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Characterization of Shiga Toxin-Producing *Escherichia coli* O26 Strains and Establishment of Selective Isolation Media for These Strains

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We characterized the carbohydrate-fermenting ability of 31 strains of Shiga toxin-producing *Escherichia coli* (STEC) O26 isolated from diarrhea patients in Aichi Prefecture, Japan, in order to establish selective isolation media for these strains. None of the 31 STEC O26 strains (24 O26:H11, 7 O26:H−) fermented rhamnose, whereas all of the other 108 STEC strains (100 O157, 8 O111) and all of the non-STEC strains except one (i.e., 58 of 59) fermented rhamnose. The great majority of the STEC O26 strains (96.8% [30 of 31]) showed very high resistance to potassium tellurite (MIC ≥ 50 μg/ml), whereas the majority of the non-STEC strains (72.9% [43 of 59]) showed very high sensitivity (MIC ≤ 1.56 μg/ml) to this compound. Accordingly, we developed a rhamnose-MacConkey (RMAC) medium in which the lactose in MacConkey medium was replaced by rhamnose, and cefixime-tellurite-RMAC (CT-RMAC) medium in which potassium tellurite (2.5 mg/liter) and cefixime (0.05 mg/liter) were added to RMAC. All of the STEC O26 strains generated colorless (rhamnose-nonfermented) colonies on both media; the vast majority of selected *E. coli* strains (95.7% [89 of 93; including 26 STEC O157, 8 STEC O111]), other than STEC O26, generated red colonies on RMAC, and most of the non-STEC strains (84.7% [50 of 59]) did not grow on CT-RMAC. We demonstrate that both the RMAC and the CT-RMAC media can be used for the isolation of STEC O26 and that CT-RMAC has better specificity for the routine isolation for STEC O26 in a laboratory.

Since the first recognized outbreak in Oregon and Michigan in the United States in 1982, Shiga toxin-producing *Escherichia coli* (STEC) O157 has emerged as a food-borne pathogen of a significant public health concern in the United States, Canada, and Europe (8, 10, 12).

In Japan, the first outbreak of STEC O157 was reported in 1990, in which two kindergartners among 319 patients died of hemolytic-uremic syndrome. After this outbreak, the reported number of STEC strains isolated was ca. 100 annually between 1991 and 1995. The number of STEC strains isolated increased abruptly to 3,021 in 1996, and ca. 2,000 strains have been isolated since 1997. STEC O157 has been the predominant O serotype among the isolated STEC strains, comprising 90.7% (476 of 525) of the total from 1991 to 1995 and 72.1% of the total in 1999; however, this predominance has been decreasing gradually in recent years (5). Accordingly, the number of STEC strains isolated other than O157 has been increasing gradually. Among these non-O157 STEC isolates, STEC O26 has been the most common serotype, comprising 18.0% (1,066 of 5,913) of the total number of STEC isolates reported from 1997 to 1999 (5). However, effective and selective isolation media for STEC O26 have not been established, whereas isolation media for STEC O157 are widely used in routine laboratory examination in many facilities.

We sought to find useful markers for detecting STEC O26 by investigating its carbohydrate-fermenting ability and to develop and evaluate selective media for STEC O26 isolation on the basis of the differences in carbohydrate fermentation.

