Shiga toxin (Stx)—producing *Escherichia coli* (STEC) cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) worldwide [1–5]. Stx are considered to be the cardinal virulence factors of STEC. These toxins consist of 2 major types, Stx1 and Stx2 [6]. Stx2 is closely related to a family of Stx2 variants or alleles (Stx2c [7], Stx2d [8], Stx2e [9], and Stx2f [10]). These respective Stx2 variants have 99.7%, 94.9%, 94.0%, and 63.4% nucleotide sequence identity in their A subunits and 95.2%, 86.6%, 79.0%, and 75.4% nucleotide sequence identity in their B subunits to the corresponding subunits of the Stx2-encoding gene [7–10]. Although Stx2c and Stx2d are produced by STEC strains isolated from humans [7, 8, 11–14], Stx2e typically is associated with pig edema disease [9, 15] and has been detected only rarely in STEC of human origin [16–19]. Stx2f has been identified in STEC isolated from feral pigeons [10], but, to date, only a single human isolate [20] has been shown to possess an stx2 variant with extensive (>99%) nucleotide sequence homology to stx2f [10].

Data suggest that the clinical outcome of STEC infection depends on the stx genotype of the infecting strain. Although the stx1 or stx1/stx2 genotypes predominate in STEC isolated from patients with uncomplicated infection (i.e., those who do not develop HUS) [21, 22], stx2 has been the most prevalent toxin genotype identified in STEC isolated from patients with HUS [21, 22]. In a study from the United States, patients infected with STEC O157 possessing stx2 but not stx1 were significantly more likely to develop systemic sequelae, including HUS, than were patients infected with STEC O157 harboring stx1 alone or stx1 and stx2 [22]. Recently, a strong statistical association also was demonstrated between the presence of the stx2 genotype and the severity of human disease, including the development of HUS and bloody diarrhea for STEC belonging to the major non-O157 serogroups, including O26, O103, O111, and O145 [23]. These data demonstrate that the stx2 genotype augments the ability of STEC to cause serious human diseases [22, 23]. In contrast to STEC containing stx2, the association of STEC that harbor stx2 variants with clinical disease is poorly understood. Specifically, the few studies performed to date on the frequency of stx2 variants in human isolates [8, 11, 12, 14, 18, 24] often were done on a relatively small scale, comprised selected groups of individuals (subjects with HUS or diarrhea who were asymptomatic carriers) and/or of STEC (O157 or non-O157), and examined a limited spectrum of stx2 variants.
To better understand the significance of STEC harboring stx2 variants in human disease, we investigated all stool samples submitted to our laboratory for routine microbiological diagnosis between 1996 and 2000, using a spectrum of polymerase chain reaction (PCR) procedures to detect stx1, stx2, and stx3 variants, including stx2a, stx2b, stx2c, and stx2f. We determined the relative frequency of STEC harboring the stx3 gene variants and their association with clinical symptoms. We also serotyped the isolates and assessed the presence of the eae gene, an important accessory virulence gene of STEC [21, 23]. Furthermore, we investigated whether or not the STEC harboring stx2 variants expressed Stx and whether or not these toxins could be detected by commercial Stx immunoassays.

Materials and Methods

Stool specimens and isolation of STEC. From January 1996 to December 2000, 5487 stool samples from patients with classic HUS (n = 510) and patients with bloody (n = 67) or watery (n = 4910) diarrhea were investigated for the presence of STEC during routine diagnostic work in the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany). Screening for and isolation of STEC from stools were performed as follows: 1 g of stool was grown in 10 mL of GN Broth Hajna (Difco Laboratories) for 6 h. E. coli O157 was sought in 1 mL of this enrichment culture by use of an immunomagnetic separation (IMS) technique and subsequent culture of magnetically separated organisms on sorbitol MacConkey (SMAC) agar and cefixime-telurite (CT)–SMAC agar, as described elsewhere [25]. To find non–fermenting STEC O157. The overnight bacterial growth was harvested into 1 L of saline, and 10⁶ cells were used in the PCR with the primer pairs KS7 and KS8 [26] and LP43 and LP44 [27], which complement the stx1, stx2, and stx3 variant genes, respectively (table 1). This PCR screening also was applied to the IMS-processed cultures from SMAC agar and CT-SMAC agar plates, to look for sorbitol-fermenting STEC O157. The PCRs were performed with the GeneAmp PCR System 9600 (Perkin Elmer–Applied Biosystems) in a volume of 50 μL that contained 5 μL of bacterial suspension (10⁶ cells), 200 μM each dNTP, 30 pmol of each primer, 5 μL of 10-fold–concentrated polymerase synthesis buffer, 1.5 mM MgCl₂, and 2.0 U of AmpliTaq DNA polymerase (Perkin Elmer–Applied Biosystems). The PCR conditions are shown in table 1. The amplification products were subjected to submarine gel electrophoresis in a 1.5% (wt/vol) agarose gel and were visualized by staining with ethidium bromide. To identify STEC in PCR-positive samples, colony blot hybridization with 100–200 well-separated colonies was performed by use of digoxigenin-labeled stx1 and stx2 probes prepared with primer pairs KS7-KS8 and LP43-LP44, respectively, from E. coli O157:H7 strain EDL 933 [28], as described by Schmidt et al. [26].

In addition to STEC isolated from patients with HUS or from patients with diarrhea without HUS, 96 STEC strains originating from asymptomatic individuals were included in the present study. These organisms were isolated between 1996 and 2000 in the Governmental Institute of Public Health Lower Saxony (Hannover, Germany) during epidemiological investigations and were submitted to our laboratory for further characterization.

stx genotyping and detection of the eae gene. The stx genotypes and the presence of the eae gene in STEC isolates were determined by PCRs used the primers and conditions shown in table 1. The strategy to detect stx2 variants was as follows: STEC that were positive in the PCR with primer pair LP43 and LP44 (which detects A subunit genes of stx2 and stx3 variants; table 1) were subjected to PCR with primer pair GK3 and GK4 [29] (table 1). The GK3-GK4

### Table 1. Polymerase chain reaction (PCR) primers and conditions used in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Target</th>
<th>PCR conditions, °C, s</th>
<th>Length of PCR product, bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS7</td>
<td>5'-CCC GGA TCC ATG AAA AAA ACA TTA TTA ATA GC-3'</td>
<td>stx1B</td>
<td>94, 30 52, 60 72, 40</td>
<td>282</td>
<td>[26]</td>
</tr>
<tr>
<td>KS8</td>
<td>5'-CCC GAA TTC AGC TAT TCT GAG TCA ACG-3'</td>
<td>stx2A and variants</td>
<td>94, 30 57, 60 72, 60</td>
<td>584</td>
<td>[27]</td>
</tr>
<tr>
<td>LP43</td>
<td>5'-ACC CTA TTT CCC GGA GTT TAC G-3'</td>
<td>stx2B</td>
<td>94, 30 52, 60 72, 40</td>
<td>260</td>
<td>[29]</td>
</tr>
<tr>
<td>LP44</td>
<td>5'-GCC TCA TCG TAT ACA CAG GAC G-3'</td>
<td>stx2B</td>
<td>94, 30 55, 60 72, 40</td>
<td>256</td>
<td>[8]</td>
</tr>
<tr>
<td>GK4</td>
<td>5'-CCA GTC ATT ATT AAA CTG-3'</td>
<td>stx3A</td>
<td>94, 30 52, 60 72, 40</td>
<td>428</td>
<td>[10]</td>
</tr>
<tr>
<td>VT2-em</td>
<td>5'-AAG AAG ATA TTT GTA GCG G-3'</td>
<td>stx2B</td>
<td>94, 30 52, 60 72, 40</td>
<td>863</td>
<td>[26]</td>
</tr>
<tr>
<td>VT2-t</td>
<td>5'-TAA GCT CAA CCA GTT CAC CAA AT-3'</td>
<td>eae</td>
<td>94, 30 52, 60 72, 40</td>
<td>280</td>
<td>[30]</td>
</tr>
</tbody>
</table>

