Enterohemorrhagic *Escherichia coli* in Human Infection: In Vivo Evolution of a Bacterial Pathogen

Alexander Mellmann, Martina Bielaszewski, Lothar B. Zimmerhackl, Rita Prager, Dag Harmsen, Helmut Tschäpe, and Helge Karch

1Institute for Hygiene, National Consulting Laboratory on Hemolytic Uremic Syndrome and IZKF Münster, and 2Department for Periodontology, University Hospital Münster, Münster, and 3National Reference Center for Salmonella and Other Enteric Pathogens, Robert Koch Institute, Branch Wernigerode, Wernigerode, Germany; and 4Department of Pediatrics, Innsbruck University Hospital, Innsbruck, Austria

(See the editorial commentary by Robins-Browne on pages 793–4)

**Background.** Enterohemorrhagic *Escherichia coli* (EHEC) cause most cases of the hemolytic uremic syndrome (HUS) worldwide. To investigate genetic changes in EHEC during the course of human infection, we analyzed consecutive stool samples and shed isolates from patients with HUS, focusing on the genes encoding Shiga toxin (*stx*) and intimin (*eae*).

**Methods.** Sequential stool samples from 210 patients with HUS were investigated for the persistence of *E. coli* strains harboring *stx* and/or *eae*. Initial stool samples were collected during the acute phase of HUS, and subsequent stool samples were collected 3–16 days later (median interval, 8 days).

**Results.** Organisms that were *stx* and *eae* positive (*stx+/eae+* strains; *n* = 137) or *stx* negative and *eae* positive (*stx–/eae+* strains; *n* = 5) were detected in the initial stool samples from 142 patients. Subsequently, the proportion of those who shed *stx+/eae+* strains decreased to 13 of 210 patients, whereas the proportion of those who shed strains that were *stx–/eae+* increased to 12 of 210 patients. Seven patients who initially excreted strains that were *stx+/eae+* shed, at second analysis, *stx–/eae+* strains of the same serotypes; they had no free fecal Shiga toxin at follow-up. Comparison of the initial and follow-up isolates from these patients with use of molecular-epidemiological methods revealed loss of *stx* genes and genomic rearrangement.

**Conclusions.** We demonstrate the loss of a critical bacterial virulence factor from pathogens during very brief intervals in the human host. These genetic changes have evolutionary, diagnostic, and clinical implications. Generation of *stx+/eae+* mutants might contribute to subclonal evolution and evolutionary success.

Gastrointestinal infections with enterohemorrhagic *Escherichia coli* (EHEC) usually manifest as acute, afebrile, painful, bloody diarrhea. EHEC are the leading precipitants of hemolytic uremic syndrome (HUS), an important cause of childhood acute renal failure [1]. *E. coli* O157:H7 is the most common EHEC serotype associated with HUS, but several non-O157:H7 serotypes, such as O26:H11, O103:H2, O111:H8, O145:H28, and O157:NM (nonmotile), have emerged as causes of this disease [2–8].

Major HUS-associated EHEC produce Shiga toxin (Stx) 1, Stx2, or Stx2c, either singly or in combinations [2, 5, 6, 9, 10]. Stxs are believed to be the major precipitants of the microvascular thrombi that form the histopathological basis of HUS [11]. However, EHEC also produce factors other than Stxs that can contribute to the pathogenesis of HUS [12]. Candidates for such non-Stx putative virulence factors include the EHEC pore-forming hemolysin [13] and intimin, encoded by *eae* [14]. Intimin mediates intimate attachment to epithelial cells in vitro and in animal models [15, 16], and it is found in almost all EHEC strains from patients with HUS [5, 6]. Enteropathogenic *E. coli* (EPEC) share intimin with the most common varieties of EHEC but lack Stx [17]. Many of the potential pathogenicity factors of EHEC are encoded by mobile genetic elements, such as plasmids, pathogenicity islands, and bacteriophages [12]. The association between DNA transfer and virulence is well documented [18]. The *stx* genes in EHEC are encoded on temperate lambdoid bacterio-

Received 25 February 2005; accepted 26 April 2005; electronically published 4 August 2005.

a A.M. and M.B. contributed equally to this article.