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MATERIALS AND METHODS

Bacterial strains. A total of 139 STEC isolates from 139 patients with diarrhea collected between 1996 and 1999 were utilized in this study. Each of these 139 STEC strains was isolated from a geographically and chronologically different case: 31 STEC O26 strains were isolated from 31 independent cases, 100 STEC O157 strains were isolated from 98 independent and 2 outbreak cases, and 8 STEC O111 strains were isolated from 8 independent cases. These 139 isolates, which had been assumed to be STEC strains from biochemical and serotyping tests, were forwarded to our institute from 17 regional health center laboratories and 5 hospitals in different parts of Aichi Prefecture in Japan over a period of 3 years. All of these STEC isolates were confirmed in our laboratory to be STEC strains by testing for the production of Shiga toxins (Stx) and for the existence of *stx* genes by use of a reverse passive latex agglutination test (VTEC-RPLA; Denka Seiken, Tokyo, Japan) (7) and the commercially available primers stx_1 (EVT-1/2) and stx_2 (EVS-1/2) (both from Takara Shuzo, Kyoto, Japan) (6, 13). All 31 STEC O26 strains (24 O26:H11 and 7 O26:H−), which produced only Stx1 and had only the stx_1 gene, were investigated for their carbohydrate fermentation abilities and to measure the MICs of potassium tellurite. In order to compare the fermenting ability and the MIC for STEC O26, 108 strains of STEC strains other than O26 (100 O157 and 8 O111) were also examined, together with 59 non-STEC *E. coli* strains (42 nonpathogenic *E. coli* and 17 diarrheagenic *E. coli* other than STEC). These 59 non-STEC *E. coli* strains had been isolated in our laboratory from stools collected from patients with diarrhea in geographically and chronologically different areas in Aichi prefecture from 1989 to 1999. After characterization of the fermenting abilities and the resistance to potassium tellurite, we developed isolation media for STEC O26. The sensitivity and specificity of the newly developed media were evaluated with 124 strains of *E. coli*, including the 31 STEC O26, 26 selected STEC O157, 8 STEC O111, and 59 non-STEC isolates, together with non-*E. coli* strains (5 strains each of *Enterobacter cloacae*, *Salmonella enterica* serovar Enteritidis, *Shigella flexneri*, and *Shigella sonnei*) isolated from the feces of patients with diarrhea. To examine the efficacy of these new media for detecting STEC O26, we investigated the lower limit of bacterial concentration by using human stools and food samples artificially spiked by each of five selected STEC O26 strains (four O26:H11 and one O26:H−).

Investigation of fermenting ability of STEC O26. The carbohydrate-fermenting ability of all of the STEC and non-STEC was examined with the following 15 carbohydrates: rhamnose, arabinose, dulcitol, glucose, inositol, lactose, maltose, mannitol, mannose, raffinose, salicin, sorbitol, sucrose, trehalose, and xylose (all from Wako Pure Chemicals, Osaka, Japan). The fermenting abilities of the strains were determined by culturing the isolates in peptone water with Andrade's indicator (1%) containing a particular carbohydrate (1%) to observe change of the color of the peptone water after incubation for 7 days at 37°C; this was done for all of the carbohydrates except for sorbitol. Sorbitol-fermenting abilities were determined after 24 h of incubation because STEC O157 is known to ferment this carbohydrate slowly over 2 to 3 days (1).

Resistance to potassium tellurite. The resistance of STEC and non-STEC isolates to potassium tellurite was determined by measuring the MIC of potassium tellurite by a routine method of agar dilution (with a final concentration of potassium tellurite of 100 to 1.56 μg/ml) (11).

Preparation of RMAC and CT-RMAC media. Based on the study of STEC O26 fermenting ability, we developed a medium in which the lactose in MacConkey medium was replaced with rhamnose. RMAC media were prepared as follows from MacConkey agar base (Difco, Detroit, Mich.), which is identical to MacConkey agar but contains no lactose. MacConkey agar base (40 g) and rhamnose (10 g) were mixed in 1 liter of distilled water and autoclaved, and then 20-ml portions of the media were dispensed into plates. On the basis of the findings of STEC O26 resistance to potassium tellurite, we also developed an RMAC medium with potassium tellurite (2.5 mg/liter) and with cefixime (0.05 mg/liter). Cefixime was added because it inhibits the growth of *Proteus* spp., which are known to be rhamnose nonfermenters like STEC O26, but does not affect the growth of *E. coli* (2, 14).

Growth of STEC O26 on RMAC and CT-RMAC media. The growth of all of the STEC O26 strains was evaluated by comparing the mean number of colonies on duplicate plates of RMAC, CT-RMAC, and desoxycholate-hydrogen sulfide-lactose (DHL), a conventional differential medium for enteric bacteria. The seeded bacterial solutions were 1:100,000 dilutions from the concentration-adjusted solutions (by the turbidity) to match the 0.5 McFarland standard (ca. 10^8 CFU/ml). From these solutions, 100 μl each of the respective strains was applied to these three media. The applicability of RMAC and CT-RMAC media for selective isolation of STEC O26 was investigated with 65 selected STEC strains (all 31 STEC O26 and 8 O111 isolates and 26 of the STEC O157 isolates), all of the non-STEC strains (59 strains), and 5 each of the non-*E. coli* strains after these bacterial solutions were inoculated onto each of these two media.

