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Chromogenic Agar Medium for Detection and Isolation of *Escherichia coli* Serogroups O26, O45, O103, O111, O121, and O145 from Fresh Beef and Cattle Feces†

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ABSTRACT

Non-O157 Shiga toxin–producing *Escherichia coli* (STEC) strains are clinically important foodborne pathogens. Unlike *E. coli* O157:H7, these foodborne pathogens have no unique biochemical characteristics to readily distinguish them from other *E. coli* strains growing on plating media. In this study, a chromogenic agar medium was developed in order to differentiate among non-O157 STEC strains of serogroups O26, O45, O103, O111, O121, and O145 on a single agar medium. The ability of this chromogenic agar medium to select and distinguish among these pathogens is based on a combination of utilization of carbohydrates, β-galactosidase activity, and resistance to selective agents. The agar medium in combination with immunomagnetic separation was evaluated and successfully allowed for the detection and isolation of these six serogroups from artificially contaminated fresh beef. The agar medium in combination with immunomagnetic separation also allowed successful detection and isolation of naturally occurring non-O157 STEC strains present in cattle feces. Thirty-five strains of the top six non-O157 STEC serogroups were isolated from 1,897 fecal samples collected from 271 feedlot cattle. This chromogenic agar medium could help significantly in routine screening for the top six non-O157 STEC serogroups from beef cattle and other food.

In the early 1980s, *Escherichia coli* O157:H7, a member of the enterohemorrhagic *E. coli* (EHEC) group, gained recognition as the causative agent for an outbreak of severe bloody diarrhea traced to consumption of improperly cooked hamburgers *(28)*. EHEC bacteria excrete potent toxins called Shiga toxins and carry genes for virulence factors, such as intimin and hemolysin, which are responsible for attaching to and effacing host epithelial cells and for lysis of red blood cells, respectively. Although *E. coli* O157:H7 is currently the most widely recognized EHEC member, strains of more than 200 other non-O157 Shiga toxin–producing *Escherichia coli* (STEC) serotypes have been implicated in cases of human disease *(5)*. Non-O157 STEC strains are similar to *E. coli* O157:H7 in that these bacteria produce Shiga toxins, can be isolated from bovine feces, and are found as contaminants on the hides and carcasses of processed beef cattle *(3)*. Many strains have been found with the same serotypes and virulence genotypes as those determined to cause human disease. According to the Centers for Disease Control and Prevention (CDC), the most common non-O157 STEC serogroups associated with human disease in the United States include O26, O103, O111, O121, O45, and O145 *(5)*. The newly emerging enteroaggregative *E. coli* serotype O104:H4 that was identified as the causative agent in a large outbreak in Germany and other European countries was not included in the top six serogroups listed by the CDC because meat animals are not reservoirs of enteroaggregative *E. coli,* including the aggregative serotype O104:H4 *(6, 30)*. It has been estimated that non-O157 STEC strains cause approximately 112,752 cases of foodborne illness in the United States annually *(29)*. The true number of illnesses caused by non-O157 STEC may be underestimated, as only about 4% of clinical laboratories routinely screen for these pathogens *(15)*. In September 2011, the Food Safety and Inspection Service, U.S. Department of Agriculture, published a notice in the Federal Register of their intent to regulate non-O157 STEC bacteria of serogroups O26, O45, O103, O111, O121, and O145 as adulterants in certain raw beef products, as had been done for *E. coli* O157:H7 several years earlier *(31)*. The isolation of non-O157 STEC in stool and foodstuffs is laborious and time consuming due to the lack of differential and selective plating media for isolation.

Over the years, varied strategies have been developed for the cultivation of pathogens. The ability to detect the

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presence of a specific enzyme using suitable substrates, in particular fluorogenic or chromogenic enzyme substrates, has led to the development of a great number of methods for the identification of microorganisms in primary isolation media. Several chromogenic agar media for the detection and isolation of *E. coli* O157:H7 are available commercially, such as Rainbow agar O157 (Biolog, Inc., Hayward, CA), BCM O157:H7 (Biosynth AG, Staad, Switzerland), Fluorocult *E. coli* O157:H7 (Merck, Darmstadt, Germany), R & F *E. coli* O157:H7 (R & F Laboratory, Downers Grove, IL), CHROMagar O157 (CHROMagar Microbiology, Paris, France), CHROMagar O26/O157 (CHROMagar Microbiology), and CHROMagar STEC (CHROMagar Microbiology), as well as a noncommercial chromogenic agar medium *(26)*. However, there is no true chromogenic agar medium commercially available to differentiate strains of all top six non-O157 serogroups of STEC on a single plate due to their variation in biochemical characteristics. At the U.S. Meat Animal Research Center (USMARC), we recently developed a unique chromogenic agar medium for the detection and isolation of strains of serogroups O26, O45, O103, O111, O121, and O145. In the present study, the USMARC chromogenic agar medium was evaluated for its efficiency in detecting and isolating STEC from artificially contaminated fresh beef and naturally contaminated cattle feces.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial cultures from the USMARC culture collection listed in Table 1, including gram-negative and gram-positive strains, were grown in nutrient broth (Difco, BD, Sparks, MD) for 16 to 18 h at 37°C. The strains of *Enterobacteriaceae* and *Pseudomonas* were streaked for isolation on violet red bile agar (Difco, BD) and tryptic soy agar (TSA; Difco, BD), respectively, while STEC strains were streaked on sorbitol MacConkey agar (Difco, BD). *Listeria monocytogenes, Staphylococcus aureus,* and lactic acid bacteria were streaked on modified Oxford, Baird-Parker, and De Man Rogosa Sharpe agar plates (Difco, BD), respectively. All the plates were incubated at 37°C for 16 to 18 h. A single colony from each plate was stabbed into nutrient soft agar (nutrient broth with 0.8% agar; Difco, BD) and served as a stock culture for the studies.

