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Evaluation of a New Chromogenic Agar Medium for Detection of Shiga Toxin-Producing *Escherichia coli* (STEC) and Relative Prevalences of O157 and Non-O157 STEC in Manitoba, Canada

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This study assesses the detection performance of CHROMagar STEC medium relative to a reference cytotoxin assay and describes the current relative prevalence of O157 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serotypes within the province of Manitoba, Canada. Over a 10-month period, 205 nonfrozen routine stool submissions to Cadham Provincial Laboratory (CPL) were used to assess the performance of CHROMagar STEC. Of the 205 stools, 14 were identified as true positives by a cytotoxin assay, with resultant CHROMagar STEC sensitivity, specificity, and positive predictive and negative predictive values of 85.7%, 95.8%, 60.0%, and 98.9%, respectively. Using a separate panel of 111 STEC strains, CHROMagar STEC was shown to support the growth of 96 (86.5%) isolates. To assess relative prevalence, attempts were made to isolate by any means all STEC strains identified at CPL over a 17-month period. Of 49 isolates (representing 86.0% of all STEC infections detected), only 28.6% were O157 STEC strains. Of the 35 non-O157 STEC strains, 29 were subjected to further molecular analysis. In contrast to earlier results from our area, carriage of stx_2 appears to have increased. Overall, although CHROMagar STEC is not recommended as a primary screen, our results indicate that it is an effective supplemental medium for the isolation of probable STEC strains. Increased isolation of these serotypes is warranted to better understand their prevalence, clinical characteristics, and epidemiology and aid in the development or enhancement of food safety control programs targeting all STEC serotypes.

Shiga toxin-producing *Escherichia coli* (STEC) emerged in North America in 1983 as a ground beef-associated outbreak of serotype O157:H7. The clear link between this serotype and subsequent large-scale outbreaks, coupled with its ease of detection on sorbitol MacConkey agar (SMAC), has led to a bias toward O157 detections in clinical and public health laboratories (1, 2). However, the non-O157 serogroups of STEC are also clearly significant in terms of public health, given the recent STEC O104:H4 outbreak in Germany and France in 2011 (3, 4) and the multitude of studies showing that the number of non-O157 STEC infections can nearly equal or sometimes surpass the number of O157 STEC infections (5–11). Since culture isolates are still the current basis for inclusion of organisms in molecular surveillance systems (12, 13), this lack of diagnostic attention and the resulting underrepresentation of isolates further hamper our ability to detect all cases of STEC disease and better understand its epidemiology.

Given the poorer understanding of non-O157 STEC and the continued need for organism isolation, new strategies are necessary for non-O157 STEC detection and isolation. The isolation and identification of non-O157 is more challenging than identifying O157 STEC as most non-O157 strains are sorbitol fermenting, limiting the effectiveness of SMAC. In this study, we evaluated CHROMagar STEC, a new chromogenic medium intended for detection of all STEC serotypes. This medium is produced by CHROMagar Microbiology (Paris, France). Using this medium, all STEC strains produce mauve colonies which are either fluorescent (non-O157) or nonfluorescent (O157) under UV light. This medium facilitates the identification and isolation of most STEC serotypes, with the added benefit that isolation of the organism would also facilitate the inclusion of non-O157 STEC strains in molecular surveillance systems. The experiments carried out within this study were meant to address two main objectives: (i) to assess the detection performance of CHROMagar STEC relative to a reference cytotoxin assay; (ii) to describe the current relative prevalence of O157 and non-O157 STEC serotypes within our local geographic area (the province of Manitoba, Canada).

MATERIALS AND METHODS

Diagnostic specimens and reference standard. Primary diagnostic specimens used in this work consisted of human stool specimens received at Cadham Provincial Laboratory (CPL), Winnipeg, Manitoba, Canada. Some assays also relied on purified STEC strains from the CPL culture collection or frozen (−80°C) Shiga-toxin (ST)-positive stools (or colony sweeps from polymyxin extraction (PECS) plates previously detected at CPL using the cytotoxin reference standard described below.

