Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with haemolytic uraemic syndrome

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Summary. The prevalence and genotype of Shiga-like toxins (SLTs) in *Escherichia coli* O157 strains from patients in Germany with haemolytic uraemic syndrome (HUS) were investigated. This was done by PCR amplification of the B-subunit genes with two primer pairs—one complementary to *slt*-IB, and the other homologous to both *slt*-IIB and *slt*-IIV:B sequences. To distinguish between *slt*-II and *slt*-IIV, the amplified DNA was digested with restriction endonucleases HaeIII and FokI. Of the 38 strains examined, 17 harboured sequences for *slt*-IIV; four contained only *slt*-IIV, three carried both *slt*-IIV and *slt*-I, and 10 strains had *slt*-IIV and *slt*-I. A further three genotypes (*slt*-I, *slt*-II, *slt*-IIV) were found in the remaining 21 strains resulting in a total of six *slt* genotypes. To determine whether the *slt* genes were expressed, and whether genotypes correlated with phenotypes, all strains were subjected to cytotoxicity assays and colony ELISA. All 38 strains displayed cytotoxic activity to Vero cells in similar quantities. The SLT-I-specific monoclonal antibody (MAb)13C4 reacted with all 10 strains in which *slt*-I sequences were identified. Colony blot ELISA with the SLT-II specific MAb11E10 detected 27 of 28 strains with *slt*-II sequences, but did not react with any of the seven strains that carried *slt*-IIV, or *slt*-I and *slt*-IIV. The high SLT variability shown here has diagnostic implications and may well have consequences for the host response in infections associated with these pathogens.

Introduction

*Escherichia coli* O157 strains are the major cause of haemorrhagic colitis and haemolytic uraemic syndrome (HUS). The Shiga-like toxins (SLTs), also called verocytotoxins, which they produce, are divided into two major groups, Shiga-like toxin I (SLT-I) and Shiga-like toxin II (SLT-II) on the basis of immunological and molecular characteristics. SLT-I toxins appear to be homogeneous and are neutralised by antibody against the Shiga toxin of *Shigella dysenteriae* type I.1-4 SLT-II toxins are heterogeneous and are not neutralised by polyclonal Shiga antitoxin or SLT-I antitoxin.5,6 Only 58% of the overall nucleotide sequence homology is shared by *slt*-I and *slt*-II.7 The SLT-II variants, SLT-IIVc and SLT-IIVhc, have been identified recently in *E. coli* O157:H7 strain E32511 and *E. coli* O157:H7 strain 7279, respectively.8,9 When compared to *slt*-II, *slt*-IIVc and *slt*-IIVhc share c. 99% and 95% homology in their respective A- and B-subunit genes. Furthermore, *slt*-IIVc and *slt*-IIVhc exhibit almost identical nucleotide sequences.9 The predicted amino-acid sequences of their B subunits are identical. Additional SLT-II variants have been described in non-O157 *E. coli* strains3,5,8,10-12 and in *Citrobacter freundii*.13 Although SLT-II variants are highly related to SLT-II at the nucleotide and protein level, they are not completely neutralised by antibodies against SLT-II. Furthermore, it has been demonstrated that SLT-IIV differs from SLT-II in its carbohydrate-binding specificities for target cell receptors.14

Different findings have been reported for the frequency of SLT-II-variants in *E. coli* O157 strains. None of the 24 *E. coli* O157 strains studied by Hii et al.15 carried *slt*-IIV sequences, whereas all eight strains analysed by Tyler et al.16 had them. However, the association between disease and individual toxins was not reported. Therefore, we examined the frequency of SLT-IIV among *E. coli* O157 strains from patients with HUS and performed a complete *slt* genotyping analysis to determine whether there was an association between individual toxins and disease.

Materials and methods

Bacterial strains

The 38 *E. coli* O157 strains used in this study are
listed in the table. They were isolated from the stools of 38 children with HUS treated in different paediatric centres in different geographical locations throughout Germany. As far as is known, there was no contact between any of the patients. Of the 38 E. coli O157 strains, 15 were identified by colony hybridisation with probes 772 and 849 as described previously,\textsuperscript{17} and 23 strains were identified by colony hybridisation with digoxigenin-labelled PCR products from the slt-IIB- or the slt-IIvB genes prepared as described previously.\textsuperscript{18} All strains gave a negative sorbitol reaction in liquid medium and on sorbitol MacConkey agar. Sorbitol-fermenting E. coli O157:H\textsuperscript{+} strains described previously\textsuperscript{19} were not examined because they all produced SLT-II.\textsuperscript{16,18} The control strains used in the slt genotyping studies were the E. coli lysogen C600(933W) that produces SLT-II, E. coli strain E32511 that produces SLT-IV, E. coli strain 1249/87 and E. coli strain 3978/91 that both produce SLT-II and -IIv, and E. coli strain C600 (H19J) that produces SLT-I.