Biochemical and enzymatic reactions. Selected STEC strains were analyzed for their carbohydrate metabolism and enzymatic reaction profiles using API 50 CHB/E and API ZYM test kits (bioMérieux, Hazelwood, MO). Reference strains, including O26:H11 strain 3392, O45:H2 strain 01E-1269, O103:H2 strain 2421, O111:NM strain 1665, O121:H19 strain 02E-2074, O145:NM strain G55578620, and O157:H7 strain ATCC 43895, were streaked on TSA (Difco, BD) and incubated at 37°C for 24 h. Colonies from TSA agar plates were grown in suspension, inoculated into test strips, and incubated, and the results interpreted according to the manufacturer's recommendations.

Medium formulation and preparation. The USMARC chromogenic agar medium was formulated based on the composition of MacConkey medium but without lactose and neutral red. The formulation of the selective differential medium was as follows: Bacto Peptone (BD, Franklin Lakes, NJ) at 17.0 g/liter, Proteose Peptone (BD) at 30 g/liter, sodium chloride (Sigma, St. Louis, MO) at 5.0 g/liter, crystal violet (Sigma) at 1.0 mg/liter, L-sorbose (Sigma) at 6.0 g/liter, D-raffinose (Sigma) at 6.0 g/liter, phenol red (Sigma) at 20 mg/liter, bromothymol blue (Sigma) at 1.5 mg/liter, and Bacto agar (BD) at 15 g/liter. All the ingredients were mixed well with 900 ml of distilled water, the pH adjusted to 7.4 $\pm$ 0.1, and the mixture autoclaved for 10 min at 115°C. The autoclaved medium was cooled to 50°C before adding 100 ml of a mixture of filter-sterilized (pH 7.1 $\pm$ 0.1) bile salts no. 3 (BD) at 3 g/liter, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (BCIG; Gold Biotechnology, St. Louis, MO) at 0.05 g/liter, isopropyl-β-D-thiogalactopyranoside (Sigma) at 0.05 g/liter, novobiocin (Sigma) at 5 mg/liter, and potassium tellurite (Sigma) at 0.125 mg/liter. The concentration of potassium tellurite can be increased for purposes of more selectivity or greater inhibition of the background flora. The base agar medium, formulated with 0.125 mg/liter of potassium tellurite, was based on the good growth of serogroup O121 strains. Phenol red has a pH range from 6.8 to 8.4, changing from yellow (acid color) to red (alkali color), while bromothymol blue has a pH range from 6.0 to 7.6, changing from yellow (acid color) to blue (alkali color) *(8)*.

Growth of bacterial strains on differential medium. The differential medium described above was inoculated separately with STEC strains and non–*E. coli* control strains (Table 1). All strains were grown in nutrient broth for 16 to 18 h at 37°C. Each strain was streaked on USMARC chromogenic agar medium. The plates were incubated at 37°C for 24 h and then at room temperature for half an hour for full color development.

