eight fecal samples (MacDonald et al., 2004).

It is possible that the pork was not the source of contamination in the products mixed with the ground beef, as *E. coli* O157:H7 is commonly associated with ground beef (Features Submission, 1992). However, pork products have been the source of STEC outbreaks in recent

years. A family in Italy ate a dry-fermented pork salami in 2004. This salami product had been contaminated with *E. coli* O157 with three major virulence genes; *stx*1*, stx*2*,* and *eae,* which caused two people to be hospitalized with bloody diarrhea and one young adult with mild diarrhea (Conedera et al., 2007; Table 2). Because multiple studies have included reports of dry-fermented salami contaminated with STEC, this pork product should be included in investigation of STEC infections with *E. coli* having the ability to survive the acidic conditions produced in the dry fermentation process (Faith et al., 1998). Other pork products, such as whole roasted pig, has been previously reported as vehicle of STEC transmission. A four-day family gathering in Ontario, Canada in 2012 resulted in fifty-nine attendees becoming sick with foodborne illness. The investigation used trace back evidence to indicate the pork from a pig roast resulted in the attendees becoming ill, with six cases demonstrating bloody diarrhea and seven cases being hospitalized. Of the twenty-nine attendees who had gone under culture isolation for STEC, eleven were positive for STEC O157:H7 (Trotz-Williams et al., 2012; Table 2). While the majority of these STEC outbreaks from swine were inconclusive as a source of contamination, they also suggest that pork products are capable of transmitting STEC foodborne illness and should be investigated further.

The prevalence of STEC in swine

Feral swine that are hunted and consumed pose a risk of directly causing foodborne illness via contaminated meat and contaminating commercial production systems for plant-based food products, like the spinach outbreak (Jay et al., 2007). In a study to determine STEC prevalence in 791 samples from wild animals (geese, roe deer, hares, moose, wild boar and gulls) hunted during the season in 2003, one sample from a wild board was STEC O157 positive

(Wahlstrom et al., 2003). A 2010 study investigated the prevalence of O157:H7 and non-O157 STEC from feral swine in southwest Spain. A total of 212 samples from feral swine killed during that season were collected for detection of STEC. *E. coli* O157:H7 prevalence was 3.3% and non-O157 STEC prevalence was 5.2%. Out of nineteen isolates characterized, four isolates carried the *stx*1 gene, twelve isolates were positive for the *stx*2 gene, and one isolate was positive for both *stx*1 and *stx*2 genes. While some *E. coli* O157:H7 isolates collected during the study had an undetermined PFGE type, it was discovered that the same PFGE type was associated with a case of diarrhea from a person living in the same area (Sanchez et al., 2010). This information indicates that the relationship between feral swine and their STEC prevalence should be investigated further to indicate the impact on public health.

The STEC prevalence in swine in commercial production systems has been reported at similar percentages from other countries. Investigation of swine as a possible reservoir of STEC and the prevalence of STEC began with a study in Japan that randomly selected 35 commercial pig farms out of 14,400 in 1997. A total of 221 pigs were selected from the 35 commercial pig farms and rectal swab samples were taken from each pig. The swabs were enriched in *Escherichia coli* broth with 25 µg/ml of novobiocin and incubated at 42ºC. This enriched rectal swab suspension was then streaked on MacConkey sorbitol agar for growth of sorbitol-negative colonies, which were used to test by slide latex agglutination test for the O157 antigen. Rectal swab samples, taken from three pigs (at 2 months, 6 months, and 9 months of age) from three individual farms, were positive for STEC O157:H7, and all 3 strains had all three virulence genes, *stx*1*, stx*2, and *eae*. The swine isolates had the same genetic markers as cattle O157:H7, and the carriage rate in swine was also similar to the carriage rate of O157:H7 in cattle, indicating that there may be a common origin of dissemination of STEC O157:H7 in food

animals in Japan (Nakazawa et al., 1999). A study of STEC O157 in fecal samples and tonsil swabs from 145 pigs in a Dutch commercial production system resulted in two isolates obtained from fecal samples (prevalence of 1.4%), and no O157 isolate was obtained from the tonsil swabs. The investigators in this study concluded that commercial production pigs could be a source of O157 strains that cause foodborne illness in humans (Heuvelink et al., 1999). In a study in Italy, 150 finisher pigs were randomly selected for fecal and carcass swab samples for detection of *E. coli* O157. Of these samples, O157 was isolated from four fecal samples and one carcass sample. None of these isolates was positive for Shiga toxin or intimin genes (Bonardi et al., 2003). Even though different detection and isolation techniques were used in each of these studies, O157 was consistently isolated in at least one rectal swab and fecal samples in all sample populations, indicating it is a possible vehicle of transmission of STEC.

The reported STEC prevalence studies in the United States is limited in comparison to the number of studies from other countries. In the National Animal Health Monitoring System's (NAHMS) studies of STEC O157 from swine populations conducted in 1995 and 2000, no STEC O157:H7 was isolated (Bush, 1997; Feder et al., 2007). In a study reported by Feder et al. (2003), colonic content samples were collected from pigs at slaughter for isolation of STEC O157. Out of the 305 colonic samples, six samples (1.9% prevalence) were positive for STEC O157:H7. (Feder et al., 2003). These studies show that differences in study design can result in different STEC prevalence rates in commercial swine populations, but the prevalence detected is generally lower than the prevalence of O157 in cattle.

The NAHMS 2000 study also detected and isolated non-O157 STEC strains. Out of 687 fecal samples collected from the healthy finisher swine from multiple states, 196 samples were positive for non-O157 STEC (a prevalence of 28.5%). Out of the 196 positive fecal samples, 219

STEC isolates belonged to serogroups of O9, O20, O91, O101, and O121, which have also been isolated from clinical cases of human illnesses, and therefore, could be of public health concern (Fratamico et al., 2004, 2008; Table 1).

A recent study trying to reduce the fecal shedding of STEC in finishing swine by the use of chlortetracycline and bacitracin found that there was no significant impact of the antimicrobials on the fecal shedding of non-O157 STEC. Of the top-7 STEC serogroups, three serogroups, O26, O103, and O145, were isolated at prevalence of 6.9%, 2.4%, and 4.8%, respectively. Further, the study found the prevalence of the Shiga toxin genes (*stx*1 and *stx*2) in enriched rectal swabs to be at 58%, much higher than the prevalence of any serogroups detected in this study, indicating that there may be more dominant *E. coli* serogroups producing these Shiga toxins and being shed in the feces of finisher pigs (Wells et al., 2013).

In conclusion, the prevalence of STEC in swine, both in the US and other countries, varies but is constantly present in the gastrointestinal tract of both domestic and feral pigs. In particular, the investigation of the type of Shiga toxins (*stx*1 or *stx*2), as well as the dominant subtypes of Shiga toxin, and top-7 STEC should be sought out further to determine the public health importance of STEC of swine origin. Prevalence of STEC in swine also has importance when STEC isolates cause edema disease in swine. Certain serogroups of STEC, particularly O138, O139, O141, and O147, are more frequently associated with edema disease in swine (Fairbrother and Gyles, 2012). These serogroups typically produce a specific subtype of Shiga toxin called Stx2e.

The prevalence of Shiga toxin subtype stx2e in edema disease in swine

Edema disease in swine is also referred to as gut edema or bowel edema. This disease can cause great losses to preweaned piglet production facilities, as it usually occurrs in pigs that are within two weeks of weaning. While this timeframe is the most common for edema disease to occur, it is seen in older herds of swine as well. Edema disease emergence in a herd usually is a result of an occasional case, however, recurrence of edema disease is common in herds with previous edema disease cases. Further, the number of deaths over the number of diagnosed piglets with edema disease can range from 50% to 90% (Casanova et al., 2018).

Swine susceptibilities to edema disease are related to a number of variables, particularly diet, stress, or the immunity of the particular piglets. Edema disease has a common characteristic of eyelid swelling, as well as unique sounds that have been defined as squeals or snoring. Neurological clinical characteristics of edema disease include ataxia, paralysis, and lack of coordination. Death is usually a result of vascular damage in the brain stem of pigs affected with edema disease. Specific cases of edema disease, ones that also include clinical signs of postweaning diarrhea, are a result of *E. coli* strains and thus indicates they play a role in pathogenesis by a production of Shiga toxin (Fairbrother and Gyles et al., 2012; Casanova et al, 2018).

Shiga toxin is a vital virulence factor in the pathogenesis of the edema disease. Of all subtypes of Shiga toxin, the most common subtype associated with the edema disease in pigs is Stx2e (Marques, 1987). This *stx*2e subtype is the second most common Shiga toxin gene subtype detected in the environment, next only to *stx*2d (Vernozy-Rozand et al., 2004; Fratamico et al., 2008). While *stx*2e is not as common in human infections as other Shiga toxin 2 subtypes, further

investigations on this subtype in human infections are warranted. A study performed phenotypic and molecular characterization of Stx2e isolates from humans and pigs and showed than human strains vary in their interactions with intestinal epithelial cells in comparison to pig strains (Sonntag et al., 2005). The human strains adhered with varying intensity to human cell lines of T84 and HCT-8 and none of the human strains adhered to pig intestinal epithelial cell lines IPEC-J2. Further, edema disease has been shown to arise at post-weaning stage, all the way to finisher pigs, as a result of differential expression of adhesion receptors on intestinal epithelial cells between newborn and adult pigs (Moredo et al., 2015). The differences in colonization factors and their host adaptations are vital in the understanding pathogenesis of both edema disease and STEC food borne illness. The most commonly reported serogroups isolated from cases of edema disease are STEC O138, O139, O141, and O147 (DebRoy et al., 2009; Table 3). 'Top-7' STEC serogroups (O26, O45, O103, O111, O121, O145, and O157) that predominantly affect public health have not been reported to produce *stx*2e in previous studies, however, a previous study hypothesized that serogroups commonly associated with human disease do not produce *stx*2e (Paton et al., 1998) Thus, thorough investigations on the prevalence of top-7 and dominant non top-7 serogroups, the virulence gene profile of isolates, and the subtype of Shiga toxin secreted by the STEC shed in swine are necessary to begin to evaluate management strategies in cases of edema disease in post weaning herds and cases of public health.

Tables

Table 1. Shiga toxin-producing *Escherichia coli* serogroups and serotypes detected and or isolated from swine feces

Authors	Most common serogroups/ serotypes	Other serogroups/serotypes	Serogroups/serotypes with *stx*$_{2e}$ involved in edema disease	Notes
Aatestrup et al., 1997	O139		O139	
Fratamico et al., 2004	O8, O20, O91, O100, O120	O2, O5, O7, O9, O11, O15, O57, O65, O68, O69, O78, O96, O101, **O121,** O152, O159, O160, O163, OX10, OX18	O2, O5, O8, O9, O11, O20, O57, O65, O68, O69, O78, O91, O100, O101, O120, O121, O159	
Kaufmann et al., 2006	O8, O9, O100	O2, O26, O65, O103, O141, O159, O180	O2, O8, O9, O26, O65, O100, O141, O159, O180	Total 630 fecal samples from healthy pigs tested, 45 STEC strains isolated.
Vu-Hac et al., 2007	O8, O149	O1, O2, O5, O8, O14, O15, O20, O23, O35, O45, O54, O60, O63, O64, O73, O80, O84, O98, O101, O103, O108 (healthy piglet), O111, O112, O114, O116 (healthy piglet), O120, O138, O139, O141, O146, O147, O157, O162, O163	O141	Total 250 strains (220 from sick piglets, 30 from healthy piglets) isolated from years 2001 to 2003.
Fratamico et al., 2008	O8, O20, O91, O100, O120	O2, O5, O7, O9, O11, O15, O57, O65, O68, O69, O78, O96, O101, **O121,** O152, O159, O160, O163, OX10, OX18	O2, O5, O8, O9, O11, O20, O65, O68, O69, O78, O100, O101, O120, **O121,** O159	All swine strains were negative for *eae* gene.

Funk, 2013	O59	O15, O20, O49, O89, O98, 115, O119, O167	O59, O15, O20, O49, O89, O115, O119, O167	All serogroup do not have eae gene. **The 1st study of STEC in US finishing swine.**
Meng et al., 2014	O20H:30, O2:H32, O100:H20	O9:H30 (4.3%), O143:H38 (3.2%), O172:H30 (3.2%), O86:H11, O76:H25, O87:H10, O114:[H30], O116:H11, O159:H16,	O20H:30, O2:H32, O100:H20, O9:H30, O143:H38, O172:H30, O86:H11, O76:H25, O87:H10, O114:[H30], O116:H11, O159:H16	All STEC isolates were $stx_{2e,}$ and all isolates were *eae* negative.
Tseng et al., 2014	**O26, O103, O111, O121,** and **O145**	O9, O20, O91, O101, **O121, O157:H7**	O138, O139, O141, and O147.	
Colello et al., 2016		O1, O2, O15, O20, O35, O69, O78, O91, **O121**, O138, O142, **O157,** O180	O1, O8, O121, O138	
Wonhee et al., 2017	O59:H21 O174:H4	**O86 (6.7%),** O100, O163, O26, O157, O8, O71, O184,	O8:H28, O59:H21 O71:H21, O86:H19 O86:H32, O174:H2 O184:H48, O100:H30	All O serotypes do not have *eae* gene, except O26 and O157.
Bardasi et al., 2017	O26, O145, O157	O103, O104, O111		High percentage positive fecal samples from qPCR, however, did not get any isolates. 31% sample positive for all 6 O groups, 28% sample positive for 5 O groups.