**PCR and restriction analysis**

The oligonucleotides used as primers were purchased from Roth, Karlsruhe, Germany. The sequences of the primers used to amplify the slt-IIB and slt-IIvB genes were GK5 (5'-ATG AAG AAG ATG TTT ATG GCG-3') and GK6 (5'-TCA GTC ATT ATT AAA CTG CAC-3'). The primers KS7 and KS8\textsuperscript{13} were used to amplify the slt-IB gene. Bacterial DNA was prepared by incubating 10 \( \mu l \) of bacterial suspension (10\textsuperscript{8} bacteria) for 10 min at 95\textdegree C. Amplifications were performed in a total volume of 50 \( \mu l \) which contained 200 \( \mu M \) deoxynucleoside triphosphates (dTTP, dCTP, dGTP and dTTP), 30 pmol of each primer, 5 \( \mu l \) of 10-fold concentrated polymerase synthesis buffer and 2.5 U of Taq DNA polymerase (Amersham Laboratories, Buckinghamshire). The samples were overlaid with 50 \( \mu l \) of mineral oil to prevent evaporation and subjected to 30 cycles of amplification. The samples were incubated at 94\textdegree C for 30 s to denature the DNA, for 1 min at 52\textdegree C to anneal the primers and at 72\textdegree C for 1 min to extend the annealed primers. Thermal cycling was performed in a thermostatically controlled waterbath. After the last cycle, the amplification products were subjected to submarine gel electrophoresis in agarose 2\% gels and visualised by staining with ethidium bromide.

To distinguish between slt-IIB- and slt-IIvB-related sequences, restriction analysis of the PCR amplification products obtained with primers GK5 and GK6 was performed according to the method of Tyler et al.,\textsuperscript{11} with minor modifications. Briefly, 10-\( \mu l \) amounts of the amplified products were digested with restrictionendonucleases HaeIII and FokI (Boehringer Mannheim GmbH, Mannheim, Germany) as recommended by the suppliers and the DNA fragments were separated and visualised as described above. It was expected from the published sequences of slt-IIB\textsuperscript{8} and slt-IIvB\textsuperscript{20} that the digestion of the slt-IIB amplicon with FokI would yield two fragments of 116 bp and 154 bp, whereas the slt-IIvB PCR product would remain undigested. Digestion of the slt-IIvB amplicon with HaeIII would yield a 128-bp and a 142-bp fragment but the slt-IIvB PCR product would remain undigested.\textsuperscript{16}

**Southern blot hybridisation**

For Southern blot analysis, total bacterial DNA was prepared as described previously,\textsuperscript{12} digested with EcoRI and electrophoresed through an agarose 0.7\% gel. The conditions for DNA transfer on to nylon membranes, capillary blotting and stringent hybridisation were as described previously.\textsuperscript{13} A 270-bp fragment of the slt-IIvB gene resulting from amplification with the primers GK5 and GK6 was labelled with digoxigenin and used as a hybridisation probe. Probe labelling was performed by the PCR programme for the slt-IIvB gene, but instead of dTTP only, a mixture of 130 \( \mu M \) dTTP and 70 \( \mu M \) digoxigenin-11-dUTP was added. Hybridisation and detection of bound probes were performed with the digoxigenin labelling and detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

**Cytotoxicity assay**

Culture filtrates of all strains were tested for cytotoxic activity on Vero cells as described previously.\textsuperscript{13} The 50\% cytotoxic dose was estimated by microscopic examination of the Vero cells after 48 h and was confirmed by staining residual Vero cells with crystal violet.\textsuperscript{13}

**Colonial blot ELISA for detection of SLTs**

The colony blot ELISA was performed in the presence of trimethoprim-sulphamethoxazole as described previously.\textsuperscript{21} The SLT-II-specific MAb 11E10\textsuperscript{22} and the SLT-I-specific MAb 13C4\textsuperscript{20} were used in the assay.