Detection of STEC strains from inoculated fresh beef tissues. A cocktail mixture of STEC strains, including O26:H11 strains 3392 and 3891, O45:H2 strain 01E-1269, O45 strain WDG-3, O103:H2 strain 2421, O111:NM strains 1665 and ECRC 3007:85, O121:H19 strain 02E-2074, an O121:H7 strain, and an O145:NM strain, was inoculated on fresh beef flanks. Each bacterial strain was grown in nutrient broth for 16 h at 37°C, the cell concentration was adjusted to approximately 1.5×10^8 CFU/ml using a spectrophotometer (Thermo Spectronic, Fisher Scientific, Pittsburgh, PA) at 600 nm, and the population level was confirmed using Enterobacteriaceae Count Plates (3M, St. Paul, MN) according to the manufacturer's recommendations. Beef flanks obtained from a local cattle processing plant were aseptically cut into 96 pieces (3 replicates of 32 pieces) with an area of 25 cm^2 (5 by 5 cm). The background bacterial population of fresh beef flanks was determined using Petrifilm aerobic count plates (3M) and was approximately 2×10^3 CFU/cm^2. A total of 96 pieces were cut and divided into three replicates. Each piece was randomly inoculated with a cocktail mixture to a total final concentration for all strains of approximately 3×10^1 CFU/cm^2 and allowed to stand for 15 min at room temperature to allow bacterial cell attachment. The inoculated 5-cm^2 beef flank square was placed in a stomacher bag and chilled for 48 h at 2 to 4°C to simulate chilling of carcasses before fabrication. Exposure to refrigeration temperature causes sublethal injury to microbial cells, which are susceptible to selective agents added to medium used for detection and/or enumeration of target organisms. Sublethal injury of bacteria can be reversed by self-repair during enrichment process in nonselective media. Therefore, an aliquot of 50 ml of tryptic soy broth (Difco, BD) was added to each chilled beef flank, and it was hand massaged and enriched at 25°C for 2 h and at 42°C for 6 h and held overnight at 4°C before immunomagnetic separation (IMS) as previously described *(4)*. The next day, a 1-ml aliquot of the enriched samples was subjected to IMS using a mixture of Dynabeads EPEC/VTEC O26, O103, O111, and O145 (5 μl of each bead). The bacterial bead complexes were captured

TABLE 1. *Growth characteristics of bacterial strains on USMARC chromogenic medium*[a]

Bacterial strain	Strain and/or source	Growth on USMARC agar medium with: 0.125 mg/liter PT	1.0 mg/liter PT
Enterobacteriaceae and *Pseudomonas*			
Citrobacter freundii	ATCC 8090	G; small, turquoise with blue center	N
Enterobacter aerogenes	ATCC 13048	F; blue-green without center	P
Enterobacter aerogenes	ATCC 23355	F; light blue-gray	P
Enterobacter cloacae	Beef isolate	G; light turquoise blue with dark center	P
Escherichia hermanii	Beef isolate	G; dark purple with magenta background	G
Hafnia alvei	ATCC 29926	F; cream with light magenta background	N
Hafnia alvei	Beef isolate	F; small, light blue	N
Klebsiella pneumonia	ATCC 13883	G; blue-green with light magenta background	N
Proteus vulgaris	ATCC 13315	N	N
Salmonella Newport	Beef isolate	G; colorless with magenta background	N
Salmonella Typhimurium	DT-104	G; colorless with magenta background	N
Serratia liquefaciens	Beef isolate	G; small cream with magenta background	P
Shigella flexneri	ATCC 12022	G; small cream with magenta background	G
Shigella sonnei	ATCC 25931	G; small purple with magenta background	P
Yersinia enterocolitica	ATCC 23715	F; light greenish blue with magenta background	N
Pseudomonas aeruginosa	ATCC 27853	G; colorless with magenta background	F
Gram-positive bacteria			
Listeria monocytogenes	1/2b, FSIS	N	N
Listeria monocytogenes	Scott A	N	N
Staphylococcus aureus	ATCC 12598	N	N
Pediococcus acidilactici	Feed isolate	N	N
Lactobacillus plantarum	NCDO 955	N	N
Shiga toxin–producing *Escherichia coli*[b]			
E. coli O15:H27	Sheep isolate	G; blue-green with dark center	P
E. coli O76:H19	Sheep isolate	F; light bluish green with dark center	N
E. coli O91:H14	Sheep isolate	F; dark greenish blue without center	P
E. coli O128:H2	Sheep Isolate	F; small, greenish blue without center	P
E. coli O26:H11	3392, human isolate	G; bright turquoise blue with lapis blue center	G
E. coli O26:H11	3891, human isolate	G; bright turquoise blue with lapis blue center	G
E. coli O26:H6	99L-210, human isolate	G; turquoise blue with hint of cobalt blue	G
E. coli O45:H2	01E-1269, human isolate	G; bluish green to light green with center	G
E. coli O45	WDG-3, cattle hide	G; bluish green to light green with center	G
E. coli O103:H2	2421, human isolate	G; light greenish to bluish green with center	G
E. coli O111:NM	1665, human isolate	G; small dark blue-green to green without center	G
E. coli O111:NM	ECRC 3007:85	G; dark blue-green to green without center	G
E. coli O121:H19	02E-2074, human isolate	G; light blue gray with light magenta background	N
E. coli O121:H7	Beef isolate	G; purple with magenta background	P
E. coli O145:NM	G55578620, human isolate	G; purple with magenta background	G
E. coli O145	Beef isolate	G; purple with magenta background	G
E. coli O157:H7	ATCC 43895	G; green to bluish green without center	G
E. coli O157:H7	FSIS 4	G; green to bluish green without center	G
E. coli O157:NM	USMARC	G; greenish blue with center	G
E. coli O157:NM	493/89, sorbitol positive	G; turquoise blue with center	G

[a] All strains were streaked on the USMARC medium, as well as on TSA for comparing amounts of growth. All plates were incubated at 37°C for 22 to 24 h and at room temperature for half an hour before observation of colony characteristics. Color profiles were the same with both concentrations of potassium tellurite. G, good; F; fair; P, poor; N, none; PT, potassium tellurite.