Reprints or correspondence: Dr. Alexander Mellmann, Institut für Hygiene, Universitätshospital Münster, Robert Koch Str. 41, 48149 Münster, Germany (mellmann@uni-muenster.de).

*Clinical Infectious Diseases* 2005;41:785–92
© 2005 by the Infectious Diseases Society of America. All rights reserved. 1058-4838/2005/4106-0003$15.00
phages [19, 20] and can be eliminated from such strains by inducing the stx-converting phages with sublethal doses of UV light, mitomycin C, or various antibiotics [19, 21, 22].

Little is known about genetic changes sustained by EHEC during human infection. Therefore, in this study, we investigated genetic changes of EHEC strains during the course of HUS. To accomplish this aim, we analyzed consecutive stool samples obtained from patients with HUS with use of molecular detection of stx and eae genes to identify strains that were stx positive and eae positive (stx+/eae+ strains) and strains that were stx negative and eae positive (stx−/eae+ strains). We compared isolates from consecutive stool samples of the same patient with regard to extended virulence profiles, plasmid profiles, and PFGE patterns. Also, we investigated molecular and phenotypic characteristics that can be used for the laboratory diagnosis of both stx+ and stx− E. coli strains associated with HUS.

PATIENTS AND METHODS

Patients. During routine diagnostic efforts conducted between 1996 and 2003, sequentially collected stool samples from 210 patients with HUS were investigated for the presence of E. coli strains harboring stx and/or eae genes. The patients were from different regions of Germany, and, except for 3 who were part of a small outbreak of HUS [23], none showed temporal or geographical linkage. HUS was defined as a case of microangiopathic hemolytic anemia (i.e., hematocrit <30% with peripheral evidence of intravascular hemolysis), thrombocytopenia (i.e., platelet count <150,000 platelets/mm³), and renal insufficiency (i.e., serum creatinine concentration greater than the upper limit of the normal range for age) [24]. The initial stool samples were collected 5–14 days after the onset of prodromal diarrhea (median interval, 9 days), which corresponds to the early HUS phase of the illness. The follow-up stool samples were collected 3–16 days later (median interval, 8 days).

Stool sample analysis. Stx in stool filtrates was detected using the Vero cell cytotoxicity assay [25]. Screening for and isolation of E. coli strains harboring target loci were performed as described elsewhere [5]. In brief, enriched stool samples were analyzed for E. coli O157 by immunomagnetic separation technique and culture of magnetically separated organisms on sorbitol MacConkey (SMAC) agar and cefixime-tellurite SMAC agar. To identify non-O157 E. coli strains associated with HUS.

Detection of other enteric bacterial pathogens in stool samples. Salmonella species, Shigella species, Yersinia enterocolitica, and Campylobacter jejuni were sought using standard procedures.

Serological investigation. Purification of lipopolysaccharides (LPs) O26 and O157 and the detection of IgM antibodies against these LPs in serum samples obtained during the acute phase of HUS were performed as described elsewhere [28].

Phenotyping methods. Isolates were serotyped using a microtiter method [29]. Biochemical identification was performed with API 20 (bioMérieux). In addition, sorbitol fermentation was detected on SMAC agar. Stx production was tested using a latex agglutination assay [30]. The enterohemolytic phenotype was sought on enterohemolysin agar [13, 31].

