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Detection of Hemolysin Variants of Shiga Toxin-Producing *Escherichia coli* by PCR and Culture on Vancomycin-Cefixime-Cefsulodin Blood Agar

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The presence of a hemolysin-encoding gene, *elyA* or *hlyA*, from Shiga toxin-producing *Escherichia coli* (STEC) was detected by PCR in each of 95 strains tested. PCR products of *elyA* from human STEC isolates of serovars frequently detected in Germany, such as O157:H−, O103:H2, O103:H−, O26:H11, and O26:H−, showed nucleotide sequences identical to previously reported ones for O157:H7 and O111:H− strains. Compared to them, four *elyA* amplicons derived from human isolates of rare STEC serovars showed identity of about 98% but lacked an *Alu*I restriction site. However, the nucleotide sequence of an amplicon derived from a porcine O138:K81:H− STEC strain was identical to the corresponding region of *hlyA*, encoding alpha-hemolysin, from *E. coli*. This *hlyA* amplicon showed 68% identity with the nucleotide sequence of the corresponding *elyA* fragment. It differed from the *elyA* PCR product in restriction fragments generated by *Alu*I, *Eco*RI, and *Mlu*I. Of the 95 representative STEC strains, 88 produced hemolysin on blood agar supplemented with vancomycin (30 mg/liter), cefixime (20 μg/liter), and cefsulodin (3 mg/liter) (BVCC). The lowest added numbers of two to six STEC CFU per g of stool or per ml of raw milk were detectable on BVCC plates after seeding of the preenrichment broth, modified tryptic soy broth (mTSB) supplemented with novobiocin (10 mg/liter), with 16 STEC strains. These strains represented the seven prevailing serovars diagnosed from German patients. However, with ground-beef samples, PCR was essential to identify the lowest added numbers of two to six STEC CFU among colonies of hemolyzing *Enterobacteriaceae*, such as *Serratia* spp. and alpha-hemolysin-producing *E. coli*. We conclude that preenrichment of stool and food samples in mTSB for 6 h followed by overnight culturing on BVCC is a simple method for the isolation and presumptive identification of STEC.

Shiga toxin-producing *Escherichia coli* (STEC) is increasingly recognized as the cause of severe diseases, such as hemorrhagic colitis and hemolytic-uremic syndrome in humans (15), edema disease in piglets (14), and diarrhea in calves (21). In addition to the major virulence factor, Shiga toxin (Stx), and its variants (30), STEC frequently produces intimin (19), which is involved in attaching of the organisms and effacing of gut mucosal cells. Furthermore, STEC secretes pore-forming hemolysins. Iron acquisition by lysis of erythrocytes and impairment of the immune response due to cytotoxicity for leukocytes are assumed to be the main pathogenic functions of the hemolysins (9).

Two different plasmid-encoded hemolysins, both members of the RTX toxin family (9, 28), have been described for STEC. Alpha-hemolysin is formed by porcine edema disease-causing STEC strains of serovars O138:K81, O139:K82, and O141:K85, which produce Stx variant 2e (14), and by *E. coli* causing urinary tract infections and septicemia (17, 22). It generates a clear, broad zone of hemolysis surrounding the colony and is visible after only 4 h on blood agar containing washed sheep erythrocytes and $CaCl_2$ (enterohemolysin agar) (4). The second hemolysin, secreted exclusively by human STEC strains, produces a narrow, turbid, hemolytic halo after overnight incubation on enterohemolysin agar (4).

The *elyA* genes in human STEC isolates of serovars O157:H7 and O111:H− are 62 to 64% identical to *hlyA*, encoding alpha-hemolysin, from *E. coli* (18, 29). Furthermore, the RTX hemolysin- or leucotoxin-encoding genes *apxIA* and *apxIIIA* of *Actinobacillus pleuropneumoniae* and *aaltA* of *A. actinomycetemcomitans* show similarities in the range of 56 to 60% with *elyA* and *hlyA*, respectively (18).