Table 2. Shiga toxin-producing *Escherichia coli* serogroups and serotypes detected and or isolated from pork products

Author	Most common serogroups/ serotypes	Other serogroups/serotypes	Serogroups/serotypes with *stx*$_{2e}$ involved in edema disease	Notes
Brooks et al., 1997		**O26:H11**, O128:H2		Isolates from mettwurst were *eae* negative.
Frydendahl, 2002	O149, O138, O139, O141 (4.1%), O8 (3.7%)	O2, O3, O4, O6, O7, O9, O15, O16, O18, O20, O21, O26, O29, O32, O45, O51, O59, O66, O77, O78, O79, O83, O88, O98, O99, O101, O115, O116, O117, O121, O128, O142, O147, O157, O158, O174, O175	Shiga toxin 2e (Stx2e): O138, O139, O141, O147,	A total of 563 *E. coli* isolates from 507 pigs and 410 herds isolated from January to August 1999 and January to December 2000. Total 42 different O groups. *E. coli* were isolated from intestinal contents of carcasses of 4-8 week old pigs with postweaning diarrhea or edema disease.
Trotz-Williams et al., 2012		O157:H7		STEC O157:H7 was isolated from patients (family gathering). Pork was the source of infection.
Colello et al., 2016	O8, O9	O1, O2, O15, O20, O35, O69, O78, O91, **O121**, O138, O142, **O157,** O180	O1, O8, O121, O138	

Mughini-Gras et al., 2016				Human STEC infections were attributed to pig just 5.7%-12.5%. Do not know O serotype.
Conedera et al., 2017		O157		**The first report of an outbreak associated with a product containing pork meat only.**
Bardasi et al., 2017	O26, O145, O157	O103, O104, O111		High percentage positive pork samples from qPCR, however, did not get any isolates. 2% samples positive for all 6 O groups, 7% samples positive for 5 O groups.
Saupe et al., 2017		O8:H19 (*stx*$_{2e}$+, *eae* -)	O8:H19 (*stx*$_{2e}$+, *eae*-)	Patient had eaten poultry liver and grilled swine meat.

Table 3. Summary of most prevalent Shiga toxin-producing *Escherichia coli* serogroups, including Shiga toxin 2e associated with edema disease

Country	Predominant O groups	O groups with *stx*2e[a]
United States	O59, O174, O8, O20, O91, O100, O120, O86, O121	O2, O5, **O8**, O9, O11, O15, **O20**, O49, O57, **O59**, O65, O68, O69, O71, O78, **O86,** O89, **O91**, **O100**, O101, O115, O119, **O120**, O121, O159, O167, **O174**, O184
Europe	O149, O8, O9, O100, O138, O139, O141	O2, **O8**, **O9**, O26, O65, **O100**, **O138**, **O139**, **O141**, O147, O159, O180
Asia	O20, O2, O100	**O2**, O9, **O20**, O76, O86, O87, **O100**, O114, O116, O143, O159, O172
South America	O8	O1, **O8**, O121, O138

[a]Serogroups in bold are the predominant serogroups

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Chapter 2 - Shiga toxin-producing *Escherichia coli* in swine feces: Detection and isolation by PCR and culture methods

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are major foodborne pathogens that cause mild to hemorrhagic colitis, which could lead to a serious complication of renal failure, called hemolytic uremic syndrome, particularly in children. Seven serogroups of STEC, O26, O45, O103, O111, O121, O145, and O157, often called top-7, account for most of the STEC-associated illness in humans in the United States. Two Shiga toxins, Shiga toxin 1 and 2, encoded by *stx*1 and *stx*2, respectively, and intimin, a protein that mediates attachment of STEC onto enterocytes, encoded by the *eae* gene, are major virulence factors involved in STEC infections. Cattle are a major reservoir of the 'top-7' serogroups, in which STEC colonize the hindgut and are shed in the feces. The feces serve as a major source of food, feed and water contaminations. In addition to cattle, other domestic and wild animals and even wild animals harbor STEC and shed them in the feces. Sources of STEC foodborne illness outbreaks have been traced to food of animal and plant origin. A study was created to detect and isolate STEC, based on culture- and PCR methods, from swine feces.

Swine fecal samples (n=598), collected from ten swine farms with finisher pigs, located in eight states, were enriched with EC broth. The enriched samples were subjected to a three-plex quantitative PCR (qPCR) assay targeting three virulence genes, *stx*1, *stx*2, and *eae*. Samples positive for either of the two Shiga toxin genes were then tested by a mPCR assay targeting top-7 serogroups (O26, O45, O103, O111, O121, O145, and O157) and O104 serogroup. Also, *stx*-positive samples were subjected to an eight-plex PCR assay, designed and validated to detect 8 serogroups (O8, O20/O137, O59, O86, O91, O100, O120, and O174) considered to be the top-8

prevalent STEC in swine feces. Samples positive for the top-7 plus O104 serogroups were subjected to serogroup-specific IMS culture method and plating on selective media for detection and isolation of top-7 serogroups of STEC. Samples positive for *stx*1 or *stx*2 gene and negative for the top-7 serogroups were directly plated onto MacConkey and Eosin-Metheylene Blue agar. Putative colonies, up to ten per sample and medium, were picked, pooled and tested for *stx* gene. If pooled colonies were positive for *stx*1 or *stx*2 gene, then each colony in the pool was tested individually to identify *stx*1 or *stx*2-carrying *E. coli*.

Of the 598 fecal samples tested by qPCR for the three major virulence genes, 155 (25.9%), 389 (65.1%), and 398 (66.6%) samples were positive for *stx*1, *stx*2, and *eae* gene, respectively. Based on the mPCR assay for the top-7 plus O104 serogroups, the three predominant serogroups detected were O26 (10.7%; 64/598), O121 (17.6%; 105/598), and O157 (11.5%; 69/598). The 8-plex PCR assay designed to detect the top-8 serogroups of swine STEC indicated a prevalence of 88.6% of O8 (530/598), 35.5% of O86 (212/598), 24.1% of O174 (144/598), 20.2% of O100 (121/598), 15.6% of O91, 4.3% of O59 (26/598), 4.2% of O120 (25/598), and 3.2% of O20/O137 (19/598). The culture method identified STEC O121 as the dominant top-7 STEC serogroup (3.8%; 23/598). None of the O157 isolates (3.5%; 21/598) carried the s*tx* gene. Isolates that were non top-7 (or O104) were tested by 12 different PCR assays that can detect 130 serogroups to identify the serogroups of STEC. The most prevalent non top-7 STEC serogroups isolated were STEC O8 and STEC O86. In conclusion, our study indicated that the major top-7 STEC was O121, and among the non-top-7 STEC, serogroups of O8 and O86 were the dominant. Interestingly, O157 serogroup implicated in outbreaks traced to pork products, was shed in the feces, but none of the isolates carried the *stx* gene. Data on the

prevalence and virulence potential of STEC from swine will be useful information to have to design management strategies that will reduce the prevalence and risk of contamination.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) reside in the hindgut of food animals and are shed in the feces, which can be a source of contamination of food resulting in foodborne illnesses in humans (Rangel et al., 2005; Gyles, 2007). STEC infections can range from mild to bloody diarrhea (hemorrhagic colitis) to renal failure (hemolytic syndrome) and even death, and the economic effect of STEC infections is impactful with an estimated cost of $280 million per year (Hoffmann et al., 2012). Ruminants are a primary reservoir of STEC, however, swine have been confirmed in previous studies as a source of STEC contamination of retail pork products (CDC, 1995). Shiga toxins (Stx), proteins secreted by *E. coli* that are cytotoxic because of inhibition of protein synthesis (Tesh et al., 1991), are major virulence factors. There are two types of Shiga toxins, Stx1 and Stx2, encoded by *stx*1 and *stx*2 genes, respectively, which are carried on a prophage (McDonough et al., 1999). Although the two Shiga toxins share some similarity in amino acid sequence and have the same mode of action, they are antigenically distinct and differ in the degree of cytotoxicity. Stx2 is more cytotoxic than Stx1, and within each, there are several subtypes. There are 3 subtypes of *stx*1: *stx*1a, *stx*1c, and *stx*1d (Zhang et al., 2002), and 7 subtypes of *stx*2: *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f, and *stx*2g (Scheutz et al., 2009). Another important virulence factor, intimin, a protein encoded by a gene called *eae*, which mediates the attachment of *E. coli* to intestinal epithelial cells is produced by a subset of STEC, called enterohemorrhagic *E. coli* (EHEC; CDC, 2019). STEC are commensals in ruminants and do not cause disease, but in swine, STEC serogroups that produce Stx2e cause s

post-weaning swine disease called edema disease (Aarestrup et al., 1997). Swine feces can directly contaminate the pork products, as well as indirectly contaminate other food products, such as produce, therefore, it is important to understand the prevalence of STEC in swine feces to assess the impact on public health. While many serogroups of STEC are pathogenic and are a concern to public health, 7 serogroups of *E. coli,* O26, O45, O103, O111, O121, O145 and O157, called top-7 STEC, account for the majority of STEC foodborne illness in the United States. In 2015, top-7 STEC caused 93.9% of culture-confirmed human STEC infections reported by the National Enteric Disease Surveillance Annual Report (CDC, 2015). These top-7 STEC have been declared as adulterants in raw ground beef and non-intact beef products by the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) (Features Submission, 1992).

A few studies in the United States, as well as in other countries, have reported on STEC prevalence in swine feces, and most of the STEC isolated belonged to non-O157 serogroups. The National Animal Health Monitoring System (NAHMS) conducted a study with pigs in the finishing phase and reported an overall STEC prevalence of 28.5%. This study detected some serogroups (O9, O20, O91, O101, and O121) that have been reported to cause human infections, as well as other serogroups (O8, O20, O91, O100, and O120) that are commonly prevalent in swine feces (Fratamico et al., 2004). Although no STEC O157 was detected by the NAHMS study, the majority of the STEC outbreaks associated with pork products over the last thirty years was attributed to STEC O157:H7 (CDC, 1995; Williams et al., 2000; MacDonald et al., 2004; Jay et al., 2007). The objectives of our study were to determine the prevalence of top-7 and other dominant non-top-7 STEC serogroups, based on PCR and culture methods, in fecal samples collected from finisher pigs from commercial production systems.

Materials and Methods

Sample and swine health data collection

A multi-site field study was conducted by collecting samples from ten different pig flows. In this study, a flow of pigs was defined as a group of swine farms that had similar health status, feeding and management procedures, and pigs were raised as age-segregated groups after weaning until they were ready for market. The study selected pig flows in eight of the top swine producing states of the US, Iowa, Minnesota, South Dakota, Nebraska, North Carolina, Oklahoma, Kansas, and Ohio. In each state, six different finishing sites (minimum site size =1,000 pigs) from each pig flow were selected and ten fecal samples from the rectum of ten finisher pigs were collected from each site close to marketing of the pigs, the earliest being three weeks. Twelve different finishing sites separate pig flows were sampled in South Dakota and Iowa. Each fecal sample was collected using a sterile glove directly from the rectum using a rectal massage technique. A total of 598 fecal samples were collected (Figure 1). After collection, fecal samples were transported in coolers with ice to the Kansas State University Pre-harvest Food Safety laboratory.

In addition to fecal sample collection, descriptive surveys were collected on the management procedures used at each site for finisher pigs selected for fecal sample collection. Dietary ingredients for possible distiller grains inclusion, a reported risk factor for fecal shedding of STEC in cattle (Jacob et al., 2010), antibiotic use and route of administration, and use of antibiotic alternative feed additives were assessed. Survey questions were submitted with each fecal sample collection kit. Any information on the descriptive survey that was not sent back, or left blank was considered as not used in that pig flow.

Escherichia coli broth (EC) enrichment

Approximately 1 g of fecal sample was inoculated into 9 ml of EC broth (Difco, ThermoFisher, Waltham, MA) and mixed thoroughly for 1 minute and the sample suspension was incubated at 40ºC for 6 hours. After incubation, the suspension was thoroughly mixed, and 1 ml was pipetted into a 2-ml centrifuge tube, boiled for 10 minutes in a water bath, centrifuged at 9,400 x g for 5 minutes and DNA was extracted using a GeneClean Turbo Kit (MP Biomedicals, Solon, OH).

Real-time PCR assay to detect Shiga toxin and intimin genes

The extracted DNA was used to perform a 3-plex real time PCR assay to detect Shiga toxin 1 (*stx*1), Shiga toxin 2 (*stx*2), and intimin (*eae*) genes (Noll et al*.,* 2015).

Multiplex PCR assay to detect top-7 STEC and serogroup O104

Any sample that was positive for *stx*1 or *stx*2 gene was then subjected to a 11-plex conventional PCR assay to detect top-7 serogroups of STEC (O26, O45, O103, O111, O121, O145, and O157) the serogroup O104, and three virulence genes (*stx*1*, stx*2, and *eae*) (Bai et al., 2012).

Design and validation of 8-plex PCR assay

The extracted DNA was subjected to an 8-plex PCR assay, designed and validated to detect eight major STEC serogroups, O8, O20/O137, O59, O86, O91, O100, O120, and O174,

based on previous studies (Fratamico et al., 2008; Funk, 2013; Frydendahl, 2002; Wonhee-Cha et al., 2018) in swine feces (Table 2; Table 3; Table 4).

Primer design.

The *wzx* gene, which encodes for the flippase required for serogroup-polysaccharide export, was used to design primers for serogroups O120, O174, O20/O137, and O100. The *wbs* gene was used to design serogroup O59. The primers for the *orf469* gene in serogroup O8 was from Li et al. (2010). The *wzy* gene for serogroups O86 and O91 were designed by Pennsylvania State University. Primers were designed to match all available sequences for the respective serogroups and to amplify the targets with distinct amplicon sizes (Table 4).

PCR reactions and visualization.

All primer stocks were prepared in 1X TE buffer (Integrated DNA Technologies, Inc., Coralville, IA) at concentrations of 100 pM/µl. Equal volumes and concentrations of the 8-primer pairs (16 primers) were mixed together. One microliter of the primer mix was used in a 20 µl PCR reaction resulting in final primer concentrations of 0.31 µM for each primer in the reaction. Each reaction also contained 10 µl of BioRad iQ Multiplex Powermix (without additional supplement), 2 µl of boiled bacterial cells, or DNA extracted from fecal, spiked fecal or enriched fecal samples, and 7 µl nuclease-free water. The PCR amplification program included a 5 min denaturation at 94° C, followed by 25 (for pure culture) or 35 cycles (for all others) of 94ºC for 30 sec, 69ºC for 30 sec, and 68ºC for 75 sec. The PCR products were run on QIAxcel Advanced (Qiagen, Germantown, MD) and data were analysed on QIAxcel ScreenGel software version 1.0.2.

Template DNA preparation.

Bacterial cultures, stored in CryoCare beads (Key Scientific Products, Stamford, TX) at -80°C, were streaked on blood agar plates (BAP, Remel, Lenexa, KS) and incubated overnight at 37°C. One or two colonies of each strain were suspended in 1 ml of distilled water and boiled for 10 min. After a quick centrifugation, 2 µl of the supernatant was used as the DNA template. GeneClean DNA extraction kit (MP Biomedicals, Solon, OH) was used for EC broth-enriched fecal samples.

