CHARACTERISATION OF BACTERIA

Genotyping of Shiga-like toxin genes in non-O157 *Escherichia coli* strains associated with haemolytic uraemic syndrome

H. RÜSSMANN, E. KOTHE, H. SCHMIDT, S. FRANKE, D. HARMSSEN, A. CAPRIOLI*
and H. KARCH†

*Institut für Hygiene und Mikrobiologie der Universität Würzburg, Bau 17, Josef-Schneider-Str. 2, 97080 Würzburg, Germany and * Laboratorio di Ultrastrutture, Istituto Superiore di Sanità, Viale Regina Elena, 229, 00161 Rome, Italy*

Summary. The pheno- and genotypes of Shiga-like toxins (SLTs) in non-O157 *Escherichia coli* strains from patients with haemolytic uraemic syndrome were determined. The clinical isolates investigated were from Italy and Germany and belonged to serotypes O22:H8, O26:H⁻, O26:H11, O91:H⁻, O111:H⁻ and O128:H⁻; one isolate was non-typable. SLT genotypes were analysed by complete nucleotide sequence analysis of the B-subunit genes. The results showed that 14 strains possessed *slt-I* alone, two contained *slt-II* alone and five isolates harboured both *slt-I* and *slt-II* genes. In only two strains were *slt-II*-related genes found, together with either *slt-I* or *slt-II*. These findings indicate that variants of SLT-II are rarely found in non-O157 *E. coli* isolates from patients with haemolytic uraemic syndrome. Polymerase chain reaction (PCR) with *Taq* cycle sequencing was found to be a suitable method for classification of *slt* genotypes.

Introduction

Haemolytic uraemic syndrome (HUS) is the leading form of acute renal failure in children. Among the various forms of HUS, the classical one, which typically follows a prodrome of acute gastroenteritis, is the most frequent. Shiga-like toxin (SLT, verocytotoxin)-producing *Escherichia coli* strains are recognised as the major cause of this disease.¹ Many serogroups of *E. coli* other than O157 have been implicated in sporadic cases of HUS.^{1–9} The first outbreak of HUS associated with non-O157 SLT-producing *E. coli* has been described recently.¹⁰ Serotyping revealed that the isolates belonged to serotype O111:H⁻.

Immunological and molecular analysis of the SLTs produced by *E. coli* have shown that, in addition to the prototypes SLT-I and SLT-II, variants of SLT-II exist both in *E. coli* O157 and non-O157 strains.¹¹ SLT-II-related toxins exhibit only partial serological cross-reactivity with SLT-II and are less toxic to HeLa cells than to Vero cells. The genes for the variants of SLT-II associated with human diseases are termed *slt-IIc*,¹² *slt-IIvhc*,¹³ *slt-IIva*,¹⁴ *vtx2ha* and *vtx2hb*.¹⁵ Recently, additional SLT-II variants have been described,

suggesting great heterogeneity among the toxins belonging to the SLT-II family.^{16,17} Because there is insufficient nucleotide heterogeneity within the *slt-II* locus to permit detection of variants with polynucleotide probes, SLT-II variants were previously undetectable. We have demonstrated the impact of polymerase chain reaction (PCR) technology and subsequent restriction endonuclease analysis by finding *slt-II*-related genes among 17 of 38 strains of *E. coli* O157 from patients with HUS.¹⁸

Little is known about the association between SLT-II variants of non-O157 *E. coli* strains and disease or their prevalence in *E. coli* strains isolated from defined diseases such as HUS. Therefore, in this study, the *slt* genotypes were determined in non-O157 *E. coli* strains associated with HUS and the combination of PCR technology with the *Taq* cycle sequencing method was examined for the rapid and precise classification of *slt* genes.