Humans are infected either by contaminated food, especially of bovine origin, such as ground beef and raw milk, or by person-to-person transmission, by the fecal-oral route (24). Detection of strains of the prominent STEC serovar O157:H7 from food and stool samples can be conducted on special media because these strains are unable to ferment sorbitol within 24 h and lack β-glucuronidase activity. However, STEC isolates from humans now comprise at least 160 different serovars with variable distributions in different countries (1, 7, 31). In Germany, STEC serovars O157:H7, O157:H−, O111:H−, O103:H2, O103:H−, O26:H11, and O26:H− prevail (7). Because of the biochemical and serological diversity, detection of major virulence factor Stx by cytotoxicity assays with Vero cells, Stx enzyme-linked immunosorbent assay (ELISA), or *stx* PCR is the method of choice for identifying STEC. For simple detection of STEC by culturing, Beutin et al. (4) described a blood agar medium called enterohemolysin agar. Unfortunately, only 74% of 54 STEC strains tested showed hemolysis on this agar (6). Additionally, the nonselective enterohemolysin agar allows concomitant flora of fecal and food specimens to overgrow STEC as well as competing hemolysis of *Enterobacteriaceae* and gram-positive bacteria.

In the present study, the occurrence of *elyA* and its variants in different STEC serovars was evaluated by restriction fragment length polymorphisms of PCR products (PCR-RFLP). Additional sequence information for *elyA* and its variants in

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TABLE 1. STEC strains tested in this study

Serovar	No. of strains	Source	*stx*/*eaeA*[a]
O157:H7	41	Human	2× stx_1/*eaeA*; 36× stx_2/*eaeA*; 5× stx_{1+2}/*eaeA*
O157:H−	11	Human	8× stx_2/*eaeA*; 3× stx_{1+2}/*eaeA*
O156:H27	1	Human	1× stx_1
O146:H−	1	Human	1× stx_{1+2}
O138:K81:H−	1	Pig (strain E57)	1× stx_{2e}
O117:H7	1	Human	1× stx_1
O113:H53	1	Human	1× stx_2
O111:H−	5	Human	3× stx_1/*eaeA*; 1× stx_2/*eaeA*; 1× stx_{1+2}/*eaeA*
O103:H2	5	Human	4× stx_1; 1× stx_2
O103:H−	2	Human	2× stx_1
O95:H−	1	Human	1× stx_1
O89:H−	1	Human	1× stx_1
O69:H−	1	Human	1× stx_{1+2}
O28:H35	1	Human	1× stx_2
O26:H12	1	Human	1× stx_1
O26:H11	5	Human	3× stx_1; 1× stx_2; 1× stx_{1+2}
O26:H−	4	Human	2× stx_1; 2× stx_2
O25:H14	1	Human	1× stx_1
O12:H−	1	Human	1× stx_1
O8:H−	1	Human	1× stx_2
O1:H−	1	Human	1× stx_{1+2}
Ont:H19	1	Human	1× stx_1
Ont:H1	1	Human	1× stx_2
Ont:H−	2	Human	1× stx_1; 1× stx_2
Orough:H11	2	Human	1× stx_1; 1× stx_2
Orough:H4	1	Raw milk	1× stx_2
Orough:H−	1	Human	1× stx_1

[a] Numbers of strains of the corresponding serovar which harbor virulence genes encoding Shiga toxin (*stx*) and intimin (*eaeA*). For specification of *stx* genes, the nomenclature proposed by Calderwood et al. (8a) was used.

strains of emerging or rare STEC serovars is provided. After short-term preenrichment, blood agar supplemented with vancomycin, cefixime, and cefsulodin (BVCC) was tested for efficient recognition of hemolysing STEC among the concomitant flora of food and stool specimens. Suspected hemolytic colonies from the modified blood agar were confirmed by PCR detection of *elyA* and *stx* genes.

MATERIALS AND METHODS

Bacterial strains and serotyping. A total of 92 STEC isolates from different patients were collected from 1993 to 1996. Stool specimens or suspected isolates were received from laboratories in different parts of Germany. The determination of O and H antigens from *E. coli* was performed as described previously (5). The STEC collection comprised 51 strains of serogroup O157 and 41 strains of other serovars (Table 1). Furthermore, the reference strain EDL 933, a human STEC isolate of serovar O157:H7 (Centers for Disease Control and Prevention, Atlanta, Ga.), porcine O138:K81:H− strain E57 (16), and an Orough:H4 isolate from raw milk were added to the strain collection.