NOTE. stx, Shiga toxin gene.

a All PCRs included 30 cycles, followed by a final extension step of 5 min at 72°C.

b stx2A, stx2B, and stx3A [10].
amplification products were digested with restriction endonucleases \( \text{Hae III} \) and \( \text{Fok I} \) (Boehringer Mannheim GmbH), to differentiate B subunit genes of \( \text{stx}_2 \) and \( \text{stx}_2c \), as described by Rüssmann et al. [11]. Isolates from which amplification products were not elicited with primers \( \text{GK3} \) and \( \text{GK4} \) were tested for the \( \text{stx}_2c \) gene by use of the primer pair \( \text{FK2} \) and \( \text{FK1} \) [30]. The isolates that did not contain \( \text{stx}_2c \), but reacted with primers \( \text{LP43} \) and \( \text{LP44} \), which suggests the presence of another \( \text{stx}_2 \) allele, were tested for the presence of the \( \text{stx}_2 \) gene with the primer pair \( \text{VT2-2} \) and \( \text{VT2-f} \) [8] (table 1). \( \text{stx}_2 \) is defined in the present study as a \( \text{stx}_2 \) variant amplified with primers \( \text{VT2-2} \) and \( \text{VT2-f} \) [8] and does not refer to the intestinal mucus--activated \( \text{Stx}2d \) toxin subtype (encoded by \( \text{stx}_2 \) variants that have been classified as \( \text{stx}_2c \) [31, 32]), as defined by Melton-Celsa et al. [33]. In addition, all STEC isolated during the study period were investigated for the \( \text{stx}_2 \) gene with the primer pair \( \text{128-1} \) and \( \text{128-2} \) [10] (table 1).

\( \text{E. coli} \) O157:H7 strain EDL933 [26, 28] was used as the positive control in the PCRs for the detection of \( \text{stx}_1, \text{stx}_2 \), and \( \text{eaE} \) genes. Strains E32511 (O157:H7; \( \text{stx}_2c \)) [34], HE250 (ONT:H12; \( \text{stx}_2d \)) [8], ED-53 (O101:H1; \( \text{stx}_2c \)) [35], and T4/97 (O128:H2; \( \text{stx}_2d \)) [10] were used as positive controls in PCRs for the detection of the respective \( \text{stx}_2 \) variants.

**Phenotypic methods.** STEC isolates were serotyped according to the method of Bockemühl et al. [36] with the use of antisera against \( \text{E. coli} \) O antigens 1–173 and \( \text{E. coli} \) H antigens 1–56. Fermentation of sorbitol was detected on SMAC agar after overnight incubation [37]. \( \text{Stx} \) production was tested by use of the Vero cell cytotoxicity assay [38] and 2 different commercial \( \text{Stx} \) assays, including an \( \text{Stx} \) EIA (Ridascreen Verotoxin; R-Biopharm, GmbH) and a latex agglutination assay (VTEC-RPLA [Verotoxin-producing \( \text{E. coli} \) reverse passive latex agglutination]; Denka Seiken). The Vero cell cytotoxicity assay was performed as described elsewhere [38]. The \( \text{Stx} \) EIA was performed according to the manufacturer’s instructions, with bacterial cultures enriched overnight in a medium that contained mitomycin C (EHEC Direct Medium; Heipha). The latex agglutination assay was performed as described by Karmali et al. [39], with supernatants of overnight cultures in Tryptic Soy Broth (Difco Laboratories) that were diluted from 1:2 to 1:128. The agglutination was examined visually after 20–24 h of incubation, and the toxin titers were expressed as the reciprocals of the highest dilutions that caused agglutination.

**Case definition.** Patients diagnosed as having diarrhea had \( \geq 3 \) semisolid or liquid stools per day. Bloody diarrhea was defined as diarrhea in which visible blood was noted in the stool. HUS was defined as hemolytic anemia (hematocrit < 30%, with evidence of the destruction of erythrocytes on a peripheral blood smear), thrombocytopenia (platelet count < 150,000 cells/mm\(^3\)), and renal insufficiency (a serum creatinine concentration that exceeded the upper limit of the normal range for age) preceded by diarrhea [40]. Asymptomatic carriers were apparently healthy individuals without diarrhea.

**Statistical analysis.** Differences between groups were assessed by use of the \( \chi^2 \) test and Yates’s corrected \( \chi^2 \) test for small numbers [41]. Epi-Info software (version 6.04b, Centers for Disease Control and Prevention and World Health Organization) was used to perform calculations. \( P < .05 \) was considered to be statistically significant.

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**Isolation of STEC and serotyping.** Between January 1996 and December 2000, stool samples from 549 (10.0%) of the 5487 individuals investigated in the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany) were positive for the \( \text{stx}_1 \) and/or \( \text{stx}_2 \) gene by PCR screening with primers \( \text{KS7-KS8} \) and \( \text{LP43-LP44} \). STEC strains could be isolated from 530 (96.5%) of 549 PCR-positive stool samples. The proportion of STEC colonies identified by colony blot hybridization with the \( \text{stx}_1 \) and \( \text{stx}_2 \) probes ranged from 0.5% to 65.0% in individual stool samples. One STEC isolate per patient was chosen randomly for further analysis. In total, 268 STEC isolates were obtained from 510 patients with HUS, and 262 isolates were obtained from 4977 patients with diarrhea who did not develop HUS. Fourteen of 262 isolates were from patients with bloody diarrhea, and 248 isolates were from 4910 patients with watery diarrhea without blood. The STEC isolation rates from patients with HUS, bloody diarrhea, and watery diarrhea were 52.5%, 20.9%, and 5.1%, respectively. Nine of 530 STEC isolates originated from 3 clusters of STEC O157 infection in families, and 11 strains were from 4 clusters of STEC O26-associated diarrhea and HUS. All the remaining 510 STEC isolates were from apparently sporadic cases of infection, without obvious geographic or temporal linkage. Among 96 STEC isolates from asymptomatic individuals provided by the Governmental Institute of Public Health Lower Saxony (Hannover, Germany), 7 originated from family members of 4 patients with HUS, and 3 were from siblings of 3 patients with diarrhea. The other 86 STEC isolates had no obvious epidemiological associations with the investigated patients. They were isolated from healthy children and adults in kindergartens, schools, factories, and community during environmental investigations. Three of these 86 STEC isolates were isolated from workers of the same meat processing plant; the other 83 were, to our knowledge, epidemiologically unrelated. Three hundred and nine of the 626 STEC isolates investigated in the present study have been described elsewhere [16, 21, 38, 42–44]. The ages of subjects from whom STEC were isolated ranged from 2 months to 73.5 years (median, 4.3 years) for patients with HUS, from 1 month to 86 years (median, 9 years) for patients with diarrhea who did not develop HUS, and from 37 months to 82 years (median, 10.5 years) for asymptomatic individuals.