E. coli strains used in the initial assay development.

Strains representing the 8 serogroups were used for the initial assay development and to determine detection sensitivity. Strains O86 and O120 were provided by *E. coli* Reference Center, Pennsylvania State University, University Park, PA; strains 5380 (O59), 6129 (O174), 5481 (O20), 6395 (O100) were provided by the FDA STEC culture collection; and strains 2089-2 (O8), 4162 (O91) were from our collection (Renter et al., 2005). For assay development, single colonies of the eight strains from BAP were cultured overnight in Luria Bertani (LB) broth individually, and 100 µl was inoculated into 10 ml LB broth and incubated for 3-5 h at 37°C until they reached an absorbance of 0.5 at 600 nm (~10^8 CFU/ml). The eight strains-cultures were mixed in equal amounts, and 1 ml of the pooled mixture was boiled for 10 min, and 2 µl of the supernatant was centrifuged at 9,400 x g for 5 min for the mPCR reactions. For the pure culture sensitivity test, six 10-fold dilutions (10^{-1} to 10^{-6}) were made from the eight strain-culture mixture, and 1 ml of each dilution was boiled and centrifuged as before for use as

template in mPCR. The same dilutions from each serogroup strain were spread-plated on MacConkey agar to determine bacterial cell concentrations (CFU/ml).

PCR assay sensitivity with pure cultures and with swine fecal sample spiked with pure cultures.

The eight serogroup strains were grown individually to an absorbance of 0.5 at 600 nm (~10^8 CFU/ml), and equal volumes of the cultures were pooled together. Serial 10-fold dilutions were made, and 1 ml of each dilution was boiled and used for the mPCR assay. For fecal samples spiked with the eight serogroup strains, the pooled cultures were prepared as before, and 1 ml of 10-fold serially diluted mixtures was inoculated into aliquots of 9 ml fecal suspensions. Fecal suspension without the eight serogroup culture mix inoculums served as an uninoculated control. Each aliquot of the fecal sample, inoculated with different dilutions of the culture mixture, was also enriched in EC broth by incubating for 6 h at 40^{o}C. DNA was extracted from inoculated fecal samples before and after enrichment. The dilutions of the eight-strains mixture used for spiking the fecal samples were also spread-plated on MacConkey agar to determine bacterial cell concentrations (CFU/ml). The experiment to determine assay sensitivity was repeated with a different fecal sample.

Culture method: Isolation of top-7 STEC and serogroup O104

Enriched fecal samples positive for any of the top-7 or O104 serogroups by 10-plex PCR assay were subjected to an immunomagnetic separation (IMS) procedure specific to the serogroup and then spread-plated onto selective media for isolation. Briefly, 980 µl of enriched sample were mixed with 20 µl of the IMS beads (Abraxis, Warminster, PA) specific for the

PCR-positive serogroup. The IMS procedure was performed according to manufacturer protocol of Kingfisher Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA). A total of 50 microliters of final IMS suspension were spread-plated onto modified Possé (MP) agar (Noll et al., 2015), or sorbitol MacConkey agar (CT-SMAC) with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L; Jacob et al., 2011) for isolation of non-O157 and O157 STEC, respectively. All plates were incubated at 37ºC for 18-24 hours. Up to 10 chromogenic colonies were picked from the MP agar and streaked onto blood agar plates (BAP: Remel, Lenexa, KS) and incubated at 37ºC for 18-24 hours. A total of 10 colonies from each plate were pooled in 50 µl of distilled water, boiled for 10 min and centrifuged at 9,400 x g for 5 min tested by mPCR assay for the six serogroups and three virulence genes. If the pooled colonies were positive for any of the serogroups, then each colony was tested individually by mPCR. From the CT-SMAC plate, up to six grey colonies (sorbitol negative) were picked and streaked onto BAP plates and incubated at 37ºC for 18-24 hours. Colonies were subjected to *E. coli* O157 latex agglutination test and a colony positive for agglutination was subjected to a 6-plex PCR assay to confirm O157 and H7 antigens and four virulence genes (Shiga toxins 1 and 2, intimin and enterohemolysin). All confirmed isolates were stored on cryogenic beads (CryoCare, Key Scientific Products, Round Rock, TX).

Culture method: Isolation of non-top-7 STEC

Enriched fecal samples positive for *stx*1 or *stx*2 gene were diluted (1 in 100 dilution) in EC broth, and 25 µl of the diluted inoculum were spread-plated onto a MacConkey agar (MAC: Remel, Lenexa, KS) and Eosin Methylene Blue (EMB: Remel, Lenexa, KS) agar plates and incubated at 37ºC for 18-24 hours for non-selective isolation of *E. coli*. Also, the enriched fecal sample was directly deposited with a sterile cotton swab onto MAC and EMB plates and then a

loop was used to streak from the inoculum spot and incubated at 37ºC for 18-24 hours for non-selective isolation of *E. coli*. A total of 10 putative colonies from MAC plates (pink, round, smooth colonies) and 10 putative from EMB plates (iridescent green and black pigmented colonies) for each sample were streaked onto BAP plates and incubated at 37ºC for 18-24 hours. The 10 colonies from each BAP were pooled in 50 µl of distilled water, boiled for 10 minutes, and centrifuged at 9,300 x g for 5 minutes. The boiled lysate was subjected to a 3-plex real-time PCR assay to detect *stx*1, *stx*2, and *eae.* If pooled colonies were positive for *stx*1 or *stx*2, then each colony was tested individually by two mPCR assays to detect the top-7 and O104, and top-8 swine STEC serogroups. Any colony positive for *stx*1 or *stx*2 but negative for the top-7, O104, or top-8 swine STEC were subjected to 12 sets of 8-12 plex PCR assays targeting non-top-7 serogroups of STEC (Ludwig, 2017). All *stx*-positive isolates were stored at -80 C in cryogenic beads.

Subtyping of Shiga toxin genes of swine STEC isolates

The non-top-7 isolates (n=213) that were positive for *stx* were subtyped by touchdown PCR method described by Shridhar et al., 2017. Because the primers designed to amplify *stx* gene did not include the subtype *stx*2e, a PCR assay outlined in Scheutz et al., (2012) was used.

Statistical analysis

The statistical analysis was performed using STATA v14.2 (StataCrop, College Station, TX). Data were considered multilevel in nature with site nested within each pig flow. Bivariate descriptive statistics of *stx*1, *stx*2, *eae* genes, prevalence of top-7 serogroups and other Shiga toxin positive serogroups were assessed by state prior to multivariable analyses. The likelihood-

ratio-chi-square test was performed to assess the unadjusted differences in prevalence proportion by state and serogroups. Generalized linear mixed models was fitted to adjust for clustering within pig flow within production system and site within pig flow. Thereafter, the multivariable-adjusted effects of state and serogroups were assessed followed by its interaction with the use of distiller's grain, direct fed microbials, copper and antibiotic in the farms. The association of each variable with the probability of a positive result was tested using mixed effects logistic regression. From the final model, marginal adjusted predicted probabilities were calculated for all the outcomes along with their 95% confidence intervals. Results were considered significant at a P-value < 0.05.

Results

PCR-based prevalence of virulence genes and STEC serogroups

Prevalence of virulence genes in feces.

Based on the 3-plex RT-PCR assay, 29.9% (179/598) of the swine fecal samples were negative for either *stx*1 or *stx*2 and 70.1% (419/598) of samples were positive for either *stx*1 or *stx*2 (Table 5). The prevalence of *stx*2 was higher than *stx*1 (65.1 vs. 25.9%). Only 20.1% (125/598) of fecal samples were positive for both Shiga toxin genes. The overall prevalence of the three virulence genes were *stx*1 (25.9%; 155/598), *stx*2 (65.1%; 389/598) and *eae* (66.5%; 398/598), respectively (Table 5).

Prevalence of top-7 and O104 STEC serogroups.

Serogroups O26, O145, and O157 were present in fecal samples from all 8 states (Table 6). Of the top-7 STEC, serogroup O121 (17.6%) was the predominant serogroup, followed by O157

(11.5%), and O26 (10.7%). However, serogroup O121 was not detected in fecal samples from the pig flow located in Oklahoma (Figure 1). The prevalence of serogroup O104 was 4.7% (28/598). Only two fecal samples, one from Iowa and another from South Dakota were positive for serogroup O111 (Figure 1). All fecal samples used for the mPCR assay were all positive for either *stx*1 or *stx*2, and the prevalence of the two genes by mPCR were 11.5 and 64%, respectively (Figure 2). The serogroups detected across the eight states (O26, O45, O104, O121, O145, and O157) were found significantly more ($P < 0.001$) than the other serogroups (Figure 3). Of the 10 pig flows sampled, the finishing diets of the three pig flows included distiller's grains. Both STEC O157 and non-O157 sergroup prevalences were higher in pig flows that used distiller's grains (Figures 4 and 5) compared to pig flows that did not use distiller's grains. Fecal samples negative for any of the top- or O104 sergroups, but positive for either *stx*1 or *stx*2, were categorized as non-top-7 and the overall prevalence of those serogroups was 30.8% (184/598).

Prevalence of top-8 swine serogroups by 8-plex PCR assay

Validation of 8-plex PCR assay with pure cultures. The specificity of each primer pair was tested individually using the mixture of the eight *E. coli* serogroups, O8, O20/O137, O59, O86, O91, O100, O120, O174. The primers only amplified the corresponding serogroup. After optimizing PCR conditions (stated in the materials and methods section), 8 distinct bands were obtained. The size of the amplicons was 562 (O86), 448 (O8), 388 (O59), 352 (O120), 317 (O174), 277 (O91), 238 (O20/O137), 193 (O100) (Figure 6).

Assay sensitivity with pure cultures and spiked fecal samples.

The initial concentration of pooled cultures of the eight serogroups was 2.3 x 10^7 CFU/ml, which was subjected to four additional ten-fold dilutions to obtain the minimum concentration of the pooled culture that amplified all 8 genes, which was 2.3 x 10^3 CFU/ml. In fecal samples spiked with different concentrations of the pooled cultures of the eight serogroups, the sensitivity of detection was 1.8 x 10^4 CFU/g before enrichment and 1.8 x 10^2 CFU/g after enrichment for 6 h in EC broth.

Prevalence of top-8 swine STEC serogroups.

Based on the 8-plex PCR assay, serogroup O8 was the most predominant with a prevalence of 88.6% (530/598), followed by followed by O86 at 35.5% (212/598), O174 at 24.1% (144/598), O100 at 20.2% (121/598), and O91 (15.6%; 93/598). The prevalence of O20/O137, O120, and O59 were less than 5% of the samples tested. Only serogroups O8 and O86 were detected in fecal samples collected from all 8 states (Table 7).

Culture method-based detection and isolation of top-7 and O104 STEC

Although several fecal samples were positive for serogroup-specific genes and Shiga toxin genes, following IMS method with plating on selective media, only a few *stx*-positive top-7 and O104 STEC (4.5%; 27/598) were detected and isolated (Table 8). The predominant STEC isolated was O121 (3.9%; 23/598). The prevalence of STEC O26 was 0.2% (1/598), STEC O103 was 0.2% (1/598), and STEC O104 was 0.3% (2/598). None of the 69 O157 isolates obtained in pure culture was positive for *stx* genes. Interestingly, 21 O157 isolates, which were positive by $rfbE_{O157}$, fermented sorbitol and produced pink pin-point colonies.

Culture method-based detection and isolation of non-top-7 STEC

Fecal samples that were positive for one or both Shiga toxin genes were used for isolation of non-top-7 STEC serogroups, including the top-8 swine STEC serogroups. Because no serogroup-specific IMS beads are available and no selective media have been developed, enriched fecal samples were inoculated directly onto MAC and EMB agar to isolate *E. coli* in pure culture and then test for Shiga toxin gene, and if positive, identify the serogroup belonging to the non-top-7 STEC.

Initially, 200 fecal samples out of the first 300 samples that were positive for *stx*1 or *stx*2 genes were plated onto MAC and EMB agar. The proportions of samples with pooled colonies positive for *stx*1 or *stx*2 gene were 405% (81/200) and 35.5% (71/200) for MAC and EMB agar, respectively (Table 9; Table 10). The total number of pure cultures belonging to top-7 and O104 and non-top-7 STEC were similar between the two media. Because neither medium was better than the other, only MAC medium was used for the remaining 300 samples. Of the 466 *stx*1 or *stx*2 positive samples, 159 samples (34%) yielded pooled colonies (pool of 10 randomly picked putative *E. coli* colonies on MacConkey agar) that were positive for *stx*1 or *stx*2 gene (Table 11). When the colonies were individually tested by 11-plex PCR (8 serogroups and 3 virulence genes), a total of 127 isolates were obtained that were positive *stx*1 or *stx*2. Of the 127 isolates, 14 belonged to the top-7 plus O104 and 113 belonged to non-top-7 STEC (Table 11). The 14 top-7 STEC belonged to only one serogroup, O121.

Among the non-top-7 STEC, a total of 19 different serogroups were identified by the 12 sets of mPCR assays with pure cultures obtained by non-selective *E. coli* culture methods. The prevalence of each of these 19 serogroups are shown in Figure 2. Of these serogroups, the most

predominant serogroups in the non-top-7 STEC isolates were O8 (19%; 111/598) and O86 (9.5%; 56/598). If multiple isolates from the same sample had the same Shiga toxin gene and the same serogroup detection by the 12 sets of mPCR assay, only one isolate was stored for subtyping of the Shiga toxin gene.

From the final model, the marginal prediction probabilities were determined with 95% CIs for the proportions of positive top-7 samples by 10-plex and O104 conventional PCR assay. Overall, the marginal mean predictions for prevalence of *E. coli* O157 was highest with the inclusion of distiller's grains in management. Further, the marginal mean predictions for prevalence of other top-7 serogroups excluding O157 was highest with the inclusion of distiller's grains in management as well. The highest prevalence of other top-7 serogroups excluding O157 that did not use distiller's grains was comparable to the lowest prevalence of other top-7 serogroups excluding O157 that did use distiller's grains in management.