Media. The supplementation of modified tryptic soy broth (mTSB) (23) with bile salts no. 3 (Difco, Detroit, Mich.) and novobiocin (Sigma, St. Louis, Mo.) was reduced to 1.12 g and 10 mg/liter, respectively. Buffered peptone water (BPW) supplemented with vancomycin (8 mg/liter), cefixime (50 μg/liter), and cefsulodin (10 mg/liter) (BPW-VCC) was prepared as described by Wallace and Jones (32).

BVCC was made from 33 g of tryptose blood agar base (Difco), 5 g of tryptose (Difco), 5 g of soluble starch (E. Merck AG, Darmstadt, Germany), 441 mg of $CaCl_2 \cdot 2H_2O$, 3 g of agar (Difco), and 970 ml of distilled water. The agar suspension was adjusted to pH 7.0 with 1 N HCl and heated at 100°C for 1 h. After the agar suspension was cooled to 50°C, 30 ml of defibrinated, sterile sheep blood (Oxoid) and sterile solutions of 30 mg of vancomycin hydrochloride (Lilly, Giessen, Germany), 20 μg of cefixime (a gift from Merck), and 3 mg of cefsulodin sodium salt (Sigma) were added. Before use, the sheep blood was washed three times with 50 ml of sterile saline. After the mixture was stirred, 25-ml quantities of BVCC were poured into petri dishes. Enterohemolysin agar was prepared as described by Beutin et al. (4).

Bacteriological examination of specimens. Fecal samples were collected from 50 healthy persons. Twenty-seven ground-beef samples were obtained from six butcher shops. Raw-milk samples originated from 53 dairy farms and two health food shops in northern Germany.

Counts of aerobic mesophilic bacteria, *Enterobacteriaceae*, and *E. coli* were determined in accordance with Section 35 of the German Federal Foods Act (10–13).

The efficiencies of preenrichment broths for the propagation of STEC strains were examined with BPW-VCC and mTSB shaken at 120 rpm and 37°C. For preenrichment of STEC strains from food and fecal specimens, 1 g or 1 ml of sample was added to 9 ml of mTSB and shaken at 120 rpm and 37°C for 6 h. Subsequently, 0.1 ml of broth culture was streaked on a BVCC plate and incubated at 37°C. Hemolytic growth was evaluated after 4 and 20 h. Colonies of hemolyzing *Enterobacteriaceae* were identified with the API 20E system (Biomerieux, Nürtingen, Germany).

With the described enrichment procedure, the detection limit for STEC was determined by seeding the food and fecal specimens with different numbers of each of 16 STEC strains. These human strains belonged to the seven predominant STEC serovars in Germany. Four of the strains were O157:H7; the remaining serovars, O157:H−, O111:H−, O103:H2, O103:H−, O26:H11, and O26:H−, were represented by two strains each.

PCR and analysis of amplicons. For release of DNA, suspensions in 50 μl of sterile bidistilled water of single hemolyzing colonies or bacterial growth from a BVCC plate were boiled for 10 min. Cell debris was centrifuged for 5 min at 8,240 × *g*. Supernatant (0.5 μl) was mixed with 40 pmol of primer (synthesized by GIBCO BRL, Eggenstein, Germany), 200 μM each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 1 U of cloned *Thermus brockianus* DNA polymerase, and assay buffer (Biometra, Göttingen, Germany). The stx_1B gene was detected by PCR as described by Rüssmann et al. (26). A 691- or 692-bp fragment of each of the stx_2AB genes and their variants was amplified with the primers stx_2-start (5′-TTT CCA TGA CRA CGG ACA GCA GTT AT-3′) and stx_2-end (5′-CTC ATT ATA CTT RGA RAA CTC AAT TTT SCC T-3′). PCR of the stx_2 amplicon was conducted in 35 cycles with denaturation for 20 s at 94°C, annealing for 60 s at 50°C, and polymerization for 60 s at 72°C.