CPL specimen collection guidelines and diagnostic protocols require that stool specimens be shipped to the laboratory without transport medium. Stools are routinely examined for *Salmonella*, *Shigella*, *Yersinia*, *Aeromonas*, and *Campylobacter*. Outbreak-associated stools are also examined for *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, and *Listeria monocytogenes*. All liquid or bloody stools are examined using a cytotoxin assay for the presence of ST or ST-producing organisms (14). During outbreak investigations, all stools are examined for ST activity, regardless of consistency. The CPL cytotoxin assay detects either free fecal ST (FST) or ST produced after polymyxin extraction (PECS) as per Karmali et al. (15). For FST, stools are diluted (1 volume of stool to 3 volumes of phosphate-buffered saline [PBS] or 1.5 stool volumes to 3 volumes of PBS for very watery stools). For PECS, Penassay broth is inoculated and

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incubated for 4 to 5 h at 37°C. Following centrifugation, pelleted cells are resuspended in polymyxin solution (1,000 units/ml) and incubated at 37°C for 30 min. Following centrifugation, the supernatant is filter sterilized (0.22-um pore size) and tested for cytotoxic activity. For both FST and PECS, cells are first examined for cytotoxic activity at approximately 19 h postinoculation and, if necessary, on a second day no later than 48 h postinoculation (16). All ST activity is confirmed by neutralization of toxin activity with rabbit polyclonal antibodies raised against ST1 and ST2. This cytotoxin assay was used as the reference standard for assessing diagnostic performance characteristics of CHROMagar STEC.

Culture methods. Cotton-tipped swabs were used to directly inoculate stool specimens to CHROMagar STEC medium without dilution. CHROMagar STEC plates were incubated in the dark at 35°C for a minimum of 24 h. Plates were routinely examined between 18 and 24 h postinoculation. Non-ST-producing *E. coli* and species other than *E. coli* began appearing on CHROMagar plates at 36 to 48 h, and reading of plates past 26 h was not productive. Occasionally, some non-STEC strains grew within 18 to 24 h, but these organisms (typically *Enterobacter* or *Proteus* species) grew as blue or colorless colonies and were easily differentiated from the mauve colonies typical of STEC. SMAC plates were incubated at 35°C for 18 to 24 h. Potential *E. coli* strains detected by either culture method were subsequently identified as *E. coli* by the Vitek 2 system (bioMérieux, Canada) and confirmed as ST producers using the above cytotoxin test. When the growth of purified isolates on CHROMagar STEC was assessed, isolates were initially grown on blood agar medium and diluted in saline to a concentration of 10^7 CFU per ml (based on a McFarland standard) (17). Ten microliters of this suspension was inoculated onto CHROMagar STEC and incubated as above for 24 h.

Serotyping and molecular methods. All *E. coli* isolates newly identified in this study were serotyped by conventional agglutination using antiserum prepared at the National Microbiology Laboratory (NML), Winnipeg, Manitoba, Canada. Detection of the stx_1, stx_2, *eaeA*, and enterohemorrhagic *E. coli* (EHEC) *hlyA* loci was performed by using the multiplex PCR described in Paton et al. (18). stx_2 subtypes were determined using PCR-restriction fragment length polymorphism (RFLP) as described by Pierard et al. (19).

RESULTS

The sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) of CHROMagar STEC were assessed over a period of 10 months using stools with receipt dates ranging from 13 June 2011 to 4 April 2012. During this time nonfrozen routine stool submissions (including liquid and/or bloody stools) were plated directly to CHROMagar STEC upon receipt and compared against the cytotoxin assay described above. Only a subset of all stools received could be evaluated, given limitations related to personnel time and CHROMagar STEC plate availability; stools that were included in the evaluation were chosen prior to the availability of cytotoxin results. In total, analysis of 205 stools from 185 individuals was possible (this stool number represented 10.9% of the 1,875 stools received at CPL for FST/PECS testing over the 10-month period). Of the 205 stools, 121 were classified as liquid, 19 were classified as liquid with blood, and 17 were formed but were accompanied by a specific physician request for ST testing. The remaining 48 stools were not classified. Twelve stools were from pediatric cases, while 193 were from adults. All plating and reading of plates were independent of and occurred prior to availability of cytotoxin results.