**Results**

**PCR strategy and restriction fragment length polymorphism for genotyping SLTs**

In an initial attempt to subdivide the strains with respect to their toxin genes, each isolate was subjected to PCR analysis with primers specific for slt-IB. As shown in the table, 10 of the 38 E. coli O157 strains showed a positive PCR result. Primers GK5 and GK6 that identify both slt-II and slt-IIvB subunit genes were then evaluated with four control strains that carried either slt-II, slt-IIv, or both. Their PCR products were analysed on agarose gels, yielding DNA bands of c. 270 bp (fig. 1A, lanes 1–4). These PCR
products were subjected to restriction endonuclease analyses. Figs. 1B and C show the fragments resulting from digestion with FokI and HaeIII, respectively. It can be seen from fig. 1B, lane 1, that the PCR product of the SLT-II-producing strain was digested by FokI into a smaller fragment, which is believed to consist of two fragments that were not separable on this gel. The PCR product of the SLT-IIv-producing strain remained undigested (fig. 1B, lane 2) and both a digested and an undigested fragment were seen from strains producing both SLT-I1 and SLT-IIv (fig. 1B, lanes 3 and 4). Fig. 1C demonstrates the results of restriction analyses with HaeIII. The PCR product from the SLT-11-producing strain remained undigested (lane 1), whereas that from the SLT-IIv-producing strain was digested (lane 2).

Southern hybridisation of genomic DNA from the four control strains producing SLT-II, SLT-IIv and both these toxins is shown in fig. 2B, lanes 3–6. DNA from the non-toxigenic E. coli strain C600 and the slt-I-expressing E. coli strain C600(H195) were included as negative controls (lanes 1 and 2 respectively). For the strains producing only SLT-I1 or SLT-IIv, a single EcoRI fragment of either 4.6 kb (fig. 2B, lane 3) or 4.9 kb (fig. 2B, lane 4) hybridised with the slt-IIB probe. For the two strains possessing sequences for both slt-II and slt-I, two EcoRI fragments hybridised with the probe (fig. 2B, lanes 5 and 6).

Prevalence of SLT-IIv, SLT-I and SLT-II in E. coli O157 strains

Restriction endonuclease analysis with HaeIII and FokI was performed on PCR products from all the strains after amplification with the primers GK5 and
Fig. 1. A, PCR amplification products with primers GK5 and GK6 to detect sft-IIB and sfr-IIuB sequences. Lane 1: E. coli C600 (933W) (sft-II); 2: E. coli E32511 (sft-IIh); 3: E. coli 1249/87 (sft-II and sft-IIh); 4: E. coli 3978/91 (sft-II and sft-IIh). Restriction fragment length polymorphism analysis of the PCR products with: B, FokI; C, HaeIII. M, DNA mol.-wt marker V (Boehringer Mannheim) is shown on the right.

Fig. 2. A, Agarose gel electrophoresis of EcoRI digested DNA fragments; B, Southern blot hybridisation with the sft-IIB probe. The isolates from which DNA samples were obtained were: lane 1, E. coli C600; 2, E. coli C600 (H19J); 3, E. coli C600 (933W); 4, E. coli E32511; 5, E. coli 1249/87; 6, E. coli 3978/91. M, DNA mol.-wt marker V (Boehringer Mannheim) is shown on the left.

Fig. 3. Determination of E. coli O157 sft-II genotypes by restriction endonuclease analysis with HaeIII. Amplification products obtained by PCR with primers GK5 and GK6 were digested with HaeIII. The following E. coli strains were amplified: lane 1, 7513/91; 2, 1249/87; 3, 3269/90; 4, 6983/91; 5, 1658/91; 6, 2406/92; 7, 3850/91; 8, 3658/93; 9, 2712/89; 10, 4847/91; 11, 5159/91. M, DNA mol.-wt marker V (Boehringer Mannheim) is shown on the left.
GK6. Analysis of the PCR products from 11 strains after digestion with HaeIII is shown in fig. 3. From this analysis: the PCR products shown in lanes 1, 4, 6, 8 and 9 are derived from slt-IIv; those in lanes 3, 7, 10 and 11 from slt-II; and the digested amplified DNA in lanes 2 and 5 contained both slt-II and slt-IIv. The results from the restriction endonuclease analyses of the PCR products obtained from the individual E. coli O157 strains are presented in the table. No discrepancy was seen between the results of the restriction endonuclease analyses performed with HaeIII and FokI.

Cytotoxic activity and SLT-II neutralisation

In liquid cultures, all the E. coli O157 isolates produced cytotoxin. In the four strains that produced only SLT-Iv, the cytotoxic activity was similar to that of the SLT-II producers, i.e., 10^5–10^7 50% cytotoxic doses/ml of culture supernate.