[b] Biochemical activity profiles are described as in Leclercq et al. *(18)*. NM, nonmotile.

using a magnetic particle processor (King Fisher 96, Thermo Electron Corp., Asheville, NC) and resuspended into 100 μl of phosphate-buffered saline (pH 7.4) with 0.05% (vol/vol) Tween 20 (Sigma). A 20-μl aliquot from one replicate per sample of the bacterial bead complexes and a similar aliquot of the enriched sample after IMS was streaked for isolation on USMARC chromogenic agar medium plates. The need for the streaking of the remaining enrichment after IMS was due to the lack of IMS beads for serogroups O45 and O121. The plates were incubated at 37°C for 24 h and then at room temperature for half an hour before four colonies with color characteristics representing each STEC serogroup were picked for confirmation using multiplex PCR *(25)*. The primer sets for serogroups O26, O45, O103, O111, O121, and O145 were previously described *(7, 10, 12, 20, 22, 24)*.

The presence of genes for the virulence factors intimin (*eae*), EHEC hemolysin (*hlyA*), and Shiga toxins (stx_1 and stx_2) carried by these serogroups was determined using a previously described multiplex PCR *(22)*. The percent recovery for each organism was calculated from the number of positive samples divided by number of inoculated samples times 100 and was compared among the organisms recovered by using WINPEPI Compare2 *(1)*.

Detection and isolation from cattle feces. Fecal samples (1,897) were collected from 271 feedlot cattle for an evaluation of the ability of the USMARC chromogenic agar medium to detect and isolate the six STEC serogroups from a natural source. All animal procedures were reviewed and approved by the USMARC Animal Care and Use Committee. Each animal was restrained in a squeeze chute at some point during June to October of 2011 to collect the samples. The fecal sample (at least 10 g) was collected from each animal as a rectal grab sample using a clean gauntlet glove (NASCO, Ft. Atkinson, WI). Feces were immediately transferred to a clean closable plastic bag (Envision, Wichita, KS) for transport to the laboratory. Each fecal sample (10 $\pm$ 0.1 g) was placed in a sterile filter bag (NASCO) with 90 ml of sterile tryptic soy broth (Difco, BD) containing 100 mM potassium phosphate buffer *(4)* (18 mM KH_2PO_4 and 82 mM K_2HPO_4, pH 7.2; Sigma) and mixed well by hand massage. All samples with added medium were enriched by incubation for 2 h at 25°C followed by 6 h at 42°C. Samples were then held at 4°C overnight and subjected to IMS the following day. Following IMS, the detection and isolation of serogroups O26, O45, O103, O111, O121, and O145 from the enriched samples were determined using the chromogenic agar medium as described for the inoculation study. The chromogenic agar medium was formulated with 0.5 mg of potassium tellurite per liter for this study, to reduce the background flora. This concentration of potassium tellurite was predetermined using the *E. coli* strains O121:H19 and O121:H7 as growth indicators. The presumptive colonies were confirmed for O-groups and virulence factors as described above.

RESULTS AND DISCUSSION

Growth and characteristics of bacterial strains on the USMARC chromogenic medium. Forty bacterial strains, including gram-negative and gram-positive strains, were cultured on the USMARC chromogenic agar medium (Table 1). The chromogenic medium was formulated based on MacConkey agar, which has been generally used for selective isolation of gram-negative enteric bacteria due to its combination of bile salts, neutral red, and lactose *(8)*. The USMARC medium was formulated by replacing lactose with sorbose and raffinose and replacing neutral red with phenol red and bromothymol blue. The formulated medium has a clear beige color. The principle of USMARC chromogenic agar medium is based on (i) fermentation of two carbohydrates (sorbose and raffinose), (ii) reaction between β-D-galactosidase and a chromogenic substrate (BCIG), and (iii) tolerance of selective agents. Fermentation of carbohydrates produces a localized pH drop, initiating a color change in the phenol red and bromothymol blue dyes. A zone of precipitated bile also may be present due to this localized drop in pH, which was easily observed for serogroup O26 after 24 h of incubation at 37°C. Isopropyl-β-D-thiogalactopyranoside was added to the medium to induce the activity of β-D-galactosidase, an enzyme that promotes lactose utilization. Using the API 50 CHB/E test kit to characterize the carbohydrate-fermenting ability of the STEC reference strains indicated that dulcitol, D-raffinose, D-saccharose, D-sorbitol, and D-sorbose had potential to serve as discriminative markers for the selective isolation of STEC serotypes O26:H11 (strain 3392), O45:H2 (strain 01E-1269), O103:H2 (strain 2421), O111:NM (strain 1665), O121:H19 (strain 02E-2074), O145:NM (strain G55578620), and O157:H7 (strain ATCC 43895) (data not shown). However, O26:H11 (strain 3392) and O121:H19 (strain 02E-2074) were the only two of the STEC reference strains that utilized sorbose. An O26:H11 strain (strain 3392) was the only one that used both sorbose and raffinose or saccharose, but a combination of using sorbose and raffinose gave a better colony color discrimination than using sorbose and saccharose.