Subtyping of Shiga toxin genes

A total of 39 *stx*1 isolates were subtyped. All 39 isolates were either *stx*1a (51.3%; 20/39) or *stx*1b subtype (48.7%; 19/39). Further, 174 isolates were used for *stx*2 subtyping by touchdown PCR assay and *stx*2e subtyping conventional PCR assay. Of the 174 isolates, most of the isolates were Shiga toxin subtype *stx*2e (95.4%; 166/174), one isolate was *stx*2a, and seven isolates could not be subtyped.

Discussion

In this study, we investigated the prevalence of Shiga toxin producing *E. coli* in finishing pigs sampled from 10 pig flows across eight different states. The prevalence of all top-7 STEC

was seen across the eight states at varying proportions, and *E. coli* O157 serogroup was one of the more prevalent top-7 serogroups in this study. However, none of the O157 isolates obtained was positive for Shiga toxin genes. Further, previous studies have isolated STEC O157 with other virulence genes (Conedera et al., 2007; Wonhee-Cha et al., 2017) (Table 1). The finisher pig flows that had the highest prevalence of *E. coli* O157, from North East Iowa and Nebraska, both disclosing that they use distiller's grains in their finishing diets. This finding agrees with studies that have investigated the association of distiller's grains in the finishing diet to fecal shedding of *E. coli* O157 in feedlot cattle (Jacob et al., 2010). Additionally, the higher prevalence of non-O157 top-6 STEC serogroups was also seen in pig flows that used distiller's grains in their finishing diets. *E. coli* O157 was one of the most dominant serogroups detected in this study (11.5%), which contrasts with previous porcine studies (Frydendahl et al., 2002; Colello et al., 2016; Tseng et al., 2014) (Table 1; Table 2). Our study is also in contrast with outbreaks of STEC infection associated with pork products, which were more often STEC O157 (Trotz-Williams et al., 2012; Jay et al., 2007). Therefore, there is a need to investigate the variability of *E. coli* O157 virulence in finisher swine populations, as well as an experimental design to show the possible increase in prevalence of *E. coli* O157 and other top-7 serogroups with the introduction of distiller's grains in the diet of swine.

The prevalence of serogroup O121 was the highest (17.6%) of all top-7 serogroups across the eight states. The high prevalence of O121 agrees with previous studies, which have shown O121 to be the most dominant serogroup of all top-7 serogroups (Fratamico et al., 2004; Fratamico et al., 2008; Tseng et al., 2014). This study and others indicate that the source of contamination of top-7 serogroups for public health can be from a variety of sources, as O121 has not been a dominant serogroup in our previous studies isolating top-7 STEC from cattle

(Noll et al., 2018). However, STEC O121 has only been considered a dominant serogroup in swine in previous studies in the United States, but not in other continents such as Europe, Asia, or South America. The only state to show no STEC O121 in our study was Oklahoma, which emphasizes studies investigating low STEC O121 prevalence across multiple finisher pig flows in Oklahoma may be indicative of regional differences in STEC O121 prevalence.

Of all non-top-7 serogroups in this study, the serogroups with highest prevalence was O8 (88.6%) and O86 (35.5%). This agrees with previous swine studies (Fratamico et al., 2004; Kaufmann et al., 2006; Fratamico et al., 2008) that have concluded that serogroup O8 was the most common serogroup (Table 1). The O8 serogroup is known to produce Shiga toxin subtype *stx*2e involved in edema disease (Table 3). Most of the isolates used for subtyping were confirmed as s*tx*2e, indicating that these populations could be at risk for edema disease. While this study confirmed one isolate with Shiga toxin subtype s*tx*2a, which is commonly isolated from cases of human infection, seven other isolates could not be subtyped.

Tables

Table 4. Gene targets, primers used, and amplicon size in the 8-plex mPCR assay to detect top-8 serogroups of Shiga toxin-producing *Escherichia coli* in swine feces

Serogroups	Gen targeted	Primer sequences	Amplicon size, Kb	Reference
O86	*Wzy*	FATTTGAGGCTGACGCGTATGGACT	562	Pennsylvania State University
		RAGCAACACTTCCAATGATCCACCC		
O8	*Orf469*	FCCAGAGGCATAATCAGAAATAACAG	448	Li et al., 2010
		RGCAGAGTTAGTCAACAAAAGGTCAG		
O59	*Wbs*	FTGGAACCCATCAACAGACCT	388	This study
		RTTCCTCCTCGAGCTGTTGTC		
O120	*Wzx*	FTGGCTTTAGGCACAGTTGTTATG	352	This study
		RAGCAATGCAACAACAAAACCAG		
O174	*wzx*	FGGAGATAAAGCTGCAGGTGAG	317	This study
		RCCAGTAAGCGGGCCTAAAAG		
O91	*wzy*	FCGCATTTAAGGACTGGCTGT	277	Pennsylvania State University
		GRTAGCAGATATGCCGACCGT		
O20/O137	*wzx*	FTCGCCAAATCAAAATGGCTAC	238	This study
		RAGGTTTGCTCTTTGGAGAGCTG		
O100	*wzx*	FTGCAACGATTATTGGTGTCG	193	This study
		RATACAAACCCGCTTGAACCA		

Table 5. Prevalence of genes encoding for major virulence factors, Shiga toxin 1 (*stx*1), Shiga toxin 2 (*stx*2), and intimin (*eae*), of Shiga toxin-producing *Escherichia coli* in swine feces (n=598) based on three-plex real-time PCR assay.

Virulence genes	No. of samples positive (%)								
	Kansas (n=60)	Minnesota (n=60)	North Carolina (n=59)	Nebraska (n=60)	Iowa (n=120)	Ohio (n=59)	Oklahoma (n=60)	South Dakota (n=120)	Total (n=598)
*stx*1		29 (48)		5 (8.0)	87 (73)	17 (28)		17 (14)	155 (26)
*stx*2	21 (35)	40 (67)	12 (20)	59 (99)	94 (78)	45 (75)	18 (30)	100 (83)	389 (65)
*stx*1 or *stx*2	21 (35)	49 (82)	12 (20)	64 (100)	112 (93)	47 (79)	18 (30)	101 (84)	419 (70)
*stx*1 and *stx*2		20 (33)		5 (8.0)	69 (58)	15 (25)		16 (13)	125 (20)
eae	20 (33)	37 (62)	42 (70)	46 (77)	98 (82)	50 (84)	40 (67)	65 (54)	398 (67)

Table 6. Prevalence of major serogroups of Shiga toxin-producing *Escherichia coli,* O26, O45, O103, O104, O111, O121, O145, O157 (top-7 plus O104) and three major virulence genes, *stx*1, *stx*2, and *eae,* in swine feces based on multiplex-PCR assay.

Serogroups and virulence genes	No. of samples positive (%)								
	Kansas (n=60)	Minnesota (n=60)	North Carolina (n=59)	Nebraska (n=60)	Iowa (n=120)	Ohio (n=59)	Oklahoma (n=60)	South Dakota (n=120)	Total (n=598)
O26	1 (2)	11 (18)	1 (2)	15 (25)	18 (15)	10 (17)	4 (7)	4 (3)	64 (11)
O45	1 (2)	4 (7)		12 (20)	6 (5)	3 (5)		1 (0.8)	27 (5)
O103	3 (5)	4 (7)		1 (2)	7 (6)	3 (5)	1 (2)	2 (2)	21 (4)
O104		1 (2)		12 (20)	10 (8)	4 (7)		1 (0.8)	28 (5)
O111					1 (0.8)			1 (0.8)	2 (0.3)
O121	3 (5)	13 (22)	10 (17)	23 (38)	37 (31)	10 (17)		9 (8)	105 (18)
O145	2 (3)	3 (5)	1 (2)	9 (15)	4 (3)	4 (7)	4 (7)	1 (0.8)	28 (5)
O157	12 (20)	6 (10)	1 (2)	11 (18)	24 (20)	6 (10)	6 (10)	3 (3)	69 (12)
stx1		24 (40)		6 (10)	73 (61)	10 (17)		8 (7)	121 (20)
stx2	24 (40)	44 (74)	12 (20)	58 (97)	99 (83)	41 (69)	15 (25)	90 (75)	383 (64)
eae	1 (2)	15 (25)	7 (12)	27 (45)	35 (29)	12 (20)	4 (7)		101 (17)
Non-top-7 (plus O104)	7 (12)	21 (35)	1 (0.2)	22 (37)	39 (33)	17 (29)	5 (8.3)	72 (60)	184 (31)

Table 7. Prevalence of major serogroups of Shiga toxin-producing *Escherichia coli* (STEC) in swine feces (n=598) based on eight-plex mPCR assay targeting serogroups O8, O120/O137, O59, O86, O91, O100, O120, and O174.

Major swine STEC serogroups	No. of samples positive (%)								
	Kansas (n=60)	Minnesota (n=60)	North Carolina (n=59)	Nebraska (n=60)	Iowa (n=120)	Ohio (n=59)	Oklahoma (n=60)	South Dakota (n=120)	Total (n=598)
O8	42 (70)	55 (92)	56 (94)	56 (94)	114 (95)	56 (94)	45 (75)	106 (88)	530 (89)
O20/O137		3 (5)		1 (2)	11 (9)	2 (3)		2 (2)	19 (3)
O59		2 (3)		1 (2)	10 (8)	2 (3)	4 (7)	7 (6)	26 (4)
O86	2 (0.3)	19 (3.2)	23 (3.8)	39 (6.5)	52 (43)	13 (22)	10 (17)	54 (45)	212 (36)
O91		11 (18)	16 (27)	4 (7)	35 (29)	3 (5)	10 (17)	14 (12)	93 (16)
O100	7 (12)	9 (15)	9 (15)	9 (15)	33 (28)	23 (38)		31 (26)	121 (20)
O120		1 (2)	5 (8)	7 (12)	10 (8)		1 (2)	1 (0.8)	25 (4)
O174		22 (37)	3 (5)	19 (32)	31 (26)	30 (50)	2 (3)	37 (31)	144 (24)

Table 8. **Prevalence of top-7 and O104 serogroups of Shiga toxin-producing *Escherichia coli* (STEC) in swine feces (n=598) based on the culture method.**

STEC serogroups	No. of samples positive (%)								
	Kansas (n=60)	Minnesota (n=60)	North Carolina (n=59)	Nebraska (n=60)	Iowa (n=120)	Ohio (n=59)	Oklahoma (n=60)	South Dakota (n=120)	Total (n=598)
O26						1 (2)			1 (0.2)
O45									0 (0)
O103	1 (2)								1 (0.2)
O104					2 (2)				2 (0.3)
O111									0 (0)
O121	3 (5)	4 (7)	7 (12)	1 (02)	2 (2)			6 (5)	23 (3.8)
O145									0 (0)
O157									0 (0)

Table 9. **Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) from Shiga toxin-positive fecal samples (n=200) that were directly plated onto MacConkey agar**

Item	Kansas (n=60)	Minnesota (n=60)	Iowa (n=120)	Oklahoma (n=60)	Total (n=300)
No. of samples positive for *stx*1 or *stx*2	21	49	112	18	200
No. of samples with pooled colonies that were positive for *stx*1 or *stx*2[a]	2	19	46	14	81
No. of pure cultures positive for *stx*1 or *stx*2[b]	2	19	32	10	63
No. of pure cultures positive for top-7 and O104 STEC[b]	2	1	0	0	3
No. of pure cultures positive for non-top-7 STEC[c]	0	18	32	10	60

[a] Determined by real-time PCR targeting *stx*1 and *stx*2 genes

[b] Determined by 11-plex conventional PCR targeting top-7 (O26, OO145, O103, O111, O121, O145, and O157) and O104 serogroups and three virulence genes (*stx*1, *stx*2 and *eae*).

[c] Isolates that were negative for the top-7 and O104, but positive for *stx*1 or *stx*2 gene.

Table 10. Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) from Shiga toxin-positive fecal samples (n=200) that were directly plated onto Eosin Methylene Blue agar

Item	Kansas (n=60)	Minnesota (n=60)	Iowa (n=120)	Oklahoma (n=60)	Total (n=300)
No. of samples positive for *stx*1 or *stx*2	21	49	112	18	200
No. of samples with pooled colonies that were positive for *stx*1 or *stx*2	6	16	39	10	71
No. of pure cultures positive for *stx*1 or *stx*2[a]	6	16	28	6	56
No. of pure cultures positive for top-7 STEC[b]	2	1	1	0	4
No. of pure cultures positive for non-top-7 STEC[c]	4	16	27	6	53

[a] Determined by real-time PCR targeting *stx*1 and *stx*2 genes

[b] Determined by 11-plex conventional PCR targeting top-7 (O26, OO145, O103, O111, O121, O145, and O157) and O104 serogroups and three virulence genes (*stx*1, *stx*2 and *eae*).

[c] Isolates that were negative for the top-7 and O104, but positive for *stx*1 or *stx*2 gene.

Table 11. Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) from Shiga toxin-positive fecal samples (n=466) that were directly plated onto MacConkey agar

Item	Kansas (n=60)	Minnesota (n=60)	Iowa (n=120)	Oklahoma (n=60)	Ohio (n=59)	South Dakota (n=120)	Nebraska (n=60)	North Carolina (n=59	Total (n=598)
No. of samples positive for *stx*1 or *stx*2	21	49	112	18	47	101	59	59	466
No. of samples with pooled colonies that were positive for *stx*1 or *stx*2	2	19	46	14	9	33	30	6	159
No. of pure cultures positive for *stx*1 or *stx*2[a]	2	19	32	10	9	33	16	6	127
No. of pure cultures positive for top-7 STEC[b]	2	1	0	0	0	6	0	5	14
No. of pure cultures positive for non-top-7 STEC[c]	0	18	32	10	9	27	16	1	113

[a] Determined by real-time PCR targeting *stx*1 and *stx*2 genes

[b] Determined by 11-plex conventional PCR targeting top-7 (O26, OO145, O103, O111, O121, O145, and O157) and O104 serogroups and three virulence genes (*stx*1, *stx*2 and *eae*).

[c] Isolates that were negative for the top-7 and O104, but positive for *stx*1 or *stx*2 gene.