STEC *eaeA* was detected by PCR as described by Schmidt et al. (27). For detection of the *estA* and *astA* genes, encoding heat-stable enterotoxins, we amplified a 155-bp fragment of *estA* by using the primers *estA*-start (5′-CCT TTC SCT CAG GAT GCT AAA CC-3′) and *estA*-end (5′-CAA GCA GGA TTA CAA CAC AAT TCA CAG-3′) as well as a 112-bp fragment of *astA* by using the oligonucleotides *astA*-start (5′-GCC ATC AAC ACA GTA TAT CCG RAG GC-3′) and *astA*-end (5′-GGT CGC GAG TGA CGG CTT TGT-3′). Amplification was conducted as described for stx_2 PCR.

Primers *hlyA*-start (5′-AGG AAG TYG TKA AGG ARC AGG AGG-3′) and *hlyA*-end (5′-CCA TCY GCG CCA TGG AAK ATA TCA-3′) were used to amplify nucleotides 2033 to 2234 of *hlyA* and nucleotides 1988 to 2186 of *elyA* and its variants, respectively. Amplification of the *hlyA* and *elyA* fragments was performed as described for stx_2 PCR, but the temperature of annealing was raised to 60°C. For typing, the *hlyA* and *elyA* amplicons were digested separately with *Alu*I, *Eco*RI, and *Mlu*I as recommended by the manufacturer (Amersham, Braunschweig, Germany).

Nucleotide sequence determination of the *hlyA* and *elyA* amplicons was performed with an automated DNA sequencer (LI-COR 4200) as recommended by the manufacturer (MWG-Biotech, Ebersberg, Germany).

RESULTS

Detection of hemolysin in STEC strains. Using primers for conserved C-terminal nucleotide sequences from *hlyA* and *elyA*, we obtained PCR products from lysates of the 95 representative STEC strains. Five amplicons revealed restriction patterns that were generated by *Alu*I, *Eco*RI, and *Mlu*I and that deviated from that of O157:H7 reference strain EDL 933. Four of the strains yielded *elyA* amplicons that were not digested by *Alu*I (Fig. 1). These strains were characterized by the presence of stx_1 genes, the absence of *eaeA*, and an association with unusual serovars, such as Ont:H−, Ont:H19, Orough:H4, and O89:H−. The *hlyA* amplification product derived from the porcine isolate of serovar O138:K81:H− was digested by *Mlu*I but not by *Alu*I and showed larger *Eco*RI subfragments corresponding to *hlyA*, encoding alpha-hemolysin (Fig. 1), than *elyA* amplicons. This strain harbored stx_{2e}, *estA*, and *astA* genes but not the *eaeA* gene.

Nucleotide sequence variations of *elyA* indicated by PCR-RFLP were confirmed by sequence analysis of the amplicons (Fig. 2). The four *elyA* amplification products which were not

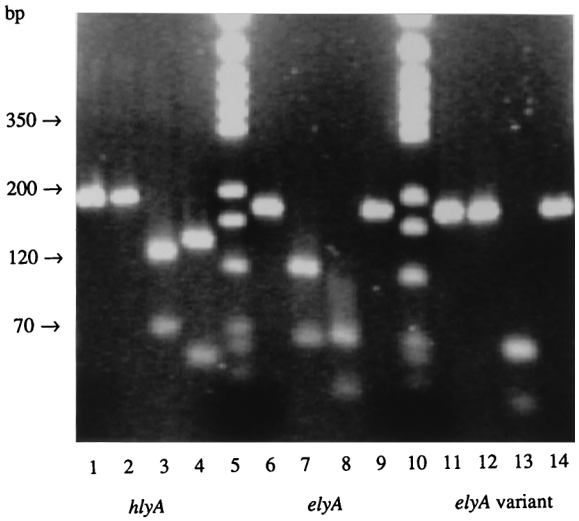

FIG. 1. PCR of *hlyA* and *elyA* as well as their subsequent typing by digestion with *Alu*I, *Eco*RI, and *Mlu*I. The corresponding amplicon of each hemolysin gene (lanes 1, 6, and 11) as well as its *Alu*I (lanes 2, 7, and 12)-, *Eco*RI (lanes 3, 8, and 13)-, and *Mlu*I (lanes 4, 9, and 14)-generated restriction fragments are shown from the left to the right. Lanes 1 to 4, products from *hlyA*; lanes 6 to 9, products from *elyA*; lanes 11 to 14, products from *elyA* variants; lanes 5 and 10, markers (pGEM-3 DNA digested separately with *Hin*fI, *Rsa*I, and *Sin*I; Promega, Mannheim, Germany). The agarose gel was documented by the Gel Doc 1000 video gel documentation system from Bio-Rad (Munich, Germany).