Detection limits of STEC O26 from stool and food samples with RMAC and CT-RMAC media. Stool samples (10 g each) obtained from five healthy human volunteers were mixed well in 200 ml of a sterilized phosphate-buffered saline (pH 7.2) and then centrifuged at 1,500 rpm for 5 min. Aliquots (5 ml each) of the resulting supernatant were dispensed into 20 sterilized plastic tubes and used for stool specimens. The specimens were seeded with five selected STEC O26 strains (four O26:H11 and one O26:H−), and 10 μl each of different concentrations of the seeded stool (10^1, 10^2, 10^3 and 10^4 organisms/ml) was applied to RMAC, CT-RMAC, and DHL media. After the plates were incubated overnight at 37°C, a maximum of five colorless colonies from each of the RMAC and CT-RMAC media and a maximum of five red colonies from DHL were picked up to identify *E. coli* by conventional biochemical tests (3), followed by serotyping of the identified *E. coli* samples with the antisera (Denka Seiken) and analysis for Stx production as described above. Commercially available pasteurized milk samples, on the other hand, were seeded with the same selected five strains of STEC O26, and 100 μl (10^0, 10^1, 10^2, and 10^3 organisms/ml) each of the seeded milk samples was cultured on RMAC and CT-RMAC media. The lowest detection limits of STEC O26 from milk samples were assessed by examining the colonies in each plate.

RESULTS

Characterization of carbohydrate-fermentimg abilities. Results of analyses of the carbohydrate-fermenting abilities of the 139 STEC and 59 non-STEC strains for the 15 carbohydrates are shown in Table 1. None of the 31 STEC O26 strains fermented rhamnose, whereas all of the other STEC strains (108 strains) and all of the non-STEC strains except one (i.e., 58 of 59 strains) fermented rhamnose. It should also be noted that none of the STEC O26 isolates (31 strains), but all of the non-STEC O26 isolates (11 strains), fermented rhamnose.

TABLE 1. Fermentation of 15 selected carbohydrates by STEC O26, O157, and O111 and by non-STEC[a]

Carbohydrate	No. of STEC strains			No. of non-STEC strains (n = 59)
	O26 (n = 31)	O157 (n = 100)	O111 (n = 8)	
Rhamnose	0	100	8	58
Arabinose	31	100	8	59
Dulcitol	0	99	8	47
Glucose	31	100	8	59
Inositol	0	0	0	2
Lactose	31	100	8	53
Maltose	31	100	7	58
Mannitol	31	100	8	59
Mannose	31	100	8	59
Raffinose	30	100	8	45
Salicin	31	0	0	33
Sorbitol	30	0	8	52
Sucrose	31	96	7	42
Trehalose	31	100	8	57
Xylose	31	100	8	58

[a] n, number of strains examined.

Measurement of potassium tellurite MICs. All of the STEC O26 strains, as well as all of the STEC O157 and O111 strains, showed high resistance to potassium tellurite (MIC ≥ 12.5 μg/ml); the great majority of the STEC O26 strains (96.8% [30 of 31 strains]) showed very high resistance to this compound (MIC ≥ 50 μg/ml). In contrast to these STEC strains, most of the non-STEC strains (50 of 59) showed high sensitivity (MIC ≤ 6.25 μg/ml) to this compound; in particular, >70% (43 of 59) of the non-STEC strains showed very high sensitivity (MIC ≤ 1.56 μg/ml).

Comparison of STEC O26 growing on RMAC and CT-RMAC. More than 75% (24 of 31) of the STEC O26 strains generated more colonies on RMAC medium than on DHL, with the average number of colonies on RMAC being 39% greater than that on DHL. In contrast, ca. 60% (18 of 31) of the STEC O26 isolates generated more colonies on CT-RMAC medium than on DHL, although the average number of colonies on CT-RMAC was 11% less than that on DHL (Table 2).

The growth and color of colonies of all of the STEC O26, the selected number of STEC O157 isolates, and all of the STEC O111 and of the non-STEC strains, together with the selected strains of the non-*E. coli* on RMAC and CT-RMAC, are shown in Table 3. It should be noted that all of the STEC O26 strains generated rhamnose-nonfermented colorless colonies on both media; all of the STEC strains other than O26 generated rhamnose-fermented red colonies on these media. It is also noteworthy that the vast majority of the non-STEC strains (55 of 59) generated red colonies on RMAC and that the majority of these non-STEC strains (50 of 59) did not grow on CT-RMAC. Among these non-STEC, four strains that generated colorless colonies on RMAC did not grow on CT-RMAC at all.

