Evaluation of Cattle for Naturally Colonized Shiga Toxin-Producing Escherichia coli Requires Combinatorial Strategies

Indira T. Kudva, Eben R. Oosthuysen, Bryan Wheeler, and Clint A. Loest

1Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, IA, USA
2Department of Animal and Range Sciences, New Mexico State University, Las Cruces, NW, USA

Correspondence should be addressed to Indira T. Kudva; indira.kudva@usda.gov

Received 5 December 2020; Revised 6 February 2021; Accepted 21 March 2021; Published 1 April 2021

Academic Editor: Elena Sorrentino

Copyright © 2021 Indira T. Kudva et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Shiga toxin-producing Escherichia coli (STEC) serogroups O157, O26, O103, O111, O121, O145, and O45 are designated as food adulterants by the U.S. Department of Agriculture-Food Safety and Inspection Service. Cattle are the primary reservoir of these human pathogens. In this study, 59 Angus crossbred heifers were tested specifically for these seven STEC serogroups using a combination of standard culture, serological, PCR, and cell cytotoxicity methods to determine if comparable results would be obtained. At the time of fecal sampling, the animals were approximately 2 years old and weighed 1000–1200lbs. The diet comprised of 37% ground alfalfa hay, 25% ground Sudan hay, and 38% ground corn supplemented with trace minerals and rumensin with ad libitum access to water. Non-O157 STEC were isolated from 25% (15/59) of the animals tested using a combination of EC broth, CHROMagar STECTM, and Rainbow Agar O157. Interestingly, the O157 serogroup was not isolated from any of the animals. Non-O157 STEC isolates were confirmed to be one of the six adulterant serogroups by serology and/or colony PCR in 10/15 animals with the predominant viable, serogroup being O103. PCR using DNA extracted from feces verified most of the colony PCR results but also identified additional virulence and O-antigen genes from samples with no correlating culture results. Shiga toxin- (Stx-) related cytopathic effects on Vero cells with fecal extracts from 55/59 animals could only be associated with the Stx gene profiles obtained by fecal DNA PCR and not culture results. The differences between culture versus fecal DNA PCR and cytotoxicity assay results suggest that the latter two assays reflect the presence of nonviable STEC or infection with STEC not belonging to the seven adulterant serogroups. This study further supports the use of combinatorial culture, serology, and PCR methods to isolate viable STEC that pose a greater food safety threat.

1. Introduction

Shiga toxin-producing Escherichia coli (STEC) is the third leading cause of foodborne illness after Campylobacter and Salmonella, implicated in 265,000 illnesses in the US and 2.8M infections globally [1–3]. A combined economic loss to public health, agriculture, and meat industry estimated at $993 million per year attributed to STEC contamination of foods and human infections prompted the declaration of commonly implicated STEC serogroups (O157, O26, O103, O111, O121, O145, and O45) as food adulterants by the USDA-Food Safety and Inspection Service (FSIS) [2, 4–9]. STEC infections are acquired through the fecal-oral route following ingestion of bacteria-contaminated food or water or after contact with infected animals and humans [10–13]. Following infection, some individuals remain asymptomatic, while others develop watery diarrhea to HUS or thrombotic, thrombocytopenic purpura [14]. No specific therapies are available for treating STEC infections in humans. STEC can infect in low doses (<10 viable bacteria) due to multiple acid tolerance and quorum sensing.
mechanisms [11, 12, 15, 16]. Virulence factors such as phage-encoded Shiga toxins (Stx) [17], Stx1 and Stx2, plasmid-encoded hemolysis (HlyA) [18], and various adhesion factors including intimin, encoded by the eae gene on the pathogenicity island locus of enterocyte effacement (LEE), play a significant role in human disease [17].

Cattle are considered the primary STEC reservoirs as most outbreaks are directly or indirectly associated with cattle [19, 20]. Cattle remain asymptomatic due to the absence of the Gb3 receptors for Stx; without uptake of toxin, there is no resulting systemic failure as observed in humans [21–23]. Although STEC can be isolated from various gastrointestinal tract sites, they persist at the rectoanal junction (RAJ) [24, 25]. Average duration of bovine O157 carriage is 30 days, although colonization of up to 1 year has been reported [26–28]. Cattle shed STEC in a seasonal pattern, with increased shedding in warmer months and decreased shedding in winter [20]. Animals shedding greater than 10^4 CFU/g feces, termed “super-shedders” contribute decreased shedding in winter [20]. Animal shedding greater than 10^4 CFU/g feces, termed “super-shedders” contribute decreased shedding in winter [20].

Researchers have used different cultures, immunomagnetic separation (IMS), and PCR methods, solely or in combination, to improve the detection of STEC in field samples although with varied success [35–44]. For instance, when real-time PCR was used to screen 573 bovine fecal samples at slaughter for Stx genes (417/573) and STEC serogroups from 114 of the 1897 bovine fecal samples tested [47–49]. To improve the selection and differentiation of the top 6 non-O157 STEC serogroups to both culture and PCR for accurate detection of the six non-O157 STEC [46]. Vero cell cytotoxicity assays have been used to predict presence of STEC in feces by correlating cytotoxicity to Stx; however, as with PCR, the results may not always result in the isolation of viable STEC (47–49). To improve the selection and differentiation of the top 6 non-O157 STEC serogroups, a chromogenic agar media was developed that enabled isolation of these serogroups from 114 of the 1897 bovine fecal samples tested [47]. Likewise, in a study evaluating 120 beef cattle, a combination of MacConkey and modified Rainbow® Agar O157 serogroups increased the recovery frequency of non-O157 STEC strains from animal feces [48].

Based on these reports, in this study, we evaluated a combination of methods to determine the occurrence of O157 and the “Big 6” non-O157 STEC in dairy cows, known to be STEC-susceptible. We compared simple fecal culture techniques followed by serology and colony PCR to direct fecal DNA PCR and Vero cell cytotoxicity assays, in order to ascertain the variability/similarity of results when using these methods to determine the presence of viable O157 and the “Big 6” non-O157 STEC in bovine fecal samples.

2. Materials and Methods

2.1. Animals and Sampling. Standard husbandry and veterinary care was provided to the animals used in this study and sampling was carried out as approved by the New Mexico State University (NMSU) Institutional Animal Care and Use Committee. Fecal samples (50 g/animal) were collected by rectal palpation from a total of 59 Angus crossbred heifers, housed at NMSU Clayton Livestock Center in January 2017 (winter), and transported overnight on ice to NADC, Ames, IA, for processing. All samples were collected into sterile Falcon tubes (Thermo Scientific, Rockford, IL) using the appropriate aseptic technique of changing gloves between samples. At the time of sampling, the animals were approximately 2 years old and weighed 1000–1200 lbs with a body condition score of 6 (BCS range 1–9, 9 being extremely fat). All heifers had been artificially inseminated in October 2016 resulting in 52/59 (88%) of the cattle being pregnant as determined by the BioPRYN test that measures pregnancy-specific protein B in serum (Biotracking, Moscow, ID). The animals were conformed and housed in single soil surface pens with partial shade covering. The diet comprised of 37% ground alfalfa hay, 25% ground Sudan hay, and 38% ground corn with ad libitum access to water.