digested by *Alu*I showed a sequence identity of 98 to 98.7% with the *elyA* fragment derived from O157:H7 and O111:H− STEC strains. Nucleotide sequences of amplicons derived from STEC strains of the prevailing serovars O157:H−, O103:H2, O103:H−, O26:H11, and O26:H− corresponded to that for the O157:H7 reference strain. The PCR product of the O138:K81:H− porcine isolate, which contained *Mlu*I subfragments, showed the same nucleotide sequence as *hlyA*, encoding alpha-hemolysin. Derived amino acid sequences of each sequenced *elyA* amplicon were identical. However, *elyA* and *hlyA* amplicons showed only 68% identities for nucleotide sequences and 72.7% identities for amino acid sequences.

Eighty-eight (92.6%) of the 95 STEC strains produced hemolysin on BVCC after 20 h. After serial propagation of these strains, they showed hemolysis on BVCC within just 4 to 6 h. Fifty (96.2%) of 52 O157 strains and 38 (88.4%) of 43 non-O157 strains lysed erythrocytes on this agar. In comparison, only 44 (84.6%) of the O157 STEC strains and 32 (74.4%) of the non-O157 STEC strains formed a hemolysis zone on enterohemolysin agar, resulting in a rate of 80% hemolyzing STEC strains on this agar. In contrast to other STEC strains, O103 strains hemolyzed on BVCC as strongly as did most alpha-hemolysin-producing *E. coli* strains. On the other hand, 2 of 13 alpha-hemolysin-producing *E. coli* strains isolated from stool specimens produced narrow and turbid zones of hemolysis overnight, like most STEC strains.

Identification of STEC strains in stool and food specimens. BPW-VCC and mTSB were tested for their efficiency as preenrichment broths for STEC isolation. In mTSB, strains grew to the stationary phase within 12 h. However, in BPW-VCC, strains grew slower and four (4.2%) of the isolates did not grow within 48 h. In BPW supplemented only with 20 μg of cefixime per liter, these four isolates grew within 48 h. However, their growth remained poor with 50 μg of cefixime per liter of BPW. These isolates belonged to serovars Orough:H−, O8:H−, O26:H−, and O138:K81:H−.

After preenrichment in mTSB, a limited number of hemolyzing *Enterobacteriaceae* lacking *stx* genes were isolated from food and fecal samples on BVCC. In 13 (26%) of 50 stool specimens, *hlyA*-containing *E. coli* colonies were confirmed by PCR-RFLP. While 11 of them secreted alpha-hemolysin on BVCC plates after 4 h of incubation, the remaining 2 produced narrow and turbid zones of hemolysis only after overnight incubation. Eleven (20%) of 55 raw-milk samples contained alpha-hemolysin-producing *E. coli* colonies characterized by a clear and broad zone of beta-hemolysis on BVCC. Each ground-beef sample contained colonies of hemolyzing *Enterobacteriaceae* that did not harbor *stx* genes. Amplicons of *hlyA* were obtained from hemolyzing *E. coli* colonies isolated from

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                1                                                                   70
                +                                                                   +
elyA            AGGAAGTCGT TAAGGAACAG GAGGTGTCAG TAGGGAAGCG AACAGAGAAA ATACAATATC GTGATTTTGA
1114/ 93        -------T-- ---------- ---------- ---------- ---------- ---------- ----------
18-6/ 95        -------T-- ---------- ---------- ---------- ---------- ---------- ----------
849/ 95         -------T-- ---------- ---------- ---------- ---------- ---------- ----------
6780/ 96        -------T-- ------G--- ---------- ---------- ---------- ---------- ----------
hlyA            -------T-- G-----G--- -----T---- -T--A--AA- ---T--A--- -CG------- -GAG--A---