The detection performance of CHROMagar STEC relative to the cytotoxin assay is shown in Table 1. Of the 14 true positives, 12 were detected by CHROMagar STEC. The sensitivity, specificity, PPV, and NPV of CHROMagar STEC were 85.7%, 95.8%, 60.0%, and 98.9, respectively. The relatively low PPV resulted from the growth of 8 *E. coli* strains on CHROMagar STEC that ultimately proved to be non-toxin producers. The stools from which these isolates originated were also negative by the cytotoxin assay, and molecular testing of the isolates demonstrated that all were negative for ST-producing genes (one isolate was identified as an enteroaggregative *E. coli* while the remaining seven were not identified as a known pathogenic type of *E. coli*).

TABLE 1 Diagnostic test performance of CHROMagar STEC relative to the cytotoxin reference standard

CHROMagar STEC result	Cytotoxin assay result (no. of samples)		
	Positive	Negative	Total
Positive	12	8	20
Negative	2	183	185
Total no. of samples	14	191	205

To assess the ability of CHROMagar STEC to support the growth of various STEC serotypes/genotypes, we expanded the panel of isolates identified above by retrieving additional purified strains, ST-positive stools, and/or PECS colony sweeps from our known-positive specimen collection. For PECS colony sweeps and ST-positive stools, we isolated organisms with the use of CHROMagar STEC or SMAC; the latter was used to ensure inclusion of STEC that might be unable to grow on CHROMagar STEC. In total, a panel of 111 STEC strains was assembled, representing 20 serogroups and 29 serotypes. This panel included 49 isolates identified from specimens received over the time frame of 7 October 2010 to 23 March 2012 (including strains identified above; these 49 isolates served the secondary purpose of demonstrating the current relative prevalence of different STEC serotypes in our geographic area and are described in greater detail below). An additional 51 non-O157 STEC strains previously identified at CPL between 2002 and 2010 were also included. The majority of these isolates (32/51) originated from a previous surveillance study at CPL that occurred between June 2002 and April 2004 (10). Finally, 11 O157 STEC strains specifically selected to represent the most common pulsed-field gel electrophoresis (PFGE) patterns seen in Manitoba were also selected. The majority of the O157 STEC strains (8/11) were identified in 2006, with the remainder isolated in 2009/2010.

Of the 111 STEC isolates tested, 96 (86.5%) grew on CHROMagar STEC in an expected manner, 2 (1.8%) grew in an inhibited manner (i.e., <10 colonies were present on the plate that were subsequently confirmed as the expected STEC strains), and the remaining 13 (11.7%) showed no growth (despite repeated attempts) (Table 2). In two situations where multiple isolates were available for some non-O157 STEC serotypes (O26:H11 and O121:H19), the inability of CHROMagar STEC to support growth was not a serotype-specific trait. For these two serotypes, respectively, 1 of 14 isolates and 3 of 16 isolates were not able to grow on the medium or showed inhibited growth. Similarly, growth of O157:H7 STEC on CHROMagar STEC was not universal as 1 of 25 O157:H7 isolates was unable to grow on the medium. This one isolate originated from a known outbreak that occurred in Manitoba in 2010. To determine whether additional isolates from this outbreak were unable to grow on CHROMagar STEC, we selected two additional outbreak isolates with the same PFGE patterns (ECXAI.0294/ECBNI.0691) plus one outbreak isolate that showed a slight vari-

TABLE 2 Ability of CHROMagar STEC to support growth of STEC

Serogroup	Serotype	Growth (no. of isolates [%])	
		Yes	No
O5	HNM	2	
O6	H34		1
O8	H8		1
O8	H9		1
O26	HNM	6	
	H21	2	
	HU	2	
	H11	13	1
O45	H2	1	
O64	HU		1
O69	H11	1	
O103	H21	3	
	H25	4	1[a]
	H2	3	
	H11	2	
	HU	1	
O108	H11	1	
O111	HNM	10	
O113	H21		1
O121	H19	13	3[a]
	H1	3	
	HNM	1	
O123	H2	1	
O145	HNM	3	
O146	H21		1
O157[b]	H7	24	1
O186	H2		1
O rough	H6		1
O rough	H21		1
Total		96 (86.5)	15 (13.5)

