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Research Paper

Shiga Toxin–Producing *Escherichia coli* in Feces of Finisher Pigs: Isolation, Identification, and Public Health Implications of Major and Minor Serogroups†

S. E. REMFRY,[1] R. G. AMACHAWADI,[1,2]* X. SHI,[3] J. BAI,[4] M. D. TOKACH,[5] S. S. DRITZ,[3] R. D. GOODBAND,[5] J. M. DEROUCHEY,[5] J. C. WOODWORTH,[5] AND T. G. NAGARAJA[3]*

[1]*Department of Clinical Sciences,* [2]*Center for Outcomes Research and Epidemiology,* [3]*Department of Diagnostic Medicine and Pathobiology,* [4]*Veterinary Diagnostic Laboratory, and* [5]*Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas 66502, USA*
(ORCID: https://orcid.org/0000-0001-9689-1124 [R.G.A.])

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ABSTRACT

Shiga toxin–producing *Escherichia coli* (STEC) are major foodborne human pathogens that cause mild to hemorrhagic colitis, which could lead to complications of hemolytic uremic syndrome. Seven serogroups, O26, O45, O103, O111, O121, O145, and O157, account for the majority of the STEC illnesses in the United States. Shiga toxins 1 and 2, encoded by stx_1 and stx_2, respectively, and intimin, encoded by *eae* gene, are major virulence factors. Cattle are a major reservoir of STEC, but swine also harbor them in the hindgut and shed STEC in the feces. Our objectives were to use a culture method to isolate and identify major and minor serogroups of STEC in finisher pig feces. Shiga toxin genes were subtyped to assess public health implications of STEC. Fecal samples ($n = 598$) from finisher pigs, collected from 10 pig flows, were enriched in *E. coli* broth and tested for stx_1, stx_2, and *eae* by a multiplex PCR (mPCR) assay. Samples positive for stx_1 or stx_2 gene were subjected to culture methods, with or without immunomagnetic separation and plating on selective or nonselective media, for isolation and identification of *stx*-positive isolates. The culture method yielded a total of 178 isolates belonging to 23 serogroups. The three predominant serogroups were O8, O86, and O121. The 178 STEC strains included 26 strains with stx_{1a} and 152 strains with stx_{2e} subtypes. Strains with stx_{1a}, particularly in association with *eae* (O26 and O103), have the potential to cause severe human infections. All stx_2-positive isolates carried the subtype stx_{2e}, a subtype that causes edema disease in swine, but is rarely involved in human infections. Several strains were also positive for genes that encode for enterotoxins, which are involved in neonatal and postweaning diarrhea in swine. In conclusion, our study showed that healthy finisher pigs harbored and shed several serogroups of *E. coli* carrying virulence genes involved in neonatal diarrhea, postweaning diarrhea, and edema disease, but prevalence of STEC of public health importance was low.

HIGHLIGHTS

- Swine harbor Shiga toxin–producing *Escherichia coli* (STEC) and shed them in the feces.
- Swine STEC could be a source of foodborne infections in humans.
- Culture method identified major and minor serogroups of STEC.
- Major serogroups of STEC included O8, O86, and O121.
- A majority of the STEC possessed Shiga toxin 2e, which is not a major public threat.

Key words: Culture method; Feces; Serogroups; Shiga toxin–producing *Escherichia coli*; Shiga toxin subtypes; Swine

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Shiga toxin–producing *Escherichia coli* (STEC) are major foodborne pathogens. Infections caused by STEC range from mild to bloody diarrhea (hemorrhagic colitis) to complications of kidney failure, resulting in hemolytic uremic syndrome, and even death *(19, 43)*. A primary virulence factor of STEC is a secreted protein called Shiga toxin (Stx), encoded by a gene carried on a prophage *(42)*. The toxin is divided into two types, Stx1 and Stx2, encoded by stx_1 and stx_2 genes, respectively, which share some amino acid homology but differ in antigenicity and degree of cytotoxicity *(54, 72)*. In addition, each toxin type has several subtypes. Shiga toxin 1 has three subtypes (Stx1a, Stx1c, and Stx1d) and Stx2 has seven subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) *(47, 66)*. Shiga toxin 2 is more cytotoxic than Stx1, and certain subtypes, Stx2a, Stx2c, and Stx2d, are more often associated with hemorrhagic colitis and complications,

* Authors for correspondence. Tel: 785-532-4356; Fax: 785-532-2252; E-mail: agraghav@vet.k-state.edu (R.G.A.). Tel: 785-532-1214; Fax: 785-532-4039; E-mail: tnagaraj@vet.k-state.edu (T.G.N.).
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including hemolytic uremic syndrome *(22, 32, 47)*. In addition to Stx, a cluster of genes located on a pathogenicity island, the locus of enterocyte effacement, encode a type III secretion system and effector proteins, which contribute to intestinal colonization and effacement lesions *(42)*. One of the genes on locus of enterocyte effacement, *E. coli* attaching and effacing gene, *eae,* encodes for an outer membrane protein called intimin, which mediates attachment of STEC to epithelial cells *(42)*. Only a subset of specific STEC serogroups referred to as enterohemorrhagic *E. coli* (EHEC) contain locus of enterocyte effacement and intimin and have the ability to induce attaching and effacing lesions in intestinal epithelium *(71)*.