One hundred and thirty-nine (51.9%) of 268 STEC isolates from patients with HUS were classic non--sorbitol-fermenting \( \text{E. coli} \) O157:H7 or O157:H7- (table 2); the remainder were sorbitol-fermenting STEC O157:H7 and non-O157 STEC isolates, which most frequently belonged to serogroup O26 (table 2). In patients with diarrhea, non-O157 STEC isolates accounted for most of the isolates, and approximately half belonged to a broad spectrum of serotypes that were not detected in patients with HUS (table 2). The 14 STEC isolates from patients with bloody diarrhea belonged to serotypes O157:H7 (11 isolates), O103:
H− (2 isolates), and O145:H− (1 isolate). STEC isolates from asymptomatic carriers belonged mostly (78.1%) to non-O157 serotypes that were not associated with HUS, but some of these isolates had serotypes that were identical to those of STEC isolates from patients with diarrhea (table 2). Three hundred and thirty-four (96.0%) of 348 non-O157 STEC isolates were detected in 226 (36.1%) of 626 isolates (table 3). Three hundred and forty-two (96.0%) of 348 non-O157 STEC isolates belonged to any of the major non-O157 STEC serogroups, including O26, O103, O111, and O145. Instead, these 78 isolates belonged to 15 other non-O157 serogroups (table 4). Thirteen (35.1%) of 37 isolates of the stx2 and stx2e genotype clustered in serogroup O128 (table 4). Five of 7 isolates of the stx2e genotype whose O antigens were typeable belonged to the serogroup O60 and the other 2 to the serogroup O8 (table 4). However, 10 (62.5%) of 16 isolates harboring stx2c and 16 (25.8%) of 62 isolates that contained stx2d were able to be autoagglutinized (table 4).

stx genotypes of STEC and the frequency of isolates harboring stx2 variants. The 626 STEC isolated belonged to 10 different stx genotypes (table 3). stx2 variants included stx2a, stx2c, and stx2e, and were detected in 226 (36.1%) of 626 isolates (table 3). stx2c was the most frequent stx2 variant. It was found in 148 (23.6%) of 626 STEC isolates but usually in combination with other stx genes, most frequently with stx2a (table 3). Specifically, 28 (4.5%) of 626 STEC isolates contained stx2c as the sole stx gene (table 3). stx2a, either alone or together with stx2c, was identified in 62 (9.9%) of 626 STEC isolates, and stx2e, always as a single stx gene, was identified in 16 (2.6%) (table 3). None of the STEC isolates in the present study contained stx2d.

Serotypes and frequency of eae in STEC harboring stx2e, stx2d, or stx2c alleles. All STEC isolates that contained stx2e only, stx2d, or stx2c were serotyped and investigated for the presence of eae (table 4). Although 19 (67.9%) of the 28 isolates of the stx2e genotype were STEC O157:H7 or O157:H−, none of the 78 STEC isolates that contained stx2d or stx2c alleles was E. coli O157 (table 4). Also, none of the latter 78 isolates belonged to any of the major non-O157 STEC serogroups, including O26, O103, O111, and O145. Instead, these 78 isolates belonged to 15 other non-O157 serogroups (table 4). Thirteen (35.1%) of 37 isolates of the stx2 and stx2e genotype clustered in serogroup O128 (table 4). Five of 7 isolates of the stx2e genotype whose O antigens were typeable belonged to the serogroup O60 and the other 2 to the serogroup O8 (table 4). However, 10 (62.5%) of 16 isolates harboring stx2c and 16 (25.8%) of 62 isolates that contained stx2d were able to be autoagglutinized (table 4).

eae was present in 22 (78.6%) of 28 STEC isolates of the stx2e genotype, each of the O157:H7/H− STEC isolates that harbor this allele (table 4). However, eae was absent from each of the 78 STEC isolates that contained stx2d or stx2c alleles (P < .000001; table 4). The absence of eae from each of the 78 STEC isolates that harbored stx2e, stx2c was also highly significant when these isolates were compared with the 193 STEC isolates of the stx2 genotype, among which 97.9% were eae positive (P < .000001; table 4), and with the 87 non-O157 STEC of the stx2 genotype (95.4% eae positive; P < .000001; table 4). The association was still significant when the 78 STEC harboring stx2d or stx2c were compared with the 9 non-O157 STEC of the stx2e genotype (33.3% eae positive; P = .0003; table 4). In the group of the 28 STEC isolates of the stx2e genotype, the frequency of eae among O157 isolates (100%) was significantly

Table 2. Serotypes of 626 Shiga toxin–producing Escherichia coli (STEC) isolated from patients with hemolytic-uremic syndrome (HUS), patients with diarrhea, and asymptomatic individuals.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>HUS</th>
<th>Diarrhea without HUS</th>
<th>Asymptomatic</th>
<th>Total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157:H7/H−, NSF</td>
<td>139 (51.9)</td>
<td>77 (29.4)</td>
<td>6 (6.3)</td>
<td>222 (35.5)</td>
</tr>
<tr>
<td>O157:H−, SF</td>
<td>39 (14.5)</td>
<td>16 (6.1)</td>
<td>1 (1.0)</td>
<td>56 (8.9)</td>
</tr>
<tr>
<td>O26:H11/H−</td>
<td>35 (13.0)</td>
<td>28 (10.7)</td>
<td>4 (1.4)</td>
<td>67 (10.7)</td>
</tr>
<tr>
<td>O145:H−</td>
<td>19 (7.1)</td>
<td>15 (5.7)</td>
<td>3 (1.1)</td>
<td>37 (5.9)</td>
</tr>
<tr>
<td>O103:H2/H18/H−</td>
<td>9 (3.4)</td>
<td>25 (9.5)</td>
<td>5 (1.8)</td>
<td>39 (6.2)</td>
</tr>
<tr>
<td>O111:H−</td>
<td>8 (3.0)</td>
<td>13 (5.0)</td>
<td>2 (1.0)</td>
<td>23 (3.7)</td>
</tr>
<tr>
<td>Others</td>
<td>19 (7.1)a</td>
<td>88 (33.6)b</td>
<td>75 (27.1)c</td>
<td>182 (29.1)</td>
</tr>
<tr>
<td>Total</td>
<td>268 (100)</td>
<td>262 (100)</td>
<td>96 (100)</td>
<td>626 (100)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of isolates. H−, nonmotile; HNT, H antigen nontypeable; NSF, non–sorbitol fermenting; O, O antigen nontypeable; Orough, autoagglutinable strains; SF, sorbitol fermenting.

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higher than that among non-O157 isolates (33.3%; \( P = .0004 \); table 4). In total, the frequency of eae among the 28 STEC isolates of the \( stx_2c \) genotype (78.6%) was significantly lower than that among the 193 STEC isolates of the \( stx_2 \) genotype (97.9%; \( P = .00004 \); table 4).

Association between STEC harboring \( stx_2 \) variants and clinical manifestations of the infection. To investigate the association between \( stx_2 \) variants and clinical manifestations of the infection, we compared the relative frequency of STEC isolates that possessed \( stx_2c \) only, \( stx_2d \) or \( stx_2e \) among STEC isolates from patients with HUS, STEC isolates from patients with diarrhea who did not develop HUS, and STEC isolates from asymptomatic individuals (table 3). Moreover, we determined the distribution of STEC harboring each of the respective \( stx_2 \) gene variants between patients with HUS and those individuals who were infected but did not develop HUS (i.e., patients with diarrhea and asymptomatic subjects; table 5), to assess the potential of such infections to progress to HUS.