Figures

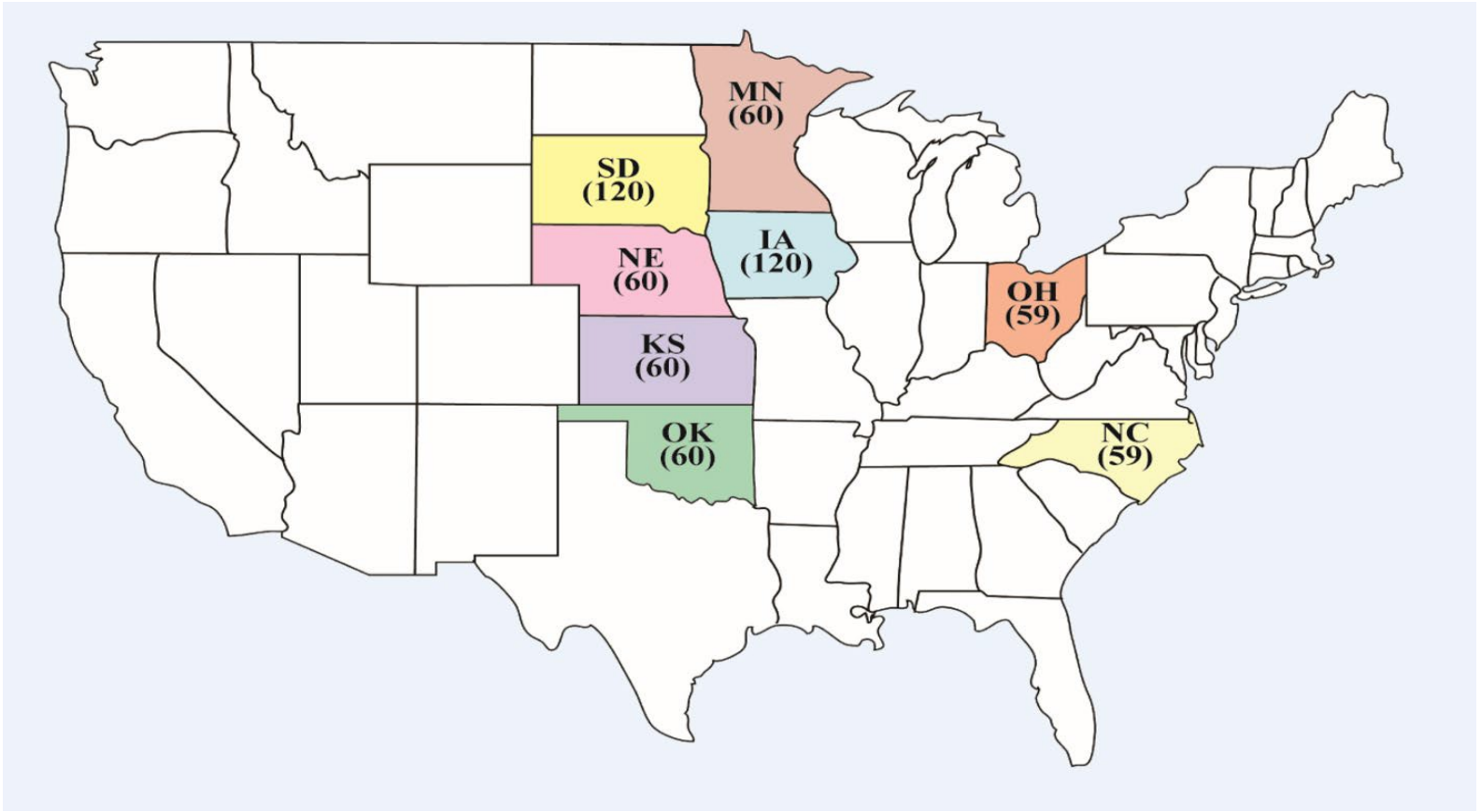

Figure 1. Number of fecal samples collected from ten pig flows in eight states, Iowa, Minnesota, South Dakota, Nebraska, North Carolina, Oklahoma, Kansas, and Ohio.

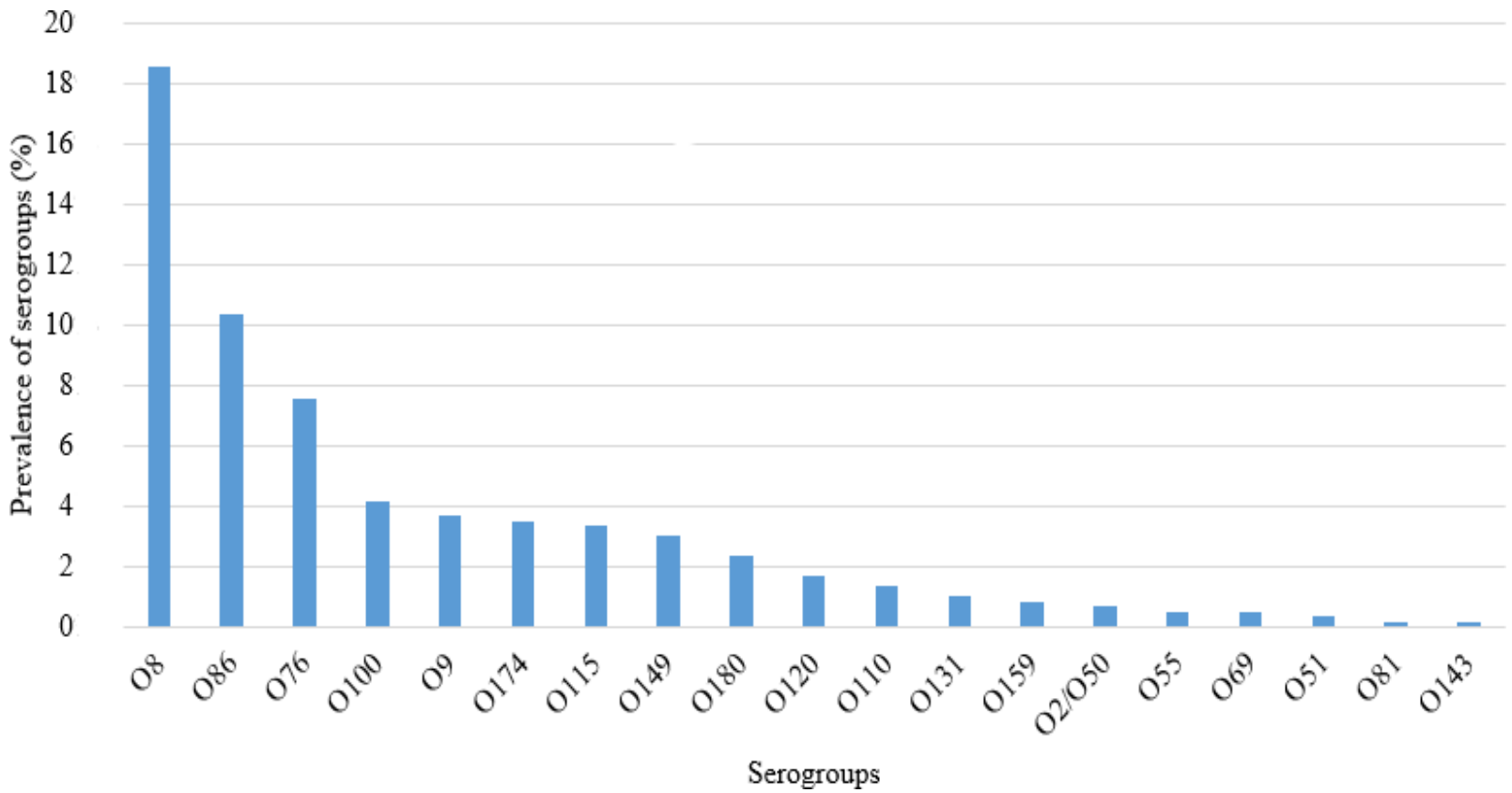

Figure 2. Identification of serogroups of Shiga toxin gene-positive *E. coli* isolates obtained from direct plating of *stx*-positive fecal samples on MacConkey or Eosin methylene blue agar based on 12 different multiplex PCR assays targeting 130 serogroups

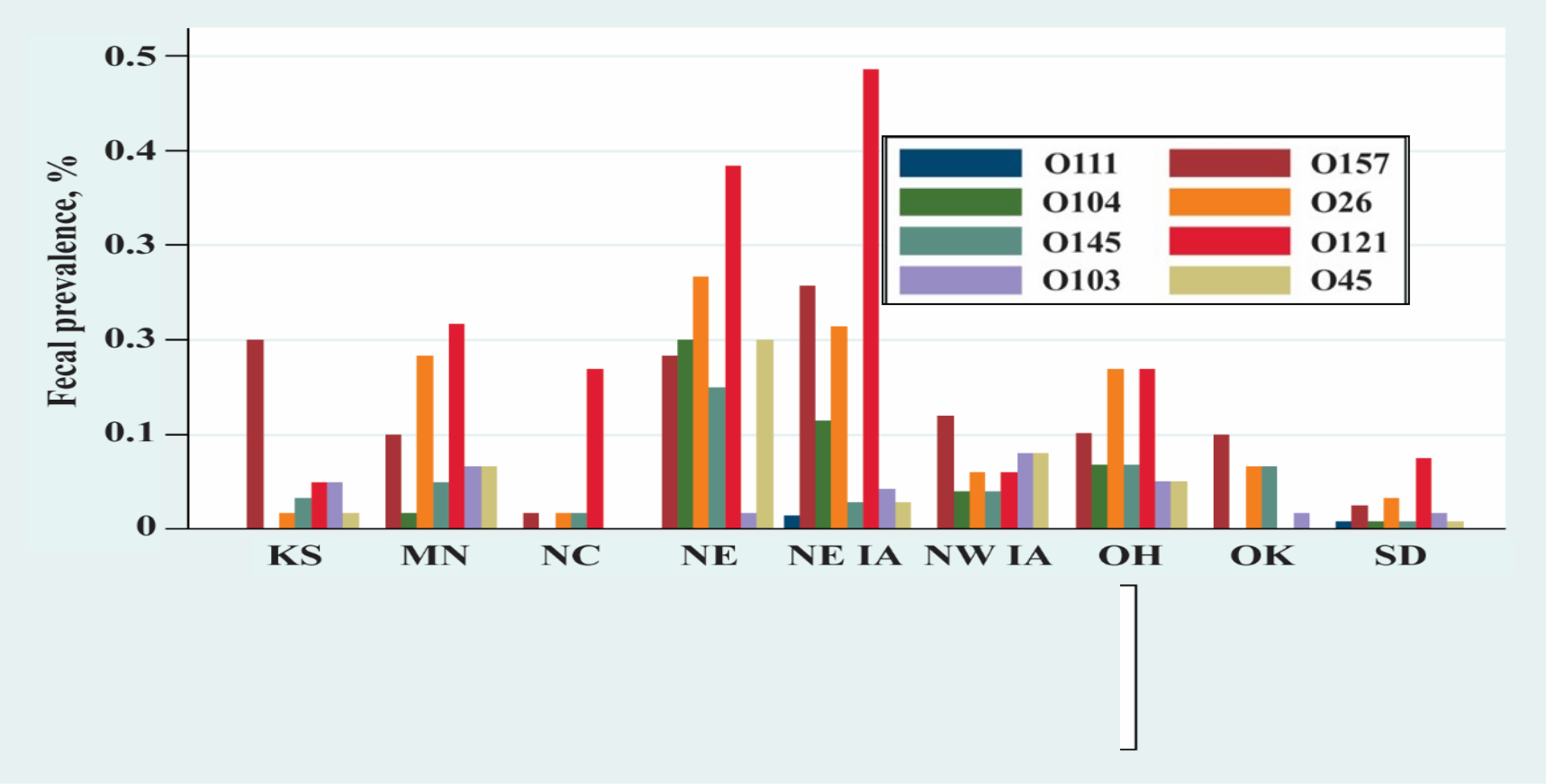

Figure 3. The fecal prevalence (%) of top-7 serogroups and O104, determined by multiplex PCR assay, across the eight states.

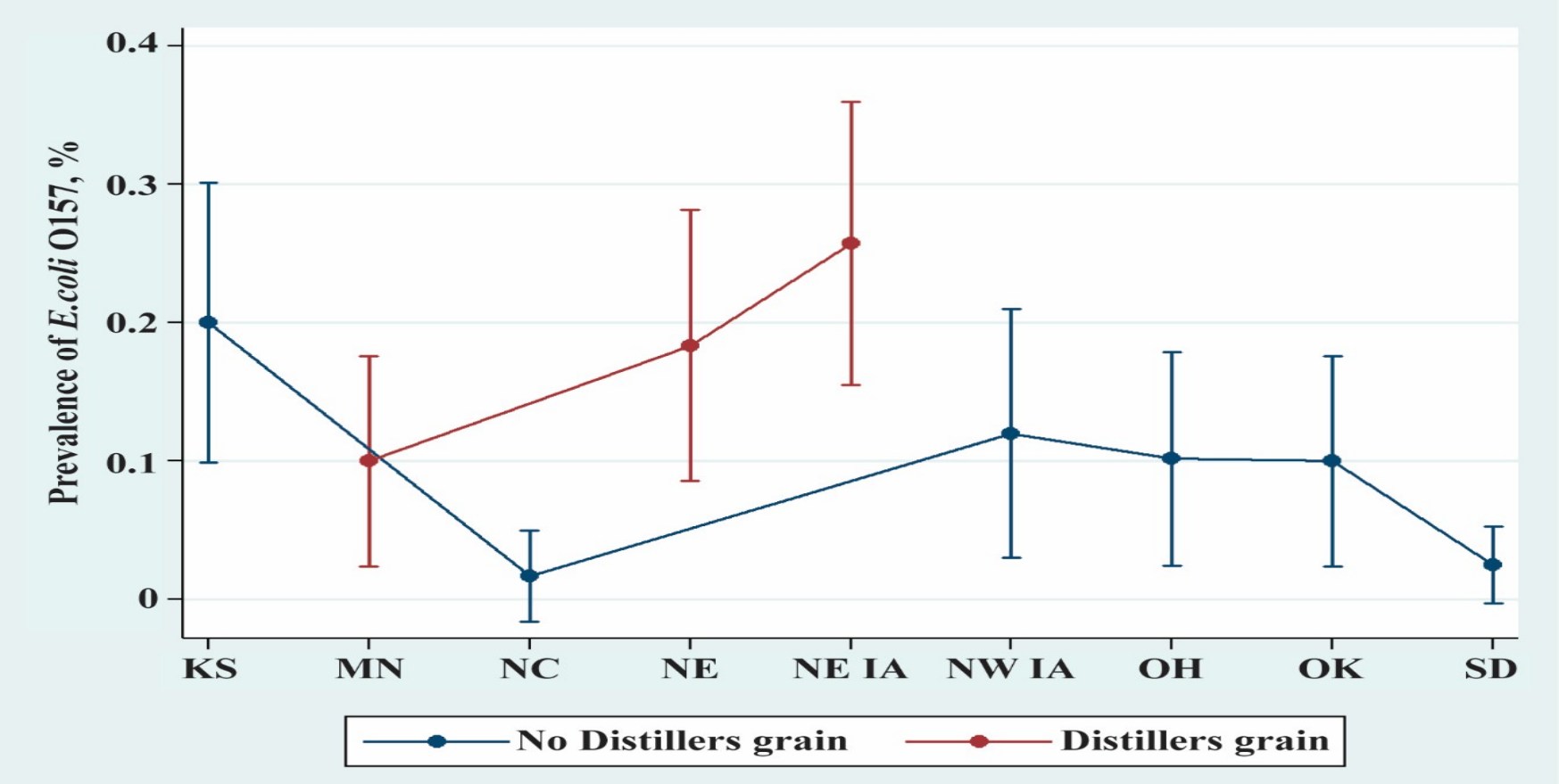

Figure 4. The prevalence (%) of *E. coli* O157 serogroup, based on multiplex PCR assay, in relation to use of distiller's grain in the finishing diet of swine.

