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-IIvB* 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-II*. A further three genotypes (*slt-I*, *slt-II*, *slt-I/slt-II*) 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-IIc and SLT-IIvhc, have been identified recently in *E. coli* O157:H[–] strain E32511 and *E. coli* O157:H7 strain 7279, respectively.^{8,9} When compared to *slt-II*, *slt-IIc* and *slt-IIvhc* share c. 99% and 95% homology in their respective A- and B-subunit genes. Furthermore, *slt-IIc* and *slt-IIvhc* exhibit almost identical nucleotide sequences.⁸ The predicted amino-acid sequences of their B subunits

are identical. Additional SLT-II variants have been described in non-O157 *E. coli* strains^{5,6,10–12} 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

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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,¹⁷ and 23 strains were identified by colony hybridisation with digoxigenin-labelled PCR products from the *slt-IB*- or the *slt-IIB* genes prepared as described previously.¹³ All strains gave a negative sorbitol reaction in liquid medium and on sorbitol MacConkey agar. Sorbitol-fermenting *E. coli* O157:H⁻ strains described previously¹⁸ were not examined because they all produced SLT-II.^{18,19} 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-IIv, *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 KS8¹³ were used to amplify the *slt-IB* gene. Bacterial DNA was prepared by incubating 10 µl of bacterial suspension (10⁴ bacteria) for 10 min at 95°C. Amplifications were performed in a total volume of 50 µl which contained 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 30 pmol of each primer, 5 µl of 10-fold concentrated polymerase synthesis buffer and 2.5 U of *Taq* DNA polymerase (Amersham Laboratories, Buckinghamshire). The samples were overlaid with 50 µl of mineral oil to prevent evaporation and subjected to 30 cycles of amplification. The samples were incubated at 94°C for 30 s to denature the DNA, for 1 min at 52°C to anneal the primers and at 72°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.*,¹⁶ with minor modifications. Briefly, 10-µl amounts of the amplified products were digested with restriction endonucleases *Hae*III and *Fok*I (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*⁹ and *slt-IIvB*²⁰ that the digestion of the *slt-IIB* amplicon

with *Fok*I would yield two fragments of 116 bp and 154 bp, whereas the *slt-IIvB* PCR product would remain undigested. Digestion of the *slt-IIB* amplicon with *Hae*III would yield a 128-bp and a 142-bp fragment but the *slt-IIB* PCR product would remain undigested.¹⁶

Southern blot hybridisation

For Southern blot analysis, total bacterial DNA was prepared as described previously,¹³ digested with *Eco*RI 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.¹³

A 270-bp fragment of the *slt-IIB* 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-IIB* gene, but instead of dTTP only, a mixture of 130 µM dTTP and 70 µM 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.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.¹³

Colony blot ELISA for detection of SLTs

The colony blot ELISA was performed in the presence of trimethoprim-sulphamethoxazole as described previously.²¹ The SLT-II-specific MAb 11E10²² and the SLT-I-specific MAb 13C4²⁰ 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

Table. Differentiation of Shiga-like toxins from *E. coli* O157 strains isolated from patients with HUS