Among the 187 known serogroups of *E. coli (20),* as many as 158 serogroups have been associated with the STEC pathotype *(8, 38, 45, 77)*. Seven serogroups, O26, O45, O103, O111, O121, O145, and O157, referred to as "top-7 STEC," are responsible for a majority of STEC infections, including several major foodborne outbreaks *(14, 34, 65)*. Ruminants are a major reservoir of STEC in which they colonize the hindgut and are shed in the feces. Several studies have shown that swine also harbor STEC and shed them in their feces *(16, 30, 31, 64, 75)*. Therefore, swine can also be a source of STEC to humans, and a few outbreaks have been linked to the consumption of pork products contaminated with STEC *(37, 74, 76)*. In cattle, STEC are commensals and do not cause disease, but in swine, STEC serogroups that produce Stx2e subtype cause edema disease *(1, 23, 33)*. Certain serogroups of STEC, particularly in swine, have been shown to carry genes that encode for enterotoxins involved in neonatal or postweaning diarrhea *(1, 24, 53)*. Although clinically relevant top-7 serogroups have been detected and isolated from swine feces, a majority of the isolates were serogroups other than the top-7 and included O8, O59, O71, O86, O100, O163, O174, and O184 *(16, 75, 76)*. Recently, we reported, by multiplex PCR (mPCR) assays, a high prevalence of serogroups of O26, O121, and O157 among the top-7 and O8, O86, O91, O100, and O174 among the non–top-7 in feces of finisher pigs *(64)*. Because of the limitation of the PCR method, which does not indicate the association of Stx genes with the serogroups present in the feces, their public health importance or potential to cause edema disease in swine could not be ascertained. Therefore, our objectives in this study were to use culture methods to identify and isolate major and minor serogroups of STEC, identify STEC serogroups that carry enterotoxin genes, and subtype the Stx gene in the STEC isolates to assess their potential to cause disease in swine and humans.

MATERIALS AND METHODS

Study design and samples. The study design and sample collections have been described previously *(64)*. In brief, 10 pig flows from eight states (Iowa, Kansas, Minnesota, Nebraska, North Carolina, Oklahoma, Ohio, and South Dakota) were included in the study. In each pig flow, six different finishing sites were randomly selected, and from each site, fecal samples from 10 finisher pigs close to marketing were collected once (60 samples per pig flow; $n = 598$ fecal samples).

Fecal sample enrichment and detection of Stx genes and top-7 and O104 serogroups. Approximately 1 g of fecal sample was suspended in 9 mL of *E. coli* broth (Difco, BD, Waltham, MA *(57)*). The sample was vortexed for 1 min and incubated at 40°C for 6 h. After incubation, 1 mL was pipetted into a 2-mL centrifuge tube. The contents were boiled for 10 min and centrifuged at 9,400 $\times$ *g* for 5 min. The DNA in the supernatant was purified by using a GeneClean Turbo kit (MP Biomedicals, Solon, OH). The purified DNA was used to detect Stx1 (stx_1), Stx2 (stx_2), and *eae* genes by real-time PCR assay *(52)*. Fecal samples positive for stx_1 or stx_2 genes were then subjected to an mPCR assay to detect serogroups of O26, O45, O103, O104, O111, O121, O145, and O157 *(2, 55)*.

Selective isolation and identification of O26, O45, O103, O104, O111, O121, O145, and O157 serogroups. Enriched fecal samples positive for any of the eight (top-7 and O104) serogroups by the mPCR assay were subjected to immunomagnetic separation (IMS) specific to the serogroup and then spread plated onto selective media for isolation. In brief, 980 μL of enriched sample was mixed with 20 μL of the IMS beads (Abraxis, Warminster, PA) specific for the PCR-positive serogroups, individually or pooled, based on the number of serogroups present *(50)*. The IMS procedure was performed according to the protocol provided by the manufacturer of the KingFisher Flex magnetic particle processor (Thermo Fisher Scientific, Waltham, MA). Fifty microliters of the final IMS suspension was spread plated onto sorbitol MacConkey agar with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) (CT-SMAC) or modified Possé agar *(51, 62)* for isolation of O157 and non-O157 STEC, respectively. All plates were incubated at 37°C for 18 to 24 h. From the CT-SMAC plate, up to six gray colonies (sorbitol negative) were picked and streaked onto blood agar plates (Remel, Lenexa, KS) and incubated at 37°C for 18 to 24 h. Colonies were subjected to *E. coli* O157 latex agglutination test, and a colony positive for agglutination was subjected to an mPCR assay to confirm O157 and H7 antigens and three virulence genes (stx_1, stx_2, and *eae*) *(3)*. From the modified Possé agar plate, up to 10 chromogenic colonies were picked and streaked onto blood agar plates and incubated at 37°C for 18 to 24 h. The colonies of the 10 isolates from each plate were pooled in 50 μL of distilled water. The solution was boiled for 10 min, centrifuged at 2,200 $\times$ *g* for 2 min, and then tested by mPCR assay for the top-7 and O104 serogroups and three virulence genes *(68)*. If the pooled colonies were positive for any of the serogroups, each isolate was tested individually by mPCR. All confirmed *stx*-positive isolates were stored in cryogenic beads (CryoCare, Key Scientific Products, Round Rock, TX).