Material and methods

Bacterial strains

The 23 non-O157 *E. coli* strains investigated in this study are listed in table I. All strains were isolated from HUS patients in Germany during 1985–1992 or in Italy during 1989–1993. Some of these strains have

Table I. The *slt* genotypes found in non-O157 *E. coli* strains associated with HUS

Strain no.	Origin	Serotype	<i>slt</i> genotype
HUS-2/85	Germany	O111:H ⁻	<i>slt-I</i>
4924/85	Germany	O111:H ⁻	<i>slt-I</i>
4321/86	Germany	O111:H ⁻	<i>slt-I</i>
3217/86	Germany	O111:H ⁻	<i>slt-I</i>
3222/87	Germany	O111:H ⁻	<i>slt-I</i>
7042/87	Germany	O111:H ⁻	<i>slt-I, slt-II</i>
5736/88	Germany	O111:H ⁻	<i>slt-I</i>
4856/88	Germany	O111:H ⁻	<i>slt-I</i>
1899/90	Germany	O111:H ⁻	<i>slt-I</i>
928/91	Germany	O111:H ⁻	<i>slt-I, slt-II</i>
78/92	Germany	O111:H ⁻	<i>slt-I</i>
472/92	Germany	O111:H ⁻	<i>slt-I, slt-II</i>
E-D31	Italy	O111:H ⁻	<i>slt-I, slt-II</i>
E-D82	Italy	O111:H ⁻	<i>slt-I</i>
E-D142	Italy	O111:H ⁻	<i>slt-I, slt-II</i>
4478/85	Germany	O26:H11	<i>slt-I</i>
E-D147	Italy	O26:H11	<i>slt-II</i>
6769/89	Germany	O26:H ⁻	<i>slt-I</i>
E-D17	Italy	O26:H ⁻	<i>slt-I</i>
469/87	Germany	O22:H8	<i>slt-I, slt-IIvhc</i>
284/89	Germany	O128:H ⁻	<i>slt-II</i>
2343/89	Germany	O91:H ⁻	<i>slt-I</i>
199/92	Germany	ONT:H ⁻	<i>slt-II, slt-IIvhc</i>

ONT, not O serotypable.

been described before.^{2,8,10,19-23} Eleven of the strains were identified by cytotoxicity assays with Vero cells¹⁹, and 12 strains by colony blot hybridisation with oligonucleotide probes 772 and 849²⁴ as described previously. Probe 849 has been shown to identify *slt-II* and its variant forms.²⁴

Amplification of *slt* genes

The primers and PCR conditions used to specifically amplify *slt* genes are given in table II. Bacterial DNA was prepared by incubating 10 µl of a bacterial suspension (10⁴ bacteria) for 10 min at 95°C. Amplifications were performed in a total volume of 50 µl containing 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 30 µmol of each primer, 5 µl of 10-fold concentrated polymerase

synthesis buffer and 2.5 U of *Taq*-DNA-polymerase (Amersham Laboratories, Buckinghamshire).

Southern hybridisation and digoxigenin labelling of the *slt-II* probes by PCR

Southern hybridisation was performed as described previously.²⁵ Briefly, labelled *slt-II*B probes were generated with *Taq*-DNA-polymerase by specifically primed incorporation of digoxigenin-11-dUTP during PCR. Generation of digoxigenin-labelled probes was performed in a total volume of 50 µl containing dATP, dCTP and dGTP at 200 µM, a mixture comprising 130 µM dTTP and 70 µM digoxigenin-11-deoxyuridine-triphosphate (Dig-11-dUTP), 30 µmol each of primer GK3 and GK4, 5 µl of 10-fold concentrated polymerase synthesis buffer and 2.5 U of *Taq*-DNA-polymerase.

Restriction endonuclease analysis

After PCR amplification with primers GK3/GK4, restriction endonuclease analysis of the products was performed with *Hae*III and *Fok*I to distinguish between *slt-II*B and *slt-II*B-related sequences. Briefly, 10-µl volumes of the amplified products were digested with *Hae*III and *Fok*I as recommended by the suppliers (Boehringer GmbH, Mannheim, Germany). The DNA fragments from restriction endonuclease digestion were separated and visualised as described above. Based on the published sequences of *slt-II*B and *slt-IIcB*,¹² restriction endonuclease digestion of the *slt-II*B PCR product with *Fok*I should yield two fragments of 115 bp and 155 bp, whereas the *slt-IIcB* PCR product should remain undigested. Conversely, restriction endonuclease digestion of the *slt-II*B-related amplification product with *Hae*III should yield a 128-bp and a 142-bp fragment. The small fragments (116 bp and 154 bp; 128 bp and 142 bp) were not separable in this gel system.

