Identification and characterisation of *Escherichia coli* strains of O157 and non-O157 serogroups containing three distinct Shiga toxin genes

SABINE FÜRST, JÜRGEN SCHEEF, MARTINA BIELASZEWSKA*, HOLGER RÜSSMANN†, HERBERT SCHMIDT and HELGE KARCH

Institut für Hygiene und Mikrobiologie der Universität Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany, * Institute of Medical Microbiology, 2nd Medical Faculty, Charles University, 150 06 Prague, Czech Republic and †Max von Pettenkofer-Institut für Hygiene und Mikrobiologie der Ludwig Maximilians Universität München, Pettenkoferstraße 9a, 80336 München, Germany

Three Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains from patients with diarrhoea were identified, each of which contained three distinct stx genes (stx1, stx2 and stx2c). The strains belonged to the serotypes O52:H19, O75:H− and O157:H− and harboured *eae* and EHEC-hly sequences. Colony-blot immun assay was used to demonstrate that both major types of Stx were expressed. The association of stx genes with either phage or phage DNA was demonstrated in all three strains. Isolated phage DNA from all strains contained *stx* sequences, but *stx2c* sequences were found only in phage DNA of two of these strains. The presence of three distinct stx genes may enhance the virulence of STEC strains and should be monitored. The observations demonstrate not only the potential of stx genes to spread within different serotypes, but also their capacity to accumulate within a single strain.

**Introduction**

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains are a widespread cause of haemorrhagic colitis and haemolytic uremic syndrome (HUS). A large body of evidence supports the hypothesis that Stx plays a major role in the pathogenesis of HUS [1, 2].

The antigenically distinct toxins Stx1 and Stx2 are produced alone or in combination by human STEC strains. In strains of *E. coli* O157 a variant of Stx2, termed Stx2c, has been identified that is antigenically related to Stx2 [3]. Nucleotide sequence similarities between *stx2* and *stx2c* were 99.6% and 94.8% in the A-subunit and B-subunit genes, respectively. Other members of the Stx2 family exist in non-O157 clinical STEC isolates. The genes for Stx1 and Stx2 are encoded on separate temperate bacteriophages [4, 5]. Phage induction may occur in response to agents causing damage to DNA, such as mitomycin C, and under such conditions Stx expression is increased.

The fact that stx genes are located in the genome of phages may also facilitate their spread by transduction and, indeed, stx genes have been found in >100 serotypes of *E. coli* [6]. Furthermore, combinations of usually two stx genes have been reported in STEC O157:H7 as well as in non-O157 STEC of human origin [7, 8]. An earlier study identified six different patterns of stx genotypes including stx1, stx2, stx2c, and combinations stx1/stx2, stx1/stx2c and stx1/stx2c in STEC O157:H7/H− strains isolated from patients with HUS [8]. Thomas *et al.* [7] found three stx genes (stx1/stx2c) in *E. coli* O157:H7/H− strains of human origin.

This study reports the finding of three STEC strains each harbouring the *stx1, stx2* and *stx2c* genes among 507 STEC isolates from patients with diarrhoea and HUS throughout Germany. It also presents evidence that the major Stx are expressed and that accessory virulence determinants and stx-phages are present in these strains.

**Materials and methods**

**Bacterial strains and phenotypic techniques**

The STEC isolates were detected in stool samples of patients with diarrhoea and HUS between 1994 and

Received 1 June 1999, revised version accepted 6 Sept. 1999.

Corresponding author: Dr H. Karch (e-mail: hkarch@hygiene.uni-wuerzburg.de).
1997 by PCR with primers GK3/GK4, LP43/LP44 and KS7/KS8 [9]. Serotyping was performed as described by Bockemühl et al. [10]. EHEC O157:H7 strain EDL933 (Stx1 and Stx2) [11], E. coli lysogen C600 (H19J) (Stx1) [4], E. coli lysogen C600 (933W) (Stx2) [12] and EHEC strain E32511 (Stx2c*) [3] were used as controls. E. coli strain DH5α (GibcoBRL) was used as indicator strain in bacteriophage-induction experiments. The Vero cell cytotoxicity assay [9] and the colony blot immunoassay [8] were performed as described previously.