2.2. Bacterial Control Strains. Following strains were used as controls to verify culture, latex agglutination, and/or PCR protocols: (i) O157 strain EDL933 (ATCC 43895: stx1+, stx2−, eae−, hlyA+). (American Type Culture Collection/ATCC, Manassas, VA), (ii) O26:U (NADC 3108: O26+, stx1+, stx2−, eae+, hlyA−) (National Animal Disease Center/NADC, Ames, IA), (iii) O45:U (NADC 3802: O45+, stx1−, stx2+, eae+, hlyA+), (iv) O103:U (NADC 3358: O103+, stx1+, stx2+, eae−, hlyA−), (v) O111:U (NADC 3309: O111+, stx1−, stx2+, eae−, hlyA−), (vi) O121:H19 (ATCC BAA2221: O121+, stx1+, stx2−, eae−, hlyA−), (vii) O121 (ATCC BAA2190: O121+, stx1+, stx2−, eae−, hlyA−), and (viii) O145:U (NADC 3196: O145+, stx1+, stx2−, eae−, hlyA−).

2.3. STEC Isolation

2.3.1. O157 Culture. Previously standardized nonenrichment and selective enrichment culture protocols were used to isolate O157 with slight modifications [49–51]. Briefly, per the protocol, 10 g fecal sample was added to 50 ml Trypticase soy broth (BD Bioscience, San Jose, Ca.) supplemented with cefixime (50 μg/liter; U.S. Pharmacopeia, Washington D.C.), potassium tellurite (2.5 mg/liter; Sigma-Aldrich Corp., St. Louis, Mo.), and vancomycin (40 mg/liter; Alfa Aesar, Haverhill, Ma.) both before and after overnight incubation of the TSB-CTV-fecal suspension at 37°C with aeration. The dilutions prepared before incubation were spread plated onto sorbitol MacConkey agar (BD Biosciences) containing 4-methylumbelliferyl-β-d-glucuronide (100 mg/liter; Sigma) (SMAC-MUG) (nonenrichment cultures). SMAC-MUG
supplemented with cefxime (50 μg/liter), potassium tellurite (2.5 mg/liter), and vancomycin (40 mg/liter) (SMAC-CTMV) was used to plate the dilutions prepared after overnight incubation (selective-enrichment cultures). Both SMAC-MUG and SMAC-CTMV plates were read after overnight incubation at 37°C, and colonies that did not ferment sorbitol or utilize 4-methylumbelliferyl-β-d-glucuronide (nonfluorescent under UV light) were further evaluated to be O157 serologically.

2.3.3. STEC Serology. Latex agglutination tests were used to serologically confirm O157 (E. coli O157 latex, Oxoid Diagnostica Reagents, Oxoid Ltd., Hampshire, UK) and the “Big 6” non-O157 (E.coli non-O157 Identification Kit, Pro-Lab Diagnostics, Ontario, Canada) serogroups.

2.4. Serogroup and Virulence Gene Profiling

2.4.1. Colony Lysates. Colonies (control and fecal isolates) selected for PCR were subcultured from selective plates onto LB plates and used to prepare colony suspensions in sterile distilled water. The suspensions were boiled for 10 min, cooled, and centrifuged, and the lysates are used as template in PCR reactions.

2.4.2. Fecal DNA Extracts. Postincubation, 5 ml of each EC broth-fecal suspension was filtered through a 40 μm filter, and the filtrate was centrifuged (5000 rpm/10 min/4°C) to collect 200–250 mg of fecal material. DNA was extracted from the fecal material using standard instructions provided with the QIAmp DNA stool kit (Qiagen, Germantown, MD). DNA yield and purity were evaluated with the Nanodrop (Life Technologies Corp., Grand Island, NY) and verified by electrophoresis on a 4% agarose gel.

2.4.3. PCR Conditions. Previously described primers [56–58] were used to amplify the wzx genes in the O-antigen cluster of non-O157 serogroups and the virulence genes as shown in Table 1. Degenerate primers targeting all variants of the stx and eae genes were also included (Table 1) [56, 58]. PCR was carried out on the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) using 10 μl of colony lysate, 200 pmol of each primer, 800 μM deoxynucleoside triphosphates, 1X diluted Ex Taq enzyme buffer, and 2.5 μl of TaKaRa Ex Taq DNA polymerase. The hot-start PCR technique was used in combination with a touchdown PCR profile [59] comprising of 20 cycles starting with an annealing temperature of 73°C with touchdown at 53°C at the end of those cycles. Additional amplification segment of 10 cycles was set, using the last annealing temperature of 53°C.

2.5. Vero Cell Cytotoxicity Assay

2.5.1. Vero Cell Culture. Vero cells (African Green Monkey Kidney cells, ATCC CCL-81), obtained from the ATCC, Manassas, Va., were grown in Dulbecco’s modified Eagle’s medium with low glucose, DMEM-LG (Invitrogen, Carlsbad, CA) with additional 10% fetal bovine serum.

2.5.2. Fecal Extract Preparation. Five ml of each EC broth-fecal suspension was filtered through a 40 μm filter, and the filtrate was centrifuged (1000xg/20 min/4°C) to collect debris-free supernatant/fecal extracts.

2.5.3. Assay for Cytotoxicity in the Absence of Antisera. The cytotoxicity assay was conducted as previously described with slight modification [60]. Vero cells were seeded at 10^5 cells/well in 24-well microtiter plates (Costar, Corning, Ma.) and incubated in the presence of 5% CO2 at 37°C for 24 h until confluency was reached. Serial dilutions (1:2 to 1:64) of the fecal extracts were prepared in DMEM-LG, and 100 μl of each dilution was added per well of the microtiter plates. Plates were incubated for 2 days at 37°C with 5% CO2. The cells were microscopically examined for cytotoxicity each day with a final read on the second day. Cytotoxic effects (visualized as detached, rounded cells) were scored 1 through 4 corresponding to <25%, 50%, 75%, and >90% cells affected. Control wells with only media were included on each test plate to verify that the cytotoxic effects observed were with the fecal extracts or purified toxins alone. Serial dilutions (1:2 to 1:256) of purified Shiga toxins (Stx-1 and Stx-2; each at a concentration of 50 ng/100 μl) from the NADC stock (NADC, Ames, IA) were tested on the Vero cells separately to validate the procedure.