The API ZYM test kits also indicated that STEC strains of serogroups O26, O45, O111, O121, and O145 carried β-D-galactosidase activity. The O121:H19 strain showed weak β-D-galactosidase activity against 2-naphthyl-β-D-galactopyranoside compared with that of the O121:H7 strain (data not shown). The reaction between β-D-galactosidase and BCIG yields 5-bromo-4-chloro-3-hydroxyindole and galactose. 5-Bromo-4-chloro-3-hydroxyindole spontaneously dimerizes and is oxidized into 5,5′-dibromo-4,4′-dichloro-indigo, an intensely blue product. The combination of the color change due to carbohydrate fermentation and the blue color from the reaction between β-D-galactosidase and BCIG resulted in differentiation among the STEC serogroups tested on the medium after 24 h of incubation at 37°C (Table 1 and Fig. 1). An O26 strain was able to ferment both sorbose and raffinose and reduced the local pH of the medium to acidic, changing phenol red and bromothymol blue to a yellow color and, combined with the β-D-galactosidase activity and bile precipitation, resulted in a bright turquoise blue with a lapis center (Fig. 1A). Representative strains from serogroups O45, O103, and O111 only fermented raffinose, but the O103 strain did not have β-D-galactosidase activity. The combination of carbohydrate fermentation and reaction of β-D-galactosidase resulted in blue-green, light green, and dark blue-green colors for the O45, O103, and O111 strains, respectively (Fig. 1B, 1C, and 1D). The light blue-gray colony color of the O121:H19 strain (Fig. 1E) was due to utilization of only sorbose in combination with a weak reaction of β-D-galactosidase activity. The O145 strains did not ferment both sorbose and raffinose but utilized amino acids and peptides from the medium and shifted the pH to alkaline, changing phenol red and bromothymol blue to red and blue colors, respectively, and combined with the β-D-galactosidase activity, resulted in a purple color. In contrast with the *E. coli* O121:H19 strain, the beef-isolated *E. coli* O121:H7 strain produced a purple color (Table 1). This strain did not utilize both sorbose and raffinose and, thus, had the same colony color as the O145:NM strain. Possé et al. *(26)* developed a differential medium using neutral red and BCIG for non-O157 STEC serogroups O26, O103, O111, and O145 and reported that O26, O103 and O111, and O145 strains produced red-purple, blue-purple, and light green colony colors, respectively.

The USMARC chromogenic agar medium did not support the growth of gram-positive bacteria, and the