Nonselective isolation of *E. coli* (without IMS) by direct plating of enriched fecal samples. Enriched fecal samples positive for the stx_1 or the stx_2 gene were directly spot inoculated with a sterile cotton swab onto MacConkey agar (MAC; Remel) and eosin–methylene blue (EMB; Remel) agar plates, and then sterile loops were used to streak from the swabbed area for isolation of *E. coli*. Also, samples were diluted (1 in 100 dilution) in *E. coli* broth, and 25 μL of the diluted inoculum was spread plated onto MAC and EMB plates. Inoculated plates were incubated at 37°C for 18 to 24 h. In total, 10 putative colonies presumptive of *E. coli* from MAC plate (pink, round, smooth colonies) and 10 putative colonies from EMB plate (iridescent green- and black-pigmented colonies) for each sample were streaked onto blood agar plates and incubated at 37°C for 18 to 24 h. The 10 colonies obtained for each sample were pooled in 50 μL

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of distilled water. The solution was boiled for 10 min and centrifuged at 2,200 × g for 2 min. The boiled lysate was subjected to a real-time PCR assay *(52)* to detect stx_1, stx_2, and *eae*. If pooled colonies were positive for stx_1 or stx_2, each colony was tested individually by mPCR assay to detect the top-7 and O104. Any colony positive for stx_1 or stx_2 but negative for the top-7 was considered as non–top-7 STEC. The serogroups of the *stx*-positive isolates were identified by 14 sets of mPCR assays targeting 137 non–top-7 serogroups of STEC *(45)*. All *stx*-positive isolates were stored at –80°C in cryogenic beads.

Subtyping of Stx genes of STEC isolates. The *stx* genes of the isolated STEC strains ($n = 178$) were subtyped by a touchdown PCR method as described previously *(69)*. In brief, each strain was grown on blood agar plates. A single colony was picked and suspended in distilled water after which it was boiled and centrifuged. The supernatant (lysate) was used to amplify stx_1 and stx_2 genes. Amplicons were purified and shipped to Genewiz, Inc. (South Plainfield, NJ) for nucleotide sequencing. The chromatogram data of each sequence were individually analyzed for conflicts, and secondary peaks and consensus sequences were produced using CLC Main Workbench software (Qiagen, Valencia, CA). The nucleotide sequences were conceptually translated to amino acid sequences, and Stx subtypes were determined based on the amino acid motifs that define each *stx* subtype *(66)*. Because the primers designed to amplify the *stx* gene did not include the subtype stx_{2e}, a PCR assay outlined was used for strains that were negative for all the other subtypes of stx_2 *(66)*.

Detection of enterohemolysin and enterotoxins. PCR assays were used to identify the following genes in all the STEC isolates ($n = 178$): *ehxA,* which encodes for enterohemolysin *(2),* and enterotoxin genes *elt, estA, estB,* and *astA,* which encode for heat-labile enterotoxin, heat stable entetrotoxins A and B, and enteroaggregative heat-stable enterotoxin 1 (EAST1), respectively *(79)*.

Statistical analysis. Statistical analysis was performed using STATA 16.1 (StataCorp, College Station, TX). Multiple mean comparisons were obtained for each variable assuming equal variances across states by Bonferroni approach, to adjust the comparison-wise error rates. Analysis was done using Probit regression on binary dependent variables assuming that the probability of a positive outcome is determined by the standard normal cumulative distribution function *(49)*. The means for number of fecal samples with pooled colonies positive for stx_1 or stx_2 gene, number of pure cultures positive for the stx_1 or stx_2 genes, and number of pure cultures positive for serogroups other than the eight serogroups obtained from direct plating of enriched fecal samples on MAC or EMB agar were compared by a two-sample t test. The Cohen's kappa statistics was used to measure the interrater agreement for numbers of pooled cultures or pure cultures positive for the *stx* gene between MAC and EMB agar plates *(46)*. Results were considered significant at a P value < 0.05.

RESULTS

Of the 598 enriched fecal samples tested by reverse transcriptase PCR assay for the three virulence genes, 419 (70.1%) samples were positive for stx_1 and/or stx_2 genes and 398 (66.7%) samples were positive for *eae* (Table 1).

Detection and isolation of STEC O26, O45, O103, O104, O111, O121, O145, and O157. Of the 419 fecal samples subjected to IMS and plating on selective media (CT-SMAC and modified Possé), 208 samples (208 of 598, 34.8%) were positive for one or more of the eight targeted serogroups (Table 1). The prevalence of the eight serogroups ranged from 15% in fecal samples collected from a pig flow in South Dakota to 75% in samples collected from a pig flow in Iowa. In total, 190 isolates (31.8% of fecal samples) positive for one of the eight serogroups were obtained in pure cultures. The five predominant serogroups isolated were O26 (38 of 598, 6.4%), O45 (24 of 598, 4.0%), O104 (25 of 598, 4.2%), O121 (62 of 598, 10.4%), and O157 (24 of 598, 4.0%). None of the fecal samples yielded an isolate positive for the O111 serogroup. Of the 190 isolates, only 25 were positive for a Stx gene, which included one strain each of O26 and O103 and 23 strains of O121 (Table 1). Both O26 and O103 were positive for stx_1 and *eae* genes, and the 23 O121 strains were positive for stx_2 and negative for *eae*. None of the O45 ($n = 24$), O145 ($n = 8$), and O157 ($n = 24$) isolates contained *stx, eae, or* $fliC_{H7}$ genes. In addition, 23 isolates were identified to be positive for stx_1 or stx_2 gene, but negative for the eight serogroups (top-7 and O104). The 23 STEC strains, identified by mPCR assays, belonged to serogroups of O2 ($n = 3$), O8 ($n = 4$), O36 ($n = 2$), O55 ($n = 2$), O76 ($n = 1$), O86 ($n = 2$), and O115 (n = 1), and eight strains were negative for any of the 137 targeted serogroups (Table 1).