2.5.4. Cytotoxicity Inhibition in the Presence of Anti-Stx1 or Anti-Stx2 Antisera. The toxin neutralization assay was performed as described previously [61] with slight
modification to determine whether the observed CPE was caused by Stx1 and/or Stx2 or other undefined factors. Briefly, microtiter plates with Vero cells and serial dilutions of the fecal extracts were set up as described above. However, in this instance, 100 μl of each fecal extract dilution was mixed with 100 μl polyclonal bovine anti-Stx1 or rabbit anti-Stx2 antisera (NADC stock) and incubated at 37°C/110rpm followed by overnight incubation at 4°C without shaking. -Q helastdilutionatwhichthefecalextractsproducedCPEon Vero cells, in the absence of antisera, was selected for this neutralization assay. A 100 μl sample from each “diluted extract-antisera” mix was added per well of the microtiter plates that were incubated and scored for protection (cells lack CPE) or no protection (cells continue to show CPE). Control wells with only media or fecal extract dilutions were included on each test plate to verify the neutralization effects if any. Additionally, purified Shiga toxins (as above), at a dilution of 1:256, were mixed with antisera and tested on the Vero cells separately to validate the procedure.

3. Results and Discussion

Transportation to processing plants and fasting increase STEC fecal shedding by colonized cattle [26, 62, 63]. STEC on hides are common sources of postharvest (after slaughter) carcass contamination; if STEC colonization of animals goes undetected, these foodborne pathogens could readily spread into packing plants, food processing plants, and consequently enter our food supply [64]. To prevent this farm to fork spread of pathogens, USDA-FSIS instituted the Hazard Analysis Critical Control Point (HACCP) program requiring slaughter facilities to decontaminate at critical carcass processing points [65]. However, efficient STEC control in cattle could enhance the success of the HACCP program [64, 66], and for this, sensitive techniques are needed to detect these foodborne pathogens present in variable concentrations in bovine feces prior to harvesting. Taking into account some of the published studies [35–40, 42–48, 60, 61, 67], we chose a combination of selective culture, serology, conventional colony PCR, direct fecal PCR methods, and cell cytotoxicity assay to detect STEC in 59 bovine fecal samples and determine if these would yield comparable results.

We cultured putative non-O157 STEC from feces of 15/59 (25%) animals tested (Table 2) using EC broth, CHROMagar STEC™, and Rainbow agar O157 plates [41, 52–55] in this study. Utilizing two different selective agar media allowed for a two-tiered differentiation of STEC from background flora. A 100 μl sample from each “diluted extract-antisera” mix was added per well of the microtiter plates that were incubated and scored for protection (cells lack CPE) or no protection (cells continue to show CPE). Control wells with only media or fecal extract dilutions were included on each test plate to verify the neutralization effects of the extract-antisera mix, if any. Additionally, purified Shiga toxins (as above), at a dilution of 1 : 256, were mixed with antisera and tested on the Vero cells separately to validate the procedure.

Table 1: Primers used in this study.

| Sequence 5′ ⟷ 3′ | Amplicon size (bp) | Reference |
|------------------|-------------------|-----------|
| Wzx158-O26-F     | GTA TCG CTG AAA TTA GAA GCC C | 158       |
| Wzx158-O26-R     | AGT TGA AAC ACC CTG ATT GGC |           |
| Wzx27-O45-F      | GTG TGT GCA TGG TGG CAT | 72        |
| Wzx27-O45-R      | TGG CCA AAC CAA CTA TGA ACT | 191       |
| Wzx191-0103-F    | ATG TTC GCT ATA TCT TCT TGG GCC | [57]     |
| Wzx191-0103-R    | TGT TCC AGG TGG TAG GAT TCG |           |
| Wbdi-O111-F      | TCA CGA TGT TGA TCA TCT GGG | 189       |
| Wbdi-O111-R      | AGG CGC TGA TGT TGT GTC TCT TAG a |           |
| Wzx189-O121-F    | GAA CCG AAA TGA TGG GTG CT | 135       |
| Wzx189-O121-R    | CAT TGA TCA GGA GAC GAC GGC G |           |
| Stx (Stx1/2)-F1  | TTT GTY ACT GTS ACA GCW GAA GCC TTA CG | 131 bp (stx1) |
| Stx (Stx 1/2)-R1 | CCC CAG TTC ARW GTR AGC TCM ACD TC | 128 bp (stx2) |
| Eae-F            | CAT TGA TCA GGA GAC GAC GGC G |           |
| Eae-R1           | CTC ATG CCG AAA TAG CCG TTM | 102       |
| O26-F            | CAATGGGCC GAA TTT TAG A | 155       |
| O26-R            | ATAAATTTTCTCGGCTGTCGC |           |
| O121             | TCGCAAAATTTGCTGGAAA | 628       |
| O121-R           | AGAAAG TGT GAA TGG CCG T |           |
| Stx1-F           | ATAAATGCGCAATTTGCGTAC | 180       |
| Stx1-R           | AGAACGCCCACGAGATCATC |           |
| Stx2-F           | GGCACCTGCTGAACTGCTGAC | 255       |
| Stx2-R           | TCGCCAGTATCTGACATGTCTG |           |
| HlyA-F           | GCCATCATCAAGGCGATGTCGC | 534       |
| HlyA-R           | AATGAGCCAAGCTGGTAAAGCT |           |