Nucleotide sequence analysis by *Taq* cycle sequencing

Before sequence analysis, digestion of the PCR products was performed with *Fok*I or *Hae*III, which

Table II. Designation and sequences of the primers and PCR conditions used for amplification of *slt* genes

Primer designation	Primer sequences	Genes amplified	PCR conditions		
			Denaturing	Annealing	Extension
KS7	5'-CCC GGA TCC ATG AAA AAA ACA TTA TTA ATA GC-3'	<i>slt-IB</i>	94°C, 30 s	52°C, 60 s 30 cycles	72°C, 40 s
KS8	5'-CCC GAA TTC AGC TAT TCT GAG TCA ACG-3'				
GK3	5'-CCC GGA TCC ATG AAG AAG ATG TTT ATG GCG-3'	<i>slt-II</i> B <i>slt-IIcB</i> <i>slt-IIvhaB</i> <i>slt-IIvhbB</i>	94°C, 30 s	52°C, 60 s 30 cycles	72°C, 40 s
GK4	5'-CCC GAA TTC TCA GTC ATT ATT AAA CTG CAC-3'				
FK1	5'-CCC GGA TCC AAG AAG ATG TTT ATA G-3'		94°C, 30 s	53°C, 60 s 30 cycles	72°C, 40 s
FK2	5'-CCC GAA TTC TCA GTT AAA CTT CAC C-3'				
FK5	5'-ATG AAG AAG ATA TTT GTA GCG-3'	<i>slt-II</i> OX3B <i>slt-II</i> O111B	94°C, 30 s	52°C, 60 s 30 cycles	72°C, 40 s
FK6	5'-TTA ATT AAA CTG CAC TTC AGC-3'				
FK7	5'-ATG AAG AAG ATG ATT ATT GCA-3'	<i>slt-II</i> vaB	94°C, 30 s	52°C, 60 s 30 cycles	72°C, 40 s
FK8	5'-TCA GTT AAA CTT CAC CTG GGC-3'				

selectively digested *slt-IIB* and *slt-IIB*-related genes, respectively, and the process was repeated to eliminate any small quantities of undigested *slt-II* or *slt-IIB*-related genes. Gel slices with the digested fragments were excised and the DNA was purified with the Prep-A-Gene kit as described by the manufacturer (BioRad, Munich, Germany). A total of 2 µg of the double-stranded PCR product was subjected to *Taq* cycle sequencing reactions with the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Darmstadt, Germany). Briefly, 9.5 µl of terminator premix, 2 µg of template DNA, 3.2 µmol each of primer GK3 and GK4 were mixed in a 0.6-ml reaction tube and made up with distilled water to a final volume of 20 µl. The tubes were placed in a thermal cycler preheated to 96°C and subjected to 25 cycles with the following parameters: 96°C for 15 s, 52°C for 15 s and 60°C for 4 min. The cycle sequencing products were extracted with phenol-chloroform and precipitated with ethanol. The resulting DNA pellets were dissolved in 4 ml of a mixture of formamide and 50 mM EDTA, pH 8.0, (5:1). Separation of sequencing products was performed on denaturing polyacrylamide 7% gels in an automatic sequencer (Model 373 A, Applied Biosystems). Nucleotide sequencing was performed in triplicate and analysed with the DNASIS program, version 2.0 from Hitachi Software (San Bruno, CA, USA). To overcome sequencing mistakes generated by *Taq*-DNA-polymerase, both strands of the PCR products were analysed with primers GK3 and GK4 as sequencing primers.

Cytotoxicity test and neutralisation assay

Culture filtrates were tested for cytotoxic activity on Vero cells¹⁹ and neutralisation assays²⁵ were performed as described previously.