Detection and isolation of STEC by direct plating of enriched fecal samples onto MAC and EMB agar. Because no serogroup-specific IMS beads are available and no selective media have been developed for serogroups other than the eight serogroups (top-7 and O104), fecal samples enriched in *E. coli* broth and positive for stx_1 and/or stx_2 genes were inoculated directly onto MAC and EMB agar. After incubation, pooled putative colonies of *E. coli* (10 colonies per sample) were tested for Stx genes, and if positive, each pure culture was tested by mPCR assay for *stx* gene and serogroup identification. Initially, 200 of 300 fecal samples from five pig flows that were positive for stx_1 and/or stx_2 genes were plated onto both MAC and EMB agar. The proportion of samples with pooled colonies (10 colonies per sample) positive for stx_1 and/or stx_2 genes were 81 (40.5%) of 200 (confidence interval [CI] = 33 to 47) and 71 (35.5%) of 200 (CI = 28 to 42) for MAC and EMB agar, respectively (Table 2). The total number of pure cultures positive for stx_1 and/or stx_2 obtained by testing individual colonies were 63 (31.5%) from MAC and 56 (28.0%) from EMB. The total number of isolates belonging to top-7 or O104 (3 for MAC and 4 for EMB) and non–top-7 STEC (60 [30%] for MAC and 53 [26.5%] for EMB) was similar between the two media (Table 2). The kappa coefficient for the proportion of samples with pooled colonies of 10 positive for stx_1 and/or stx_2 genes and total number of pure cultures positive for stx_1 and/or stx_2 obtained by testing individual colonies from MAC or EMB was 0.89 or higher, indicating almost perfect agreement between the two media (Table 2). Because neither medium was better than the other, only MAC medium was used for the remaining 300 samples.

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TABLE 1. *Prevalence of eight serogroups (top-7 and O104) of Shiga toxin–producing* Escherichia coli *(STEC) in swine feces (*n *= 598) based on the culture method involving serogroup-specific immunomagnetic beads*[a]

STEC gene and serogroup	Kansas (n = 60)	Minnesota (n = 60)	Iowa: Pig flow 1 (n = 60)	Iowa: Pig flow 2 (n = 60)	Oklahoma (n = 60)	Ohio (n = 59)	South Dakota: Pig flow 1 (n = 60)	South Dakota: Pig flow 2 (n = 60)	Nebraska (n = 60)	North Carolina (n = 59)	Total (n = 598)
No. (%) of fecal samples positive for stx_1 and/or stx_2[b]	21 A (35.0)	49 BC (81.7)	59 C (98.3)	53 BC (88.3)	18 A (30.0)	47 BC (79.7)	56 BC (93.3)	45 B 75.0)	59 C (98.3)	12 A (20.3)	419 (70.1)
No. (%) of fecal samples positive for *eae*	20 A (33.3)	37 BC (61.7)	57 D (95.0)	41 BC (68.3)	40 BC (66.7)	50 CD (84.7)	37 BC (61.7)	28 AB (46.6)	46 CD (76.7)	42 BCD (71.2)	398 (66.7)
No. (%) of fecal samples positive for one or more of the eight serogroups[c]	14 AB (23.3)	26 BC (43.3)	45 D (75)	22 AB (36.7)	10 A (16.7)	23 ABC (39.0)	10 A (16.7)	9 A (15.0)	38 CD (63.3)	11 AB (18.6)	208 (34.8)
No. (%) of serogroup-positive pure cultures isolated	17 AB (28.3)	21 AB (35.0)	40 CD (66.7)	10 A (16.7)	12 AB (20)	25 BC (41.7)	6 A (10.1)	8 A (13.3)	42 D (70)	9 A (15.3)	190 (31.8)
No. of *stx*-positive isolates/total no. of isolates belonging to eight targeted serogroups[d]											
O26	0/1	0/6	0/9	0	0/4	1/8	0/3	0/1	0/6	0	1/38
O45	0/1	0/5	0/1	0/3	0	0/1	0	0/1	0/12	0	0/24
O103	1/1	0/2	0/3	0	0	0/2	0/1	0	0	0	1/9
O104	0	0	0/8	0/2	0	0/4	0	0	0/11	0	0/25
O111	0	0	0	0	0	0	0	0	0	0	0
O121	3/3	4/6	2/17	0/4	0	0/8	1/1	5/6	1/9	7/8	23/62
O145	0	0/1	0	0/1	0/4	0/2	0	0	0	0	0/8
O157	0/11	0/1	0/2	0	0/4	0	0/1	0	0/4	0/1	0/24
Total	4/17	4/21	2/40	0/10	0/12	1/25	1/6	5/8	1/42	7/9	25/190
No. of *stx*-positive isolates/total no. of isolates belonging to serogroups other than the eight targeted serogroups[e]											
O2			1				2				3
O8			4								4
O36			1				1				2
O55									2		2
O76			1								1
O86									2		2
O115		1									1
Unidentified			2	1	2	0	1	1	1	0	8
Total (%)	0	1	9	1	2	0	4	1	5	0	23 (3.9)

[a] Means within a row sharing the same letter are not statistically different ($P > 0.05$).
[b] Tested by real-time PCR assay *(52)*.
[c] Tested by multiplex PCR assay targeting eight serogroups (O26, O45, O103, O104, O111, O121, O145, and O157) and three virulence genes (stx_1, stx_2, and *eae*) *(57)*.
[d] Isolates positive for *stx* gene but negative by multiplex PCR for the eight serogroups (O26, O45, O103, O104, O111, O121, O145, and O157).
[e] Serogroups identified by using 14 sets of mPCR assays targeting 140 serogroups of STEC other than the top-7 and O104 serogroups *(45)*.