1Degenerate nucleotide codes are as follows: Y (C, T); S (C, G); W (A, T); R (A, G); M (A, C); D (A, G, T).
| Animal number | Number of fluorescent colonies shown as CFU/ml | Colony lysate PCR | Fecal–DNA PCR | Vero cell assay |
|---------------|-----------------------------------|-----------------|----------------|----------------|
|               | Latex agglutination-based serogroup of non-O157 isolates |                |                |                |
|               | O103 | O111 | O121 | O145 | O26 | O45 | eae | stx | stx1 | stx2 | hlyA | CPE dilution | CPE scores with anti-Stx1 sera | No CPE with anti-Stx2 sera |
| 5602 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 5620 | $7 \times 10^3$ | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 5657 | $5 \times 10^4$ | O103 | – | – | – | – | – | – | – | – | – | – | – | – |
| 5724 | – | – | – | – | – | – | – | – | – | – | – | – | 0 | 3 |
| 5743 | – | – | – | – | – | – | – | – | – | – | – | – | 16 | 1 |
| 5759 | $1 \times 10^4$ | NT | – | – | – | – | – | – | – | – | – | – | – | 2 |
| 5760 | $1 \times 10^6$ | O103 | + | – | – | – | + | + | + | + | 8 | 1 |
| 5771 | $4 \times 10^4$ | NT | – | – | – | – | – | + | + | + | + | 8 | 1 |
| 5776 | $5 \times 10^4$ | NT | – | – | – | – | – | + | + | + | + | 8 | 1 |
| 5787 | – | – | – | – | – | – | – | – | – | – | – | 0 | 4 |
| 5800 | – | – | – | – | – | – | – | – | – | – | – | 4 | 1 |
| 5825 | $4 \times 10^4$ | O103 | + | – | – | – | + | + | + | + | 4 | 1 |
| 5845 | – | – | – | – | – | – | – | – | – | – | + | 2 | 2 |
| 5868 | – | – | – | – | – | – | – | – | – | – | + | 2 | 2 |
| 5874 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 5875 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 5889 | – | – | – | – | – | – | – | – | – | – | + | 4 | 1 |
| 5901 | – | – | – | – | – | – | – | – | – | – | + | 8 | 1 |
| 5915 | – | – | – | – | – | – | – | – | – | – | + | 0 | 0 |
| 5916 | $4 \times 10^4$ | O103 | + | – | – | – | + | + | + | + | 0 | 0 |
| 5922 | – | – | – | – | – | – | – | – | – | – | + | 4 | 3 |
| 5935 | $2.4 \times 10^4$ | O103 | + | – | – | – | + | + | + | + | 4 | 3 |
| 5938 | $6.5 \times 10^6$ | O26 | – | – | – | – | + | + | + | + | 8 | 1 |
| 5939 | – | – | – | – | – | – | – | – | – | – | + | 2 | 2 |
| 5949 | – | – | – | – | – | – | – | – | – | – | + | 8 | 1 |
| 5950 | – | – | – | – | – | – | – | – | – | – | + | 8 | 3 |
| 5964 | – | – | – | – | – | – | – | – | – | – | + | 8 | 3 |
| 5966 | – | – | – | – | – | – | – | – | – | – | + | 8 | 1 |
| 5982 | $3.5 \times 10^8$ | O103 | + | – | – | – | + | + | + | + | 0 | 2 |
| 5985 | $3 \times 10^9$ | NT | – | – | – | – | + | + | + | + | 8 | 1 |
| 5996 | – | – | – | – | – | – | – | – | – | – | + | 4 | 3 |
| 6009 | – | – | – | – | – | – | – | – | – | – | + | 4 | 3 |
| 6011 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6012 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6013 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6017 | $2 \times 10^7$ | O103 | + | – | – | – | + | + | + | + | 2 | 1 |
| 6044 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6049 | – | – | – | – | – | – | – | – | – | – | + | 0 | 3 |
| 6069 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6082 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6089 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6101 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6120 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6122 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6130 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| 6137 | – | – | – | – | – | – | – | – | – | – | + | 2 | 1 |
| Animal number | Number of mauve, fluorescent colonies shown as CFU/ml<sup>1</sup> | Latex agglutination-based serogroup of non-O157 isolates | Colony lysate PCR | Fecal-DNA PCR | Vero cell assay | No CPE<sup>4</sup> with anti-Stx1 sera | No CPE<sup>4</sup> with anti-Stx2 sera |
|---------------|---------------------------------------------------------------|--------------------------------------------------------|------------------|---------------|----------------|---------------------------------|----------------------------------|
| 6141          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | CPE dilution<sup>3</sup> CPE scores | -                              | -                                |
| 6148          | 2 × 10<sup>5</sup>                                            | NT                                                     | -                | -             | + + + + 2 2 n y | -                              | -                                |
| 6150          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 2 3 n n | -                              | -                                |
| 6153          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 2 2 n y | -                              | -                                |
| 6171          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 0 3 y y | -                              | -                                |
| 6179          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 4 3 y y | -                              | -                                |
| 6507          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 8 1 n y | -                              | -                                |
| 6509          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 2 4 y y | -                              | -                                |
| 6529          | 5 × 10<sup>4</sup>                                            | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 4 3 n y | -                              | -                                |
| 6542          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 2 1 y y | -                              | -                                |
| 6561          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 2 1 n y | -                              | -                                |
| 6582          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 0 3 y y | -                              | -                                |
| 6584          | -                                                             | O103 O111 O121 O145 O26 O45 eae stx stx1 stx2 hlyA O103 O111 O121 O145 O26 O45 eae Stx stx1 stx2 hlyA | -                | -             | + + + + 0 3 y y | -                              | -                                |

<sup>1</sup>Putative non-O157 STEC forming mauve, fluorescent colonies on CHROMagar STEC<sup>TM</sup>, and postenrichment in EC broth were further analyzed by serology and colony PCR.<sup>2</sup>NT, not typed to either of the "Big 6" non-O157 STEC serogroups by latex agglutination.<sup>3</sup>The last dilution at which CPE still observed on the Vero cells was selected when setting up the toxin neutralization assays.<sup>4</sup>y, yes; n, no; n/a, not applicable as no CPE was observed when at undiluted concentrations.
O121 were isolated from 9, 2, and 1 bovine fecal samples, respectively, with O26 and O121 being coisolated with other serogroups (Table 2). Colonies that appeared to be non-O157 STEC, based on the phenotype on the CHROMagar STEC™ and Rainbow agar O157 plates, were also isolated from 5/15 animals, but these could not be assigned to either of the "Big 6" non-O157 STEC based on serology and colony PCR (‘NT’; Table 2). Interestingly, O157 was not isolated from any of the animals tested, despite using a sensitive, selective-enrichment protocol capable of detecting 1 CFU O157/10g feces (Table 2) [50, 51, 68, 69]. This may have correlated with the reported seasonal variation in O157 shedding by cattle as these samples were collected in winter when cattle shed O157 in very low numbers [39, 70].

Colony PCR revealed that all the non-O157 STEC isolates carried the eae and hlyA genes (Table 2). The serogroups O103 and O26 did not match the virulence gene profiles of the respective control strains. The O121 isolate from animal #6529 matched one of the control O121 strains lacking all four virulence genes; however, the O121 antigen gene could not be amplified from this isolate, suggesting possible mutations within this gene (Table 2). Considering that the primers successfully amplified the corresponding regions from the control strains suggests that we mostly isolated variant serogroups.

