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NOTES

Rapid Detection and Isolation of Shiga-Like Toxin (Verocytotoxin)-Producing *Escherichia coli* by Direct Testing of Individual Enterohemolytic Colonies from Washed Sheep Blood Agar Plates in the VTEC-RPLA Assay

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By combining the enterohemolysin test and the VTEC-RPLA test (specific for the detection of Shiga-like toxin I [SLT-I], SLT-II, and SLT-IIc), single colonies of SLT-producing *Escherichia coli* were found to constitute between 0.03 and 68.1% of the coliform flora in human stool cultures and were isolated and characterized within 72 to 96 h.

Some types of Shiga-like toxin (SLT)-producing *Escherichia coli* (SLTEC) are important human pathogens causing hemorrhagic colitis and hemolytic uremic syndrome (HUS). The detection of these pathogens from patients' stool samples can be complicated when SLTEC strains are present in low numbers and also if phenotypical traits for their identification are absent (4, 8). About 90% of SLTEC strains isolated from humans exhibit a typical enterohemolytic phenotype on washed sheep blood agar which can be employed as a diagnostic marker for their identification (1, 3). In order to establish a rapid identification and isolation system for SLTEC from human stool, we combined the enterohemolysin test as a microbiological screening system for SLTEC with the VTEC-RPLA test as a rapid detection system for SLT production.

The SLTEC strains and the *E. coli* hemolysins are described elsewhere (2, 3, 7). Washed sheep blood agar plates were prepared in our laboratory (3) and compared with commercially available enterohemolysin agar plates (9) for the detection of different *E. coli* hemolysins (3, 7). No difference between both types of plates for detection of different hemolytic phenotypes was found. All SLTEC strains were analyzed for Vero cell toxicity as described previously (3, 5). *slt-I* and *slt-II* genes were detected by DNA-DNA hybridization and by *slt-I*-, *slt-II*-, and *slt-IIe*-specific PCR (2, 3). *slt-II* and *slt-IIc* were distinguished as described previously (6).

For the isolation of fecal SLTEC, a small amount of stool was inoculated into a tube containing 5 ml of sterile tryptic soy broth. The stool culture was incubated without shaking for 20 to 22 h at 37°C. The next day, the grown culture was serially diluted 10-fold in phosphate-buffered saline (PBS), pH 7.2. From each dilution (10^0 to 10^{-7}), 0.1 ml was spread with a glass rod on Endo agar (Merck, Darmstadt, Germany) and enterohemolysin agar. The plates were incubated for 20 to 22 h at 37°C. The enterohemolysin agar plates were recorded for hemolysis after 3 h of incubation (indicating only α-hemolysin) and after overnight incubation (indicating all types of hemolysins). The titer of coliform bacteria was calculated by count-

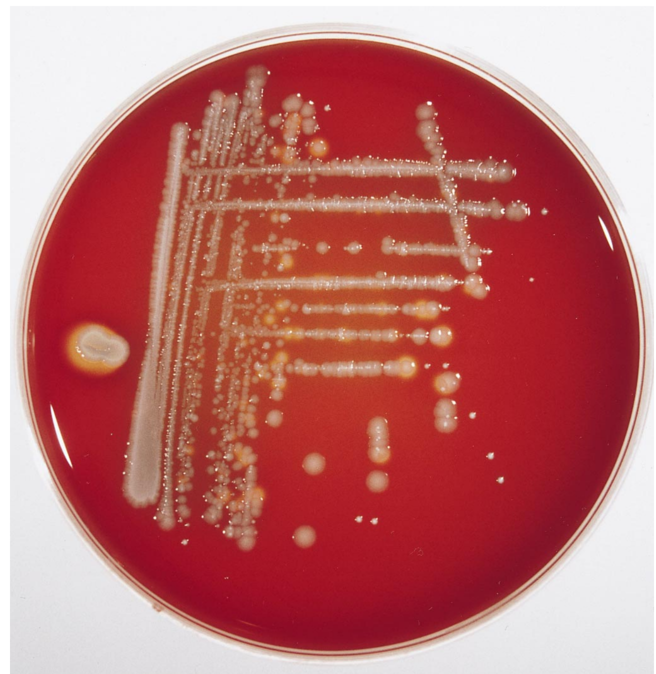

FIG. 1. Enterohemolysin agar plate inoculated with the stool culture of patient 6 (Table 2) after incubation for 22 h at 37°C. Colonies of *E. coli* O157:H7 become visible by their enterohemolytic phenotype after overnight incubation.