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TABLE 2. *Detection and isolation of Shiga toxin–producing* Escherichia coli *(STEC) from Shiga toxin–positive fecal samples (*n = 200*) that were directly plated onto MacConkey or eosin–methylene blue agar*

Item	No. of fecal samples[a] cultured on MacConkey agar, $n = 200$ (%; 95% confidence interval)	No. of fecal samples[a] cultured on eosin–methylene blue agar, $n = 200$ (%; 95% confidence interval)	P value[b]	Kappa coefficient[c]
No. of fecal samples with pooled colonies of 10 that were positive for stx_1 and/or stx_2[d]	81 (40.5; 33–47)	71 (35.5; 28–42)	0.30	0.89
No. of pure cultures positive for stx_1 and/or stx_2	63 (31.5; 25–38)	56 (28.0; 21–34)	0.44	0.91
No. of pure cultures positive for the eight STEC serogroups[e]	3	4	—[f]	—
No. of pure cultures positive for serogroups other than the eight STEC	60 (30.0; 23–36)	53 (26.5; 20–32)	0.43	0.91

[a] Include fecal samples collected from five pig flows located in the states of Kansas, Minnesota, Iowa, and Oklahoma.
[b] Based on two-sample t test.
[c] Based on kappa statistic.
[d] Determined by real-time PCR *(52)*.
[e] Determined by 11-plex conventional PCR targeting eight serogroups (O26, O45, O103, O104, O111, O121, O145, and O157) and three virulence genes (stx_1, stx_2, and *eae*) *(57)*.
[f] —, not computed due to complete agreement.

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Results from detection and isolation of STEC from all *stx*-positive samples ($n = 419$) from the 10 pig flows that were directly plated onto MAC medium are shown in Table 3. Of the 419 samples positive for stx_1 and/or stx_2 (70.1%) tested by the 11-plex PCR, 159 samples (26.7% of the total fecal samples) yielded pooled colonies (pool of 10 randomly picked putative *E. coli* colonies) that were positive for stx_1 and/or stx_2 genes. When the colonies from the *stx*-positive pools were individually tested by 11-plex PCR (O26, O45, O103, O104, O111, O121, O145, and O157 serogroups and stx_1, stx_2, and *eae* genes), a total of 154 pure cultures were obtained that were positive for stx_1 and/or stx_2. Overall, the number of samples that yielded pooled colonies positive for either stx_1 or stx_2 and the number of pure cultures obtained positive for stx_1 or stx_2 were different among 10 flows ($P < 0.05$; Table 3). The prevalence of *stx*-positive serogroups ranged from 3.3% in samples from pig flow in Kansas to 43.3% in samples collected from one of the pig flows in Iowa and South Dakota. Of the 154 isolates obtained, 14 (2.3% of the total fecal samples) belonged to one or more of the eight serogroups (top-7 and O104) and the remaining 140 (23.4% of the total fecal samples) belonged to other serogroups (Table 3). All 14 top-7 STEC were identified as serogroup O121.

Among the 140 STEC isolates subjected to mPCR assays, serogroups were identified for 113 isolates and 27 isolates were negative for the targeted 137 serogroups (Table 4). Among the 20 serogroups identified, three serogroups, O8 (31 of 140), O86 (19 of 140), and O100 (9 of 140), were the most predominant. The prevalence of all 23 STEC serogroups and unidentified serogroups, isolated with or without IMS, expressed as the proportion of the total fecal samples tested ($n = 598$) in the study are shown in Figure 1. The three predominant serogroups of STEC isolated in pure cultures from 598 fecal samples were O8 (5.9%), O121 (4.3%), and O86 (3.2%) (Fig. 1).

Stx gene types and subtypes. In total, 178 STEC strains, which included 26 stx_1 strains and 152 stx_2 strains, were subtyped (Table 5). If a fecal sample yielded more than one isolate by both culture methods (IMS and direct plating) and the isolates belonged to the same serogroup and had the same Stx type, only one isolate from that sample was used for subtyping. None of the 178 strains contained both stx_1 and stx_2. The two strains of stx_1-positive O26 and O103 were also positive for *eae* gene. Of the 152 stx_2-positive serogroups identified, only serogroup O51 ($n = 2$) was positive for *eae* gene. All 26 stx_1 strains contained stx_{1a} subtype. All 152 stx_2-positive strains, which included 122 strains of identified serogroups and 30 strains of unknown serogroups, contained stx_{2e} subtype. The 27 strains of O121 obtained with or without IMS method, which is one of the top-6 non-O157 STEC involved in human infections, also contained stx_{2e} (Table 5).

Prevalence of enterohemolysin and enterotoxin genes. Only two serogroups, O26 and O103, were positive for the *ehxA* gene (Table 5). Among the enterotoxin genes tested, *astA* (EAST1) was the most prevalent (74 of 178, 41.6%), followed by heat-stable enterotoxins *estA* (30 of 178, 16.9%) and *estB* (22 of 178, 12.4%) (Table 5). None of the STEC strains contained the heat-labile enterotoxin gene (*elt*). Serogroups O26 and O103, which are two of the top-7 STEC involved in human infections and positive for stx_1, were negative for the four enterotoxin genes (*astA, estA, estB,* and *elt*). All the other stx_1-positive serogroups, except O149, were positive for the EAST1 gene. Serogroup O121, another top-7 STEC and positive for stx_{2e}, was positive for EAST1 gene and negative for the other three genes. Shiga toxin 2–positive serogroups of O2, O36, O51, and O143 were negative for all the enterotoxin genes. Of the 30 stx_{2e}-positive strains of unknown serogroups, 6 were positive for EAST1 and 15 were positive for each of two heat-stable enterotoxins (Table 5). Two strains of O159 serogroup, 2 of the 35 strains of O8 serogroup, and 5 strains of the 30