Results

PCR strategy and restriction fragment length polymorphism for genotyping SLTs

To subdivide the 23 *E. coli* strains according to their *slt* genes, each isolate was subjected to PCR analysis with the primers listed in table II. No PCR product was obtained with the primer pairs FK1/FK2, FK5/FK6 and FK7/FK8. However, 20 of the 23 *E. coli* strains showed a positive PCR result with the *slt-IB*-specific primer pair KS7/KS8. Nine of the 23 isolates yielded a positive PCR result with primers GK3/GK4 that identify both *slt-IIB* and the related subunit genes *slt-IICB*, *vtx2haB* and *vtx2hbB*. Five of these nine isolates were of *E. coli* serotype O111:H⁻, one was *E. coli* O22:H8, one was *E. coli* O128:H⁻, one was *E. coli* O26:H11 and one strain was not typable. The PCR products of these nine isolates analysed on agarose gels yielded DNA bands of c. 270 bp.

Restriction endonuclease analysis

For further characterisation, the 270-bp DNA bands of the nine isolates were subjected to restriction endonuclease analyses with *Hae*III and *Fok*I, which selectively digest *slt-II*-related genes and *slt-II*, respectively. Fig. 1, lanes 1–6, shows representative fragment patterns resulting from digestion with *Fok*I, and lanes 7–12 with *Hae*III. It can be seen that the PCR products of four isolates were digested with *Fok*I into two smaller fragments (fig. 1, lanes 1, 3, 4 and 6). The digestion patterns of these PCR products with *Hae*III are depicted in fig. 1, lanes 7, 9, 10 and 12. The PCR fragment of only the *E. coli* O22:H8 strain 469/87 remained undigested with *Fok*I (fig. 1, lane 2), but was digested with *Hae*III (fig. 1, lane 8). Fig. 1, lanes 5 and 11, shows both digested and undigested fragment bands for the PCR products of strain 199/92

Fig. 1. Agarose gel electrophoresis of representative fragments obtained by PCR with primers GK3/GK4 which were subsequently subjected to restriction endonuclease analysis with *Fok*I (lanes 1–6) or *Hae*III (lanes 7–12). The PCR fragments analysed were from the following strains: lane 1, 7042/87; 2, 469/87; 3, 928/91; 4, 472/92; 5, 199/92; 6, 284/89; 7, 7042/87; 8, 469/87; 9, 928/91; 10, 472/92; 11, 199/92; 12, 284/89; M, DNA mol.-wt markers.

<u>strain</u>	1	50
	<u>atgaagaagatgtttatggcggttttatttgcattagcttctgtaa</u> <u>atgc</u>	
472/92	-----	
284/89	-----	
199/92 ^a	-----	
199/92 ^b	-----t-----	
469/87	-----t-----	
		100
	<u>aatggcgggcggtattgtgctaaaggtaaaattgagttttccaagtata</u> <u>atg</u>	
472/92	-----	
284/89	-----	
199/92 ^a	-----	
199/92 ^b	-----c-----	
469/87	-----c-----	
		150
	<u>aggatgacacatttacagtgaagggtgacgggaaagaatactggacc</u> <u>agt</u>	
472/92	-----	
284/89	-----	
199/92 ^a	-----	
199/92 ^b	--a--t--c--a--a--g--c-----	
469/87	--a--t--c--a--a--g--c-----	
		200
	<u>cgctggaatctgcaaccgttactgcaaagtgctcagttgacaggaat</u> <u>gac</u>	
472/92	-----	
284/89	-----	
199/92 ^a	-----	
199/92 ^b	-----c-----	
469/87	-----c-----	
		250
	<u>tgtcacaatcaaataccagttacgtgtgaatcagggtccggatttgct</u> <u>gaag</u>	
472/92	-----	
284/89	-----	
199/92 ^a	-----	
199/92 ^b	-----	
469/87	-----	
		270
	<u>tgcagtttaataatgactga</u>	
472/92	-----	
284/89	-----	
199/92 ^a	-----	
199/92 ^b	-----	
469/87	-----	

