BACTERIAL CHARACTERISATION

Pheno-genotyping of verotoxin 2 (VT2)-producing Escherichia coli causing haemorrhagic colitis and haemolytic uraemic syndrome by direct analysis of patients' stools

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Summary. The subtype of verotoxin 2 (VT2) found in 22 VT2-positive stool samples from severely diseased Italian and German children with haemorrhagic colitis or haemolytic uraemic syndrome, or both, and that produced by the corresponding VT-producing Escherichia coli (VTEC) strains isolated from the stools were studied by cytotoxicity sero-neutralisation assays and by polymerase chain reaction (PCR) amplification of the VT2 B-subunit gene, followed by restriction fragment length polymorphism (RFLP) analysis. The free faecal toxin was serotyped as the classical VT2 in 21 stool samples, and as the VT2 variant VT2c in one. For all but one of the VTEC isolates, the toxin phenotype was consistent with the type of VT produced in vivo and found in the corresponding stool samples. Genotyping was in agreement with phenotyping for those strains harbouring a single type of VT2 gene. Three O157:H7 isolates carrying both VT2 and VT2c genes had the VT2 phenotype, instead of the expected VT2c phenotype. Direct PCR analysis of stools detected VT genes in only 11 of 20 VT-positive stool samples suggesting that the Vero cell cytotoxicity assay is more sensitive in diagnosing VTEC infection. Immunological and genetic subtyping of VT2 performed directly on stools samples from patients with haemolytic uraemic syndrome could be a useful complementary approach to understanding the role of the different types of VT in this syndrome.

Introduction

Infection with verotoxin (VT)-producing Escherichia coli (VTEC) has been closely associated with haemorrhagic colitis (HC), which may be followed by severe systemic complications, such as haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura.1,2

Verotoxins, also known as Shiga-like toxins (SLTs), constitute a family of related cytotoxins that can be divided into two groups. One is represented by VT1, biochemically and antigenically similar to the Shiga toxin of Shigella dysenteriae 1.2-4 The other, typified by VT2 (SLT II), the toxin encoded by bacteriophage 933w,1 is not neutralised by antisera to Shiga toxin.2,3 Two other toxins antigenically related to VT2 have been characterised recently and included in the VT2 group—the VT produced by the strains pathogenic to pigs, called SLT IIv,5 or VTe,6 and the toxin produced by the human strains E32511 and B2F1, designated as VT2v6 or SLTIIC.7 According to the recent nomenclature proposal of O'Brien et al.8 the terms VT2, VT2c and VTe will be adopted in this report. The variants of VT2 are cytotoxic for Vero cells, but VTe is not active against HeLa cells, and VT2c shows reduced toxicity for this cell line when compared with the classical VT2.3-6 Serological differences between VT2 and VT2c have also been observed. Although polyclonal antiserum raised against VT2c neutralised both VT2c and VT2 at the same dilution, the neutralising titres of polyclonal or monoclonal anti-VT2 antibodies against the homologous toxin were higher than those against VT2c.9 The differences in toxicity to various cell lines and in neutralisation experiments are due to minor differences in the amino-acid sequence of the B subunit of the two toxins.6,7,9

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VT2 subtyping has been performed by both serological and genetic methods,6, 9, 10 but the occurrence of the different pheno-genotypes among VTEC of different serotype and from different sources has been poorly investigated,6, 9, 11, 12 and the respective roles of VT2 and VT2c in the pathogenesis of human disease have still to be elucidated. Moreover, conflicting data have been reported concerning the most important serotype in human disease. In Canada, Tyler et al.,4 reported that all seven E. coli O157:H7 isolates examined in their study had the VT2c genotype, whereas Hii and colleagues8 did not find the VT2c pheno-genotype in any of their 23 O157:H7 strains. However, examined in their study had the VT2c genotype, but the occurrence examined in their study had the VT2c genotype, whereas Hii and colleagues8 did not find the VT2c pheno-genotype in any of their 23 O157:H7 strains.

The purpose of this study was to determine the toxin phenotype and genotype of VT2-producing E. coli strains causing severe disease in Italian and German children. VT2-positive stool samples and the corresponding VTEC isolates were studied by seroneutralisation assays with VT2 and VT2c specific antisera and polymerase chain reaction (PCR) amplification of the VT2 B-subunit gene followed by restriction fragment length polymorphism (RFLP) analysis.6, 10, 12 The use of these typing procedures directly on stool samples provided information on the pheno-genotype of the infecting strain even in those cases in which it could not be isolated, but VT or VT-specific DNA sequences were detectable in faeces.

Materials and methods

Clinical specimens and bacterial strains

Stool specimens were from 22 sporadic and epidemiologically unrelated cases of HC or HUS, or both, enrolled in the Italian and German nationwide surveillances of HUS.13-15 They had been examined for VTEC by screening lactose-fermenting colonies by the Vero cell assay,13 or by the colony blot hybridisation technique with synthetic oligonucleotide probes complementary to VT-specific gene sequences, or both.16 The VT2 genotype of four of the O157 strains has been reported already.15 The presence of free cytotoxins had been assessed by the Vero cell assay as described previously15, 17 with neutralising antisera to VT1, VT2 (group), and Clostridium difficile cytotoxin. Patients' sera were examined by ELISA for antibodies to the lipopolysaccharides (LPS) of the three major VTEC serogroups O157, O26 and O11113, 11 and the results were confirmed by immunoblotting.15, 18 Stool specimens were kept frozen until PCR and VT2-subtyping sero-neutralisation assays had been performed.