[a] Inhibited growth for the O103:H25 isolate and 1 of 3 O121:H19 isolates.
[b] Eleven of these O157 isolates represent the most common PFGE types found in our area and include the following: ECXAI.0256/ECBNI.0012; ECXAI.0854/ECBNI.0130; ECXAI.0001/ECBNI.0012; ECXAI.1497/ECBNI.0012; ECXAI.1551/ECBNI.0004; ECXAI.1186/ECBNI.0299; ECXAI.1128/ECBNI.0284; ECXAI.0001/ECBNI.0297; ECXAI.0170/ECBNI.0154; ECXAI.2250/ECBNI.0676; ECXAI.0294/ECBNI.0691.

ation in its PFGE pattern (designated ECXAI.2488/ECBNI.0691). None of these three additional isolates was able to grow, indicating a consistent inability of this outbreak strain to grow on the medium. Given that these three isolates would represent a recent clonal expansion of a common ancestral strain, they were not included as part of the denominator of 111 STEC strains to avoid unnecessarily skewing percentages.

Following the assessment of diagnostic performance, we used the opportunity of this study to describe the relative prevalence of STEC serotypes currently circulating in our geographic area and the molecular characteristics of these isolates. As noted above, to accomplish this objective, we utilized the 14 isolates identified as part of the CHROMagar STEC assessment and then broadened the time frame by retrieving from −80°C storage all other ST-positive stools (and/or stocked colony sweeps from PECS plates) that had been identified and stocked at CPL between 7 October 2010 and 23 March 2012. In total, over this period of time, specimens from 57 patients had been identified as ST positive by cytotoxicity (i.e., the 14 cytotoxin-positive individuals above plus a further 43 ST-positive stools). Stool specimens/colony sweeps for five of these patients were not found, and for a further three patient specimens no organism could be isolated from either the frozen stool or the colony sweeps (by either SMAC or CHROMagar STEC). For the remaining 49 patients, we were able to isolate STEC using either CHROMagar STEC or SMAC medium (Table 3). Only one colony per patient was typed; therefore, the number of STEC isolates examined is equivalent to the number of infected individuals and would generally represent the predominant serotype present in a given individual. This group of isolates represents 86.0% (49/57) of the cytotoxin-positive patients identified at CPL over a 15-month period. A subset of 29 non-O157 isolates (all with recent isolation dates falling within the time frame of 2010 to 2012) was also characterized for the presence of four virulence factor genes (*stx*$_1$, *stx*$_2$, *eaeA*, and EHEC *hlyA*) typically associated with STEC (Table 4).

TABLE 3 STEC serotypes, detected by any method, in Manitoba, Canada, with specimen receipt dates from 7 October 2010 to 23 March 2012

Serogroup[a]	Serotype	No. of isolates (%)
O8	H9	1 (2)
O26	H11	4 (8.2)
	HNM	2 (4.1)
	H21	2 (4.1)
	HU	2 (4.1)
O69	H11	1 (2)
O103	H21	2 (4.1)
	H2	1 (2)
	HU	1 (2)
O108	H11	1 (2)
O111	HNM	3 (6.1)
O121	H19	8 (16.3)
	H1	3 (6.1)
O123	H2	1 (2)
O157	H7	14 (28.6)
O186	H2	1 (2)
O rough	H6	1 (2)
O rough	H21	1 (2)

[a] Samples are ordered numerically by serogroup.

DISCUSSION

SMAC agar has been the standard for identification and isolation of O157 STEC; however, this medium does not facilitate the isolation of non-O157 serogroups, given their typical sorbitol-fermenting phenotype. Additionally, some O157 STEC strains have also been identified that ferment sorbitol and hence would also not be readily identified on SMAC agar (20). Over the past 15 years, various chromogenic agars have been developed to attempt to overcome some of these issues. CHROMagar O157 (CHROMagar, Paris, France) was developed primarily for the detection of O157 STEC. The manufacturers of this medium have recently devel-