                71                                                                  140
                +                                                                   +
elyA            ATTCA***GA ACCGGTGG*A ATTCCTTATG ATGTAATAGA TAATCTTCAT TCTGTTGAAG AGCTCATTGG
1114/ 93        ---------- ---------- ---------- ----G----- ---------- ---------- -A--------
18-6/ 95        ---------- ---------- ---------- ----G----- ---------- ---------- -A--------
849/ 95         ---------- ---------- ---------- ----G----- ---------- ---------- -A--------
6780/ 96        ---------- ---------- ---------- ----G----- ---------- ---------- -A--------
hlyA            -----CTCAT -T-AA---T- -AAAT---AC -*-AG-CT-- ---CT-AT-- --C--G---- -A--T-----

                141                                                                 203
                +                                                                   +
elyA            CGGAAAACAT GATGATGAAT TCAAAGGCGG TAAGTTTAAT GATATATTCC ATGGCGCAGA TGG
1114/ 93        ------G--- ---------- ---------- ---------- -----C---- ---------- ---
18-6/ 95        ---------- ---------- ---------- ---------- -----C---- ---------- ---
849/ 95         ---------- ---------- ---------- ---------- ---------- ---------- ---
6780/ 96        ---------- ---------- ---------- ---------- -----C---- ---------- ---
hlyA            GACC-CG-G- -CC--CA-G- -TTTT---A- ---A----C- -----C---- -------G-- ---
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FIG. 2. Nucleotide sequences of *elyA* and *hlyA* amplicons. *elyA* represents strains of serovars O157:H7 (28), O111:H− (29), and O157:H−, O103:H2, O103:H−, O26:H11, and O26:H−; strain 1114/93 was serovar Ont:H−; strain 18-6/95 was serovar Orough:H−; strain 849/95 was serovar O89:H−; strain 6780/96 was serovar Ont:H19; and *hlyA* represents strains used by Kuhnert et al. (18) and strain E57 (serovar O138:K81:H−). Dashes represent nucleotides identical to *elyA*. Asterisks symbolize gaps in the aligned nucleotide sequence.

15 ground-beef samples (55.6%). Three of these samples showed beta-hemolysis after 4 h on BVCC. Mixtures of hemolyzing *Serratia* spp. and *E. coli* colonies were detected in seven samples. *Serratia* spp. as the only hemolyzing colonies were isolated from 11 (40.7%) of 27 ground-beef samples. They were easily distinguished from *E. coli* colonies by their white color and their musty smell after 24 h of incubation. Hemolyzing *Citrobacter freundii* was isolated from one ground-beef specimen.

To determine the limit of detection of STEC by preenrichment with mTSB and consecutive plating on BVCC, 16 STEC strains of the seven predominant serovars in Germany were added separately to preenrichment broth with five specimens each of ground beef, raw milk, and stool. Even the smallest amounts of 2.3 CFU of STEC added per g of stool and 6.2 CFU of STEC added per g of ground beef and per ml of raw milk were detected. However, detection of STEC by culturing on BVCC was hampered in ground-beef samples and one stool specimen by hemolysis of *Serratia* spp. and non-STEC *E. coli*, respectively. In these, the inoculated STEC colonies were recognized by PCR of genes encoding Stx and hemolysin from single hemolytic colonies or total growth.

Determination of the concomitant flora resulted in total counts of 1.2×10^6 to 4.8×10^8 aerobic mesophilic bacteria per g of stool, counts of 5.3×10^6 to 4.3×10^7 per g of ground beef, and counts of 1.63×10^3 to 4.4×10^7 per ml of raw milk. *E. coli* counts varied from 2.1×10^5 to 4.3×10^7 per g of stool, from 3 to 750 per g of ground beef, and from <0.3 to 9.3 per ml of raw milk. The numbers of *Enterobacteriaceae* in ground-beef samples ranged from 2.4×10^4 to 5×10^5 per g.