PCR using DNA extracted from feces (‘fecal-DNA PCR’) matched the serogroup results of colony PCR for most isolates and amplified serogroup antigens from additional samples (Table 2). Serogroups matched between the two PCR methods for isolates from animals 5620, 5657, 5760, 5825, 5916, 5935, 5938, 5982, and 6017; however, the results did not match for animal 6529 (Table 2, Supplementary Figure 1). The O103-antigen gene was also amplified from fecal DNA samples of 12 additional animals including 5776, 5787, 5845, 5868, 5901, 5915, 5985, 6069, 6120, 6171, 6542, and 6582 (Table 2). Thus, going by fecal DNA PCR alone, 21/59 (36%) animals could be considered as positive for non-O157 STEC. Virulence genes were also amplified in various combinations, from all fecal DNA extracts (Table 2), which could suggest that 100% of the animals were colonized with non-O157 STEC. However, fecal-DNA PCR results did not always correspond with the isolation of viable non-O157 STEC and hence may reflect the presence of genetic material left over from recent colonization or the presence of other STEC besides the seven adulterant serogroups targeted by our assays. Thus, results based solely on fecal DNA PCR need to be interpreted in the context of other tests and not independently.

Greater numbers of virulence genes were observed using fecal-DNA PCR in our study (Table 2, Supplementary Figure 1). This may be due to the presence of free Stx-converting bacteriophages or other STEC DNA in the absence of viable bacteria (false positives) as previously reported [71, 72]. Virulence profiles within serogroups have been recorded as being highly variable in field samples, over time, and between locations [73–75]. Hence, our observed variation in virulence profiles of STEC isolated compared to the control strains is not novel. We also observed a higher incidence of O103 by fecal DNA PCR than by culture, 36% versus 15%; O26 was isolated from 2 animal samples and O121 from 1 animal sample by culture only (Table 2). Such discrepancies between PCR and culture observed in other studies and again may be indicative of relatively older colonization versus ongoing infections with viable adulterant STEC that are more likely to contaminate the environment, hides, and hence the carcass at slaughter [46, 74, 76].

Vero cell cytotoxicity assay was used to evaluate the presence of functional toxins in the fecal samples (47, 48). Purified Shiga toxins, used as controls, demonstrated cytotoxicity on Vero cells at 1:64 dilution for Stx1 and 1:256 dilution for Stx2, which was neutralized with the corresponding antisera (Supplementary Figure 2) thus validating the test. Similar cytopathic effects (CPE) were observed with fecal extracts from 58/59 animals in Vero cell cytotoxicity assays, of which 95% (55/58) was neutralized with polyclonal anti-Stx1 and/or anti-Stx2 antisera (Table 2, Supplementary Figure 3). This Stx-related CPE could be associated with isolation of STEC and/or amplification of toxin genes via fecal DNA PCR; other STEC serogroups not targeted in our study may have also contributed to the presence of Stx in the fecal extracts (Table 2). Interestingly, CPE caused by fecal extracts from animal #6122 was neutralized with both anti-Stx1 and anti-Stx2 antisera in the absence of viable STEC or amplification of Stx genes suggesting remnant toxins from a relatively older infection with no current trace of viable STEC or DNA (Table 2). In contrast, no CPE was observed with fecal extracts from animal #5915, although the stx2 gene was amplified from the same sample (Table 2, Supplementary Figure 2), indicating a possibly nonfunctional gene. Additionally, non-Stx factors in the fecal extracts, such as viruses, may have caused CPE observed with 3/58 fecal extracts (animals #6044, #6069, and #6148) that were not neutralized with the anti-Stx antisera (Supplementary Figure 3) [77, 78]. These variations indicate that Vero cell cytotoxicity assays require verification through neutralization steps and correlation with culture/PCR results.

STEC O103 was the predominant non-O157 STEC to be isolated from the dairy cattle evaluated in this study (Table 2). This serogroup has become one of the common non-O157 STEC to be isolated from cattle in the US and globally [37, 39, 44]. For instance, thirty calves from a closed herd in Canada were found to harbor at least one of the seven major STEC serogroups with the predominant being O103 (75.8%) and O157 (70%) [73]. Analysis of composite calf feces collected from 12 dairy farms in New Zealand identified STEC O26 (33%) to be the most prevalent serogroup, followed by O45 (25%), O103 (17%), and O121 (9%) [79]. STEC O103 is also being increasingly associated with outbreaks in the US; after the venison-related O103 outbreak in 2010, three recent multistate outbreaks were associated with O103 contaminated ground beef, ground bison meat, and clover sprouts following investigations by the Centers for Disease Control and Prevention and USDA-FSIS [80–83]. This makes our observation epidemiologically relevant as well.
4. Conclusions

In summary, non-O157 STEC were isolated from 25% (15/59) of the animals tested using a combination of EC broth, CHROMagar STEC™, and Rainbow agar O157 in this study. The two different selective agar media, used sequentially in this study, enabled differentiation of STEC from background flora and into tentative serogroups based on colony color and fluorescence phenotype. Serology and/or colony PCR was subsequently used to confirm the serogroup of the tentative non-O157 STEC as one of the “Big 6” STEC adulterants in 10/15 animals. The predominant viable non-O157 STEC serogroup isolated was O103. PCR using DNA extracted from feces verified most of the colony PCR results but also identified additional virulence and O-antigen genes from samples with no correlating culture results. Similarly, Stx-related CPE on Vero cells with fecal extracts from 35/59 animals could only be associated with the Stx gene profiles obtained with fecal DNA PCR and not culture results. Differences between culture versus fecal DNA PCR and cytotoxicity assay results suggest that the latter two assays, while alluding to the presence of STEC, may not always reflect an ongoing, viable infection with the seven adulterant STECs. Hence, this study validates that a combination of fecal culture methods are needed to distinctly isolate viable “Big 6” non-O157 STEC that pose a food safety threat. Culture methods cannot be substituted with fecal PCR or cytotoxicity assays alone which at the most could be used as primary screens to identify samples likely to harbor STEC.

Data Availability

No data were used to support the findings of this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to acknowledge Dr. A.F. Summers, NMSU, for access to feedlot cattle, expert technical assistance provided by Miss Hannah Mazon, Intern, USDA/1890 National Scholars Program, and Miss Erika Biernbaum, ORISE Postgraduate, Dr. I. T. Kudva’s laboratory. This research was supported in part by the New Mexico Agriculture Experiment Station and by the USDA Innovation Fund (#692-0142-601) awarded to Dr. I. T. Kudva and the USDA-ARS CRIS project (5030-32000-112–00D).