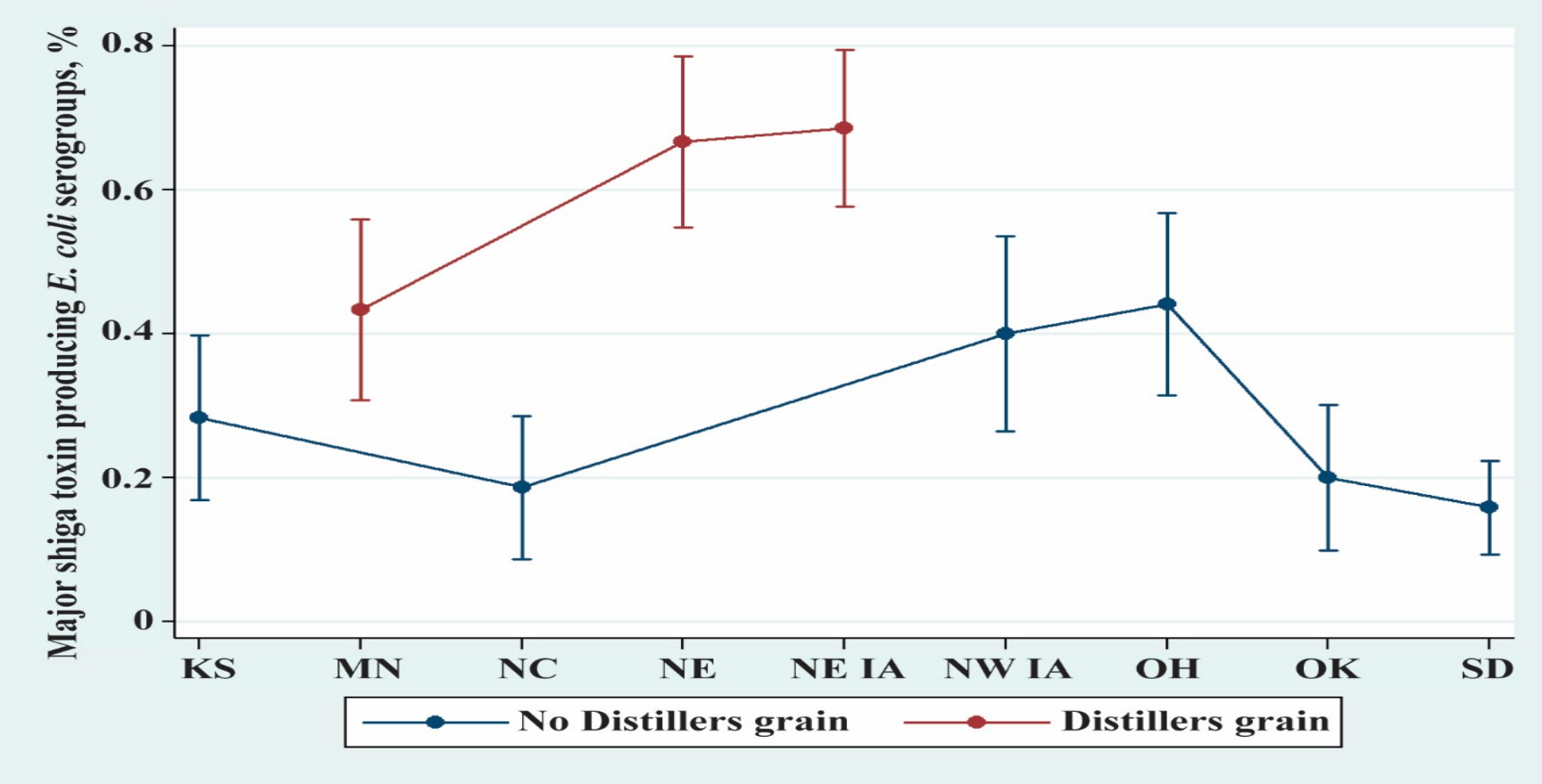

Figure 5. The prevalence (%) of non-O157 *E. coli* serogroups, based on multiplex PCR assay, in relation to use of distiller's grain in the finishing diet of swine.

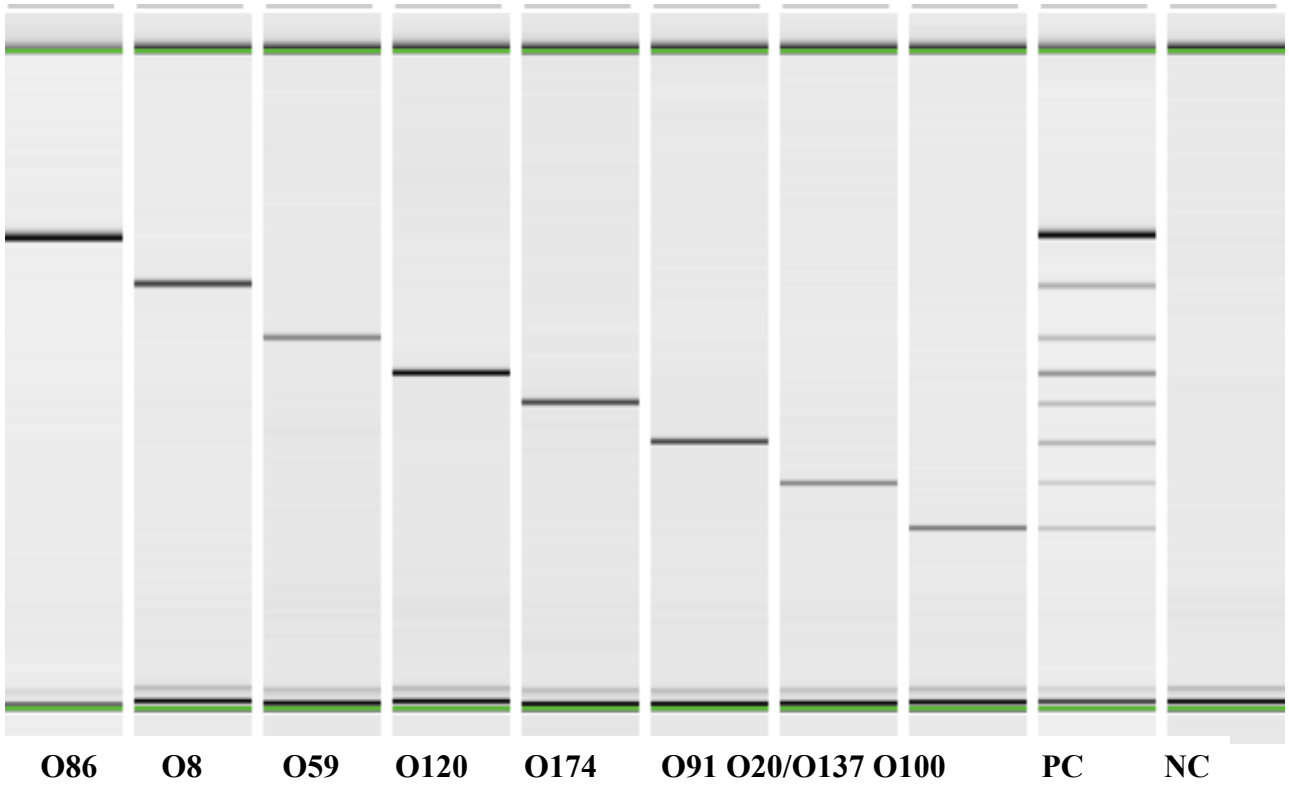

Figure 6. Qiaxel image of the amplicons of the 8 serogroups-specific gene of individual and pooled Shiga toxin-producing *Escherichia coli* amplified by the multiplex PCR assay.

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Chapter 3 - Shiga toxin-producing *Escherichia coli* in wheat grains: Detection and isolation by PCR and culture methods

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are major foodborne pathogens that cause mild to hemorrhagic colitis, which could lead to a serious complication of renal failure, called hemolytic uremic syndrome, particularly in children. Seven serogroups of STEC, O26, O45, O103, O111, O121, O145, and O157, often called top-7, account for most of the STEC-associated illness in humans in the United States. Shiga toxin 1 and 2, encoded by *stx*1 and *stx*2, respectively, and intimin, a protein that mediates attachment of STEC onto enterocytes, encoded by *eae* gene, are major virulence factors involved in STEC infections. Cattle are a major reservoir of the 'top-7' serogroups, in which STEC colonize the hindgut and are shed in the feces. The feces serve as a major source of food, feed and water contaminations. In addition to cattle, other domestic and wild animals and even wild animals harbor STEC and shed them in the feces. Sources of STEC foodborne illness outbreaks have been traced to food of animal and plant origin. A study was created to detect and isolate STEC, based on culture- and PCR methods, from wheat grains.

Wheat grain samples (n=626), collected from different regions of the country and transported to the laboratory, were enriched using two different media, modified Buffered Peptone Water with pyruvate (mBPWp) and *Escherichia coli* (EC) broth, and subjected to a multiplex PCR (mPCR) assay to detect the top-7 serogroups (O26, O45, O103, O111, O121, O145, and O157) and three major virulence genes (*stx*1, *stx*2, and *eae*). Samples positive by PCR for any of the top-7 serogroups and or *stx* genes were then cultured using serogroup-specific immunomagnetic separation (IMS) and plating on selective media for detection and isolation of

top-7 STEC. Based on the mPCR assay, the prevalence of the top-7 serogroups in wheat grain samples enriched by mBPWp, were 0.2% of O26 (1/626), 0.6% of O45 (4/626), 0.2% of O103 (1/626), and 0.6% of O157 (4/626), and the prevalence of virulence genes were 0.2% of *stx*1 (1/626), 0.2% of *stx*2 (1/626), and 0.8% of *eae* (5/626). The prevalence of top-7 serogroups, enriched in EC broth, were 0.2% of O26 (1/626), 1.9% of O45 (12/626), 0.5% of O103 (3/626), and 1.0% of O157 (6/626), and that of virulence genes were *stx*1 (0.3%; 2/626), *stx*2 (1.3%; 8/626), and *eae* (1.1%; 7/626). The number of wheat grain samples positive for STEC serogroups and or virulence genes by PCR method (36/626; 5.8%) was higher in samples enriched by EC broth than mBPWp broth (15/626; 2.4%). Based on culture methods, the top-7 serogroups prevalent in wheat grain samples enriched in mBPWp were 0.2% of O26 (1/626), 0.5% of O45 (3/626), 0.2% of O103 (1/626), and 0.6% of O157 (4/626), and none of the isolates was positive for any of the three virulence genes. In wheat grain samples enriched in EC broth, the prevalence of the top-7 serogroups were 0.2% of O26 (1/626), 0.8% of O45 (5/626), 0.3% of O103 (2/626), and 0.8% of O157 (5/626), with no detection of virulence genes. The number of wheat grain samples positive for STEC serogroups and/or virulence genes, by culture method, was higher in samples enriched by EC broth than mBPWp broth (5/36 and 0/15, respectively). None of the isolates of the top-7 serogroups by either enrichment method was positive for the Shiga toxin genes. A total five isolates that carried Shiga toxin 2 gene were isolated from wheat grains enriched in EC broth. The five isolates were confirmed as serogroups O8 (0.64%; 4/626) and O130 (0.16%; 1/626) by PCR. Our study shows that wheat grains were contaminated with the top-7 serogroups of *E. coli,* but none of the isolates carried the Shiga toxin genes. The two *stx*2-positive serogroups that were isolated, O8 and O130, are not major STEC pathogens and have only been implicated in sporadic diarrheal cases in animals and humans.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC), particularly serogroups, O26, O45, O103, O111, O121, O145, and O157, collectively called "top-7", are major foodborne pathogens in the United States. These serogroups have been labeled as adulterants in ground beef and non-intact beef products by the United States Department of Agriculture and Food Safety and Inspection Service (USDA FSIS) (Features Submission, 1992). Infections caused by STEC can vary from mild to bloody diarrhea, and in severe cases can result in serious complications, such as hemolytic uremic syndrome (HUS). Two types of exotoxins, called Shiga toxins, Stx1 and Stx2, encoded by *stx*1 and *stx*2, respectively, are major virulence factors. Severe STEC infections are more often linked to Stx2 than Stx1 (Mayer et al., 2012). *E. coli* O157 was first identified as a pathogen in 1982, and the serotype O157:H7 is the most common STEC serotype impacting public health in North America (CDC, 2014). As many as 150 serogroups of *E. coli* can produce Shiga toxins and cause infections, more often as sporadic infections, in humans. Information on these serogroups, in terms of prevalence and virulence potential, is limited due to lack of detection and isolation methods. Ruminants, particularly cattle, are a major reservoir of STEC. They harbor the organisms in the hindgut and shed them in the feces, which serves as the major source of contamination of food of animal and plant origin (Los et al., 2018; NandaKafle et al., 2018).