Detection limits of STEC O26 from stool and food samples with RMAC and CT-RMAC media. All of the five selected STEC O26 strains seeded in the stool specimens were detected on RMAC and CT-RMAC at a lower concentration (10^3/ml) but were not detected on DHL even at the highest concentration (10^4/ml) (Table 4). It is noteworthy that three of these five

TABLE 2. Percentages of the number of colonies generated on RMAC and CT-RMAC compared to those generated on DHL

Isolate no.	Serotype	% Colonies (versus DHL)		No. of colonies on DHL
		RMAC[a]	CT-RMAC[b]	
1	O26:H11	114	100	51
2	O26:H11	100	84	100
3	O26:H11	158	48	40
4	O26:H11	142	104	50
5	O26:H11	193	118	45
6	O26:H11	355	113	31
7	O26:H11	92	70	120
8	O26:H11	150	90	20
9	O26:H11	156	106	48
10	O26:H11	114	88	51
11	O26:H11	96	90	125
12	O26:H11	194	72	32
13	O26:H11	93	47	70
14	O26:H11	89	57	148
15	O26:H11	107	93	95
16	O26:H11	111	90	100
17	O26:H11	74	46	125
18	O26:H11	124	45	55
19	O26:H11	120	97	90
20	O26:H11	85	107	72
21	O26:H11	172	114	95
22	O26:H11	138	102	139
23	O26:H11	165	96	112
24	O26:H11	125	103	208
25	O26:H−	95	130	40
26	O26:H−	113	39	38
27	O26:H−	111	101	90
28	O26:H−	203	128	40
29	O26:H−	198	89	46
30	O26:H−	256	124	25
31	O26:H−	77	72	130

[a] Median = 120; mean ± standard deviation = 139 ± 59.
[b] Median = 93; mean ± standard deviation = 89 ± 26.

STEC O26 strains were detected at an even lower concentration (10^2/ml) on CT-RMAC. All of the colonies grown on CT-RMAC were colorless, and these colonies were all identified as STEC O26 by conventional biochemical tests and serological screening procedures, along with analyses for Stx production, whereas colonies on the RMAC media were a mixture of both red and colorless colonies. Some of these colorless colonies were identified as *Pseudomonas aeruginosa* by using the API 20E system (Biomérieux, Marcy l'Étoile, France). In contrast to stool specimens with a variety of enteric bacteria, for the spiked milk samples with virtually no other bacteria all of the five seeded STEC O26 strains were detected even with a lower concentration (10 organisms/ml) on both RMAC and CT-RMAC, except for one isolate which grew only at a higher concentration of 10^2/ml on CT-RMAC (Table 5).

DISCUSSION

Characterization of the carbohydrate-fermenting ability of the 139 STEC isolates (31 O26, 100 O157, and 8 O111) revealed that either rhamnose, dulcitol, or salicin could serve as a discriminative marker for the selective isolation of STEC O26 (Table 1). Among these three carbohydrates, rhamnose was considered to be most discriminative because none of the STEC O26 strains, but all of the STEC strains (except for STEC O26) and all but one of the non-STEC strains did ferment this carbohydrate. This rhamnose-nonfermenting characteristic of STEC O26 was a further confirmation of our previous study with 85 STEC O26 strains isolated from patients with unrelated diarrhea in six different prefectures in Japan during 1996 and 1997 (4). This finding was very interesting because a sorbitol-nonfermenting characteristic is a well-known discriminative property of STEC O157 (9). Char-

TABLE 3. Growth and color of colonies of STEC O26 and selected enteric bacteria on RMAC and CT-RMAC

Organism	Serotype	No. of strains examined	No. of colonies on:			
			RMAC		CT-RMAC	
			Red	Colorless	Red	Colorless
STEC	O26	31	0	31	0	31
	O157	26	26	0	26	0
	O111	8	8	0	8	0
Non-STEC	O157	13	13	0	3	0
	O26	12	9	3	5	0
	Other	34	33	1	1	0
Non-*Escherichia coli*						
Enterobacter cloacae		5	4	1	0	1
Salmonella enterica serovar Enteritidis		5	5	0	0	0
Shigella flexneri		5	0	5	0	2
Shigella sonnei		5	1	4	0	0