Supplementary Materials

Supplementary Figure 1. Representative colony (a) and fecal DNA (b) PCR results are shown for animal 5760. PCR reactions were analyzed by electrophoresis on a 4% agarose gel and loaded in lanes 1–13 as 100 bp ladder, O103, O111, O121, O145, O26, O45, eae, stx1, stx2, hlyA, and 2-log ladder. Amplicon sizes in bp, as expected, are shown. Supplementary Figure 2. Vero cell cytotoxicity assay. I, assay controls with media only (a) and media and antisera (b) on Vero cells are shown. II, diluted Stx and diluted Stx with anti-Stx sera are shown as described in the inserted legends. Cytopathic effects of Stx1 at 1:64 dilution (c) and Stx2 at 1:256 dilution (d) on the Vero cells and also the protection of the Vero cells in the presence of antisera, anti-Stx1 (d) and anti-Stx2 (f), are shown. III, absence of cytotoxic effects with undiluted fecal extracts from animal 5951 is shown (g, h). All images were captured at 10x magnification using an inverted microscope. Supplementary Figure 3. Vero cell cytotoxicity assay with fecal extracts. Examples of various effects of fecal extracts on Vero cells are shown as described in the inserted legends. Images were captured at 10x magnification using an inverted microscope. (Supplementary Materials)

References

[1] D. M. Tack, L. Ray, P. M. Griffin et al., “Preliminary incidence and trends of infections with pathogens transmitted commonly through food - foodborne diseases active surveillance network, 10 U.S. Sites, 2016-2019,” MMWR. Morbidity and Mortality Weekly Report, vol. 69, no. 17, pp. 509–514, 2020.
[2] E. Scallan, P. M. Griffin, F. J. Angulo, R. V. Tauxe, and R. M. Hoekstra, “Foodborne illness acquired in the United States-unspecified agents,” Emerging Infectious Diseases, vol. 17, no. 1, pp. 16–22, 2011.
[3] S. E. Majowicz, E. Scallan, A. Jones-Bitton et al., “Global incidence of human shiga toxin-ProducingEscherichia coli infections and deaths: a systematic review and knowledge synthesis,” Foodborne Pathogens and Disease, vol. 11, no. 6, pp. 447–455, 2014.
[4] S. Hoffmann and E. Scallan Walter, “Acute complications and sequelae from foodborne infections: informing priorities for cost of foodborne illness estimates,” Foodborne Pathogens and Disease, vol. 17, no. 3, pp. 172–177, 2020.
[5] S. Hoffmann, M. B. Batz, and J. G. Morris, “Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens,” Journal of Food Protection, vol. 75, no. 7, pp. 1292–1302, 2012.
[6] R. L. Scharff, “Economic burden from health losses due to foodborne illness in the United States,” Journal of Food Protection, vol. 75, no. 1, pp. 123–131, 2012.
[7] T. J. Barrett, H. Lior, J. H. Green et al., “Laboratory investigation of a multistate food-borne outbreak of Escherichia coli O157:H7 by using pulsed-field gel electrophoresis and phage typing,” Journal of Clinical Microbiology, vol. 32, no. 12, pp. 3013–3017, 1994.
[8] USDA, Shiga toxin-producing Escherichia coli in certain raw beef products, U. S. Department of Agriculture-Food Safety and Inspection Service, Washington D.C, 2011.
[9] USDA, FSIS Verification Testing for Non-o157 Shiga Toxin-Producing Escherichia coli (Non-o157 STEC) under Mt60, Mt52, and Mt53 Sampling Programs. FSIS NOTICE-40-12, U. S. Department Of Agriculture-Food Safety And Inspection Service, Washington D.C, 2012.
[10] J. B. Kaper, J. P. Nataro, and H. L. T. Mobley, “Pathogenic Escherichia coli,” Nature Reviews Microbiology, vol. 2, no. 2, pp. 123–140, 2004.
[11] J. C. Paton and A. W. Paton, “Pathogenesis and diagnosis of shiga toxin-producing Escherichia coli infections,” Clinical Microbiology Reviews, vol. 11, no. 3, pp. 450–479, 1998.
[12] B. P. Bell, M. Goldoft, P. M. Griffin et al., “A multistate outbreak of Escherichia coli O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers,” *JAMA*, vol. 272, no. 17, pp. 1349–1353, 1994.

[13] P. E. Ray and X.-H. Liu, “Pathogenesis of Shiga toxin-induced hemolytic uremic syndrome,” *Pediatric Nephrology*, vol. 16, no. 10, pp. 823–839, 2001.

[14] T. K. Davis, N. C. Van De Kar, and P. I. Tarr, “Shiga toxin/verocytotoxin-producing Escherichia coli infections: practical & scientific perspectives,” *Microbiology Spectrum*, vol. 2, 2014.

[15] C. L. Mayer, C. S. Leibowitz, S. Kurosawa, and D. J. Stearns-Kurosawa, “Shiga toxins and the pathophysiology of hemolytic uremic syndrome in humans and animals,” *Toxins*, vol. 4, no. 11, pp. 1261–1287, 2012.

[16] M. Rivas, I. Chinen, E. Milwesbsky, and M. Masana, “Risk factors for shiga toxin-producing Escherichia coli-associated human diseases,” *Microbiology Spectrum*, vol. 2, 2014.

[17] D. Karpman and A. L. Stahl, “Enterohecromorphic Escherichia coli pathogenesis and the host response,” *Microbiol Spectr*, vol. 2, 2014.

[18] H. Schmidt, L. Beutin, and H. Karch, “Molecular Analysis of the plasmid-encoded hemolysin of Escherichia coli O157:H7 strain EDL 933,” *Infection and Immunity*, vol. 63, no. 3, pp. 1055–1061, 1995.

[19] A. K. Persad and J. T. LeJeune, “Animal reservoirs of shiga toxin-producing Escherichia coli,” *Microbiol Spectr*, vol. 2, 2014.

[20] I. T. Kudva and C. J. Hovde, *Escherichia coli O157:H7 in Ruminants-A Review*, Vol. 2, Research Signpost, Trivandrum, India, 1998.

[21] T. E. Besser, B. L. Richards, D. H. Rice, and D. D. Hancock, “Escherichia coli O157[ratio]* H7* infection of calves: infectious dose and direct contact transmission,” *Epidemiology and Infection*, vol. 127, no. 3, pp. 555–560, 2001.

[22] I. M. Prumboom-Brees, T. W. Morgan, M. R. Ackermann et al., “Cattle lack vascular receptors for Escherichia coli O157:H7 Shiga toxins,” *Proceedings of the National Academy of Sciences*, vol. 97, no. 19, pp. 10325–10329, 2000.

[23] Y. Nguyen and V. Sperandio, “Enterohecromorphic E. coli (EHEC) pathogenesis,” *Frontiers in Cellular and Infection Microbiology*, vol. 2, p. 90, 2012.

[24] S. W. Naylor, J. C. Low, T. E. Besser et al., “Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157:H7 in the bovine host,” *Infection and Immunity*, vol. 71, no. 3, pp. 1505–1512, 2003.