Strain no.	Serotype	Distribution of <i>slt</i> genotypes			Reactivity by colony blot ELISA with MABs		Reference for strains
		<i>slt-I</i>	<i>slt-II</i>	<i>slt-IIv</i>	13C4 (SLT-I)	11E10 (SLT-II)	
1193/89	O157:H ⁻	+	-	-	+	-	This study
6578/91	O157:H7	+	-	-	+	-	This study
4326/93	O157:H ⁻	+	-	-	+	-	This study
1071/87	O157:H7	-	+	-	-	+	27
3269/90	O157:H7	-	+	-	-	+	27
4740/91	O157:H7	-	+	-	-	+	27
3850/91	O157:H ⁻	-	+	-	-	+	27
4847/91	O157:H7	-	+	-	-	+	This study
5159/91	O157:H ⁻	-	+	-	-	-	This study
0728/92	O157:H7	-	+	-	-	+	This study
3574/92	O157:H7	-	+	-	-	+	This study
4444/92	O157:H7	-	+	-	-	+	This study
4991/92	O157:H7	-	+	-	-	+	This study
3112/93	O157:H ⁻	-	+	-	-	+	This study
3355/93	O157:H ⁻	-	+	-	-	+	This study
3797/93	O157:H7	-	+	-	-	+	This study
4372/93	O157:H7	-	+	-	-	+	This study
7513/91	O157:H7	-	-	+	-	-	This study
6983/92	O157:H7	-	-	+	-	-	This study
2406/92	O157:H7	-	-	+	-	-	This study
3658/93	O157:H7	-	-	+	-	-	This study
3268/90	O157:H7	+	+	-	+	+	27
5769/87	O157:H7	+	+	-	+	+	27
5288/91	O157:H7	+	+	-	+	+	This study
7576/92	O157:H ⁻	+	+	-	+	+	This study
2712/89	O157:H ⁻	+	-	+	+	-	This study
7443/91	O157:H ⁻	+	-	+	+	-	This study
5291/92	O157:H ⁻	+	-	+	+	-	This study
1249/87	O157:H7	-	+	+	-	+	This study
1658/91	O157:H7	-	+	+	-	+	27
3978/91	O157:H ⁻	-	+	+	-	+	This study
4841/91	O157:H7	-	+	+	-	+	This study
5130/91	O157:H7	-	+	+	-	+	This study
5289/91	O157:H7	-	+	+	-	+	This study
5650/91	O157:H7	-	+	+	-	+	This study
6537/91	O157:H ⁻	-	+	+	-	+	This study
2403/92	O157:H7	-	+	+	-	+	This study
4218/93	O157:H7	-	+	+	-	+	This study

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-II 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-II-producing strain remained undigested (lane 1), whereas that from the SLT-IIv-producing strain was digested (lane 2). As expected, the PCR products of the two strains producing both SLT-II and SLT-IIv consisted of both a digested and an undigested fragment band (lanes 3 and 4).

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(H19J) were included as negative controls (lanes 1 and 2 respectively). For the strains producing only SLT-II 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-II*B probe. For the two strains possessing sequences for both *slt-II* and *slt-IIv*, 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 *slt-II*B and *slt-II*vB sequences. Lane 1: *E. coli* C600 (933W) (*slt-II*); 2: *E. coli* E32511 (*slt-II*v); 3: *E. coli* 1249/87 (*slt-II* and *slt-II*v); 4: *E. coli* 3978/91 (*slt-II* and *slt-II*v). 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 *slt-II*B 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 *slt-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 *Hae*III 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 *Hae*III and *Fok*I.

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-IIv, 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-IIv*, *slt-I/slt-II*) 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.²³ 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.^{24–26} 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 electro-

phoresis and DNA fingerprinting have provided epidemiological evidence that *E. coli* O157 strains are highly related.^{27,28} The relatively low degree of genetic variation observed in the O157 population suggests that the pathogen probably arose from a single clone.²⁸ 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.^{9,29} Thomas *et al.*³⁰ 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.*¹⁵ carried *slt-IIv* sequences, whereas all eight strains examined by Tyler *et al.*¹⁶ possessed *slt-IIv*. In strains from Britain, an oligonucleotide homologous to *slt-IIv* sequences reacted with 109 of 176 isolates,³⁰ 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.*¹⁶ 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 *Hae*III, *Rsa*I and *Nci*I in differentiating between SLT-II and SLT-IIv.¹⁸ In our study, *Hae*III was confirmed as the most suitable enzyme for the digestion of *slt-IIvB*. To digest *slt-IIv* selectively, we introduced *Fok*I. This was found to be superior to *Rsa*I, for minimal amounts of the amplified DNA remained undigested and the genotype of SLT could be predicted correctly. The genotype as predicted by *Fok*I digestion was confirmed by immunological detection of SLT-II. Further detailed study on variants of SLT-II is required to determine the complete nucleotide sequence, in order to assess whether or not the SLT-IIv of *E. coli* O157 strains is a homogeneous toxin.

Our findings on SLT diversity may be of epidemiological value. *E. coli* O157 strains which have been shown to have highly related DNA fingerprints²⁷ 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.²⁵ Therefore, antitoxic immunity appears to be important in the immune response against *E. coli* O157-associated

disease. Because little is known at present about the prevalence of SLT-specific antibodies in man, SLT-

diversity might contribute to the high virulence of *E. coli* O157 strains.

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