Genotyping methods. The PCR strategy to detect stx was described elsewhere [5, 32, 33]. Detection and characterization of eae was performed as described elsewhere [26, 34, 35]. The EHEC hemolysin gene (EHEC-hlyA) and the sfpA gene [36] were detected using primer pairs hlyA1-hlyA4 [13] and sfpA-U–sfpA-L [37], respectively. The cytotoxic distending toxin (CDT)–V gene cluster was detected as described elsewhere [38]. E. coli O157:H7 strain EDL933 [39], E. coli O26:NM strain 5720/96 [10], and SF E. coli O157:NM strain 493/89 [38] were used as positive controls in PCRs. flhC restriction fragment–length polymorphism (RFLP) analysis was performed as described elsewhere [40].

Plasmid profiles and PFGE analysis. Plasmid profiles were determined as described elsewhere [9]. PFGE was performed using XbaI-digested genomic DNA from Salmonella braenderup strain H9812 (Centers for Disease Control and Prevention) as a size marker [41]. Restriction fragment patterns of genomic DNA were analyzed with BioNumerics software, version 4.0 (Applied Maths BVBA) and analyzed with the criteria of Tenover et al. [42].

Statistical analysis. Statistical analysis was performed using 2-tailed Fisher’s exact test. P values <.05 were considered to be statistically significant.

RESULTS

Analysis of initial stool specimens from patients with HUS for stx+ and/or eae+ E. coli. Initial cultures of stool samples from 137 of 210 patients with HUS were positive for stx and eae genes (table 1). From the initial stool cultures for 2 patients that tested positive for stx but not eae, stx+/eae− strains of serotypes O91:H21 and O113:H21 were isolated. Stool cultures from 5 additional patients were eae+ and stx−. Fecal filtrates from 2 patients in which neither stx+ nor eae− strains were

786 • CID 2005;41 (15 September) • Mellmann and Bielaszewska et al.
detected were found to contain Stx activity. In all other patients, the Vero cell assay and genetic analysis agreed (table 1). Stools from all patients were negative for Salmonella species, Yersinia enterocolitica, and Campylobacter jejuni.

**Follow-up stool analysis.** To determine whether the pathogen population changes over time within individual patients, we analyzed follow-up stool samples. In 1 of the 64 subjects with neither stx- and/or eae-harboring *E. coli* nor detectable Stx activity in their initial stools, an stx-positive, eae-positive *E. coli* O157:H7 strain was subsequently identified (table 1), suggesting intermittent shedding. The 5 patients shedding stx-/eae+ *E. coli* strains in their initial stools had identical strains at follow-up (table 1). Of the 137 patients who had stx- and eae-positive strains in their initial stools, only 12 (8.8%) shed the infecting strains in follow-up stools. In 118 patients (86.1%), the follow-up stool samples were negative for free Stx, as detected by the Vero cell cytotoxicity assay (table 1). In the remaining 7 patients who shed stx+/eae+ strains of serotypes O26:H11/NM (6 patients) or O157:NM (1 patient) in their initial stool samples, the follow-up stool samples yielded stx-/eae+ strains of the same serotype (tables 1 and 2). Together, this replacement of an stx+/eae+ organism by an stx-/eae+ organism of the same serotype in a follow-up stool sample was observed in 6 (28.6%) of 21 patients infected with *E. coli* O26, 1 (3.3%) of 30 patients infected with SF *E. coli* O157:NM, but none of 86 patients infected with EHEC strains of other serotypes including O157:H7 (table 1). Thus, the conversion from stx+ to stx− strains was significantly more frequent among *E. coli* O26 (6 of 21) than among EHEC of all other serogroups combined (1 of 116) (P<.001, by 2-tailed Fisher’s exact test), suggesting that the stx prophage may be less stable in EHEC O26 than in other EHEC strains. Of interest, this process occurred independently in all 3 patients who were affected during a small EHEC O26:H11 outbreak [23] (patients A, C, and E; table 2), raising the possibility that stx lability is a function of individual strains. There was free fecal Stx in the initial stool samples but not in the subsequent stool samples (table 2). Cultures of initial and subsequent stool samples from each of these 7 patients were negative for other bacterial enteric pathogens. Acute-phase serum samples, which were available from 6 of these patients, demonstrated IgM antibodies to cognate (but not heterologous) LPS (table 2).