[25] J. E. Keen, W. W. Laegreid, C. G. Chitko-Mckown, L. M. Durso, and J. L. Bono, “Distribution of shiga-toxigenic Escherichia coli O157 in the gastrointestinal tract of naturally O157-shedding cattle at necropsy,” *Applied and Environmental Microbiology*, vol. 76, no. 15, pp. 5278–5281, 2010.

[26] N. Fegan, P. Vanderlinden, G. Higgs, and P. Desmarchelier, “The prevalence and concentration of Escherichia coli O157 in faeces of cattle from different production systems at slaughter,” *Journal of Applied Microbiology*, vol. 97, no. 2, pp. 362–370, 2004.

[27] D. A. Widiash, N. Idl, K. Omoe, S. Sugii, and K. Shinagawa, “Duration and magnitude of faecal shedding of Shiga toxin-producing Escherichia coli from naturally infected cattle,” *Epidemiology and Infection*, vol. 132, no. 1, pp. 67–75, 2004.

[28] J. Y. Lim, J. Li, H. Sheng, T. E. Besser, K. Potter, and C. J. Hovde, “Escherichia coli O157:H7 colonization at the rectoanal junction of long-duration culture-positive cattle,” *Applied and Environmental Microbiology*, vol. 73, no. 4, pp. 1380–1382, 2007.

[29] F. Omissakin, M. MacRae, I. D. Ogden, and N. J. C. Strachan, “Concentration and prevalence of Escherichia coli O157 in cattle feces at slaughter,” *Applied and Environmental Microbiology*, vol. 69, no. 5, pp. 2444–2447, 2003.

[30] L. Matthews, J. C. Low, D. L. Gally et al., “Heterogeneous shedding of Escherichia coli O157 in cattle and its implications for control,” *Proceedings of the National Academy of Sciences*, vol. 103, no. 3, pp. 547–552, 2006.

[31] W. C. Cray and H. W. Moon, “Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7,” *Applied and Environmental Microbiology*, vol. 61, no. 4, pp. 1586–1590, 1995.

[32] E. A. Dean-Nystrom, B. T. Bosworth, W. C. Cray, and H. W. Moon, “Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves,” *Infection and Immunity*, vol. 65, no. 5, pp. 1842–1848, 1997.

[33] S. W. Naylor, D. L. Gally, and J. Christopher Low, “Enterohemorrhagic *E. coli* in veterinary medicine,” *International Journal of Medical Microbiology*, vol. 295, no. 6-7, pp. 419–441, 2005.

[34] R. A. Mir, T. A. Weppelmann, M. Kang et al., “Association between animal age and the prevalence of Shiga toxin-producing Escherichia coli in a cohort of beef cattle,” *Veterinary Microbiology*, vol. 175, no. 2-4, pp. 325–331, 2015.

[35] D. A. Dargatz, J. Bai, B. V. Lubbers, C. A. Kopral, B. An, and G. A. Anderson, “Prevalence of Escherichia coli O-types and Shiga toxin genes in fecal samples from feedlot cattle,” *Foodborne Pathogens and Disease*, vol. 10, no. 4, pp. 392–396, 2013.

[36] Z. D. Paddock, J. Bai, X. Shi, D. G. Renter, and T. G. Nagaraja, “Detection of Escherichia coli O104 in the feces of feedlot cattle by a multiplex PCR assay designed to target major genetic traits of the virulent hybrid strain responsible for the 2011 German outbreak,” *Applied and Environmental Microbiology*, vol. 79, no. 11, pp. 3522–3525, 2013.

[37] A. B. Ekiri, D. Landblom, D. Doetkott, S. Olet, W. L. Shelver, and M. L. Khaita, “Isoanalysis and characterization of Shiga toxin-producing escherichia coli serogroups O26, O45, O103, O111, O113, O121, O145, and O157 shed from range and feedlot cattle from postweaning to slaughter,” *Journal of Food Protection*, vol. 77, no. 7, pp. 1052–1061, 2014.

[38] K. Verstraete, E. Van Coillie, H. Werbrouck et al., “A qPCR assay to detect and quantify Shiga toxin-producing *E. coli* (STEC) in cattle and on farms: a potential predictive tool for STEC culture-positive farms,” *Toxins*, vol. 6, no. 4, pp. 1201–1221, 2014.

[39] D. M. A. Dewsbury, D. G. Renter, P. B. Shrirdhar et al., “Summer and winter prevalence of shiga toxin-producing Escherichia coli (STEC) O26, O45, O103, O111, O113, O121, O145, and O157 in feces of feedlot cattle,” *Foodborne Pathogens and Disease*, vol. 12, no. 8, pp. 726–732, 2015.

[40] L. W. Noll, P. B. Shrirdhar, D. M. Dewsbury et al., “A comparison of culture- and PCR-based methods to detect six major non-O157 serogroups of shiga toxin-producing Escherichia coli in cattle feces,” *PLoS One*, vol. 10, Article ID e0135446, 2015.

[41] B. D. Parsons, N. Zelyas, B. M. Berenger, and L. Chui, “Detection, characterization, and typing of shiga toxin-producing Escherichia coli,” *Frontiers in Microbiology*, vol. 7, p. 478, 2016.

[42] C. A. Cull, D. G. Renter, D. M. Dewsbury et al., “Feedlot- and pen-level prevalence of enterohemorrhagic Escherichia coli in feces of commercial feedlot cattle in two major U.S. Cattle
feeding areas,” *Foodborne Pathogens and Disease*, vol. 14, no. 6, pp. 309–317, 2017.

[43] Z. R. Stromberg, G. L. Lewis, L. G. Schneider et al., “Culture-based quantification with molecular characterization of non-O157 and O157 enterohemorrhagic *Escherichia coli* isolates from rectoanal mucosal swabs of feedlot cattle,” *Foodborne Pathogens and Disease*, vol. 15, no. 1, pp. 26–32, 2018.

[44] A. Dixon, N. Cernicchiaro, R. G. Amachawadi, X. Shi, C. A. Cull, and D. G. Renter, “Longitudinal characterization of prevalence and concentration of shiga toxin-producing *Escherichia coli* serogroups in feces of individual feedlot cattle,” *Foodborne Pathogens and Disease*, vol. 17, no. 10, 631 pages, 2020.

[45] E. Hofer, R. Stephan, M. Reist, and C. Zweifel, “Application of a real-time PCR-based system for monitoring of O26, O103, O111, O145 and O157 Shiga toxin-producing *Escherichia coli* in cattle at slaughter,” *Zoonoses and Public Health*, vol. 59, no. 6, pp. 408–415, 2012.