TABLE 3. *Detection and isolation of Shiga toxin–producing* Escherichia coli *(STEC) from Shiga toxin gene–positive fecal samples (*n *= 419) that were directly plated onto MacConkey agar*[a]

Item	Kansas (n = 60)	Minnesota (n = 60)	Iowa: Pig flow 1 (n = 60)	Iowa: Pig flow 2 (n = 60	Oklahoma (n = 60)	Ohio (n = 59)	South Dakota: Pig flow 1 (n = 60)	South Dakota: Pig flow 2 (n = 60)	Nebraska (n = 60)	North Carolina (n = 59)	Total (n = 598)
No. (%) of samples with pooled colonies positive for stx_1 and/or stx_2[b]	2 A (3.3)	19 BCD (31.7)	26 CD (43.3)	20 BCD (33.3)	14 ABC (23.3)	9 AB (15.3)	17 ABCD (28.3)	16 ABCD (26.7)	30 D (50.0)	6 AB (10.2)	159 (26.7%)
No. (%) of pure cultures positive for stx_1 and/or stx_2[c]	2 A (3.3)	23 C (38.3)	26 C (43.3)	18 BC (30.0)	11 ABC (18.3)	11 ABC (18.6)	26 C (43.3)	14 ABC (23.3)	17 ABC (28.3)	6 AB (10.2)	154 (25.6%)
No. (%) of pure cultures positive for the eight STEC serogroups[c]	2	1	0	0	0	0	1	5	0	5	14 (2.3)
No. (%) of pure cultures positive for serogroups other than the eight serogroups[d]	0	22 BCD	26 D	18 BCD	11 ABC	11 ABC	25 CD	9 AB	17 BCD	1 A	140 (23.4)

[a] Means within a row sharing the same letter are not statistically different ($P > 0.05$).
[b] Determined by real-time PCR targeting stx_1 and stx_2 genes *(52)*.
[c] Determined by 11-plex conventional PCR targeting eight serogroups (O26, O145, O103, O104, O111, O121, O145, and O157) and three virulence genes (stx_1, stx_2, and *eae*) *(68)*.
[d] Isolates that were positive for stx_1 or stx_2 gene, but negative for the eight serogroups.

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TABLE 4. *Detection of serogroups of Shiga toxin–producing* Escherichia coli *(STEC), other than the top-7 (O26, O45, O103, 111, O121, O145, and O157), isolated by direct plating of Shiga toxin–positive fecal samples (*n *= 419) on MacConkey agar by 14 sets of multiplex PCR assays*[a]

			Iowa				South Dakota				
Serogroup	Kansas (n = 60)	Minnesota (n = 60)	Pig flow 1 (n = 60)	Pig flow 2 (n = 60	Oklahoma (n = 60)	Ohio (n = 59)	Pig flow 1 (n = 60)	Pig flow 2 (n = 60)	Nebraska (n = 60)	North Carolina (n = 59)	Total (n = 598)
O2			2								2
O8		9	5	6		7	2	1	1		31
O9			1	1	1		3				6
O36			4				1	1			6
O51		1					1				2
O55									1		1
O69			2								2
O76		1	2	3							6
O81			1								1
O86						1	8	3	6	1	19
O100			1	1		1	4	2			9
O110			1								1
O115		3		2							5
O120		1									1
O131							2				2
O143				1							1
O149		3	2	1							6
O159			1						1		2
O174		2		2		2					6
O180		2	2								4
Unidentified			2	1	10		4	2	8		27
Total (%)	0 (0)	22 (36.7)	26 (43.3)	18 (30.0)	11 (18.3)	11 (18.6)	25 (41.7)	9 (15.0)	17 (28.3)	1 (1.7)	140 (23.4)

[a] Serogroups identified by using 14 sets of mPCR assays targeting 137 serogroups of STEC other than the top-7 and O104 serogroups *(45)*.

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unidentified serogroups were positive for all three enterotoxin genes (*astA, estA,* and *estB*).

DISCUSSION

The fecal samples (n = 598) used in this study were from 10 pig flows located in the top swine-producing states in the United States. The PCR method detected 208 samples (34.8%) as positive for one or more of the eight STEC serogroups (O20, O45, O103, O104, O111, O121, O145, and O157). The culture method (IMS followed by plating on selective media) yielded a total of 190 serogroup-positive isolates in pure cultures. Among the eight targeted STEC serogroups, the five predominant serogroups detected were O26, O45, O104, O121, and O157. However, only a small number of the isolates (25 of 190) were positive for the *stx* gene. A similar high prevalence of STEC serogroups negative for *stx* gene have also been reported in feces of cattle *(51)*. Absence of *stx* gene, which is carried on a mobile genetic element (prophage), in a known STEC serogroup suggests either the loss of the phage or the organism has the potential to acquire the phage to become Shiga toxigenic *(5, 15)*. Also, it is possible these serogroups had H types, which are not known to carry the prophage. Interestingly, none of the O157 isolates obtained in this study was positive for Stx gene or H7 gene, indicating that O157 strains had other H type and not pathogenic, unlike O157:H7. This is in contrast to prevalence in cattle in which the O157 isolates are almost always positive for one or both *stx* and H7 genes *(51)*. In a 2000 National Animal Health Monitoring System study that involved 687 fecal samples collected from finisher pigs in 13 top swine-producing states, none of the *stx*-positive isolates obtained belonged to the O157 serogroup *(30)*. In another National Animal Health