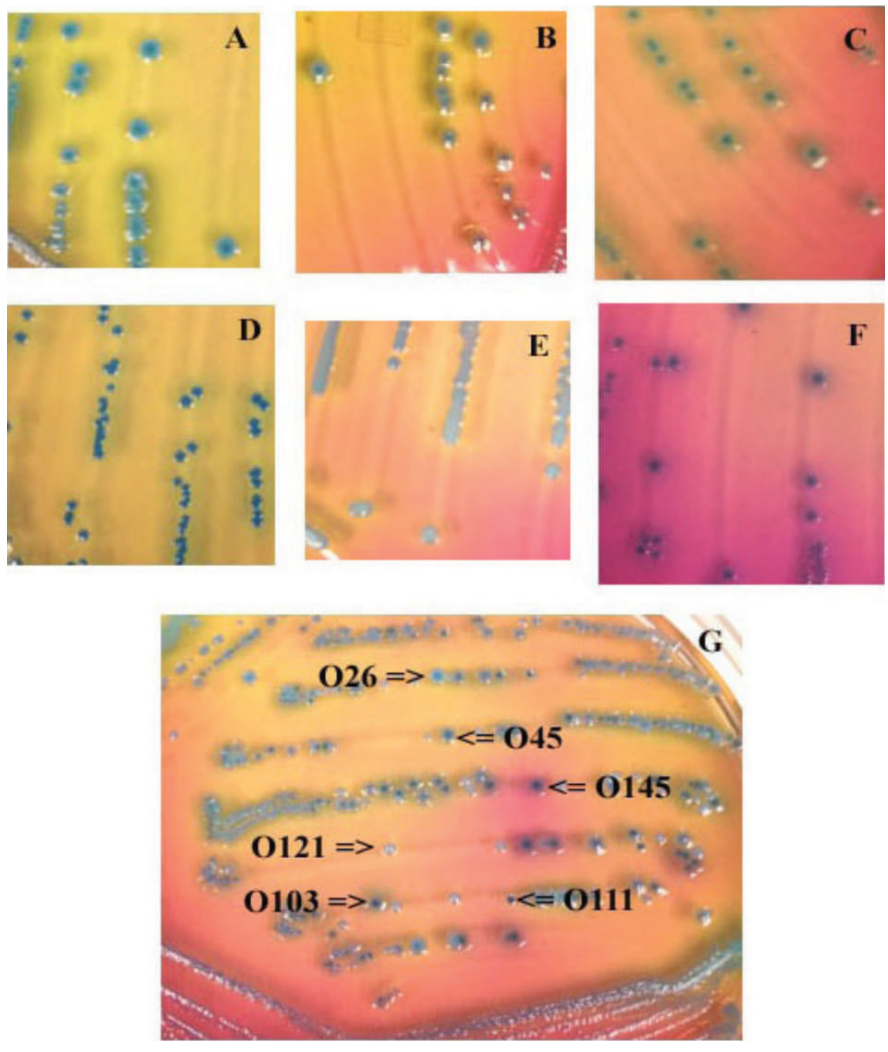

FIGURE 1. *Serogroup color profiles on the USMARC chromogenic medium are as follows: O26, turquoise blue with lapis blue center (A), O45, bluish green to light green with center (B), O103, light green to blue-green with center (C), O111, dark blue-green without center (D), O121, light blue gray without center (E), O145 or O121:H7, purple and magenta halo or background (F), and mixed serogroups O26, O45, O103, O111, O121, and O145 (G).*

growth of *L. monocytogenes, S. aureus,* and lactic acid bacteria was completely inhibited. However, some bacteria in the *Enterobacteriaceae* family grew on this medium with colony color characteristics similar to those of non-O157 STEC serogroup strains tested. For example, *Citrobacter freundii* and *Enterobacter cloacae* showed good growth with the medium containing 0.125 mg of potassium tellurite per liter and produced a turquoise colony color with a center, similar to serogroup O26 strains. With an increase in the amount of potassium tellurite to 1.0 mg/liter, the growth of *C. freundii* was inhibited and poor growth of *E. cloacae* was observed. The presumptive colonies can be picked for biochemical identification or confirmed to be serogroup O26 using PCR as described previously. The growth of most of the *Enterobacteriaceae,* with the exceptions of *Escherichia hermanii* and *Shigella flexneri,* was inhibited by increasing the amount of potassium tellurite to 1.0 mg/liter (Table 1). However, at 1.0 mg of potassium tellurite per liter, only non-O157 STEC strains of serogroups O26, O45, O103, O111, and O145 grew well. The serotype O121:H7 strain grew poorly, while the serotype O121:H19 strain did not grow when the USMARC chromogenic agar medium was formulated with 1.0 mg of potassium tellurite per liter. This was not unexpected, as different strains of *E. coli* are known to vary in their resistance to potassium tellurite *(11).* The MICs of tellurite (ranging from 0.05 to 400 mg/liter) for a wide range of 543 STEC strains were investigated *(14),* and 78% of STEC strains were found to require a MIC of 3.1 mg/liter, while 22% of tested strains required MICs below 3.1 mg/liter. Orth et al. *(21)* found that the ability of STEC strains to grow on tellurite-containing media is due to the presence in the majority of non-O157 STEC strains of *ter* genes that encode tellurite resistance. The presence of the *ter* gene cluster was also associated with the presence of *eae* genes *(21),* which is in agreement with the study of Fukushima et al. *(14),* where 94% of STEC isolates that grew in the presence of tellurite were *eae* positive.