In 2009, an outbreak of STEC O157:H7 was linked to consumption of prepackaged cookie dough, a product prepared with raw wheat flour. The outbreak resulted in 77 people meeting the case definition of STEC infection, and 21 of those cases were confirmed as STEC infections by PFGE patterns. Of those 21 isolates, 19 were *stx*2 positive, one was positive for

both *stx*1 and *stx*2 positive, and one isolate was *stx*1 positive only (Neil et al., 2012). Because prepackaged cookie dough has multiple ingredients, the wheat flour was implicated but not confirmed as the source of contamination (Neil et al., 2012). Flour is considered less likely to get contaminated because of minimal water content, compared to other ingredients in prepackaged cookie dough, and lower water content does not support bacterial growth (Crowe et al., 2017). However, a STEC outbreak of dry dough mix occurred in 2016, across nine states, that resulted in 13 cases of STEC infection. *E. coli* O157:H7 was isolated from the dry dough mix in this outbreak (Gieraltowski et al., 2017). In the investigation by the Minnesota Department of Agriculture, 17 samples of dry dough mix from the outbreak were collected, with seven samples identified as carrying non-O157 STEC serogroups, with one isolate positive for *stx*1 and six isolates positive for *stx*2. Furthermore, the FDA collected six samples of dry dough mix and identified one isolate of *stx*2 positive O8:H28 (Gieraltowski et al., 2017).

The STEC outbreak reports were linked to wheat flour products and the investigation said the flour itself could not be implicated, as it is a non-sterile product that is exposed to a variety of environmental contaminants and the product is made of more ingredients than just wheat flour (Gieraltowski et al., 2017). A wheat flour outbreak occurred in the United States between 2015-2016 across 24 states. A total of 63 people developed STEC infections and O121 or O26 were identified as the serogroups involved. Of the 63 cases, 17 were hospitalized, and one developed HUS. Traceback evidence concluded that all isolates from these cases were genetically related and were traced to a General Mills facility in Kansas City, MO (CDC, 2016). A study by Crowe et al. (2017) to identify exposures associated with this outbreak, and samples of clinical cases and flour samples associated with this outbreak were collected. Both clinical case samples and flour samples collected had genomic DNA extracted to identify if these cases had the same

PFGE pattern as the outbreak strain. Out of the 56 cases that were identified with STEC by this method, 55 cases were identified as STEC O121 and one was identified as STEC O26. Forty isolates out of the 55 STEC O121 isolates were used for whole genome sequencing and were identified as closely related genetically. In 2016, another wheat flour outbreak occurred in Canada, across six provinces that resulted in 30 cases of confirmed *E. coli* O121 infection with eight cases resulting in hospitalizations. The Public Health Agency of Canada investigated and confirmed that isolates from all 30 cases had the same genetic fingerprint as the isolate from the flour (PHAC et al., 2017). Additionally, British Columbia in Canada had an outbreak of *E. coli* O121 infection from flour in 2017. This outbreak resulted in six cases who had consumed flour. A sample of the flour that was collected was positive for the same genetic strain of *E. coli* O121 identified in all six cases (BCCDC et al., 2017). Flour contamination has also been investigated outside the United States (Mäde et al., 2017). This study collected flour samples from Germany between the years of 2014 and 2017, enriched using a two-step procedure for STEC, detection by PCR, and then isolated STEC by culture methods. Further, Mäde et al., 2017 collected data on the results of detection and isolation in combination with grain species and flourmills. Thirty-eight of 98 samples (39%) were positive for STEC by three-plex real time PCR assay, and 17 of 88 (19%) were positive for STEC by culture methods. This study had an objective of overall detection of STEC, so the STEC isolates detected were not serogrouped. The isolation and detection of STEC was not dependent on the species of grain, however there was evidence indicating the detection and isolation of STEC was clustered with four of the flourmills (Mäde et al., 2017). The point at which flour gets contaminated is not known, but it is possible the cereal grains are contaminated before or at the time of flour milling.

Reports of microbial contamination of cereal grains are limited (Laca et al., 2006; Los et al., 2018). Bacterial contamination of cereal grains can occur before harvest, during harvest, during transportation, in storage, or at the flour mill (Gilbert et al., 2010). The source of bacterial contamination could be air, dust, soil, water, insects, birds and animal feces (Laca et al., 2006). At the flour mill, the source of contamination may be from the encrusted grain dust and residues inside the grain bins (Berghofer et al., 2003). Most of the bacteria that contaminate cereal grains are harmless non-pathogenic bacteria. However, grains can also be contaminated with pathogens such as *Escherichia coli, Salmonella, Bacillus,* etc. (Los et al., 2018). Our objectives were to detect and isolate STEC from wheat grain samples collected from different regions of the US.

Materials and Methods

Wheat grain samples

Non-cleaned wheat grain samples (n=626) collected from different regions of the country were delivered to the Kansas State University Pre-Harvest Food Safety Laboratory. Samples were assigned laboratory accession numbers and stored at -80ºC until processed. Two days before sample processing, samples were removed from the -80ºC freezer and placed in a refrigerator for samples to thaw. A day before processing, samples were removed from the refrigerator and 50 g of the sample were weighed, labeled, and placed into an individual Whirl-Pak Vertical Filter Bag (Nasco Fort Atkinson, WI). All weighed samples were left at room temperature overnight for additional thawing.

Inoculation of wheat grain samples with STEC to validate STEC detection

Preparation of STEC inoculum.

A single colony of a top-7 STEC strain cultured on sheep blood agar (BAP: Remel, Lenexa, KS) was inoculated into 9 ml of Luria-Beratni (LB) broth and incubated overnight at 37° C. One hundred microliters of the broth culture were pipetted into 9 ml LB broth and incubated for approximately 3 hours at 37° C to reach an absorbance of 0.4 at 600 nm (approx.10^8 CFU per ml). The culture was then serially diluted ten-fold in LB broth to achieve an expected concentration ranging from 10^7 to 10^1 CFU per ml.

Inoculation of wheat grain samples.

Twenty-five gram of wheat grain sample (confirmed negative for top-7 *E. coli* serogroups and *stx*1*, stx*2*,* and *eae* genes by PCR) were suspended in 222.5 ml of modified Buffer Peptone water with pyruvate (mBPWp, BAM Media M192a; HiMedia) in a Whirl-Pak Vertical Filter Bag (Nasco) and inoculated with 2.5 ml of serially diluted culture to obtain 10^1, 10^2, or 10^3 CFU per g of wheat grain inoculum. The same procedure was also used with the *Escherichia coli* broth enrichment method. Inoculation of wheat grains at the 3 different concentrations was used to see what enrichment method could detect STEC at a lower concentration. The final bacterial concentration spiked for each strain of STEC is shown in Table 12.

Detection of STEC in spiked samples by PCR.

The spiked wheat grain samples were mixed in a stomacher (Bagmix 400, Interscience, Mourjou, France) for 1 minute and incubated at 37° C for 5 hours. After 5 hours of incubation, one ml of each acriflavin (10 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L) (BAM Manual M192a) were added and incubated for an additional 18 hours. After incubation, 1 ml of the sample enriched was pipetted into a 2-ml centrifuge tube, boiled for 10 minutes in a water

bath and centrifuged at 9,400 x g for 5 minutes. The boiled lysate was then subjected to a 10-plex PCR assay to detect top-7 serogroups of STEC (O26, O45, O103, O111, O121, O145, and O157) and the three major virulence genes (*stx*1*, stx*2, and *eae*) (Bai et al., 2012).

Ten milliliters of spiked sample were pipetted into a tissue culture flask (T-75; Thermo Scientific, Waltham, MA) containing 90 ml of EC broth (Oxoid, Thermo Scientific, Waltham, MA) and then incubated at 42^{o} C for 18-24 hours. One milliliter of the sample was pipetted into a 2-ml centrifuge tube, boiled for 10 minutes in a water bath and centrifuged at 9,400 x g for 5 minutes and DNA was extracted using the GeneClean Turbo Kit (MP Biomedicals). The extracted DNA was then used to perform a 10-plex PCR to detect top-7 serogroups of STEC (O26, O45, O103, O111, O121, O145, and O157) and 3 major virulence genes (*stx*1*, stx*2, and *eae*).

Detection of STEC in received wheat grain samples by PCR

Modified Buffer Peptone Water with Pyruvate (mBPWp) enrichment method.

Fifty grams of wheat grains were suspended in 450 ml of mBPWp in a filter bag and then pulsed in a stomacher for 1 minute. After stomaching, the inoculated sample was incubated at 37^{o} C for 5 hours, and 1 ml of each of acriflavin (10 mg/L), cefsulodin (10 mg/L), and vancomycin (8 mg/L) (ACV supplement) were added, and followed by incubation, for an additional 18-24 hours. After incubation, 1 ml of the sample was pipetted into a 2-ml centrifuge tube, boiled for 10 minutes in a water bath and centrifuged at 9,400 x g for 5 minutes. The boiled lysate was then subjected to 10-plex PCR to detect top-7 serogroups of STEC and three major virulence genes. Samples positive for any of the top-7 serogroups or three virulence genes were then subjected to culture method for isolation.

E. coli broth (EC) enrichment method.

Fifty grams of wheat grains were suspended in 450 ml of mBPW and then pulsed in a stomacher for 1 minute. The suspension was incubated at 37° C for 5 hours, and ACV supplement was added and incubated for 18-20 hours. After incubation, 10 ml of the sample was pipetted into a tissue culture flask containing 90 ml of EC broth and then incubated at 42° C for 18-24 four hours. One milliliter of the sample was pipetted into a 2-ml centrifuge tube, boiled for 10 minutes in a water bath and centrifuged at 9,400 x g for 5 minutes and DNA was extracted. The extracted DNA was used to perform a 10-plex PCR to detect top-7 serogroups of STEC and three major virulence genes. Samples positive for any of the top-7 serogroups or three virulence genes were then subjected to culture method for isolation.

Culture method for isolation of top-7 STEC serogroups

Enriched samples that were PCR positive for the top-7 serogroups were subjected to immunomagnetic separation (IMS) procedure specific to the serogroup that was detected and then spread-plated onto selective media for isolation. Briefly, 980 µl of enriched sample were mixed with 20 µl of the IMS beads (Abraxis®, Warminister, PA) specific for the PCR positive serogroup. The IMS procedure was performed according to the manufacturer protocol of Kingfisher Flex Magnetic Particle Processor (Thermo Scientific). Fifty microliters of final the IMS suspension were spread-plated on modified Possé (MP) agar (Noll et al., 2018), or sorbitol MacConkey agar (CT-SMAC) with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L), for isolation of non-O157 and O157 STEC, respectively. The plates were incubated at 37° C for 18-24 hours. Up to ten chromogenic colonies were picked from the MP agar and streaked onto BAP and incubated at 37° C for 18-24 hours. The colonies were pooled in 50 µl of distilled water,

boiled for 10 min, centrifuged at 9,300 x g for 5 min, and then tested by 10-plex PCR assay to identify non-O157 serogroups and the three virulence genes. If the pooled colonies were positive for any of the serogroups, then each colony was tested individually by 10-plex PCR to identify the serogroup and virulence genes. All confirmed isolates were stored in cryogenic beads (CryoCare, Key Scientific Products, Round Rock, TX). Up to six grey colonies (sorbitol negative) were picked from the CT-SMAC agar and streaked onto BAP plates and incubated at 37ºC for 18-24 hours. Colonies were subjected to *E. coli* O157 latex agglutination test and a colony positive for agglutination was subjected to a 6-plex PCR assay (Bai et al., 2010) to confirm O157 and H7 antigens and 4 virulence genes (*stx*1*, stx*2*, eae* and *ehxA*). All confirmed isolates were stored in cryogenic beads.

Culture method for Isolation of non-top-7 STEC

Fifty microliters of enriched sample, positive for *stx*1 and or *stx*2 gene but negative for any of the top-7 serogroups, were spread-plated onto a MacConkey agar (MAC) and Eosin methylene blue (EMB) agar plates and incubated at 37º C for 18-24 hours for non-selective isolation of *E. coli*. Ten putative colonies from MAC plates (pink, round, smooth colonies) and ten from EMB plates (iridescent green and black pigmented colonies) were streaked onto BAP and incubated at 37º C for 18-24 hours. The ten colonies from each MAC or EMB plate were pooled in 50 µl of distilled water, boiled for 10 minutes, and centrifuged at 9,300 x g for 5 minutes. The boiled lysate was subjected to 10-plex PCR assay to confirm that the isolate was negative for top-7 serogroups but positive for either Shiga toxin gene. Colonies positive for *stx*1*, stx*2*,* and or *eae* were confirmed by a three-plex real-time PCR assay (Noll et al., 2015). All

confirmed colonies were stored in cryogenic beads. Identification of the serogroup was by multiplex PCR assays targeting non-top-7 serogroups of STEC (Ludwig, 2017).

Results

The spiked samples were reproduced ten times for each concentration of each serogroup, and the number of positive samples were based on 10-plex PCR results (Table 12). For serogroup O26 spiking, both mBPWp and EC culture methods were identical in ability to detect O26 and *stx*1 (both detected 10 times out of 10 at all CFU per g; spiking levels). The EC method detected *eae* 10 times out of 10 samples at 18 CFU per g, while the mBPWp method detected *eae* 9 times out of 10 samples at 18 CFU per g. For serogroup O45 spiking, both mBPWp and EC culture methods were identical in ability to detect *eae* and *stx*1 10 times out of 10 at all CFU per g. The EC method detected O45 10 times out of 10 samples at all CFU per g, while the mBPWp method detected O45 9 times out of 10 samples at 230 CFU per g and 2,300 CFU per g, and only 5 times out of 10 at 23 CFU per g. For serogroup O121 spiking, both mBPWp and EC culture methods were identical in ability to detect O121 and *stx*2 (both detected 10 times out of 10 at all CFU per g; spiking levels). The EC method detected *eae* 10 times out of 10 samples at 170 CFU per g and 1700 CFU per g, and only 9 times out of 10 at 17 CFU per g, while the mBPWp method detected *eae* 0 times out of 10 at all CFU per g. For serogroup O157 spiking, both mBPWp and EC culture methods were identical in ability to detect O157, *stx*1, *stx*2, and *eae* (both detected 10 times out of 10 at all CFU per g; spiking levels).