TABLE 4. Detection limit of STEC O26 in stool samples

Isolate no.	Medium	No. of colonies identified as STEC O26/total no. of colonies[a] with an inoculum[b] of:			
		10^1	10^2	10^3	10^4
1	RMAC	0/5	0/5	2/5	5/5
	CT-RMAC	NG	2/2	5/5	5/5
	DHL	NT	0/5	0/5	0/5
2	RMAC	0/5	0/5	3/5	4/5
	CT-RMAC	NG	1/1	5/5	5/5
	DHL	NT	0/5	0/5	0/5
3	RMAC	0/5	1/5	3/5	5/5
	CT-RMAC	NG	2/2	5/5	5/5
	DHL	NT	0/5	0/5	0/5
4	RMAC	0/5	0/5	1/5	4/5
	CT-RMAC	NG	NG	5/5	5/5
	DHL	NT	0/5	0/5	0/5
5	RMAC	0/5	0/5	1/5	4/5
	CT-RMAC	NG	NG	5/5	5/5
	DHL	NT	0/5	0/5	0/5

[a] NG, no growth; NT, not tested.
[b] The inoculum sizes were 10^1, 10^2, 10^3, and 10^4 seeded organisms/ml of stool sample, as indicated.

TABLE 5. Number of STEC O26 colonies from milk samples on RMAC and CT-RMAC

Isolate no.	Medium	No. of STEC O26 colonies with an inoculum[a] of:			
		10^0	10^1	10^2	10^3
1	RMAC	0	2	10	103
	CT-RMAC	0	2	8	73
2	RMAC	0	3	24	212
	CT-RMAC	0	2	26	180
3	RMAC	0	1	22	187
	CT-RMAC	0	1	17	137
4	RMAC	0	6	28	233
	CT-RMAC	0	6	24	190
5	RMAC	0	2	11	150
	CT-RMAC	0	0	3	99

[a] The inoculum sizes were 10^0, 10^1, 10^2, and 10^3 seeded organisms/ml of milk, as indicated.

acteristics of STEC strains unable to ferment a specific carbohydrate, depending on their serotype, could therefore be used as important discriminative indices for establishing an isolation medium for a specific STEC O serotype. However, more detailed examinations and characterization of carbohydrate-fermenting ability of other STEC O serotypes remain to be elucidated.

Since we found that all of the STEC O26 strains, like STEC O157 (MIC $\geq$ 12.5 μg/ml), were resistant to potassium tellurite (15), we decided to add this compound to suppress the growth of not only the majority of the *E. coli* strains but also most of the gram-negative bacteria constituting the normal flora of human feces. Another critically important way to effectively isolate STEC O26 from fecal samples is to suppress the growth of *Proteus* spp., which are frequently found in human feces and also are rhamnose nonfermenters and thus generate colorless colonies on RMAC. We found that cefixime at a concentration of 0.05 mg/liter effectively suppresses the growth of *Proteus* spp. without affecting the growth of STEC O26 (data not shown). We therefore developed the CT-RMAC medium, which contains both potassium tellurite and cefixime in the RMAC medium. Because potassium tellurite and cefixime effectively inhibited the growth of bacteria other than STEC O26, STEC O26 can specifically be isolated as a colorless colony on CT-RMAC. Accordingly, CT-RMAC is considered to be more useful than RMAC for isolating STEC O26 from human stools containing a large number of and a wide variety of enteric bacteria. In fact, we have observed in the study of stool cultures seeded with STEC O26 that only the colorless colonies of STEC O26 were generated on CT-RMAC. In contrast to the colorless colonies on the CT-RMAC, we observed that a mixture of colorless colonies of STEC O26 and *P. aeruginosa* and a large number of red colonies of a variety of other bacteria in fecal flora were generated on RMAC. However, RMAC might be useful for its good sensitivity when STEC O26 is being detected in a food specimen supposed to have fewer contaminating bacteria. Among the 93 *E. coli* strains other than STEC O26 (i.e., a selected 26 STEC O157 isolates, all 8 O111 isolates, and all 59 non-STEC isolates), only 4 *E. coli* strains (3 non-STEC O26 and 1 O124) developed colorless colonies on RMAC; however, none of these grew on CT-RMAC. These results also suggest that CT-RMAC can be used as a primary medium for a selective isolation of STEC O26 from fecal samples. Even though an isolate of *Enterobacter cloacae* and two of the five *Shigella flexneri* strains generated colorless colonies on both RMAC and CT-RMAC media, these bacteria can be easily differentiated from STEC O26 by biochemical characterization with the use of conventional differentiation media for enteric bacteria, such as triple sugar iron agar, sulfide indole motility, and Voges-Proskauer semisolid agars (3).

In conclusion, both the RMAC and the CT-RMAC media, which we developed based on the rhamnose-nonfermenting characteristic of STEC O26, are very useful and effective isolation media for STEC O26, with CT-RMAC having better specificity for the isolation from stool samples.

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