**PCR**

PCR reactions for detection of EHEC-specific sequences were performed with 10³ bacterial cells. Primers KS7 and KS8 were used to amplify the B-subunit gene of Stx1 [9] and JS1 (5′-cat gaa gaa gat gtt tgg ccc g-3′) and JS2 (5′-ctc agt cat taa act gca c-3′) to amplify the B-subunit gene of Stx2 and Stx2 variants. GK3 and GK4 [9] were initially used to identify stx1 and stx2, but have the same binding sites in the respective B-subunit genes as JS1 and JS2. HaeIII and FokI were used to differentiate between stx1 and stx2, as described previously [8]. SK1 and SK2 target the conserved region of eae and plasmid-specific sequences were detected with primers lhA1 and lhA4, wakr-B and wakr-F and D1 and D13R [9].

**Bacteriophage techniques**

Bacteriophage DNA was prepared by standard methods. For bacteriophage induction, a single colony of bacterial lysogen was grown in Trypticase Soy Broth (Oxoid) containing 5 mM CaCl₂ to an OD₆₀₀ of 0.5. Then mitomycin C was added to a final concentration of 0.5 μg/ml and incubation was continued overnight in the dark. Cultures were centrifuged (15 min, 5000 g) and supernatants were filter-sterilised through membrane filters (Schleicher and Schill, Dassel, Germany) of pore size 0.2 μm.

A double-layer agar method was used to investigate plaque formation. Plaque hybridisation was performed according to standard methods with the use of digoxigenin-11-dUTP labelling and detection kit (Boehringer GmbH, Mannheim, Germany).

**Standard DNA techniques**

Preparation and digestion of chromosomal DNA, labelling of gene probes and Southern blot hybridisation with stx1 and stx2 probes were performed as described previously [9]. Nucleotide sequencing was performed by Taq cycle sequencing with an automated sequencer (Model 377A, Applied Biosystems, Weiterstadt, Germany).

Nucleotide sequence analyses and searches for homologous DNA sequences in database libraries were performed with the programme package HUSAR (Heidelberg Unix Sequence Analysis Resources, German Cancer Research Center, Heidelberg, Germany) and the Lasergene software package (DNAStar, Madison, WI, USA).

**Results**

**Characterisation of E. coli isolates carrying multiple stx genes**

Multiple stx genes – stx₁, stx₂ and stx₂c – were identified in three clinical isolates of E. coli. This was achieved with separate PCR reactions with primers KS7/KS8 and JS1/JS2, followed by restriction analysis of PCR products derived from primers JS1/JS2 with HaeIII and FokI. Serotyping showed that the three strains belonged to serotypes O52:H19, O75:H₁ and O157:H⁻. The strains were designated 99, 4799 and 17069, respectively (Table 1). Culture filtrates of all strains were highly cytotoxic to Vero cells (10⁻⁷–10⁻⁵ 50% cytotoxic doses/ml of filtrate) (Table 1). In addition, all strains were subjected to colony-blot immunoassay with either the Stx1-specific monoclonal antibody (MAb) 13C4 or the Stx2-specific MAb BiEO [14]. Both antibodies reacted with all three strains, indicating that the stx genes were expressed (Table 1).

Analysis of accessory virulence factors, as described for E. coli O157 [15], revealed that all three strains harboured eae gene and were positive for EHEC-hly genes. Whereas E. coli O157:H⁻ strain 17069 possessed both the kaiP and espP genes, the non-O157 isolates were negative for both of these genes (Table 1).

Southern hybridisation of EcoR1-digested genomic DNA was performed with total DNA isolated from all three strains and control strains. With the stx₁ probe, a single DNA fragment of 8.5 kb hybridised in tests with DNA from control strain C600 (H19J) (Table 1) and single fragments of 10.0, 4.8 and 7.2 kb hybridised with DNA from strains 99, 4799 and 17069, respectively (Table 1).

With the stx₂ probe, hybridisation occurred with single DNA fragments of 4.8 kb and 5.8 kb from control strains C600 (933W) and E32511 (Table 1), respectively. Two hybridised fragments of different sizes were detected in tests with strains 17069 (5.5 and 7.5 kb) and 99 (4.2 and 9.0 kb) (Table 1). In the case of strain 4799, four fragments of 4.5, 7.0, 8.2 and 12.5 kb hybridised with the stx₂ probe (Table 1).