\( stx_2c \) was the only \( stx_2 \) variant associated with HUS. However, the proportion of STEC isolates that possessed the \( stx_2c \) genotype was similar among isolates from patients with HUS (3.7%), isolates from patients with diarrhea who did not develop HUS (5.0%), and isolates from asymptomatic individuals (5.2%) (table 3). Moreover, no significant difference was found in the distribution of the 28 STEC isolates of the \( stx_2c \) genotype between patients with HUS (10 [35.7%] of 28) and those who did not develop HUS (patients with diarrhea or asymptomatic subjects; 18 [64.3%] of 28; \( P = .21 \)) (table 5). This lack of association applied to the 19 isolates of O157 serogroup (table 4), 5 of which were associated with HUS, and to the 9 non-O157 isolates of the \( stx_2c \) genotype (table 4), 5 of which were associated with HUS (data not shown). In addition, the proportions of STEC isolates of the \( stx_2c \) genotype that contained the eae gene were comparable among isolates from patients with HUS (7/10) and among isolates from individuals who did not develop HUS (5/18; data not shown). Of 13 patients with diarrhea from whom STEC of the \( stx_2c \) genotype were isolated (table 3), the stool sample of 1 patient was bloody; the infecting strain belonged to the serotype O157:H7. The other 12 patients, 8 of whom were infected with STEC O157:H7/H1 and 4 with non-O157 STEC, had diarrhea without visible blood.

In contrast to \( stx_2c \), the \( stx_2d \) gene was identified in none of 268 STEC isolates from patients with HUS but was present in 41 (15.6%) of 262 STEC isolated from patients with diarrhea (\( P < .000001 \)) and in 21 (21.9%) of 96 STEC isolated from asymptomatic individuals (\( P < .000001 \)) (table 3). The signifi-

**Table 3.** Shiga toxin gene (\( stx \)) genotypes of 626 Shiga toxin–producing *Escherichia coli* (STEC) and their relative frequency among isolates from patients with hemolytic-uremic syndrome (HUS), patients with diarrhea, and asymptomatic individuals.

<table>
<thead>
<tr>
<th>( stx ) genotype</th>
<th>HUS</th>
<th>Diarrhea without HUS</th>
<th>Asymptomatic*</th>
<th>Total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>( stx_1 )</td>
<td>10 (3.7)</td>
<td>111 (42.4)</td>
<td>45 (46.8)</td>
<td>166 (26.5)</td>
</tr>
<tr>
<td>( stx_2 )</td>
<td>147 (54.9)b</td>
<td>41 (15.7)b</td>
<td>5 (5.2)b</td>
<td>193 (30.8)</td>
</tr>
<tr>
<td>( stx_1 + stx_2 )</td>
<td>26 (9.7)</td>
<td>9 (3.4)</td>
<td>6 (6.3)</td>
<td>41 (6.5)</td>
</tr>
<tr>
<td>( stx_2c )</td>
<td>10 (3.7)c</td>
<td>13 (5.0)c</td>
<td>5 (5.2)c</td>
<td>28 (4.5)</td>
</tr>
<tr>
<td>( stx_2d )</td>
<td>6 (2.2)</td>
<td>14 (5.3)</td>
<td>6 (6.3)</td>
<td>26 (4.2)</td>
</tr>
<tr>
<td>( stx_2e )</td>
<td>68 (25.4)</td>
<td>17 (6.5)</td>
<td>3 (3.1)</td>
<td>88 (14.0)</td>
</tr>
<tr>
<td>( stx_1 + stx_2d )</td>
<td>1 (0.4)</td>
<td>4 (1.5)</td>
<td>1 (1.0)</td>
<td>6 (1.0)</td>
</tr>
<tr>
<td>( stx_1 + stx_2c )</td>
<td>0 (0)d,e</td>
<td>15 (5.7)d,e</td>
<td>10 (10.4)d,e</td>
<td>25 (4.0)</td>
</tr>
<tr>
<td>( stx_1 + stx_2d )</td>
<td>0 (0)d,f</td>
<td>26 (9.9)d,f</td>
<td>11 (11.5)d,f</td>
<td>37 (5.9)</td>
</tr>
<tr>
<td>( stx_2 )</td>
<td>0 (0)d</td>
<td>12 (4.6)d</td>
<td>4 (4.2)d</td>
<td>16 (2.6)</td>
</tr>
<tr>
<td>Total</td>
<td>268 (100)</td>
<td>262 (100)</td>
<td>96 (100)</td>
<td>626 (100)</td>
</tr>
</tbody>
</table>

*NOTE.* Data are no. (%) of isolates. The \( \chi^2 \) test and Yates’s corrected (YC) \( \chi^2 \) test were used for calculations. \( P < .05 \) was considered to be statistically significant.

*3, 2, and 3 of the isolates of genotypes \( stx_2c, stx_2d, stx_2c + stx_2d, \) and \( stx_1 + stx_2 \), respectively, originated from asymptomatic contacts of 4 patients with HUS and 3 patients with diarrhea. Three of 10 STEC of the \( stx_2c \) genotype were isolated from workers of the same meat processing plant.

*\( \beta \) The relative frequency of \( stx_2c \) among STEC from patients with HUS vs. diarrhea, \( P = .000001 \), \( \chi^2; 1 \) df, 88.95; HUS vs. asymptomatic, \( P = .000001 \), \( \chi^2; 1 \) df, 71.62; and diarrhea vs. asymptomatic, \( P = .009 \), \( \chi^2; 1 \) df, 6.84.

*\( \gamma \) The relative frequency of \( stx_2d \) among STEC from patients with HUS vs. diarrhea, \( P = .049 \), \( \chi^2; 1 \) df, 0.48; HUS vs. asymptomatic, \( P = .74 \); YC \( \chi^2, 0.11 \); and diarrhea vs. asymptomatic, \( P = .86 \); YC \( \chi^2, 0.03 \).

*\( \delta \) The relative frequency of all STEC with \( stx_2d \) among STEC from patients with HUS vs. diarrhea, \( P = .000001 \), \( \chi^2; 1 \) df, 45.46; HUS vs. asymptomatic, \( P < .000001 \), \( \chi^2; 1 \) df, 51.52; and diarrhea vs. asymptomatic, \( P = .17 \), \( \chi^2; 1 \) df, 1.90.

*\( \epsilon \) The relative frequency of \( stx_2 \) among STEC from patients with HUS vs. diarrhea, \( P = .00007 \), \( \chi^2; 1 \) df, 15.79; HUS vs. asymptomatic, \( P = .00001 \); YC \( \chi^2, 24.94 \); and diarrhea vs. asymptomatic, \( P = .12 \), \( \chi^2; 1 \) df, 2.36.

*\( \zeta \) The relative frequency of STEC harboring \( stx_2d \) among STEC from patients with HUS vs. diarrhea, \( P = .00001 \), \( \chi^2; 1 \) df, 27.97; HUS vs. asymptomatic, \( P < .000001 \); YC \( \chi^2, 27.88 \); and diarrhea vs. asymptomatic, \( P = .067 \), \( \chi^2; 1 \) df, 0.18.