Based on the initial 10-plex PCR assay, the top-7 serogroup prevalence by the mBPWp method in received wheat grain samples tested (Table 13) was 1.6% (10/625). The prevalence of each serogroup was O26 (0.2%; 1/626), O45 (0.6%; 4/626), O103 (0.2%; 1/626), and O157

(0.6%; 4/626) detected, and the prevalence of virulence genes were 0.2% of *stx*1 (1/626), 0.2% of *stx*2 (1/626), and 0.8% of *eae* (5/626). The one *stx*1 positive sample was also intimin positive, and the one *stx*2 positive sample had no other virulence gene detection. The top-7 serogroup positive samples came from five states (Colorado, Indiana, Kansas, Missouri, and Ohio) and two O45 positive samples came from unidentified states. The one *stx*1 positive sample came from Maryland and the one *stx*2 positive sample came from Montana (Table 13).

Further, the initial 10-multiplex PCR assay detected top-7 serogroup prevalence in wheat grain samples by EC broth method (Table 14) as 2.6% (16/626), with those being 3 non-O157 serogroups and O157. The individual prevalence of each serogroup was O26 (0.2%; 1/626), O45 (1.9%; 12/626), O103 (0.5%; 3/626), and O157 (1.0%; 6/626), with virulence gene detection of *stx*1 (0.3%; 2/626), *stx*2 (1.3%; 8/626), and *eae* (1.1%; 7/626). Both *stx*1 positive samples were also intimin positive, and all *stx*2 positive samples had no other virulence gene or serogroup detection. None of the EC enriched samples detected top-7 serogroups positive for Shiga toxin genes, and ten samples (1.6%; 10/626) were non-top-7. The top-7 serogroup positive samples came from seven states (Colorado, Indiana, Kansas, Missouri, Ohio, South Dakota, and Virginia) and two O45 positive samples came from unidentified states. The two *stx*1 positive samples came from Maryland and Missouri, and the *stx*2 positive samples came from four states (Montana, North Dakota, Oklahoma, and Texas) (Table 14).

Based on culture methods, the top-7 serogroup prevalence by the mBPWp method (Table 15) in wheat grain samples isolated by IMS (n=10) was 90% (9/10). The prevalence of each serogroup was O26 (0.2%; 1/626), O45 (0.5%; 3/626), O103 (0.2%; 1/626), and O157 (0.6%; 4/626) isolated. None of the mBPWp enriched sample isolates detected top-7 serogroups positive for Shiga toxin genes or intimin. Further, all four of the O157 isolates were positive for

rfbE by 6-plex PCR assay, and negative for H7 antigens and the three virulence genes. The top-7 serogroup positive isolates came from five states (Colorado, Indiana, Kansas, Missouri, and Ohio) and one O45 positive samples came from an unidentified state. (Table 15).

The top-7 serogroup prevalence by the EC method (Table 16) in wheat grain samples isolated by IMS method (n=22) was 81.3% (13/22). The prevalence of each serogroup was O26 (0.2%; 1/626), O45 (0.8%; 5/626), O103 (0.3%; 2/626), and O157 (0.8%; 5/626) isolated. None of the EC enriched sample isolates detected top-7 serogroups positive for Shiga toxin gene. Further, all five of the O157 isolates were positive for *rfbE* by 6-plex PCR assay, and negative for H7 antigens and the three virulence genes detected. The top-7 serogroup positive isolates came from seven states (Colorado, Indiana, Kansas, Missouri, Ohio, South Dakota, and Virginia) and two O45 positive samples came from unidentified states. (Table 16).

Prevalence of the non-top-7 STEC positive samples from mBPWp enrichment method (n=2) was 0.0% (0/2), as none were isolated on MAC and EMB, and prevalence of the non-top-7 STEC positive samples from EC enrichment method (n=8) was 62.5% (5/8). All five isolates were confirmed *stx*2 positive, with no other virulence gene detection. These five isolates came from three states (North Dakota, Oklahoma, and Texas). After twelve multiplex PCR assays to confirm a total of 130 serogroups of *E. coli,* the five isolates were confirmed as serogroups O8 (0.64%; 4/626) and O130 (0.16%; 1/626) (Table 16).

Discussion

Wheat grains have the capability of being contaminated with STEC and may have implications on public health, especially if wheat grains are heavily exposed to different contaminated environments. Since this study design was blind to the source of the wheat grains

and their environmental exposure, no conclusions can be made for serogroup prevalence being related to wheat grain treatment. No top-7 STEC were isolated from wheat grain samples in this study, which could be a result of wheat grains samples not being stored near where cattle are being housed and managed. The wheat grain samples were collected for this study using a convenience sampling strategy. Convenience sampling is commonly used, as it creates simplicity within sample collection and samples can be facilitated in a short duration of time. However, there are disadvantages with this strategy, as studies using convenience sampling tend to have a lack of power for reduced sample size. Further, our study is not representative of any particular state or region within the United States. In this study, the conclusion that can be made is that STEC does exist in wheat grains in the United States. Future research is needed to have more descriptive data on the handling and environmental exposure of the wheat grains, as well as having a larger sample size from each state and region of the United States to increase overall power of the study.

Additional studies in detection of Shiga toxin-producing *E. coli* in flour have also been performed to evaluate flour as a vehicle for transmission. Kindle et al. 2019 created a study to analyze 70 flour samples sold from various production systems from Switzerland. These samples were enriched and screened for Shiga toxin genes like in our study, however, Kindle et al. enriched flour at a 1:10 ratio. Out of the 70 samples analyzed, 9 were positive for Shiga toxin gene by real time PCR, and 8 were positive by culture methods used in this study. There were three serogroups typeable (O11, O103, and O146), and three isolates were non-typeble for serogroups. With the indication that STEC in this study is contaminated with O103 and O146, a top-7 serogroup for public health and a serogroup frequently isolated from humans in

Switzerland, indicates that flour could be a novel vehicle for transmission of STEC infection with consumers being at risk.

Some STEC outbreaks associated with wheat flour have been seen internationally, as the 2016/2017 outbreak of STEC O121 in Canada. This outbreak was over the span of three days from a single facility and was the same serogroup as the United States outbreak, STEC O121. Understanding the level of contamination is critical to investigating the cause of contamination of the flour products, so Gill et al., 2019 created a study to quantify the foodborne pathogen in these flour samples. They performed quantification data on recalled flour samples, as well as 8 retail flour samples, to examine exposure risk and the infectivity of strains. They collected recalled flour samples and used Most Probably Number (MPN) estimates to enumerate the concentration at 3 different analytical units of recalled flour. At the conclusion of this study, all retail flour samples were negative for STEC, even at the 5 x 100 g, and there were low enough levels of STEC O121 in the recalled flour to indicate no evidence of fecal contamination in the recalled flour. Further, Gill et al. also indicated that larger analytical units may be required to consistently detect STEC O121 in these flour samples at the level detected in the investigation. Since no STEC O121 was detected in the wheat grain samples in our study, it may be important to use larger analytical units up to several hundred grams in future studies for detection and isolation. It is important to note that the most commonly seen non-top-7 serogroup that produced Shiga toxin seen in this study was *E. coli* O8, which is a common serogroup isolated from food sources (Werber et al., 2008), and serotypes of STEC O8 have been associated with HUS (Galli et al., 2010) indicating prevention methods are needed. This serogroup is also commonly isolated from swine (Tseng et al., 2014; Sonntag et al., 2005). This could be a result of wheat grains being exposed to swine fecal matter, where the wheat grains may get contaminated. Post study

investigation involving the subtyping of the *stx*2 gene in the STEC O8 isolates may further indicate the wheat grains were exposed to swine, as *stx*2e is a subtype of Shiga toxin most commonly isolated from swine feces (Tseng et al., 2014). Investigations of the handling and exposure of these wheat grains is critical in producing prevention methods. These prevention methods could include more developed biosecurity methods in treatment of wheat grains, transport and handling, and preparing product for consumption are also critical to preventing food borne illness.

Tables

Table 12. Detection of serogroup-specific gene, *stx*1, *stx*2, and *eae* genes in wheat grain samples inoculated with *Escherichia coli* O26, O45, O121 or O157 and subjected to two enrichment methods

Serogroups, strains, and targeted genes	Bacterial concentration (CFU per g)	No. of samples positive by multiplex PCR (n=10)							
		Enriched in modified buffered peptone water with pyruvate				Enriched in *Escherichia coli* broth			
		Serogroup-specific gene	*stx*1	*stx*2	*eae*	Serogroup-specific gene	*stx*1	*stx*2	*eae*
E. coli O26 2017-11-F150B1 (*wzx*$_{O26}$, *stx1* and *eae)*	18	10*	10*	-	9	10	10	-	10
	180	10	10	-	10	10	10	-	10
	1,800	10	10	-	10	10	10	-	10
E. coli O45 2017-5-242E(*wzx*$_{O45}$, *stx1* and *eae*)	23	5	10	-	10	10	10	-	10
	230	9	10	-	10	10	10	-	10
	2300	9	10	-	10	10	10	-	10
E. coli O121 FNW19M93 (*wbqE* $_{O121}$ and *wbqF*$_{O121}$, *stx2,* and *eae)*	17	10	10	-	0	10	10	-	9*
	170	10	10	-	0	10	10	-	10
	1700	10	10	-	0	10	10	-	10
E. coli O157 CDC 380-94(*rfbE*$_{O157}$, *stx1, stx2,* and *eae)*	26	10	10	10	10	10	10	10	10
	260	10	10	10	10	10	10	10	10
	2600	10	10	10	10	10	10	10	10

Table 13. Wheat grain samples, enriched in modified buffered peptone water with pyruvate, positive for top-7 *Escherichia coli* serogroups O26, O45, O103, O111, O121, O145, and O157, and three major virulence genes, *stx1, stx2,* and *eae* based on PCR method of detection.

Wheat grain samples		Top-7 serogroups and major virulence genes										Non-top-7 STEC[a]
State	No. of samples	O26	O45	O103	O111	O121	O145	O157	*stx*1	*stx*2	*eae*	
Alabama	5											
Arkansas	13										1	
Colorado	22							1				
Illinois	1											
Indiana	13			1								
Kansas	186		1					3			1	
Maryland	19								1		1	1
Minnesota	25											
Missouri	36	1										
Mississippi	1											
Montana	30									1		1
North Carolina	5										1	
North Dakota	55											
Nebraska	30											

Ohio	1		1									
Oklahoma	57										1	
South Dakota	49											
Texas	24											
Virginia	4											
Not available	50		2									
Total	**626**	**1**	**4**	**1**	**0**	**0**	**0**	**4**	**1**	**1**	**5**	**2**

[a] Samples positive for *stx*1 or *stx*2, but negative for the top-7 serogroup

Table 14. Wheat grain samples, enriched in *Escherichia coli* broth, positive for top-7 serogroups, O26, O45, O103, O111, O121, O145, and O157, and three major virulence genes, *stx1, stx2,* and *eae,* of *Escherichia coli* based on PCR method of detection

Wheat grain samples		Top-7 serogroups and major virulence genes										Non-top-7[a]
State	No. of samples	O26	O45	O103	O111	O121	O145	O157	*stx1*	*stx2*	*eae*	
Alabama	5											
Arkansas	13										1	
Colorado	22							2				
Illinois	1											
Indiana	13			1								
Kansas	186		4					4			1	
Maryland	19								1		1	1
Minnesota	25											
Missouri	36	1	1	1					1		2	1
Mississippi	1											
Montana	30									1		1
North Carolina	5										1	
North Dakota	55									1		1
Nebraska	30											
Ohio	1		1									

Oklahoma	57									2	1	2
South Dakota	49			1								
Texas	24									4		4
Virginia	4		1									
Not available	50		5									
Total	**626**	**1**	**12**	**3**	**0**	**0**	**0**	**6**	**2**	**8**	**7**	**10**

[a] Samples positive for *stx*1 or *stx*2, but negative for the top-7 serogroups

Table 15. Culture method of isolation and identification of STEC serogroups from wheat grain samples, enriched in modified buffered peptone water with pyruvate, that were PCR- positive for serogroups, O26, O45, O103, O111, O121, O145, and O157, and three major virulence genes, *stx1, stx2,* and *eae*.

Wheat grain samples		Top-7 serogroups and major virulence genes										Non-top-7[a]
State	No. of positive samples by 10-plex[b]	O26	O45	O103	O111	O121	O145	O157	*stx*1	*stx*2	*eae*	
Alabama	0											
Arkansas	1											
Colorado	1							1				
Illinois	0											
Indiana	1			1								
Kansas	4		1					3				
Maryland	1											
Minnesota	0											
Missouri	1	1										
Mississippi	0											
Montana	1											
North Carolina	1											

North Dakota	0											
Nebraska	0											
Ohio	1		1									
Oklahoma	1											
South Dakota	0											
Texas	0											
Virginia	0											
Not available	2		1									
Total	**15**	**1**	**3**	**1**	**0**	**0**	**0**	**4**	**0**	**0**	**0**	**0**

[a] Samples positive for *stx*1 or *stx*2, but negative for the top-7 serogroups

[b]Samples initially positive by 10-plex PCR positive for top-7 serogroups

Table 16. Culture method of isolation and identification of *Escherichia coli* serogroups from wheat grain samples, enriched in *Escherichia coli* broth, that were PCR-positive for the top-7 serogroups, O26, O45, O103, O111, O121, O145, and O157, and three major virulence genes, *stx1, stx2,* and *eae.*

Wheat grain samples		Top-7 serogroups and major virulence genes										
State	No. of positive samples by10-plex	O26	O45	O103	O111	O121	O145	O157	*stx*1	*stx*2	*eae*	Non-top-7[a]
Alabama	0											
Arkansas	1										1	1
Colorado	2							2				
Illinois	0											
Indiana	1			1								
Kansas	8							3				
Maryland	1											
Minnesota	0											
Missouri	5	1										
Mississippi	0											
Montana	1											
North Carolina	1										1	1

North Dakota	1									1		1[b]
Nebraska	0											
Ohio	1		1									
Oklahoma	3									1	1	2[c]
South Dakota	1			1								
Texas	4									3		3[c]
Virginia	1		1									
Not available	5		3									
Total	**36**	**1**	**5**	**2**	**0**	**0**	**0**	**5**	**0**	**5**	**3**	**8**

[a] Samples positive for *stx*1 or *stx*2, but negative for the top-7 serogroups

[b] The *stx*2 isolate confirmed as serogroup O130

[c] The *stx*2 isolates confirmed as serogroup O8

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