TABLE 4 Molecular characterization of non-O157 STEC isolates

Serotype	No. of isolates	Virulence gene profile			
		hlyA	*eaeA*	stx_1	stx_2
O69:H11	1	+	+	+	−
O26:HNM	1	+	+	+	+
	1	−	+	+	−
O26:H21	2	+	+	+	+
O26:H11	3	+	+	+	+
	1	+	+	+	−
O186:H2	1	+	+	+	−
O123:H2	1	+	+	+	+
O121:H19	8	+	+	−	+
O121:H1	3	+	+	−	+
O111:HNM	3	+	+	+	+
O108:H11	1	+	+	−	+
O103:H21	2	+	+	+	+
O103:H2	1	+	+	+	+

oped a similar medium, CHROMagar STEC, designed to facilitate the isolation of both O157 and non-O157 STEC strains. In this study, we evaluated the detection performance of this medium, and we present a summary of the relative prevalence of STEC serotypes seen within the province of Manitoba over a 17-month period, which emphasizes the frequent occurrence of non-O157 STEC in ST-positive stool specimens.

In our evaluation, CHROMagar STEC had a sensitivity and specificity of 85.7% and 95.8%, respectively, relative to our routinely used, reference diagnostic cytotoxin assay. Although the sensitivity of the medium is relatively high, it did miss some ST-positive stools. This observation suggests that it is most suitable as a supplemental medium for isolating STEC from known positive stools, as opposed to being used on its own as a primary screen. Additionally, the relatively low PPV of 60% occurs as some non-STEC strains are able to grow on the medium; therefore, any suspect STEC isolated on the medium must be verified for the presence of ST. A literature search at the time of writing revealed no other evaluations of CHROMagar STEC for the detection of STEC from human stools; therefore, we were unable to compare our values to other published work. Additional evaluations of the medium would be necessary to further verify and establish the expected detection performance when it is used for human clinical diagnostics.

As part of determining the detection performance of CHROMagar STEC, we also assessed the extent to which the growth of different STEC serotypes was supported by the medium. Although most STEC strains grow on CHROMagar STEC (86.5% of all isolates tested), it is not universally supportive of growth of all STEC strains. Failure to grow was not serotype specific (i.e., some strains of a given serotype will grow, while others will not, including some strains of O157:H7), indicating that the genetic characteristic(s) that governs growth does not correlate precisely with serotype. We identified only one other study that evaluated the growth characteristics of a panel of STEC strains on CHROMagar STEC as part of an evaluation focused on food testing (21). Similar to our percentage of 11.7% (13.5% including isolates showing inhibited growth), Tzschoppe et al. (21) found that CHROMagar STEC failed to support the growth of 17 (13.6%) of 125 enterohemorrhagic *E. coli* isolates. As per our results, they also found that failure to support growth was not serotype specific although there was a trend toward a lesser likelihood of growth for strains within the O103:H2 serotype and sorbitol-fermenting O157:H7. Both our results and those of Tzschoppe et al. (21) confirm that it is always necessary to validate growth of an isolate of interest if CHROMagar STEC is being contemplated as a supplemental agar to facilitate the isolation of specific strains of STEC (e.g., during outbreak investigations when an isolate has been identified through other means).

A secondary objective of this study was to assess the relative prevalence of STEC serotypes. Of the 49 isolates characterized, the O157:H7 serotype was still the most common STEC serotype isolated; however, its prevalence relative to all other non-O157 serotypes as a whole was only 28.6%. This may actually be an overestimate as more severe cases of disease, typically associated with O157 STEC (7), may be more likely to present to emergency departments or clinics. The common occurrence of non-O157 STEC emphasizes the importance of considering these serotypes in ST diagnostic approaches.

In comparison to data collected over the previous 15 years in Manitoba, O157:H7 appears to have continually decreased relative to non-O157 STEC. Using a consistent diagnostic approach, in the late 90s, 50% of ST-positive stools in Manitoba were associated with O157 STEC (9), 37% were in 2002 to 2004 (10), and 29% were associated with O157 STEC in the current study time frame. A downward trend in the proportion of O157 STEC strains relative to non-O157 types has also been observed generally in Canada (2). However, this downward trend is not necessarily global as the relative proportion of O157 strains in Australia has varied from 39% to 74% over a 9-year period with no clear upward or downward trend. Again, enhanced diagnostics and surveillance of non-O157 types are necessary to better understand the overall epidemiological patterns of all STEC types.