**Genotypic and phenotypic characteristics of consecutive stool samples**

*Table 1.* Analysis of consecutive stool samples from 210 patients with hemolytic uremic syndrome by means of PCR screening and colony blot hybridization with probes complementary to the stx, stx2, and eae genes and by the Vero cell assay.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Findings, by initial stool specimen result</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) of patients</td>
<td>stx+/eae+/Vero+</td>
</tr>
<tr>
<td>O26:H11/NM (21), O103:H2 (6), O111:H8/NM (8), O145:H28 (11), O157:NM (30), O157:H7 (61)</td>
<td>137 (65.2)</td>
</tr>
<tr>
<td>No. of patients</td>
<td>12</td>
</tr>
<tr>
<td>Second specimen result</td>
<td>stx+/eae+/Vero+</td>
</tr>
<tr>
<td>No. of patients</td>
<td>118</td>
</tr>
<tr>
<td>Serotype (no. of isolates)</td>
<td>NA</td>
</tr>
<tr>
<td>O26:H11/NM (6), O157:NM (1)</td>
<td>7</td>
</tr>
</tbody>
</table>

**NOTE.** eae+, eae gene present; eae−, eae gene absent; NA, not applicable; stx+, stx gene present; stx−, stx gene absent; Vero+, positive Vero cell assay result; Vero−, negative Vero cell assay result.

a stx types included stx1, stx2, and/or eae-harboring E. coli nor detectable Stx activity in their initial stools, an stx-positive, eae-positive *E. coli* O157:H7 strain was subsequently identified (table 1), suggesting intermittent shedding. The 5 patients shedding stx−/eae+ *E. coli* strains in their initial stools had identical strains at follow-up (table 1). Of the 137 patients who had stx- and eae-positive strains in their initial stools, only 12 (8.8%) shed the infecting strains in follow-up stools. In 118 patients (86.1%), the follow-up stool samples were negative for free Stx, as detected by the Vero cell cytotoxicity assay (table 1). In the remaining 7 patients who shed stx+/eae+ strains of serotypes O26:H11/NM (6 patients) or O157:NM (1 patient) in their initial stool samples, the follow-up stool samples yielded stx−/eae+ strains of the same serotype (tables 1 and 2). Together, this replacement of an stx+/eae+ organism by an stx−/eae+ organism of the same serotype in a follow-up stool sample was observed in 6 (28.6%) of 21 patients infected with *E. coli* O26, 1 (3.3%) of 30 patients infected with SF *E. coli* O157:NM, but none of 86 patients infected with EHEC strains of other serotypes including O157:H7 (table 1). Thus, the conversion from stx+ to stx− strains was significantly more frequent among *E. coli* O26 (6 of 21) than among EHEC of all other serogroups combined (1 of 116) (P<.001, by 2-tailed Fisher’s exact test), suggesting that the stx prophage may be less stable in EHEC O26 than in other EHEC strains. Of interest, this process occurred independently in all 3 patients who were affected during a small EHEC O26:H11 outbreak [23] (patients A, C, and E; table 2), raising the possibility that stx lability is a function of individual strains. There was free fecal Stx in the initial stool samples but not in the subsequent stool samples (table 2). Cultures of initial and subsequent stool samples from each of these 7 patients were negative for other bacterial enteric pathogens. Acute-phase serum samples, which were available from 6 of these patients, demonstrated IgM antibodies to cognate (but not heterologous) LPS (table 2).
**Table 2. Replacement of enterohemorrhagic *Escherichia coli* strains by *stx*-negative, *eae*-positive variants of the same serotypes within individual patients with hemolytic uremic syndrome.**