Fig. 2. Nucleotide sequences of the *slt-IIB*- and *slt-IIB*-related genes present in *E. coli* non-O157 strains. The published¹² *slt-IIB* sequence is shown in the top line and compared with that of those B-subunit genes present in O111:H⁻ strain 472/92, O128:H⁻ strain 284/89, O22:H8 strain 199/92 and O22:H8 strain 469/87. The gene sequences are shown in descending order as described. For strain 199/92, both the *slt-IIB*- and the *slt-II*-related genes were sequenced and indicated ^a and ^b respectively. The nucleotides are numbered above the sequence.

resulting from restriction endonuclease digestion with *FokI* and *HaeIII*, respectively. Overall, it was predicted from these restriction endonuclease analyses that the PCR products consisted of *slt-IIB* in seven strains, *slt-IIB*-related genes in one strain, and a mixture of *slt-II* and *slt-II*-related genes in the other.

Southern blot hybridisation

In addition to restriction endonuclease analyses, Southern hybridisation of genomic DNA from all nine strains possessing *slt-II* or *slt-II*-related genes was performed with the *slt-IIB* probe. DNA samples from the non-toxicogenic *E. coli* C600 and the *slt-I*-expressing strain *E. coli* C600 (H19J) were included as negative controls. In the positive control strain *E. coli* C600 (933W), which harbours *slt-II*, a single *EcoRI* fragment of 4.6 kb hybridised with the *slt-IIB* probe. The other positive control strain, which possesses *slt-IIC*, showed a fragment of 4.9 kb when hybridised with the *slt-IIB* probe. In *EcoRI*-digested DNA of strain 469/87, a single fragment of 3.9 kb hybridised. In contrast, seven *E. coli* strains (7042/87, 928/91, 472/92, E-D31, E-D142, E-D147 and 284/89) demonstrated a single fragment of 4.6 kb. In genomic DNA of strain 199/92, two *EcoRI* fragments of 3.9 kb and c. 7 kb hybridised with the probe. This is consistent with the presence of two genes belonging to the *slt-II* family.

Nucleotide sequence analysis by the *Taq* cycle sequencing method

To definitively subtype the B-subunit genes, the fragments resulting from PCR with primers KS7/KS8 and GK3/GK4 were subjected to nucleotide sequence analysis. Before sequence analysis, the PCR product obtained from amplification with the primer pair GK3/GK4 was digested with *FokI* and *HaeIII*, as described above. The undigested fragments consisting of pure *slt-II* or *slt-IIB*-related genes were subjected to nucleotide sequence analysis. Comparing the sequences present in the 23 *E. coli* strains, 100% homology with *slt-IB* or *slt-IIB* was revealed in all strains. Two strains possessed *slt-IIB*-related genes in addition to *slt-IB* or *slt-IIB* (fig. 1, table I). The B-subunit genes of these strains possessed sequences that differed in three nucleotide bases from *slt-IICB* but were identical to the published¹³ *slt-IIvhcB* sequence (fig. 2).

Cytotoxic activity and neutralisation assay

To determine whether the *slt* genes were expressed, and if genotyping correlated with current immunological SLT detection methods, all strains were subjected to cytotoxicity assays and the neutralisation test. When culture supernates were tested for cytotoxicity, all 23 *E. coli* isolates yielded similar cytotoxic activity (10^4 – 10^6 CD50/ml of culture supernate). However, in the two strains expressing

SLT-IIvhc, the cytotoxic activity was not neutralised by antibodies against SLT-I or SLT-II or both.

Discussion

Shiga-like toxin (SLT)-producing *E. coli* strains are increasingly recognised as human pathogens causing a wide spectrum of illness including HUS. The great importance for public health lies in the fact that HUS leads to irreversible renal failure in a number of patients, necessitating dialysis or renal transplantation with the concomitant risks. The non-O157 *E. coli* strains isolated from patients with HUS belong to several different serogroups, *E. coli* O111 being predominant among the isolates from Italy and Germany. Both *E. coli* O157 and non-O157 strains have been shown to produce one or both of the two prototype SLTs—SLT-I and SLT-II. Much attention has been addressed previously to variant forms of SLT-II which were first characterised on a molecular basis in the *E. coli* O91:H21 strain B2F1 associated with HUS.¹⁵ More recently, SLT-II variants have been described in an *E. coli* O111 strain from a patient with HUS¹⁷ and in an *E. coli* OX3 strain isolated from a case of sudden infant death syndrome.¹⁶