Colony blot ELISA

To determine whether the slt genes were expressed, and whether genotyping correlated with current immunological SLT-detection methods, all strains were subjected to colony ELISA with either the SLT-I-specific MAb 13C4 or the SLT-II-specific MAb 11E10. MAb 13C4 reacted with all 10 strains in which slt-I sequences were found (slt-I, slt-I/slt-II, slt-I/slt-IIv) as shown in the table. MAb 11E10 reacted with 27 of 28 E. coli strains in which slt-II sequences (either as the sole genotype or with slt-I or slt-IIv) were identified. It was of considerable interest that MAb 11E10 did not recognise any of the four strains with slt-IIv alone or the three strains with both slt-I and slt-IIv sequences.

Discussion

The frequency of infections by SLT-producing E. coli O157 continues to increase worldwide. There is evidence from animal models that SLTs may play a crucial role in the pathogenesis of both intestinal and extra-intestinal manifestations of E. coli O157 infection. In addition to SLT-I and SLT-II, there is a growing interest in variants of SLT-II that previously were undetectable because of their high nucleotide homology to SLT-II. By means of PCR amplification and subsequent restriction endonuclease analysis, we have demonstrated the impact of SLT-IIv in clinical isolates of E. coli O157. Of the 38 E. coli O157 strains tested, 17 contained slt-IIv sequences, either alone or in combination with slt-I or slt-II. In total, six combinations of slt genotypes were detected: slt-I, slt-II, slt-IIv, slt-I/slt-II, slt-I/slt-IIv and slt-II/slt-IIv. These findings may have implications for the host response in E. coli O157 infections.

The considerable toxin diversity is an interesting phenomenon because multilocus enzyme electrophoresis and DNA fingerprinting have provided epidemiological evidence that E. coli O157 strains are highly related. The relatively low degree of genetic variation observed in the O157 population suggests that the pathogen probably arose from a single clone. At first sight, this genomic homogeneity appears to be in contrast to the SLT heterogeneity. The simplest explanation for SLT diversity would be that an ancestral isolate contained slt-I, slt-II and slt-IIv. Loss of any one or two slts would result in the six genotypes found. Loss of slt genes has been reported in both E. coli O157 and non-O157 toxin producers. Thomas et al. reported recently on E. coli O157 strains reacting with oligonucleotides specific for the three toxins. However, of the E. coli O157 strains investigated here, no isolate was found that harboured all three toxins.

Molecular methods involving PCR, oligonucleotide DNA probes and Southern hybridisation have been used to subdivide the SLT-II family. Variable results have been reported on the prevalence of SLT-IIv. None of the 24 E. coli O157 strains studied by Hii et al. carried slt-IIv sequences, whereas all eight strains examined by Tyler et al. possessed slt-IIv. In strains from Britain, an oligonucleotide homologous to slt-IIv sequences reacted with 109 of 176 isolates, an SLT-IIv frequency similar to our findings. The variation in prevalence may depend on the methodology used for SLT-IIv identification or the source of the strains. We adopted the strategy of Tyler et al. to identify SLT-IIv, i.e., the use of primers homologous to SLT-II and SLT-IIv and distinguishing between the slt genes by restriction enzyme analysis. Initial investigations in our laboratory compared the restriction enzymes HaeIII, RsaI and NcoI in differentiating between SLT-II and SLT-IIv. In our study, HaeIII was confirmed as the most suitable enzyme for the digestion of slt-IIv. To digest slt-IIB selectively, we introduced FokI. This was found to be superior to RsaI, for minimal amounts of the amplified DNA remained undigested and the genotype of SLT could be predicted correctly.

The genotype as predicted by FokI digestion was confirmed by immunological detection of SLT-IIv. In our study, HaeIII was confirmed as the most suitable enzyme for the digestion of slt-IIv. To digest slt-IIB selectively, we introduced FokI. This was found to be superior to RsaI, for minimal amounts of the amplified DNA remained undigested and the genotype of SLT could be predicted correctly.

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Our findings on SLT diversity may be of epidemiological value. E. coli O157 strains which have been shown to have highly related DNA fingerprints might be subdivided by their toxin genotype. However, as slt genes may be easily lost during storage or in vivo, further studies on the value of SLT genotyping as an epidemiological marker need to be done. SLT genotype heterogeneity may also have an important influence on the host response, because anti-SLT antibodies efficiently neutralise the cytotoxic effect by interference with receptor binding. Therefore, antitoxic immunity appears to be important in the immune response against E. coli O157-associated
References