<table>
<thead>
<tr>
<th>Patient, stool specimen</th>
<th>Age, months</th>
<th>Specimen collection, days after onset</th>
<th>PCR result, by primer</th>
<th>Colony blot hybridization result, by probe</th>
<th>Serotype of the isolate</th>
<th>Free fecal Stx result</th>
<th>Serum anti-026 IgM</th>
<th>Serum anti-0157 IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>5</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>12</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>9</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:H11</td>
<td>Pos</td>
<td>Present</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>16</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:NM</td>
<td>Neg</td>
<td>Present</td>
</tr>
<tr>
<td>C</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>7</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:H11</td>
<td>Pos</td>
<td>Present</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>14</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:H11</td>
<td>Neg</td>
<td>NS</td>
</tr>
<tr>
<td>D</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>7</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:H11</td>
<td>Pos</td>
<td>Present</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>5</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:H11</td>
<td>Pos</td>
<td>Present</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>13</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:H11</td>
<td>Neg</td>
<td>NS</td>
</tr>
<tr>
<td>F</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>9</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:NM</td>
<td>Pos</td>
<td>NS</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>13</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O26:NM</td>
<td>Pos</td>
<td>NS</td>
</tr>
<tr>
<td>G</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>8</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O157:NM</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
<td>11</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>O157:NM</td>
<td>Neg</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NOTE.** NA, not applicable; Neg, negative; NM, nonmotile; NP, not performed; NS, no serum was available; Pos, positive; Stx, Shiga toxin.

a Interval between the onset of prodromal diarrhea and the collection of the stool specimen.

b In total, 302–406 colonies from each stool specimen that was investigated were subjected to colony blot hybridization. Isolate was defined as positive for Stx if a stool filtrate diluted 1:50 displayed a cytotoxic effect on Vero cells and as negative for Stx if no cytotoxic effect on Vero cells was observed with a stool filtrate diluted 1:50 or 1:10.

c Isolate was defined as positive for Stx if a stool filtrate diluted 1:50 or 1:10 displayed a cytotoxic effect on Vero cells and as negative for Stx if no detectable cytotoxic effect on Vero cells was observed with a stool filtrate diluted 1:50 or 1:10.

**stx+ and stx− E. coli isolates.** One stx+/eae+ isolate from the initial stool sample and 1 stx−/eae+ isolate from the follow-up stool sample for each of these 7 patients with HUS were further analyzed. *HhaI* RFLP analysis of *fliC* PCR products demonstrated that the 9 O26:H11 isolates and 3 O26:NM isolates shared a *fliC* restriction pattern that was identical to that of the H11 type strain Su4321–41 (data not shown). Also, each of these isolates possesses *eae*-encoding β-intimin (table 3). The stx-containing isolates produced Stx1 and/or Stx2, in accordance with their stx genotypes, whereas no Stx was detected in culture supernatants of any stx− strains (table 3). All 12 O26 isolates, whether or not they possessed stx, contained the EHEC-*hlyA* gene and displayed an enterohemolytic phenotype. In contrast, the stx+ and stx− SF *E. coli* O157:NM isolates produced the same *fliC* RFLP pattern H7 and possessed *eae γ* and EHEC-*hlyA* genes but did not express the enterohemolytic phenotype. Also, both contained a plasmid-borne *sfpA* gene encoding a sorbitol-fermenting EHEC fimbriae and the *cdtA, cdtB,* and *cdtC* genes encoding CDT-V.

**Plasmid profiles and PFGE analysis.** The consecutive stx+/eae+ and stx−/eae+ E. coli O26 isolates from patients A, B, C, and E had identical plasmid profiles (both within the same patient and among the patients). In contrast, the consecutive isolates from patient F differed from each other by 2 plasmids (table 3). The stx+ and stx− E. coli O26 isolates from patient D shared a 90-kb plasmid, but the stx− isolate lacked the 75-kb plasmid that was present in the stx+, positive strain (table 3). PFGE demonstrated that, with a single exception (patient F), the intrapatient stx+/eae+ isolates and the stx+/eae− isolates differed by only 2 or 3 bands. The first isolate and the follow-up isolate from patient F differed by >7 bands, possibly because of a coinfection.