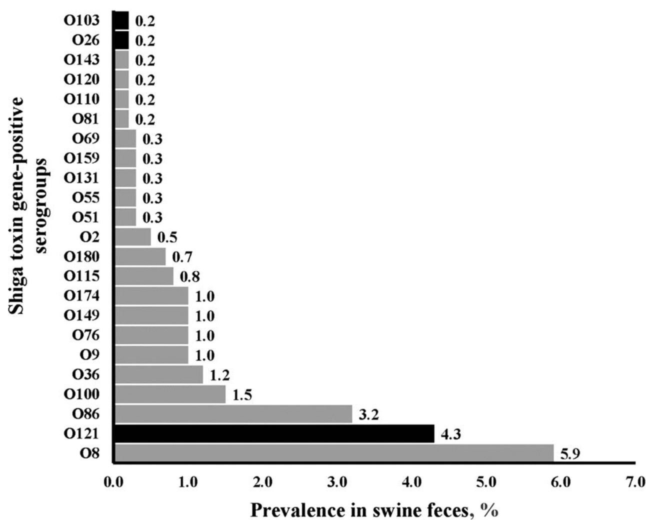

FIGURE 1. *Prevalence of top-7 (dark bars) and non–top-7 (gray bars) serogroups of Shiga toxin gene–positive* Escherichia coli *isolates as proportion of fecal samples (*n *= 598) obtained by cultured method.*

TABLE 5. *Prevalence of Shiga toxin gene subtypes, intimin, and enterotoxin genes in* Escherichia coli *strains isolated from swine feces*

Serogroup	No. of strains	Shiga toxin gene		Intimin gene, *eae*	Enterohemolysin gene, *ehxA*	Enterotoxin gene[a]			
		Type	Subtype			*astA*	*elt*	*estA*	*estB*
O26	1	stx_1	stx_{1a}	1	1				
O103	1		stx_{1a}	1	1				
O110	1		stx_{1a}			1			
O69	2		stx_{1a}			2			
O180	4		stx_{1a}			4			
O115	5		stx_{1a}			5			
O76	6		stx_{1a}			6			
O149	6		stx_{1a}						
O81	1	stx_2	stx_{2e}			1			
O120	1		stx_{2e}			1			
O143	1		stx_{2e}						
O51	2		stx_{2e}	2					
O55	2		stx_{2e}			2			
O159	2		stx_{2e}			2		2	2
O131	2		stx_{2e}			2			
O2	3		stx_{2e}						
O9	6		stx_{2e}					2	2
O174	6		stx_{2e}					2	
O36	7		stx_{2e}						
O100	9		stx_{2e}			3		6	
O86	19		stx_{2e}						1
O121	26		stx_{2e}			26			
O8	35		stx_{2e}			13		3	2
Unidentified	30		stx_{2e}			6		15	15

[a] *astA,* enteroaggregative heat-stable enterotoxin 1 (EAST1); *elt,* heat-labile enterotoxin (LT); *estA,* heat-stable enterotoxin A (STa); *estB,* heat-stable enterotoxin (STb).

Monitoring System study that tested swine feces for *E. coli* O157:H7, serogroup O157 was isolated from 106 (4.2%) of 2,526 fecal samples and only five (0.2%) isolates contained the *stx* gene *(26)*. In studies that have reported isolation of O157 from swine feces, detection of isolates with *stx* gene is a rare occurrence *(16, 18, 27)*. Interestingly, outbreaks of STEC infection associated with pork products were more often with O157 than other STEC serogroups *(18, 37, 74)*.

The predominant *stx*-positive serogroup among the top-7 was O121 (23 of 25 *stx*-positive isolates). In studies that have reported prevalence and characteristics of STEC in finisher pigs in the United States, serogroup O121 was not detected or detected at a much lower rate *(16, 30, 75)*. The difference is likely because of the use of O121-specific IMS beads in our study, which likely enhanced the probability of isolation from feces. The mPCR assay, designed to detect the top-7 serogroups and three virulence genes in cattle feces, also included serogroup O104 *(55, 68)* because in 2011, O104:H4, a hybrid pathotype of STEC and enteroaggregative *E. coli,* was involved in a major foodborne outbreak in Europe *(6)*. Also, O104 serogroup-specific IMS beads are commercially available and culture method for selective isolation and identification from cattle feces have been described previously *(68)*. Although serogroup O104 strains were isolated in this study (25 of 598, 4.2%), none of them possessed *stx* genes.

Because no IMS beads are commercially available and no selective media have been developed for isolation of STEC other the top-7 and O104, fecal samples positive for *stx* gene were directly plated onto media (MAC and EMB) that are selective and somewhat differential for *E. coli*. Other studies have used commercial chromogenic agar such as CHROMagar or Rainbow agar *(13, 16, 75)*. Use of different media can affect the ability to isolate different STEC serogroups. Some of the commercial chromogenic agar media contain a selective agent, tellurite, which is inhibitory to certain STEC strains *(36, 56)*. Neither MAC nor EMB allows phenotypic identification of any particular serogroup or pathotype of *E. coli*. In the initial comparison of the two media, neither medium was superior to the other; therefore, MAC was chosen as the medium for isolation for the remaining fecal samples. The nonselective isolation method allowed us to obtain a total of 154 isolates (25.8% of the total fecal samples) positive for *stx* genes in pure cultures, which included 14 top-7 STEC (all O121) and 140 non–top-7 STEC. Of all the non–top-7 STEC serogroups identified in this study, the serogroups with the highest prevalence were O8 (35 of 598, 5.9%), O86 (19 of 598, 3.2%), O100 (9 of 598, 1.5%), and O36 (7 of 598, 1.2%). The predominance of *E. coli* O8 in swine feces agrees with previous studies *(30, 31, 44)*. Serogroup O8 is one of the common serogroups involved in postweaning diarrhea in weaned piglets *(33, 35)*. It is not surprising that a small proportion of strains were unidentified because even with