DISCUSSION

The described PCR method proved efficient for detecting hemolysin genes from *E. coli*: *elyA* from STEC strains and *hlyA* from *E. coli* producing alpha-hemolysin. PCR-RFLP showed that all human STEC isolates harbored *elyA*. In comparison to the results for the O157:H7 reference strain, PCR-RFLP and subsequent nucleotide sequencing of *elyA* amplicons revealed only four STEC strains with minor sequence variations. These strains were of rare non-O157 serovars associated with stx_1 genes and lacked *eaeA*. However, among the STEC strains, *hlyA* was restricted to an O138:K81:H− strain. Alpha-hemolysin of this porcine strain was associated with stx_{2e} and *estA* genes, as shown by Meyer and Karch (20). In the present study, *astA*, encoding a second heat-stable enterotoxin, was detected in this strain. The close association of *elyA*, located on the 94- to 103-kb STEC virulence plasmid (28), and *stx* genes, harbored by a lysogenic lambdoid phage (25), was remarkable. This situation could also be true for *hlyA* from porcine strains containing stx_{2e}.

The different rates of detection of hemolysin from STEC by PCR (100%) and by culturing on BVCC (92.6%) might have been due to the repression of gene expression under growth conditions in the laboratory, faulty transport of hemolysin to the cell surface, or mutations of *elyA* not targeted by the PCR method described here. In comparison to STEC strains of rare serovars, a slightly higher proportion of strains of serogroup O157 showed hemolysis on BVCC and enterohemolysin agar. Essential ingredients of blood agar are required to detect hemolysis of STEC. These include calcium (2) and washed sheep blood (4). An increase in the vancomycin concentration to 250 mg per liter of BVCC allowed us to recognize the hemolysis of two additional strains in our STEC collection. Presumably, vancomycin facilitated the secretion of hemolysin through an increase in the permeability of the cell wall. In previous studies, the rates of detection of hemolyzing STEC on enterohemolysin agar varied from 97.6% (3) to 75.2% (7). In the present study, BVCC was superior to enterohemolysin agar for the detection of hemolysis by STEC. After serial propagation of STEC on BVCC, hemolysin production was observed during the logarithmic growth phase, as has been reported for other hemolysins of the RTX type (9).

A vancomycin (8 mg/liter), cefixime (50 μg/liter), and cefsulodin (10 mg/liter) supplementation which differed from that in BVCC was used in BPW-VCC for preenrichment of STEC (32). The higher quantities of cefixime in BPW-VCC caused 4.2% growth inhibition of the strains in our culture collection. This inhibition was avoided by use of mTSB instead of BPW-VCC for preenrichment of STEC. After preenrichment with mTSB, the frequent association of Stx production with the formation of hemolysin allowed us to isolate most STEC strains from special blood agar, such as BVCC. The antibiotic supplements of BVCC allowed us to detect resistant, hemolyzing *Enterobacteriaceae* as *E. coli*, *C. freundii*, and *Serratia* spp. after 16 h of incubation but suppressed the growth of gram-positive bacteria, *Proteus* spp., and *Pseudomonas* spp. However, after 24 h of incubation, colonies of *Serratia* spp. differed in color, shape, and odor from other species of *Enterobacteriaceae* in ground-beef samples. As members of the *Proteae*, *Serratia* spp. produce a type of hemolysin different from the RTX cytolysins (8).

Examining 180 fecal non-STEC *E. coli* isolates from healthy children and patients, Bettelheim (3) identified 3 (1.7%) weakly hemolyzing and 52 (28.9%) alpha-hemolysin-producing strains. Beutin et al. (4) detected no weakly hemolyzing strains but 40 (15%) alpha-hemolysin-producing strains among 267 fecal non-STEC *E. coli* isolates from 200 healthy infants. In good accordance with these studies of fecal non-STEC *E. coli* isolates, we detected such strains in 4% of fecal samples from healthy patients by the production of a narrow, turbid, hemolytic halo and in 22% of such fecal samples by the production of strong hemolysis.

In conclusion, BVCC considerably facilitated the isolation, presumptive identification, and enumeration of most STEC strains in stool and raw-milk specimens. In ground-beef specimens and a few stool specimens, STEC strains were sensitively detected by PCR of *stx* and *elyA* genes from single, weakly hemolyzing colonies grown on BVCC. Thus, preenrichment of stool and food samples in mTSB for 6 h followed by subculturing on BVCC may be recommended for the isolation and presumptive identification of STEC strains. The identities of such strains should be confirmed by proof of *stx* genes and Stx itself.

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