**stx− E. coli as the only pathogen in patients with HUS.** Five of 210 patients with HUS had, in both initial and follow-up stool samples, *E. coli* strains that possessed *eae* but lacked stx and stx, genes (table 1). The eae+ isolates from initial and follow-up stool samples from each of these 5 patients belonged to the same serotype (table 1) and shared identical genotypic
and phenotypic characteristics (table 4). Notably, except for stx genes, the genotypic and phenotypic characteristics of these stx−/eae+ isolates were identical to those identified in stx+ isolates of the corresponding serotypes. Culture supernatants of these strains were not toxic to Vero cells, suggesting that they did not contain stx genes that might have been undetectable with our PCR protocol. Accordingly, initial and follow-up stool samples from these patients contained no free fecal Stx, as demonstrated by the Vero cell assay. None of these patients were infected with Salmonella species, Shigella species, Y. enterocolitica, or C. jejuni.

**DISCUSSION**

Genomic alterations in an infecting pathogen, in the course of an acute infection, have multiple implications. From a diagnostic standpoint, 2 observations are particularly important. First, the timing of the stool sample collection may be critical for finding Stx-producing strains in patients with HUS. Tarr et al. [44] demonstrated that, if the stool samples of patients with HUS were cultured within 6 days after the onset of diarrhea for EHEC O157:H7, the recovery rate was nearly 100%. This rate decreased to 33.3% in stool samples collected >6 days after the onset of diarrhea. In our study, we analyzed stool samples obtained from patients with HUS that were usually collected >7 days after the onset of diarrhea, and we screened the samples for EHEC by the detection of Stxs or stx. At initial examination, 141 (96.6%) of 146 strains were detected using such methods, whereas only 13 (52.0%) of 25 were detected in follow-up stool samples. This confirms a previous report [44] that EHEC are difficult to identify in patients’ feces late in illness. It also demonstrates that, in such cases, stx- and Stx-independent procedures are required to detect strains that might have lost their stx genes. An efficient method for the detection of stx−/eae+ strains is colony blot hybridization with a probe complementary to the eae conserved region. Although this procedure is labor intensive and time consuming, it is presently used to recover stx−/eae+ strains in our laboratories. However, such an approach is not without pitfalls, because it would miss strains of serotypes O91:H21 and O113:H21, which were present in 2 patients in our study. Therefore, a combination of stx and eae probes is required for the colony blot hybridization to identify members of the known spectrum of pathogenic E. coli in patients with HUS. Second, the loss of stx genes in 6 of 21 EHEC O26 and in 1 of 30 SF EHEC O157:NM but in none of 61 EHEC O157:H7 strains suggests that this phenomenon is associated with particular non-O157:H7 serotypes, especially O26:H11/NM. In this context, the finding in a clinical microbiological laboratory of an stx−/eae+ E. coli O26 strain in a patient with HUS should lead a treating physician to consider such a patient as potentially infected with an EHEC strain and to follow therapeutic procedures recommended for patients infected with EHEC (e.g., to avoid antibiotic therapy). Of interest, in our study, the tendency of the stx gene loss was correlated with EHEC serogroup rather than with the median time interval between the collection of initial and follow-up stool samples. Indeed, this time interval was shorter (median interval, 6 days) for patients infected with E. coli O26 and O157:NM strains that lost their stx genes (table 2) than it was for all other patients (median interval, 8 days). Although less likely, an alternative to the loss of stx genes by the 6 EHEC O26 and 1 SF O157:NM strains during infection might be that these patients were infected with a mixed population of stx+ and stx− strains from the same source and that the stx+ strains overwhelmed the stx− strains during the acute phase of the illness but did not persist for as long as the stx− strains.