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the conventional serological method, a proportion of *E. coli* isolates are untypeable *(21)*. It is possible that the unidentified serogroups belong to the 13 serogroups of STEC that were not targeted in the mPCR assays *(45)*. Also, a couple of STEC serogroups, O14 and O57, which do not contain O-antigen biosynthesis gene clusters, are negative in PCR assays *(20, 39)*. It would be of interest to have these unidentified strains whole genome sequenced for in silico serogrouping *(41)*.

A majority of the STEC isolates from swine feces (152 of 178, 85.4%) carried stx_2 gene. Of the two Stx types, Stx2 is more cytotoxic and more commonly associated with severity and complications of human STEC illnesses than Stx1 *(22, 47, 60)*. Shiga toxin 2, particularly in association with intimin, results in a higher risk for severe infections *(12)*. Only 1 of the 22 stx_2-positive serogroups, O51, carried intimin (*eae*) gene. Intimin is the best-characterized adhesin that mediates attachment and induces effacement of enterocytes *(71),* but there are other adhesins in STEC, such as autoagglutinating adhesin, encoded by *saa* gene, that can mediate attachment *(58, 59)*. The rarity of *eae* gene in swine STEC strains agrees with other reports *(4, 70, 76)*. The 26 strains belonging to eight serogroups carrying Stx1 gene possessed stx_{1a}, a subtype that has the potential to cause severe infections particularly in association with intimin (*eae*) gene *(14)*. Only two serogroups, O26 and O103, were positive for *eae* gene, and these two serogroups are the two most commonly implicated in human STEC infections, next only to O157 *(14, 65)*.

Interestingly, all 152 stx_2-positive isolates, which included both identified (n = 122) and unidentified (n = 30) serogroups, carried the stx_{2e} subtype. All strains of serogroup O121 (n = 26), which is one of the top-7 serogroups involved in human STEC illnesses, also carried stx_{2e} gene. The same serogroup isolated from cattle feces or human clinical cases generally carries a *stx* subtype other than stx_{2e} *(68)*. Shiga toxin 2e is the most common subtype of Stx found in *E. coli* isolated from swine feces and pork products *(4, 11, 30),* which is likely because Stx2e-producing *E. coli* is the cause of edema disease, characterized by subcutaneous and submucosal edema, neurological signs, and death, in weaned piglets. In contrast to swine, Stx2e-producing *E. coli* is rarely isolated from other animals *(11)*. Although several stx_{2e}-positive strains, none except O8 belonged to the O groups frequently involved in edema disease, which are O8, O138, O139, O141, and O147 *(1, 23, 35, 76)*.

Besides Stx genes, several strains were positive for genes that encode for heat-stable enterotoxigenic (*estA* and *estB*) and enteroaggregative (*astA*) enterotoxins, which are the toxins involved in *E. coli* causing neonatal diarrhea and postweaning diarrhea in swine *(23, 29, 33, 40, 79)*. Interestingly, none of the strains carried the heat-labile enterotoxin gene (*elt*), which agrees with reports on stx_{2e}-producing *E. coli* isolated from swine and pork products *(11)*. The prevalence of Shiga toxin gene–positive *E. coli* strains harboring enterotoxin genes in swine feces is not uncommon *(4, 7, 28, 33, 53, 63, 67, 78, 80)*.

Shiga toxin 2e–producing *E. coli* are not a major threat for causing human infections, but they have been isolated from feces of healthy humans and in sporadic cases of mild diarrhea *(9, 10, 32, 48, 61, 70)*. A couple of stx_{2e}-positive serogroups, O60 and O101, which are more frequently reported to cause human infections, were not detected in this study *(32)*. Certain serotypes producing Stx2e, such as O9:H$^-$, O101:H$^-$, and O51:H49, have been associated with hemolytic uremic syndrome, a serious complication of STEC infection *(25, 73)*. There is evidence that stx_{2e}-positive *E. coli* involved in human infections have acquired specific virulence determinants to facilitate adaptation to the human host *(70)*. In addition, several stx_{2e}-positive strains also contained genes that produce enterotoxins (EAST1 or heat-stable enterotoxin), which have the potential to cause diarrhea in humans.

Our findings in this study show that swine in the finishing phase harbored, and shed in the feces, several serogroups of STEC that included both top-7, implicated in human infections, and non–top-7. However, only a small proportion of the serogroup-positive strains isolated carried Stx gene and a majority did not carry the gene that codes for intimin, a major colonizing factor. Shiga toxin gene–positive O157, the most common serogroup of human STEC infections, including infections linked to pork, was not detected in swine feces. A majority of STEC strains isolated in the study carried Stx gene subtype 2e, which is not considered to be of major public health importance and is only involved in sporadic cases of mild diarrhea. Our study shows that healthy pigs in the finishing phase harbor and shed several serogroups of *E. coli* carrying virulence genes involved in neonatal diarrhea, postweaning diarrhea, and edema disease, but prevalence of STEC that are of public health threat is low.

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