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TABLE 1. Sensitivity of the VTEC-RPLA assay for detection of SLT-I (VT1) and SLT-II (VT2) in polymyxin B extracts from single enterohemolysin agar-grown SLTEC colonies

Strain (reference no.)[a]	SLT gene(s)[b]	Serotype	Phenotype[c]	VTEC-RPLA test result	
				VT1	VT2
H19 (3)	*slt-I*	O26:H11	Ehly	1:64[d]	–[e]
IP29580 (3)	*slt-I*	O26:H11	Ehly	1:64	–
CB1745 (1)	*slt-I*	O26:H11	Ehly	1:64	–
CB4169 (3)	*slt-I*	O111:H$^-$	Ehly	1:64	–
CB168 (3)	*slt-I*	O111:H$^-$	Ehly	1:64	–
C3075/69 (3)	*slt-I*	O114:H4	Hly$^-$	1:64	–
CB5131	*slt-I*	ONT[f]	Ehly	1:16	–
CB5500	*slt-I*	O-rough	Ehly	1:64	–
CB2467 (1)	*slt-II*	O157:H7	Hly$^-$	–	1:64
CB2114 (1)	*slt-II*	O157:H7	Ehly	–	1:16
CB569[g]	*slt-II*	O157:H7	Ehly	–	–
CB1716 (1)	*slt-II*	O157:H7	Ehly	–	1:32
CB993 (1)	*slt-II*	O145:H$^-$	Ehly	–	1:4
CB2724 (1)	*slt-II*	O30:H2	Ehly	–	1:2
CB3838	*slt-II*	O40:H2	Hly$^-$	–	1:1
CB2851 (1)	*slt-IIc*	O157:H$^-$	Ehly	–	1:2
CB2255 (1)	*slt-IIc*	O157:H7	Ehly	–	1:4
CB2394 (1)	*slt-IIc*	O157:H7	Ehly	–	1:1
O31	*slt-IIc*	OX3:H21	Hly$^-$	–	1:4
C1403-83 (3)	*slt-I*, *slt-II*	O157:H7	Ehly	1:64	1:8
CB571 (3)	*slt-I*, *slt-II*	O157:H7	Ehly	1:64	1:32
C3007/85 (3)	*slt-I*, *slt-II*	O111:H$^-$	Ehly	1:32[d]	1:2
PH	*slt-I*, *slt-II*	O111:H$^-$	Ehly	1:32	1:2
94CD2	*slt-I*, *slt-II*	O48:H21	Ehly	1:64	1:16
CB2910 (1)	*slt-I*, *slt-IIc*	O157:H$^-$	Ehly	1:64	1:2
H201/89	*slt-I*, *slt-IIc*	O22:H8	Ehly	1:64	1:2
CB3026	*slt-I*, *slt-IIc*	O157:H$^-$	Ehly	1:64	1:8
E32511 (3)	*slt-II*, *slt-IIc*	O157:H7	Ehly	–	1:64
CB573 (3)	*slt-II*, *slt-IIc*	O157:H7	Ehly	–	1:16
H416/89	*slt-II*, *slt-IIc*	O91:H21	Ehly	–	1:64
CB295 (3)	*slt-IIe*	O139:K12:H1	α-Hly	–	–
DG269/1 (2)	*slt-IIe*	O9:H$^-$	Hly$^-$	–	–
E57 (2)	*slt-IIe*	O138:K81	Hly$^-$	–	–

[a] Strains for which no references are given are from this study.
[b] *slt-I*, *slt-II*, *slt-IIc*, and *slt-IIe* were detected by DNA hybridization and by SLT-specific PCR as described in the text.
[c] Ehly, enterohemolytic; α-Hly, α-hemolytic; Hly$^-$, no hemolysis.
[d] Highest dilution of polymyxin B extract which gave a positive reaction in the VTEC-RPLA test.
[e] Negative in the VTEC-RPLA test.
[f] ONT, O antigen not typeable.
[g] This strain was weakly positive for VT2 in multiple-colony extracts (dilution 1:2) but negative for VT2 when single colonies were tested (three individual colonies were tested in three separate tests).

ing lactose-fermenting coliform colonies from the appropriate dilutions of the stool culture on Endo agar. Hemolytic colonies on enterohemolysin agar were counted, and their numbers were calculated as a percentage of the total coliform counts. Some stool cultures yielded only a few hemolytic bacteria which were only visible as small hemolytic spots in the bacterial cell lawn. In this case, bacteria grown in the hemolysis zones were further purified to single colonies by streaking them out on fresh enterohemolysin agar plates, and this was followed by overnight incubation at 37°C.

The VTEC-RPLA test (reverse passive latex agglutination test for the detection of verocytotoxins 1 [VT1] [SLT-I] and VT2 [SLT-II]; Unipath Limited, Basingstoke, United Kingdom) was used for the detection of SLT production (9). We employed this assay for the detection of SLT production in *E. coli* grown on enterohemolysin agar for 22 h at 37°C. A loopful of bacteria was taken from enterohemolysin agar and suspended for SLT extraction in 1 ml of PBS, pH 7.2, containing 5,000 U of polymyxin B (Sigma, Deisenhofen, Germany) per ml. Extracts from single colonies of bacteria were prepared in 100-μl volumes. The extracts were incubated for 30 min at 37°C, and this was followed by centrifugation for 20 min at 1,300 × *g*. The supernatant in dilutions from 1:1 to 1:64 was tested by the VTEC-RPLA test according to the producer's instructions. The test was performed in 96-well (U-bottom) microtiter plates (Becton Dickinson Labware, Oxnard, Calif.). After 24 h of incubation, the reaction mixtures were examined visually for agglutination.