After STEC O157:H7, the two most common STEC serotypes identified during our study period were O121:H19 (eight isolates) and O26:H21 (four isolates), consistent with the two most common non-O157 STEC strains identified in this area in 2002 to 2004 (10). Of the 11 non-O157 serogroups identified in our study, the four most common serogroups, O121, O26, O103, and O111 (with, respectively, 11, 10, 4, and 3 isolates), accounted for 80% of the non-O157 isolates. Our results are consistent with the global prevalence of the O26 serogroup in general and specifically the O26:H11 serotype (11, 21a, 22, 23). Our frequent identification of O121:H19 was consistent with the common occurrence of the O121 serogroup in the United States (22) and the O121:H19 serotype in at least one European country (Switzerland) (23). However, this serogroup or serotype was not identified in the Australian OzFoodNet surveillance program (11), suggesting that reservoirs and transmission patterns for some non-O157 serotypes differ from country to country. Vally et al. (11) have noted different risk factors when O157 and non-O157 serotypes are compared as a group. Variations in prevalence for serotypes such as O121 suggest that these differences likely also extend to individual non-O157 STEC. Increased effort at isolation of non-O157 types would assist in furthering our understanding of the epidemiological patterns associated with individual serotypes in different areas.

In contrast to our earlier study (10), stx_2, alone or in combination with stx_1, was common in the non-O157 STEC isolates examined in this investigation. In 2002 to 2004, only 9 of 32 (28.1%) non-O157 STEC isolates carried stx_2 alone or with stx_1 versus 25 of 29 (86.2%) isolates in the present study ($P = 0.0142$; chi-square, 6.011). For the most common non-O157 STEC seen in our area, O26:H11, this shift also occurred. In 2002 to 2004, all 9 O26:H11 isolates examined carried stx_1 alone, while only 1 of 4 isolates in 2010 to 2012 showed this molecular characteristic. Given limited sample sizes and the observation that most of the serotypes identified in 2002 to 2004 were not present in 2010 to 2012, it is not clear if this overall shift is due to a temporal replacement of strains with different molecular characteristics or whether existing strains in our area have newly acquired stx_2.

Among the isolates we identified in 2010 to 2012, the percentage of non-O157 STEC isolates carrying stx_2 alone (12 of 29 [41.4%] isolates) is similar to that seen in other studies (ranging from 21% to 55% of non-O157 STEC isolates examined) (7, 22–25). Our results vary in the percentage of isolates carrying only stx_1 compared to those with both stx_1 and stx_2. All publications we identified report stx_1-carrying isolates as outnumbering those carrying both genes (37% to 61% of non-O157 STEC for the former compared to 2% to 36% of isolates carrying both genes) (7, 22–25). For our isolates the percentages were reversed with 13.8% of isolates carrying stx_1 versus 44.8% with both genes. Five of the above-cited studies indicated isolation dates, and, with the exception of Käppell et al. (23), all used organisms isolated prior to 2002. More recent surveillance data from these areas would be necessary to determine whether the molecular characteristics we identified in Manitoba are localized to our area or whether the acquisition and spread of stx_2 genes have increased. In general, if shifts of this kind are more widespread, additional research would be needed to understand the underlying determinants and their associated clinical and public health implications.

Although CHROMagar STEC is not recommended as a primary screen, our results indicate that it is an effective supplemental medium for the isolation of probable STEC. Given that current evidence suggests that there is an increasing prevalence of non-O157 STEC relative to O157:H7, coupled with the occurrence of several recent outbreaks associated with non-O157 STEC, the use of this medium in conjunction with other selective media will facilitate the isolation of many STEC serotypes. Increased detection and isolation of non-O157 STEC serotypes will also facilitate their inclusion in molecular surveillance systems enhancing the early detection and prevention of STEC outbreaks associated with non-O157 serotypes. In general, increased isolation of these serotypes is warranted to better understand their prevalence, clinical characteristics, and epidemiology and aid in the development or enhancement of food safety control programs targeting all STEC serotypes.

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