Recent epidemiological studies have verified the frequent presence of SLT-II-related toxins among *E. coli* O157 isolates.^{18, 26, 27} In this study, non-O157 *E. coli* isolates from patients with HUS were investigated to analyse the variability within the *slt-II* locus and to determine the prevalence of *slt-II* variants. The findings demonstrated that all strains had classical *slt-I* or *slt-II*, or both. Only two strains harboured *slt-II*-related genes and these were found together with either *slt-I* or *slt-II*. Even taking into account the small sample size, the infrequent detection of *slt-II* variants argues against the idea that these variants represent an important toxin component in non-O157 *E. coli*.

Comparison of the nucleotide sequences of the B subunit genes of the SLT-II variants with published sequences revealed 100% homology to *slt-IIvhcB*, a gene which was first described in *E. coli* O157:H7 strain 7279.¹³ The *Taq* cycle sequencing method, which takes advantage of thermostable DNA polymerase, was found to be a potentially attractive method for precise genotyping of the genes belonging to the *slt-II* family. However, if *Taq*-polymerase-mediated sequencing is performed before sequence analysis, digestion of the PCR products with *FokI* and *HaeIII* must be performed to exclude sequence errors due to the simultaneous presence of *slt-II*- and *slt-II*-related genes in the PCR products. Because of its automation and its reduced template requirement, this method can be recommended for large-scale sequencing of *slt* genes in clinical isolates.

Other isolates of the three serogroups (O91, O111, O128) analysed here have been *slt* genotyped by other investigators,^{14, 15, 17} and have yielded different results. In *E. coli* O128:B12 strain H.I.8, *slt-IIva* was identified

and shown to have the greatest divergence from *slt-II* described to date.¹⁴ The A-subunit gene exhibits 69.5%, and the B-subunit gene 78.1% nucleotide sequence homology to the corresponding genes of *slt-II*. We were unable to identify *slt-IIva* in *E. coli* O128 strain 284/89, even with the use of primers specific for *slt-IIvaB*. Interestingly, the B-subunit gene of the *E. coli* O128 isolate analysed in the present study possessed 100% homology to *slt-IIB* sequences. Paton *et al.* recently reported an *E. coli* O111 isolate from a patient with HUS that produced an SLT-II variant.¹⁷ In this study, *E. coli* O111:H⁻ was the most frequent non-O157 serotype associated with sporadic cases of HUS, being found in 12 of 23 isolates. However, *slt-II*-related genes were not detected in any of these strains. Ten of the 15 *E. coli* O111 strains had only *slt-I* sequences. In the remaining five strains, *slt-I* was present with a toxin gene that had 100% homology with the *slt-IIB* gene. Therefore, it is most likely that the toxin present in the *E. coli* O111 strains sequenced in our study was SLT-II. Additional SLT-II-related toxins with the greatest nucleotide sequence homology to *slt-II* have been shown to be present in *E. coli* O91:H21 strain B2F1.¹⁵ Two variant forms of *slt-II*,

vtx2ha and *vtx2hb*, are both present in strain B2F1. They demonstrated 95.5% and 96.6% nucleotide sequence homology for the B-subunit genes, respectively, when compared to *slt-IIB*. The *E. coli* O91 strain 2343/89 investigated in the present study possessed only the *slt-I* gene. However, the presence of an *slt-II*-related gene very similar to *vtx2ha* was verified in *E. coli* O22:H8 strain 469/87 and in the ONT strain 199/92. Both isolates were shown to harbour, in addition to *slt-IIvhc*, either *slt-I* (strain 469/87) or *slt-II* (strain 199/92).

Despite the use of advanced genotyping procedures, the prototype SLTs, and not the SLT-II variants, have been shown here to be predominant in non-O157 *E. coli* HUS isolates. However, it will be of interest to compare the prevalence of SLT-II variants in isolates from patients with diarrhoea and from asymptomatic carriers. This would enable a definitive statement to be made concerning their possible association with the pathogenesis of non-O157-associated disease.

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