*\( \theta \) The relative frequency of \( stx_2 \) among STEC from patients with HUS vs. diarrhea, \( P = .0004 \), \( \chi^2; 1 \) df, 12.56; HUS vs. asymptomatic, \( P = .005 \); YC \( \chi^2, 7.78 \); and diarrhea vs. asymptomatic, \( P = .09 \); YC \( \chi^2, 0.01 \).
Table 4. Serotypes and frequency of the gene encoding intimin (eae) in Shiga toxin–producing *Escherichia coli* (STEC) harboring Shiga toxin gene (stx) stx2e, only, stx2ad, or stx2d, compared with STEC of the stx2d genotype.

<table>
<thead>
<tr>
<th>stx genotype</th>
<th>O157 serogroup</th>
<th>Non-O157 serogroups</th>
<th>Total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of isolates</td>
<td>No. (%) eae positive</td>
<td>No. of isolates</td>
</tr>
<tr>
<td>stx2e</td>
<td>19</td>
<td>19 (100.0)</td>
<td>9</td>
</tr>
<tr>
<td>stx2d</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>stx2 + stx2d</td>
<td>0</td>
<td>NA</td>
<td>37</td>
</tr>
<tr>
<td>stx2e</td>
<td>106</td>
<td>106 (100.0)</td>
<td>16</td>
</tr>
<tr>
<td>stx2d</td>
<td>83*</td>
<td>83 (95.4)</td>
<td>193</td>
</tr>
</tbody>
</table>

NOTE. H*, nonmotile; HNT, H antigen nontypeable; NA, not applicable; ONT, O antigen nontypeable; Orough, autoagglutinable strains. The χ² test and Yates’s corrected (YC) χ² test were used for calculations. *P < .05 was considered to be statistically significant.

*STECharboring stx2ad(stx2ad + stx2d)or stx2e vs. STECharboring stx2d, P = .0003; YC χ², 13.16.

*STECharboring stx2e(stx2ad + stx2d) or stx2e vs. STECharboring stx2d, P < .000001, χ²: 1 df, 77.34. STECharboring stx2ad(stx2ad + stx2d) or stx2e vs. STECharboring stx2d, P < .00001, χ²: 1 df, 252.44. STECharboring stx2e vs. STECharboring stx2d, P = .00004; YC χ², 16.96.

*P = .0004; YC χ², 12.40.

*Serotypes O23:H* + O50:HNT, O60:H19, O77:H2, O92:H1NT, O120:H1NT, O145:H* (2 strains), and Orough:H1NT.


*Serotypes O8:H1NT, O8:H* , O60:H2, O60:H* (4 strains), and Orough:H1NT (9 strains).


Significantly higher frequency among isolates from patients with diarrhea and among isolates from asymptomatic individuals, compared with that among isolates from patients with HUS, was observed for the 25 STEC isolates that harbored stx2d as the sole stx gene (5.7%, 10.4%, and 0% of isolates from subjects with diarrhea, without symptoms, and with HUS, respectively; P = .00007 and P = .000001 for isolates from patients with diarrhea and for isolates from asymptomatic subjects, respectively, vs. isolates from patients with HUS; table 3) and for the 37 STEC isolates that contained stx2d together with stx2 (9.9%, 11.5%, and 0% of isolates from patients with diarrhea, asymptomatic subjects, and patients with HUS, respectively; P < .000001 for both isolates from patients with diarrhea and isolates from asymptomatic subjects vs. isolates from patients with HUS; table 3). However, no significant difference was observed in the relative frequency of STEC harboring stx2d among STEC isolates from patients with diarrhea (15.6%) and among STEC isolates from asymptomatic individuals (21.9%; P = .17; table 3). The lack of significant association with diarrhea versus asymptomatic infection applied to the 25 STEC isolates containing stx2d only and to the 37 STEC isolates containing stx2ad and stx2e (table 3). All patients with diarrhea from whom STEC isolates containing stx2d were isolated had diarrhea without visible blood. The highly significant association with uncomplicated infection (including nonbloody diarrhea or asymptomatic infection) versus HUS was confirmed for both the respective groups of the stx2d-containing STEC isolates on the basis of the distributions of such isolates between patients with HUS (0%) and those infected individuals who did not develop HUS (100%; P < .000001; table 5).

Similar to stx2ad, stx2e was absent from all 268 STEC isolated from patients with HUS but was present in 12 (4.6%) of 262 STEC isolates from patients with diarrhea (P = .0004) and in 4 (4.2%) of 96 STEC isolated from asymptomatic individuals (P = .005; table 3). Also, in STEC harboring stx2d, the difference in the relative frequency of stx2d-harboring STEC among patients with diarrhea (4.6%) and among STEC from asymptomatic subjects (4.2%) was not statistically significant (P = .90; table 3). All 12 patients infected with STEC harboring stx2d had nonbloody diarrhea. The association of STEC harboring stx2d with uncomplicated infection that did not progress to HUS was highly significant when the distribution of the 16 stx2d-harboring STEC between patients with HUS (0/16) and those individuals who did not develop HUS (16/16) was compared (P < .000001; table 5).

In contrast to STEC harboring the stx2 variants, STEC of the stx2 genotype were significantly associated with HUS. The relative frequency of such strains among STEC from patients with HUS was 54.9%, whereas that among isolates from patients with diarrhea who did not develop HUS was 15.7% (P < .000001), and that among asymptomatic individuals was 5.2% (P < .000001; table 3). Moreover, 147 (76.2%) of 193 STEC isolates of the stx2 genotype originated from patients with HUS, but only 46 (23.8%) of these strains were isolated from subjects in which the infection did not progress to HUS (P < .000001; table 5). Also, in contrast to all stx2 variants that were not significantly associated with diarrhea versus asymptomatic infection (table 3), the relative frequency of STEC of the stx2 genotype among isolates from patients with diarrhea (15.7%) was significantly higher than that among STEC from
asymptomatic individuals (5.2%; P = .009; table 3). Seven of 41 patients with diarrhea from which STEC isolates of the stx2 genotype were recovered had stools with visible blood.

When we compared the relative risk of infections with STEC harboring stx2 and various stx2 variants to progress to HUS, we found that the proportion of patients that developed HUS after infection with STEC of the stx2 genotype (76.2%; table 5) was significantly higher than the proportion of patients who developed HUS after infection with STEC of the stx3 genotype (35.7%; P = .00001; table 5). Similarly, the proportion of patients who developed HUS after infection with STEC of the stx3 genotype (35.7%; table 5) was significantly higher than the proportion of those who developed HUS after infection with STEC harboring stx2d (0%; P = .000004; table 5) or stx2e (0%; P = .019; table 5).