Detection of STEC strains from inoculated fresh beef tissues. The strains of all six serogroups that were inoculated onto surfaces of fresh beef were recovered with the USMARC chromogenic agar medium (Table 2). The recovery rates of bacteria of the six serogroups ranged from 54.2 to 97.9%. Strains of serogroup O26 were the most frequently recovered from inoculated fresh beef after chilled storage for 48 h at 2 to 4°C, followed by strains of serogroups O103, O111, and O145. Strains of serogroups O45 and O121 were recovered the least often due to two factors. First, there are no commercial IMS beads available for serogroups O45 and O121. IMS has been shown to be an

TABLE 2. *Recovery of six serogroups of non-O157 STEC strains from a cocktail mixture inoculated onto fresh beef flanks using USMARC chromogenic medium*[a]

Replication	No. of samples (n = 96)	No. (%) of samples positive for serogroup[b]:					
		O26	O45	O103	O111	O121	O145
A	32	32	21	30	23	14	20
B	32	32	18	32	29	18	32
C	32	30	26	22	27	20	32
Total	96	94 (97.9) A	65 (67.7) C	84 (87.5) B	79 (82.3) B	52 (54.2) C	84 (87.5) B

[a] USMARC chromogenic agar medium was formulated with 0.125 mg of potassium tellurite per liter.
[b] The percentages for positive samples with the same letter are not significantly different ($P > 0.05$).

effective and important tool for the isolation of STEC bacteria, especially *E. coli* O157:H7 strains, from complex sample matrices, including bovine feces *(19),* soils *(16),* milk *(27),* and ground beef *(2, 33).* Second, the background flora and/or other serogroups competed with each other either in the enrichment medium or on the chromogenic agar plate. Because colony colors are based upon carbohydrate fermentation and pH indicators, adjacent colonies could influence different shades of colony colors due to different microenvironments around the colonies. It should be noted that background microflora can confound the recovery of non-O157 STEC. Compared with the results with the USMARC agar medium, it was difficult to differentiate among serogroup O26 (red-purple), O103 (blue-purple), and O111 (blue-purple) when the differential medium developed by Possé et al. *(26)* was used because background flora caused all colony colors to change to blue-purple to purple (data not shown).

Detection and isolation of the top six non-O157 STEC serogroups from cattle feces. Although recovery of non-O157 STEC strains inoculated onto surfaces of beef flanks was successful, an additional study was performed to examine the detection and isolation of the naturally occurring top six non-O157 STEC serogroups from the heavy background microflora of cattle feces using the same colony color profiles described above (Table 1 and Fig. 1). The medium was formulated with a predetermined amount of 0.5 mg of potassium tellurite per liter instead of the 0.125 mg of potassium tellurite per liter of the basal medium in order to overcome high background flora of fecal samples, but it could still detect serogroup O121. The top six STEC serogroups represent only a minor component of cattle's fecal microflora *(9, 19),* and most cattle secrete less than 100 CFU/g of STEC bacteria *(23).* In this study, isolates of serogroups O26, O45, O103, O111, O121, and O145 were detected and isolated from cattle feces (Table 3). A total of 114 isolates of the top six serogroups were recovered from 1,897 cattle fecal samples. Serogroup O103 bacteria were the most commonly isolated among the serogroups. Of the 114 top six serogroup isolates, 35 were determined to be STEC, carrying either the stx_1 or stx_2 gene. Twenty isolates belonged to serogroup O26, which was the most frequently isolated STEC serogroup in this study, followed by serogroup O103. Serogroup O111 bacteria were the least frequently isolated from this study, even though IMS was used to concentrate bacteria of this serogroup from enriched samples. The low recovery rate of serogroup O111 bacteria may be due to their low population levels in fecal samples, so that serogroup O111 bacteria could not compete well with background microflora. It was also found that the Dynabeads for O111 bacteria have a low affinity, causing weak antibody-antigen complexes to remain intact during the IMS procedure *(32).* The detection and isolation of serogroups O45 and O121 may be underestimated, as discussed above. The USMARC chromogenic agar medium has a limitation in the detection of non-O157 STEC strains. Because differentiation of the agar medium among serogroups for non-O157 STEC strains is based on the utilization of carbohydrates, β-galactosidase activity, and toleration of selective agents and non-O157 STEC strains and other strains of *E. coli* are diverse between themselves, strains that have the same aforementioned properties can grow on this medium and have the same color characteristics as strains of the top six non-O157 STEC serogroups. For example, 33 isolates of serogroup O26 were isolated from cattle feces, but only 20 isolates were Shiga toxigenic strains (Table 3). Of 20 Shiga toxigenic strains, 19 isolates had a turquoise colony color, while 6 strains that were non-Shiga toxigenic (*hlyA*, *eae*) also had a turquoise colony color. Therefore, the USMARC chromogenic agar medium provided color profiles that helped with routine screening among serogroups of the non-O157 STEC bacteria when compared with the commonly used sorbitol MacConkey agar, which mostly produces pink to red colonies for non-O157 STEC strains. The number of colonies picked from sorbitol MacConkey agar is higher than the number picked from the USMARC chromogenic agar medium due to the one colony color, which subsequently increases the time for further characterizations. The presumptive colonies from the USMARC chromogenic agar medium need to have their O-groups identified and their virulence factors determined using PCR as described above. Isolates from other agar media, such as CHROMagar O26/O157, CHROMagar STEC, or Rainbow, are also presumptive colonies, and further identifications and characterizations are needed. As mentioned previously, a high background microflora can interfere with the detection and isolation of STEC due to utilization of carbohydrates causing pH changes around the colonies, which limits the detection of target organisms. This interference, however, can be alleviated using treatment with hydrochloric acid