An agreement between SLT genotype and the toxin type indicated by the VTEC-RPLA assay was found by testing 33 SLTEC strains carrying different SLT genes. The VTEC-RPLA test specifically reacted with all strains carrying the *slt-I*, *slt-II*, and *slt-IIc* genes but not with strains carrying only *slt-IIe* or with SLT-negative strains.

The sensitivity of the VTEC-RPLA assay for the examination of single colonies of SLTEC was tested (Table 1). While *slt-I*-carrying strains showed generally high SLT-I toxin titers, low SLT-II titers were associated with some *slt-II* and with all *slt-IIc* strains. This might be due to the fact that low amounts of SLT-II are produced by some SLTEC strains or to differences in the sensitivity of the VTEC-RPLA test towards SLT-I and SLT-II. With single colony extracts, the end point dilution at which SLTs could still be detected was sometimes lower than that with multiple colony extracts.

TABLE 2. Isolation by VTEC-RPLA assay of SLTEC strains from stool cultures on enterohemolysin agar

Description of patient providing sample			Verocytotoxicity[a]	% Ehly^{+b}	No. of colonies tested	VTEC-RPLA test result		SLTEC type isolated from stool		SLT gene(s)
No.[c]	Sex	Clinical status				VT1	VT2	Serotype	Phenotype	
1	Female	HUS	+	0.03	5[d]	–[e]	1:16–1:32[f]	O157:H$^-$	Ehly	*slt-II*, *slt-IIc*
2	Male	HUS	+	1.0[g]	2	1:64	1:32	O157:H7	Ehly	*slt-I*, *slt-II*
3	Female	HUS	+	30.8[g]	2	–	1:32–1:64	O157:H7	Ehly	*slt-II*, *slt-IIc*
3	Female	HUS	+	1.1[g]	2	–	1:16–1:64	O157:H7	Ehly	*slt-II*, *slt-IIc*
4	Male	Enteritis	+	68.1	2	1:16–1:64	1:16–1:64	O157:H$^-$	Ehly	*slt-I*, *slt-II*
5	Male	HUS	–	0.05	8[d]	–	1:2–1:8	O133:H$^-$	Ehly	*slt-IIc*
5	Male	HUS	–	ND[h]				None		
6	Female	HUS	+	17.4	2	–	1:32	O157:H7	Ehly	*slt-II*
7	Male	Healthy	+	1.0[g]	2	–	1:16	O157:H7	Ehly	*slt-II*, *slt-IIc*
8	Male	Enteritis	+	49.4	2	1:16–1:32	–	ONT:H^{-i}	Ehly	*slt-I*

[a] +, verocytotoxic; −, not verocytotoxic.
[b] Ehly$^+$, enterohemolytic phenotype.
[c] Two stool samples were obtained from patients 3 and 5.
[d] SLTEC strains were detected only as hemolytic spots in the lawn of nonhemolytic organisms.
[e] −, negative VTEC-RPLA result.
[f] The range of reactivity in the VTEC-RPLA test with individual colonies is indicated.
[g] Presence of α-hemolytic *E. coli.*
[h] ND, not detected.
[i] ONT, O antigen not typeable.

SLTEC strains were isolated from human stool by the combined use of enterohemolysin agar and the VTEC-RPLA test (Table 2). The titer of coliform bacteria in stool cultures varied between 6.2×10^6 and 1.8×10^9 bacteria per ml. Verocytotoxicity was detected in 8 of 10 stool cultures. Seven stool cultures yielded large numbers of colonies of hemolytic bacteria, and two stool cultures yielded only a few hemolytic bacteria which were visible only as hemolytic spots in the lawn of nonhemolytic organisms. These were first purified to single hemolytic colonies before they were taken for the VTEC-RPLA test. The percentage of enterohemolytic bacteria varied from 0.03 up to 68.1% of the total coliform counts. Low numbers of enterohemolytic colonies (<1% of the coliform counts) were only detectable when dilutions of the stool culture were examined for enterohemolytic colonies. Higher numbers of enterohemolytic colonies could also be detected by streaking the stool culture onto enterohemolysin agar plates (Fig. 1).

All 10 stool cultures additionally harbored nonhemolytic bacteria, and 4 stool cultures also yielded α-hemolytic *E. coli*. A representative number of enterohemolytic colonies from each stool culture was tested for similarity by examining phenotypical traits like fermentation of lactose and sorbitol, β-D-glucuronidase activity on Flurocult *E. coli* O157:H7 agar (Merck), growth on Simmons citrate agar, and agglutination with *E. coli* O-specific rabbit antisera. By these examinations, the enterohemolytic colonies obtained from each individual stool culture were found to be indistinguishable from each other. SLTEC *E. coli* O157 was isolated from six patients, and two patients yielded other SLTEC types. All SLTEC isolates were verocytotoxic and carried one or more SLT genes (Table 2). None of the nonhemolytic and α-hemolytic bacteria which were isolated from the 10 stool cultures were positive for verotoxins or SLT genes. By the combined use of the enterohemolysin agar and the VTEC-RPLA test, human pathogenic SLTEC strains were able to be isolated from stool and characterized for their SLT types within 72 to 96 h.

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