Association between infections with STEC harboring stx2 variants and the age of patients. To determine whether infections with STEC harboring different stx2 variants were associated with particular age groups, we compared the ages of patients infected with STEC with the stx2a, stx2b, or stx2e alleles (table 5). As indicated by median ages of patients and by proportions of patients of <5 and >18 years old, patients infected with STEC harboring stx2d or stx2e alleles were substantially older than patients infected with STEC of the stx2b genotype (table 5). Only 13 (16.7%) of 78 patients infected with STEC with stx2a or stx2d alleles were <5 years old, whereas 14 (50.0%) of 28 patients infected with STEC possessing the stx2b genotype belonged to this age group (P = .0005; table 5). Moreover, 50 (64.1%) of 78 patients infected with STEC harboring stx2d or stx2e were >18 years old, whereas only 6 (21.4%) of 28 patients infected with STEC of the stx2b genotype were adults (P = .0001; table 5). In comparison, the proportion of adults among patients infected with STEC of the stx2e genotype (3.1%; table 5) was significantly lower than that among patients infected with STEC of the stx2e genotype (21.4%; P = .0004) and that among patients infected with STEC harboring stx2a or stx2e (64.1%; P < .000001; table 5). Also, the proportion of patients <5 years old among patients infected with STEC of the stx2e genotype (87.6%; table 5) was significantly higher than that among patients infected with STEC harboring stx2a (90.9%; P = .000003) and that among patients infected with STEC harboring stx2b or stx2d (64.1%; P < .000001; table 5).

Toxin production by STEC possessing stx2 variants and detection of Stx2 variants by commercial immunoassays. To investigate whether the STEC harboring various stx2 variants produced Stx and whether these toxins were detectable by commercially available Stx assays, culture supernatants of the 50 STEC that contained stx2a, stx2b, or stx2e, as the sole stx genes and were isolated from patients with diarrhea or HUS (table 3) were tested for the Vero cell cytotoxicity and for their reactivity in the Stx EIA and the latex agglutination assay (table 6). All 23 isolates of the stx2b genotype produced the toxin, as detected by the Vero cell assay and by the latex agglutination assay. However, 3 of 23 Stx2c producers tested negative in the Stx EIA (table 6). Among the 15 STEC of the stx2e genotype, only 10 produced Stx2d, as demonstrated by their cytotoxicity for Vero cells and by positive results in the Stx EIA and the latex agglutination assay (table 6). The remaining 5 isolates that contained stx2e as their sole stx gene were not cytotoxic for Vero cells and tested negative in both immunoassays. Stx2e was produced by 11 of 12 isolates harboring stx2e, but in only 7 of them was the toxin detectable by the Stx EIA and by the latex agglutination assay (table 6). Altogether, 44 (88.0%) of 50 STEC isolates that contained stx2a, stx2b, or stx2e as the sole stx genes produced the respective Stx, and the toxins of 37 (84.1%) and 40 (90.9%) of these 44 strains could be detected by the Stx EIA and by the

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**Table 5.** Association of Shiga toxin–producing *Escherichia coli* (STEC) harboring Shiga toxin 2 gene (*stx*2) variants with clinical manifestations of the infection and with age of patients, compared with STEC of the *stx*2 genotype.

<table>
<thead>
<tr>
<th>stx genotype</th>
<th>Total no. of isolates</th>
<th>Isolates from patients with HUS, no. (%)a</th>
<th>Isolates from patients without HUS (with D or A), no. (%)</th>
<th>P</th>
<th>χ², 1 df</th>
<th>Age of patients, median years (range)</th>
<th>Patients ≤ 5 years old, no. (%)b</th>
<th>Patients &gt; 18 years old, no. (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx2a</td>
<td>28</td>
<td>10 (35.7)</td>
<td>18 (64.3)</td>
<td>.21</td>
<td>1.54</td>
<td>3 (9 mo–64 y)</td>
<td>15 (50.0)</td>
<td>6 (21.4)</td>
</tr>
<tr>
<td>stx2b</td>
<td>25</td>
<td>0 (0)</td>
<td>25 (100.0)</td>
<td>&lt;.000001</td>
<td>50.00</td>
<td>38.5 (9 mo–86 y)</td>
<td>4 (16.0)</td>
<td>16 (64.0)</td>
</tr>
<tr>
<td>stx2a + stx2b</td>
<td>37</td>
<td>0 (0)</td>
<td>37 (100.0)</td>
<td>&lt;.000001</td>
<td>74.00</td>
<td>28 (4 mo–66 y)</td>
<td>5 (13.5)</td>
<td>25 (67.6)</td>
</tr>
<tr>
<td>stx2c</td>
<td>16</td>
<td>0 (0)</td>
<td>16 (100.0)</td>
<td>&lt;.000001</td>
<td>32.00</td>
<td>19.5 (14 mo–59 y)</td>
<td>4 (25.0)</td>
<td>9 (56.3)</td>
</tr>
<tr>
<td>stx2d</td>
<td>193</td>
<td>147 (76.2)</td>
<td>46 (23.8)</td>
<td>&lt;.000001</td>
<td>105.71</td>
<td>3.5 (2 mo–30 y)</td>
<td>169 (87.6)</td>
<td>6 (3.1)</td>
</tr>
</tbody>
</table>

NOTE. A, asymptomatic; D, diarrhea; HUS, hemolytic-uremic syndrome. The χ² test and Yates’s corrected (YC) χ² test were used for calculations. P < .05 was considered to be statistically significant.

a Relative risk of HUS development was as follows: *stx*2a vs. *stx*2b, P = .00001; χ²; 1 df, 19.45. *stx*2a vs. *stx*2b (*stx*2a/*stx*2b or *stx*2a/*stx*2b); P = .000004; YC χ², 21.43. *stx*2b vs. *stx*2d, P = .019; YC χ², 5.50.

b Proportions of patients <5 years old among patients infected with STEC containing *stx*2a (*stx*2a/*stx*2a or *stx*2a/*stx*2b) or *stx*2b vs. *stx*2a, P = .00003; YC χ², 21.67; and *stx*2b vs. *stx*2b (*stx*2a/*stx*2a or *stx*2a/*stx*2b) or *stx*2b, P < .000001; YC χ², 1 df, 126.60.

c Proportions of patients >18 years old among patients infected with STEC containing *stx*2a (*stx*2a/*stx*2a or *stx*2a/*stx*2b) or *stx*2b vs. *stx*2a, P = .0004; YC χ², 12.61; and *stx*2b vs. *stx*2b (*stx*2a/*stx*2a or *stx*2a/*stx*2b) or *stx*2b, P < .000001; YC χ², 1 df, 126.06.

d Median age of all 62 patients infected with STEC possessing *stx*2a (*stx*2a/*stx*2a or *stx*2a/*stx*2b) was 31.5 years.

e The proportion of patients <5 years old among all 78 patients infected with STEC harboring *stx*2a (*stx*2a/*stx*2a or *stx*2a/*stx*2b) or *stx*2b was 16.7%.

f The proportion of patients >18 years old among all 78 patients infected with STEC harboring *stx*2a (*stx*2a/*stx*2a or *stx*2a/*stx*2b) or *stx*2b was 64.1%.

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variants of the infection to microangiopathic sequelae such as HUS has been shown to represent a risk factor for the progression of symptoms in patients during a 5-year period. However, STEC with the presence of stx1 and stx2 alleles, respectively (table 6). The inference that STEC harboring different stx alleles have different abilities to cause HUS has several diagnostic implications. Most important, it demonstrates the importance of isolating of STEC from stool cultures that are positive for stx by PCR screening or for Stx by EIA screening [45]. This should be followed by rapid subtyping of stx2 genes, because the information about the stx2 allele, combined with the information about the presence of eae in the STEC isolate, has considerable predictive value for the treating physician to assess the risk of HUS development in a patient who presents with STEC infection, if our findings can be validated in prospective studies.