TABLE 3. *Isolation of naturally occurring serogroups O26, O45, O103, O111, O121, and O145 from feces of feedlot cattle*[a] *using USMARC chromogenic agar medium*[b]

Serogroup	No. of isolates	Virulence factor[c] profile	Characteristics
O26	6	*eae*	Light or pale green without center
O26	6	*hlyA*, *eae*	Turquoise blue without center
O26	1	*hlyA*, *eae*	Purple with magenta background
O26	13	*hlyA*, *eae*, stx_1	Turquoise blue with dark blue center
O26	1	*hlyA*, *eae*, stx_1	Light green with center
O26	6	*hlyA*, *eae*, stx_2	Turquoise blue without center
O45	1	*eae*	Bluish green to light green with center
O45	1	*hlyA*, *eae*, stx_1	Bluish green to light green with center
O45	1	stx_2	Light turquoise blue without center
O103	65	*hlyA*, *eae*	Purple with magenta background
O103	4	*hlyA*, *eae*, stx_1	Light green to bluish green with center
O103	2	*hlyA*, *eae*, stx_1	Light green to bluish green with light turquoise center
O111	1	*hlyA*, *eae*, stx_1	Turquoise blue without center
O121	2	*hlyA*, *eae*, stx_2	Pale green without center but with magenta background
O145	2	*hlyA*, *eae*, stx_1	Purple with magenta background
O145	1	*hlyA*, *eae*, stx_1	Turquoise bluish green without center
O145	1	*hlyA*, stx_2	Purple with magenta background

[a] Fecal samples (1,897) were collected from 271 feedlot cattle.
[b] USMARC chromogenic medium was formulated with 0.5 mg of potassium tellurite per liter to reduce background flora but allow the growth of *E. coli* O121 strains.
[c] The following virulence genes of the isolates were determined using multiplex PCR *(22)*: *eae,* intimin; *hlyA,* EHEC hemolysin; stx_1 and stx_2, Shiga toxins.

(0.125 N for 30 s), which has been reported to be an effective method for isolation of STEC from feces, food, and environmental samples *(13)* by taking advantage of the characteristic tolerance of low-pH environments exhibited by many strains of STEC *(17)*. Serial dilution before surface plating on this agar medium also helps to reduce the amount of colonies on the plate, which helps in the differentiation of colony colors among serogroups. However, the sensitivity of detection may be lost if target organisms from samples are at low levels in the beginning.

When compared with the colony color profiles of STEC strains from Table 1 and Figure 1, most of the isolates from cattle feces had color profiles similar to those of the reference strains on the USMARC chromogenic agar medium, except for strains of serogroups O111 and O121. There were certain strains that developed different color characteristics than the reference strains. For example, one isolate each from STEC serogroups O26, O45, and O145 had colors of light green, light turquoise blue, and turquoise blue-green instead of bright turquoise blue, bluish green, and purple, respectively (Tables 3 and 1). STEC strains of serogroups O111 and O121 from cattle feces did not have the same colony color characteristics as the reference strains on the USMARC chromogenic agar medium. The medium detected both STEC and non-STEC strains of serogroups O26, O45, O103, O111, O121, and O145 (Table 3), and hence, downstream characterization of STEC, such as PCR for virulence factors, is required. Interstrain phenotypic variability was observed in some cases. For example, light green to blue-green colonies were observed to be serogroup O26, O45, O103, O121, or even O157 (Tables 1 and 3). Therefore, presumptive isolates should be confirmed using serogroup-specific PCR and genetic characterization of virulence genes, such as *stx* and *eae,* in order to properly identify STEC strains of the top six serogroups.

In conclusion, the non-O157 STEC serogroups are a challenging problem because, unlike *E. coli* O157:H7, they have no unique or distinguishing physiological features or phenotypic characteristics common to all pathogenic strains to readily discriminate them from other *E. coli* strains. The application of the USMARC chromogenic medium allows selective detection and isolation of STEC serogroups based on the carbohydrates selected and β-galactosidase activity, as well as tolerance to selective agents. The colony color profiles of STEC strains of serogroups O26, O45, O103, O111, O121, and O145 on the medium described in this study could be used to significantly improve screening of the top six non-O157 STEC serogroups. The USMARC chromogenic agar medium can be used in routine diagnostic laboratories to screen for STEC in food products and feces from animals and humans. Confirmation of all presumptive colonies using biochemical characterizations and/or PCR of O-groups is recommended.

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