[46] P. S. Ekong, M. W. Sanderson, P. B. Shridhar et al., “Bayesian estimation of sensitivity and specificity of culture- and PCR-based methods for the detection of six major non-O157 *Escherichia coli* serogroups in cattle feces,” *Preventive Veterinary Medicine*, vol. 161, pp. 90–99, 2018.

[47] N. Kalchayanand, T. M. Arthur, J. M. Bosilevac, J. E. Wells, and T. L. Wheeler, “Chromogenic agar medium for detection and isolation of *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from fresh beef and cattle feces,” *Journal of Food Protection*, vol. 76, no. 2, pp. 192–199, 2013.

[48] R. Fan, K. Shao, X. Yang et al., “High prevalence of non-O157 Shiga toxin-producing *Escherichia coli* in beef cattle detected by combining four selective agars,” *BMC Microbiology*, vol. 19, p. 213, 2019.

[49] I. T. Kudva, P. G. Hatfield, and C. J. Hovde, “Effect of diet on the shedding of *Escherichia coli* O157:H7 in a sheep model,” *Applied and Environmental Microbiology*, vol. 61, no. 4, pp. 1363–1370, 1995.

[50] I. T. Kudva, C. W. Hunt, C. J. Williams, U. M. Nance, and C. J. Hovde, “Evaluation of dietary influences on *Escherichia coli* O157:H7 shedding by sheep,” *Applied and Environmental Microbiology*, vol. 63, no. 10, pp. 3878–3886, 1997.

[51] I. T. Kudva, P. G. Hatfield, and C. J. Hovde, “Characterization of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep,” *Journal of Clinical Microbiology*, vol. 35, no. 4, pp. 892–899, 1997.

[52] J. J. Hirvonen, A. Siitonen, and S.-S. Kaukoranta, “Usability and performance of CHROMagar STEC medium in detection of Shiga toxin-producing *Escherichia coli* strains,” *Journal of Clinical Microbiology*, vol. 50, no. 11, pp. 3586–3590, 2012.

[53] C. C. Conrad, K. Stanford, T. A. McAllister, J. Thomas, and T. Reuter, “Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces,” *Journal of Microbiological Methods*, vol. 105, pp. 22–30, 2014.

[54] B. Verhaegen, K. De Reu, M. Heyndrickx, and L. De Zutter, “Comparison of six chromogenic agar media for the isolation of a broad variety of non-O157 shigatoxin-producing *Escherichia coli* (STEC) serogroups,” *International Journal of Environmental Research and Public Health*, vol. 12, no. 6, pp. 6965–6978, 2015.

[55] USDA, *Morphologies of representative strains from six non-O157 shiga toxin-producing *Escherichia coli* (STEC) grown on modified Rainbow agar*, U. S. Department of Agriculture-Food Safety and Inspection Service, Office of Public Health Science, Washington, DC, 2019.
[70] K. Stanford, R. P. Johnson, T. W. Alexander, T. A. McAllister, and T. Reuter, "Influence of season and feedlot location on prevalence and virulence factors of seven serogroups of Escherichia coli in feces of western-Canadian slaughter cattle," *PLoS One*, vol. 11, Article ID e0159866, 2016.

[71] A. Martínez-Castillo and M. Muniesa, "Implications of free Shiga toxin-converting bacteriophages occurring outside bacteria for the evolution and the detection of Shiga toxin-producing Escherichia coli," *Frontiers in Cellular and Infection Microbiology*, vol. 4, p. 46, 2014.

[72] P. Quirós, A. Martínez-Castillo, and M. Muniesa, "Improving detection of Shiga toxin-producing Escherichia coli by molecular methods by reducing the interference of free Shiga toxin-encoding bacteriophages," *Applied and Environmental Microbiology*, vol. 81, no. 1, pp. 415–421, 2015.

[73] J. Hallewell, T. Reuter, K. Stanford, E. Topp, and T. W. Alexander, "Monitoring seven potentially Pathogenic Escherichia coli Serogroups in a closed herd of beef cattle from weaning to finishing phases," *Foodborne Pathogens and Disease*, vol. 13, no. 12, pp. 661–667, 2016.

[74] N. Cernicchiaro, C. A. Cull, Z. D. Paddock et al., "Prevalence of Shiga toxin-producing Escherichia coli and associated virulence genes in feces of commercial feedlot cattle," *Foodborne Pathogens and Disease*, vol. 10, no. 10, pp. 835–841, 2013.

[75] S. A. Ison, S. Delannoy, M. Bugarel et al., "Genetic diversity and pathogenic potential of attaching and effacing Escherichia coli O26:H11 strains recovered from bovine feces in the United States," *Applied and Environmental Microbiology*, vol. 81, no. 11, pp. 3671–3678, 2015.

[76] Z. R. Stromberg, N. W. Baumann, G. L. Lewis et al., "Prevalence of enterohemorrhagic Escherichia coli O26, O45, O103, O111, O121, O145, and O157 on hides and pre-intervention carcass surfaces of feedlot cattle at harvest," *Foodborne Pathogens and Disease*, vol. 12, no. 7, pp. 631–638, 2015.

[77] A. Hansa, R. B. Rai, K. Dhama, M. Y. Wani, M. Saminathan, and G. J. Ranganath, "Isolation of bovine coronavirus (bcoV) in vero cell line and its confirmation by direct FAT and RT-PCR," *Pakistan Journal of Biological Sciences*, vol. 16, no. 21, pp. 1342–1347, 2013.

[78] N. Income, N. Kosoltanapiwat, S. Taksinoros, P. Leaungwutiwong, and I. F. Chavez, "Molecular identification of enteroviruses from cattle and goat feces and environment in Thailand," *Applied and Environmental Microbiology*, vol. 85, no. 5, Article ID e02420, 2019.

[79] C. M. Ross, D. Rapp, V. M. Cave, and G. Brightwell, "Prevalence of Shiga toxin-producing Escherichia coli in pasture-based dairy herds," *Letters in Applied Microbiology*, vol. 68, no. 2, pp. 112–119, 2019.

[80] CDC, Outbreak of E. coli Infections Linked to Ground Beef Centers for Disease Control and Prevention Food Safety Alert, 2019.

[81] CDC, Outbreak of E. coli Infections Linked to Ground Bison Produced by Northfork Bison Distributions, 2019.

[82] CDC, Outbreak of E. coli Infections Linked to Clover Sprouts. Centers for Disease Control and Prevention, 2020.

[83] J. M. Rounds, C. E. Rigdon, L. J. Muhl et al., "Non-O157 shiga toxin-producing Escherichia coli associated with venison," *Emerging Infectious Diseases*, vol. 18, no. 2, pp. 279–282, 2012.