Discussion

The presence of the stx2 genotype in an infecting STEC isolate has been shown to represent a risk factor for the progression of the infection to microangiopathic sequelae such as HUS [22, 23]. However, the clinical significance of STEC harboring variants of stx2 is unknown. The present study represents the first attempt to classify STEC harboring the presently known stx2 variants with respect to their capacity to cause extraintestinal manifestations in humans. We found that STEC harboring stx2, only, stx2d, or stx2e, accounted for 14.3% of the 530 STEC isolated from patients during a 5-year period. However, STEC with different stx2 alleles differed markedly in their association with HUS. Specifically, stx2 was the only stx2 variant associated with HUS, but the risk of developing HUS after infection with STEC of the stx2 genotype was significantly lower than that after infection with STEC of the stx2 genotype. In contrast to STEC harboring stx2d, STEC possessing stx2d or stx2e alleles were not associated with HUS in the present study. However, such strains accounted for 20.2% of 262 STEC isolated from patients with diarrhea who did not develop HUS.

The finding of stx2d or stx2e alleles in 26.1% of 96 STEC from asymptomatic individuals of the same population during the same time period has 2 important implications. First, it supports the association of STEC harboring stx2d or stx2e with uncomplicated infection. Second, the comparable frequency of stx2d and stx2e alleles among STEC isolated from patients with diarrhea (20.2%) and among STEC isolated from asymptomatic subjects (26.1%) raises the question about the etiological role of the E. coli containing these genes in the diarrhea of the patients from which these strains were isolated. This question cannot be answered in our study, which was focused on the detection of STEC and subtyping of their stx genes and did not investigate systematically the presence of other enteric pathogens in the patients. Hence, on the basis of our data, we cannot exclude the possibility that some of the patients from whom STEC harboring stx2d or stx2e were isolated might have been coinfected with obligate bacterial or viral diarrheagenic pathogens that were, in fact, causative agents of the disease. Ideally, future studies will encompass a broader spectrum of intestinal pathogens to evaluate the etiological role of STEC harboring stx2d or stx2e in diarrhea and thus better understand the pathogenic potential of such strains for humans. The finding of stx2d in 21.9% of STEC from asymptomatic subjects in our study is not surprising, compared with the presence of this stx2 variant in 65% of 37 STEC isolated from asymptomatic carriers in Switzerland [14]. The presence of stx2e in STEC isolated from asymptomatic individuals has not been reported elsewhere, to our knowledge.

### Table 6. Toxin production by Shiga toxin–producing Escherichia coli (STEC) harboring Shiga toxin gene (stx) stx2d, stx2e, or stx2e and detection of Stx2c, Stx2d, and Stx2e by commercial Shiga toxin (Stx) immunoassays.

<table>
<thead>
<tr>
<th>stx genotype</th>
<th>Total no. of isolates cytotoxic for Vero cells</th>
<th>No. of isolates cytotoxic in Stx EIA</th>
<th>No. of Vero cell cytotoxic isolates positive in latex agglutination assay</th>
<th>Titer with Stx2 latex reagent, geometric mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx2e</td>
<td>23</td>
<td>23</td>
<td>20</td>
<td>1:10 (1:4–1:16)</td>
</tr>
<tr>
<td>stx2d</td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>1:4 (1:2–1:8)</td>
</tr>
<tr>
<td>stx2e</td>
<td>12</td>
<td>11</td>
<td>7e</td>
<td>1:4 (1:2–1:8)</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>44</td>
<td>37</td>
<td>1:6 (1:2–1:16)</td>
</tr>
</tbody>
</table>

* Only STEC isolated in the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany) from patients with diarrhea or patients with hemolytic-uremic syndrome were investigated for toxin production.

b Ridascreen Verotoxin (R-Biopharm): a mixture of monoclonal antibodies against Stx1 and Stx2 was used as an Stx reagent.

c Verotoxin-producing E. coli reverse passive latex agglutination (Denka Seiken): latex particles sensitized with polyclonal antibodies against Stx1 and Stx2 were used as Stx1 and Stx2 reagent, respectively.

# Only isolates from the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany) from patients with diarrhea or patients with hemolytic-uremic syndrome were investigated for toxin production.

The presence of stx2 in an infecting STEC isolate has been shown to represent a risk factor for the progression of the infection to microangiopathic sequelae such as HUS [22, 23]. However, the clinical significance of STEC harboring variants of stx2 is unknown. The present study represents the first attempt to classify STEC harboring the presently known stx2 variants with respect to their capacity to cause extraintestinal manifestations in humans. We found that STEC harboring stx2, only, stx2d, or stx2e, accounted for 14.3% of the 530 STEC isolated from patients during a 5-year period. However, STEC with different stx2 alleles differed markedly in their association with HUS. Specifically, stx2 was the only stx2 variant associated with HUS, but the risk of developing HUS after infection with STEC of the stx2 genotype was significantly lower than that after infection with STEC of the stx2 genotype. In contrast to STEC harboring stx2d, STEC possessing stx2d or stx2e alleles were not associated with HUS in the present study. However, such strains accounted for 20.2% of 262 STEC isolated from patients with diarrhea who did not develop HUS.

The finding of stx2d or stx2e alleles in 26.1% of 96 STEC from asymptomatic individuals of the same population during the same time period has 2 important implications. First, it supports the association of STEC harboring stx2d or stx2e with uncomplicated infection. Second, the comparable frequency of stx2d and stx2e alleles among STEC isolated from patients with diarrhea (20.2%) and among STEC isolated from asymptomatic subjects (26.1%) raises the question about the etiological role of the E. coli containing these genes in the diarrhea of the patients from which these strains were isolated. This question cannot be answered in our study, which was focused on the detection of STEC and subtyping of their stx genes and did not investigate systematically the presence of other enteric pathogens in the patients. Hence, on the basis of our data, we cannot exclude the possibility that some of the patients from whom STEC harboring stx2d or stx2e were isolated might have been coinfected with obligatory bacterial or viral diarrheagenic pathogens that were, in fact, causative agents of the disease. Ideally, future studies will encompass a broader spectrum of intestinal pathogens to evaluate the etiological role of STEC harboring stx2d or stx2e in diarrhea and thus better understand the pathogenic potential of such strains for humans. The finding of stx2d in 21.9% of STEC from asymptomatic subjects in our study is not surprising, compared with the presence of this stx2 variant in 65% of 37 STEC isolated from asymptomatic carriers in Switzerland [14]. The presence of stx2e in STEC isolated from asymptomatic individuals has not been reported elsewhere, to our knowledge.