**Plaque hybridisation**

The extent to which stx genes were associated with phages was investigated in induction experiments. Cultures of strains 17069, 99, 4799 and control strains C600 (H19J) and C600 (933W) were induced with
mitomycin C. Plaque formation on strain DH5α was observed with phage lysates from both control strains and with strain 17069 of O157:H−, but not with lysates from the other two strains. Hybridisation analysis with stx1 and stx2 probes showed that c. 5% of the plaques formed by the phage lysate of strain 17069 hybridised with the stx1 probe, whereas the remaining 95% hybridised with the stx2 probe.

Hybridisation of phage DNA

Hybridisation of EcoRI-restricted phage DNA from strains 17069, 99 and 4799 was performed with stx1 and stx2 probes to determine whether the stx genes in the strains were located in the genome of prophages. DNA from phages H19J and 933W was also hybridised. With the stx1 probe, a single DNA fragment (8.5 kb) of control phage H19J (Table 1) and of phage DNA prepared from strains 17069 (7.2 kb), 99 (10.0 kb) and 4799 (4.8 kb) hybridised, but no fragment from control phage 933W (Table 1). With the stx2 probe, a single fragment of control phage 933W (4.8 kb) and of phage DNA from strains 17069 (5.5 kb) and 4799 (8.2 kb) – but no fragment from control phage H19J or phage DNA from strain 99 – hybridised (Table 1).

Sequencing of stx genes

Sequencing of stx1 PCR products of all three strains obtained with primers KS7/KS8 and subsequent analysis yielded 100% identity with stx1. After amplification with primers JS1 and JS2, PCR products of all strains were cleaved with HaeIII before analysis of stx2 sequences. The undigested fragment, when subjected to nucleotide sequence analysis, revealed 100% concordance with the stx3B sequence reported by Schmitt et al. [3]. Characterisation of the stx3B sequence was performed on the undigested fragment after treatment of the PCR products with FokI. The stx3B sequence in the three strains investigated was 100% identical to the stx3B sequences published by Schmitt et al. [3].

Discussion

Although STEC belonging to serogroups O52 and O75 have been isolated from patients with diarrhoea [16] and HUS [10], these strains belonged to serotypes different from those found in the present study – namely O52:H25 [16] and O75:H5 [10] – and produced either Stx2 or Stx1 only. STEC of serogroups O52 and O75 have rarely been isolated from patients with HUS or diarrhoea in our laboratory in recent years. The fact that only three (0.59%) of 507 STEC strains analysed in our laboratory between 1994 and 1997 possessed a combination of three different stx genes suggests that such strains are rare among clinical STEC isolates. This is consistent with the finding of Thomas et al. [7], who showed that isolates possessing a combination of stx1, stx2 and stx3 genes occurred with a low frequency in the O157:H7/H− STEC group.
Although Southern hybridisation of chromosomal DNA with the stx1 and stx2 probes showed the presence of stx1 and stx2/stx2c genes in all three strains, only isolated phase DNA from strains 17069 and 4799 hybridised with the stx2 probe. This finding suggests that in strain 99 a Stx2-converting phase is not inducible by mitomycin C or that the stx2 gene in that strain is not phase-encoded. After mitomycin C induction, only strain 17069 formed plaques and these hybridised with stx1 and stx2 probes. Plaque formation was not observed with strains 99 and 4799. The simplest explanation for this phenomenon is that the indicator strains used in the plaque assay were not sensitive to these phases, because of a lack of specific receptors.

The extent to which multiple stx genes in one strain of STEC can modulate the level of virulence is not known and the specific role of each of Stx1, Stx2 and Stx2c in the pathogenesis of human disease has not yet been well defined. On the one hand, it is conceivable that strains possessing a combination of three different stx genes are more virulent than those harbouring only one, or two, stx genes – assuming that all three genes are expressed. On the other hand, the presence of stx genes may be a biological disadvantage, in the sense that such strains are more susceptible to phage-inducing agents in the intestine and this leads to the lysis of bacterial host cells. Consistent with the latter hypothesis is the fact that all three patients had uncomplicated diarrhoea.

The observations reported here suggest that Stx genotyping and phenotyping will become important for monitoring various STEC strains. Changes in toxin patterns can be monitored by the techniques described in this study. Characterisation of STEC strains expressing multiple stx genes will be an important topic for further investigations concerning virulence and epidemiology of STEC.

We thank Barbara Plaschke for excellent technical assistance. This work was supported by grant Ka 717/3-1 from the Deutsche Forschungsgemeinschaft.

References