Except for the 2 SF E. coli O157:NM strains, all stx−/eae+ E. coli strains could be distinguished on enterohemolysin agar. The enterohemolytic phenotype is produced by EHEC hemolysin (EHEC-Hly) [13]. Its structural gene, EHEC-hlyA [13], was present in each of the stx−/eae+ E. coli strains. However, the mechanism underlying its nonexpression in SF E. coli O157:NM is unknown. Therefore, our data demonstrate that EHEC-Hly production is independent of Stx production in stx−/eae+ E. coli strains O26:H11 and O145:H28, even though the pro-
duction of EHEC-Hly is a useful marker for the detection of EHEC, as proposed by Beutin et al. [31].

From the standpoint of pathogenesis, it is particularly interesting that, in 5 patients with HUS, stx−/eae+ E. coli strains were the only pathogens identified. It is possible that these strains could cause disease by a yet-to-be determined Stx-independent mechanism. Alternatively, these organisms might have colonized these patients without being involved in the pathogenesis of the underlying HUS. In some studies, stx−/eae+ E. coli strains have been isolated from healthy infants [26, 43], but strains of serotype O157:H7/NM were not found, and O26:H11 strains were extremely rare [43]. In view of the instability of the stx genotype that we have demonstrated, it also seems plausible that the stx−/eae+ strains might have directly descended from Stx-producing E. coli strains, which had been present in these patients earlier in the infection. In this scenario, they would have lost these loci before our analysis and, as such, would represent isogenic mutants of Stx-producing strains that have replaced the original EHEC strains.

Our data offer an insight into mechanisms of pathogen evolution. Maintaining the phage encoding stx may be lethal to the bacterial host cell. Survival might be favored by loss of the phage, because such stx− progeny of stx+ progenitors are less prone to lysis. Mammalian host signals, such as those initiated by hydrogen peroxide, can induce Stx-encoding prophages [45]. By generating stx− mutants, a strain can survive without automatically lysing and carrying the burden of toxin production. The loss of stx-encoding phage can thus offer a selective advantage. Molecular typing methods demonstrated the relatedness of the strains within single patients, which suggests that the change in genotype is caused by the loss of relatively little genome. Even though the exact mechanisms by which stx genes are lost are still unclear, these data might explain the genome rearrangement observed by means of PFGE. A spontaneous loss of stx1 or stx2 genes by EHEC isolates during laboratory subcultures that resulted in nontoxicogenic derivatives, can occur in particular non-O157 serotypes [46], and recently, spontaneous loss of both stx1 and stx2 genes in vitro has also been described in an E. coli O157:H7 clinical isolate [47]. We refer to this phenomenon as clonal turnover, which defines changes in clonal composition characterized by an appearance of new clonal genotypes and loss of old ones [46]. Clonal turnover can be the result of selection for a mutant and subsequent clonal replacement within individual patients or of massive genomic rearrangements, producing the appearance of a new clone. The dynamics of these processes and their function in the evolution of these pathogens warrant further investigation. However, compared with the slow clonal turnover in H. pylori [48–50], real-time, rapidly emergent mutations occur in enterohemorrhagic E. coli during human infection.

Acknowledgments

We thank Philip I. Tarr for fruitful and extensive discussions of the manuscript.

Financial support. The Bundesministerium für Bildung und Forschung (BMBF) Project Network of Competence Pathogenomics Alliance (BD no. 119523 to M.B.), BMBF Verbundprojekt (no. 01KI 9903 to M.B.), and the Interdisciplinary Center of Clinical Research Münster (IZKF, Project no. K2/061/04 to A.M.).

Potential conflicts of interest. All authors: no conflicts.

References

43. Beutin L, Marches O, Bettelheim KA, et al. HEP-2 cell adherence, antigen aggregation, and intimin types of attaching and effacing Escherichia coli.