The inference that STEC harboring different stx2 alleles have different abilities to cause HUS has several diagnostic implications. Most important, it demonstrates the importance of isolating of STEC from stool cultures that are positive for stx by PCR screening or for Stx by EIA screening [45]. This should be followed by rapid subtyping of stx2 genes, because the information about the stx2 allele, combined with the information about the presence of eae in the STEC isolate, has considerable predictive value for the treating physician to assess the risk of HUS development in a patient who presents with STEC infection, if our findings can be validated in prospective studies. In
this respect, stx genotyping appears to offer an advantage to serotyping among non-O157 STEC, because STEC harboring stx2d or stx2e belong to a broad spectrum of non-O157 serotypes, but the association between uncomplicated infection that does not show a tendency to progress to HUS and the specific stx2 alleles persists, independently of serotype. For laboratories that cannot use PCR to subtype stx genes in STEC, it is particularly noteworthy that Stx2c, Stx2d, and Stx2e produced by most patients’ STEC isolates investigated in this study could be detected by commercially available Stx immunoassays, including EIA and the latex agglutination assay. The inability of the commercial assays to detect Stx2e in 4 producers of this toxin is in agreement with the observation by Beutin et al. [46], who could not detect Stx2e in STEC isolated from pig edema disease using the VTEC-RPLA assay. The reason for the inability of both commercial assays used in this study to detect Stx2e in some of the toxin producers could be either inadequate in vitro production of toxin by such isolates or antigenic differences in the toxin molecules that diminish the recognition of these toxins by antibodies used in the respective commercial immunoassays. In fact, a lower sensitivity of the Stx2 latex agglutination reagent for the detection of Stx2c, compared with that for the detection of Stx2, was observed by Karmali et al. [39] and appears to be the case also for Stx2d and Stx2e, as suggested by the low latex agglutination titers (≈1:8) that we observed in STEC producing these toxins in the present study. From a diagnostic standpoint, such low toxin titers in the latex agglutination assay may suggest production of an Stx2 variant, rather than of the classic Stx2, by the isolate. However, considering the important predictive value of the information about the Stx2 type produced by an STEC isolate, commercial immunoassays that use specific antibodies that would differentiate Stx2 from its variants and identify the respective Stx2 variants should be developed and evaluated. However, it should be remembered that, although toxin detection assays represent valuable adjuncts to culturing, it is crucial to isolate STEC that can be further characterized for clinical, epidemiological, and analytical purposes. Such assays should not be used in lieu of standard microbiologic assessments.

An additional limitation in the use of toxin detection assays is that not all STEC that harbor stx2 variants produce the respective toxins under laboratory conditions. In our study, the lack of the toxin production was most pronounced in STEC harboring stx2d as the sole stx gene. One-third of these isolates (5/15) did not produce Stx2d in vitro, as demonstrated by the absence of the Vero cell cytotoxicity in their culture supernatants. Similarly, lack of toxin production was observed in 1 of 12 STEC that harbored stx2e. Although reasons for the lack of the in vitro toxin production in these STEC are unclear, there are several possible explanations. First, the proteins might not be expressed because mutations in stx genes introduce changes in reading frames or stop codons. Second, the genes might not be expressed under the growth conditions used. Third, the toxins were synthesized but were not released from the bacterial cells because of a defect in a toxin export mechanism. However, because the environment in the intestine differs substantially from laboratory conditions, the lack of Stx production in vitro does not exclude the possibility that the toxins were not produced by these STEC in vivo during infection. Investigations are in progress to determine the reason(s) for the inability of the respective isolates to produce the toxins in vitro.

The lack of the association of STEC harboring stx2d or stx2e with HUS, as demonstrated in the present study, may have several explanations. The first is the universal absence of the eae gene from all STEC that contain stx2d or stx2e, as observed in this study and reported by Piérad et al. [8, 18]. eae is an important accessory virulence gene of STEC isolated from patients with HUS [21], and its presence in STEC O157 and STEC of the 4 major non-O157 serogroups (O26, O103, O111, and O145) has been associated strongly with the ability of such STEC to cause severe human disease, including HUS [23]. Alternatively, Stx2d and Stx2e might be less toxic than Stx2 or Stx2c for humans. Naturally occurring Stx2 variants differ in their virulence in a mouse model [47]. Although not directly comparable to human infection, E. coli that expressed Stx2d had lower toxicity than isogenic clones that expressed Stx2c when administered intraperitoneally to mice [47]; oral virulence of 1 of the Stx2d-expressing clones for streptomycin-treated mice was significantly lower than that of both clones that expressed Stx2c [47]. Reduced pathogenicity of STEC harboring stx2d for humans is also suggested by the high frequency of isolation of such STEC from stool samples of asymptomatic individuals [14], an observation that was also confirmed in our study. Another possible reason for the lack of the association of STEC harboring stx2d or stx2e with HUS in our study might be the fact that such STEC infected mostly adults. Only 13 of 78 individuals infected with STEC harboring stx2d or stx2e were < 5 years old and thus of the age that has been shown to represent a significant risk factor for the development of systemic complications after STEC infection [1]. In this context, it is noteworthy that the previous rare reports on human infections with STEC harboring stx2e, mainly have described adult patients [17–19]. The reason why such STEC have a tendency to infect adults rather than young children remains unknown. However, this relationship is obviously complex. For example, stx7-positive, stx2-negative STEC are clearly capable of causing HUS [48], and there have been rare reports of STEC harboring stx2d [13, 49] and stx2e [19] being isolated from patients with HUS. In our study, we cannot exclude the possibility that some of the patients with HUS from whom STEC were not recovered, probably because the isolation was not attempted until presentation with HUS, might have been infected with STEC harboring these stx2 variants.

Similar to the etiological role of STEC containing stx2d or stx2e in diarrhea, the epidemiology of infections caused by such strains is presently unknown. A recent report of the high preva-
lence of stx<sub>2d</sub> in <i>E. coli</i> from the normal intestinal microflora of sheep [13] and the finding of stx<sub>2d</sub> in selected human isolates that belonged to the same serotypes as ovine stx<sub>2d</sub>-harboring STEC [13] suggest that sheep could be a reservoir of stx<sub>2d</sub>-harboring STEC for humans. This would be consistent with our finding of STEC containing stx<sub>2d</sub> in 3 workers from a meat processing plant. The serogroups identified in stx<sub>2d</sub>-harboring STEC isolated from patients in this study (O8 and O60) and in other studies (O101 and O9 [17, 19]) have not been associated with pig edema disease [15]. However, the isolation of Stx2e-producing STEC of serogroup O101 from healthy pigs [50] and the finding that such strains demonstrate a high degree of genetic relatedness to a human stx<sub>2d</sub>-harboring STEC O101 isolate [35] suggest that healthy pigs might be a potential reservoir of stx<sub>2d</sub>-harboring STEC for humans.

Stx<sub>f</sub> is a newly described Stx variant that we identified in STEC recovered from feral pigeons [10]. The observation that stx<sub>f</sub> of a pigeon isolate is almost identical to a stx<sub>f</sub> variant gene identified previously in an <i>E. coli</i> strain isolated in Canada from a patient with diarrhea [20] prompted us to investigate all human STEC in the present study for the presence of stx<sub>f</sub>. The uniform absence of stx<sub>f</sub> from the 626 STEC suggests that stx<sub>f</sub> may have minimal, if any, link to pathogenicity in humans. However, an investigation is in progress to look for stx<sub>f</sub> in human <i>E. coli</i> isolates that are similar to pigeon stx<sub>2d</sub>-harboring STEC [10], eae positive but negative for other stx genes.

In conclusion, STEC possessing different stx<sub>2</sub> variants differ in their capacity to cause HUS. Although infection with STEC of the stx<sub>2a</sub> genotype can progress to HUS, STEC harboring stx<sub>2a</sub> or stx<sub>2e</sub> genes are not associated with HUS but represent a significant part of STEC from patients with uncomplicated diarrhea and are found frequently in asymptomatic STEC carriers. Such isolates lack eae and have a tendency to infect adults, rather than young children. The presence of stx<sub>2d</sub> or stx<sub>2e</sub> in an STEC isolate, combined with the absence of the eae gene, may thus predict mild disease with a minimal risk of HUS development; prospective studies will be needed to test the predictive value and clinical utility of stx genotyping efforts.

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References