Verotoxin typing

The cytotoxic titre of a sample was defined as the highest dilution inducing a cytopathic effect after incubation with the Vero cell monolayer for 3 days at 37°C. Neutralising antisera were prepared as described previously,13 with E. coli strains C600(933w)4 and E32511/HSC, the derivative of strain E32511, which produce only VT2c.8 VT2 subtyping was performed by sero-neutralisation of cytotoxicity to Vero cells according to the method of Hii and co-workers.8 Briefly, doubling dilutions of stool filtrates or VTEC culture supernates were mixed with constant amounts of rabbit antisera to VT2 and VT2c, diluted to have the same neutralising activity towards the homologous toxin. To evaluate the decrease in the toxin titre of the samples, antisera were diluted to obtain only a partial neutralisation, and the residual cytotoxicity for Vero cells was measured. Specimens neutralised to the same extent by both antisera were considered to contain VT2, whereas those neutralised more efficiently by VT2c antiserum were considered to contain VT2c alone or in combination with VT2. Results were confirmed for each sample by determining the ratio of the cytotoxic titre on Vero cells to that on HeLa cells;8 values > 8 were taken as evidence of the presence of VT2c in the sample. Stool filtrates also containing VT1 or C. difficile cytotoxin, or both, were treated with the corresponding neutralising antisera17 before the VT2-typing assay was performed.

VTEC genotyping

PCR was performed with the oligonucleotide primer pair GK3/GK4, which amplifies the B-subunit gene of both VT2 and VT2c. The nucleotide sequences of the GK3/GK4 primers have been described previously.10 Bacterial DNA was prepared by incubating 10 μl of bacterial suspension (108 bacteria) for 10 min at 95°C. DNA was isolated from stool specimens by the method of Brian et al.14 The stool samples were heated before DNA was purified on silica particles.19 PCR was performed as described previously.10 The amplification products were subjected to submarine gel electrophoresis on agarose 1.5% gels and visualised by staining with ethidium bromide. RFLP was performed as described by Tyler et al.6 with minor modifications.10 Briefly, 15-μl portions of the amplified products obtained by PCR were subjected to restriction endonuclease digestion with RsaI (Pharmacia LKB, Uppsala, Sweden), as recommended by the supplier. The DNA fragments obtained were separated and visualised as described above.

Results

Determination of VT type by sero-neutralisation assays

Twenty-two stool samples showing a cytotoxic activity completely or partially neutralised by VT2c antiserum and available in a sufficient amount to
perform the typing assays were included in the study. Four samples also contained VT1, and three contained the cytoxin of *C. difficile*, but these toxic activities were neutralised with specific antisera before the VT2 subtyping tests. The neutralisation assays performed on free faecal toxins (table) showed that in all except one of the stool samples the cytotoxicity was neutralised to the same extent by both antisera, indicating the presence of classical VT2. Only one stool specimen, from a German patient, was positive for VT2c. This was the only sample that consistently showed reduced toxicity towards HeLa cells when compared with Vero cells (data not shown). Because no antiserum was available that neutralised VT2c without neutralising VT2, the possibility that VT2 was also present in that specimen could not be excluded. However, the reduced toxicity for HeLa cells seemed to exclude this possibility.

VTEC strains were isolated from 14 of the 22 stool samples and their characteristics are shown in the table. Nine strains belonged to serotype O157:H7, one to O157:H+, three to O111:H− and one to O120:H19. Analysis of patients’ sera for serogroup-specific anti-LPS antibodies (table) showed evidence of infection with *E. coli* O157 in four additional cases, and with *E. coli* O111 in one. Antibodies to *E. coli* O157 LPS were found also in the patient whose stool yielded *E. coli* O120. Seroto-neutralisation assays showed that 13 of the 14 isolates produced a toxin belonging to the VT2 group, and for 12 of them the subtyping test showed that it was the classical VT2. Only the O157:H7 strain from patient HUS/3658 produced VT2c. There was good agreement between the type of VT2 produced by each VTEC isolate *in vitro* and that of the toxin produced *in vivo* and found in the corresponding stool sample. The sole discrepancy was observed for patient HUS/58 whose stool contained both VT1 and VT2, whereas the derived *E. coli* O111:H− strain produced VT1 only.

### Table. Toxin phenotype and genotype of VTEC associated with HC or HUS, or both, in Italian and German children, as determined by the analysis of *E. coli* strains and stool samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stool sample*</th>
<th>VTEC isolate</th>
<th>Serum antibodies to</th>
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<tbody>
<tr>
<td></td>
<td>Phenotype</td>
<td>Genotype</td>
<td>Serotype</td>
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<td>O111:H−</td>
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*Typing performed directly on stool samples.
I. sample from an Italian patient; G. sample from a German patient; ND, not determined.

**PCR amplification and RFLP analysis of VT genes**

Restriction endonuclease analysis was performed on the PCR products from all the strains after amplification of the VT2 B-subunit gene with the primers GK3/GK4. The 288-bp PCR products from representative strains producing VT2, VT2c or both toxins are shown in the figure (panel a). Panel b shows the same products after digestion with *RsaI*. As expected, the enzyme produced a 216-bp fragment from the VT2 B-subunit gene (lane 1), and a smaller 148-bp fragment from the VT2c gene (lanes 3-5). Both fragments were observed when the strain examined carried both VT2 and VT2c genes (panel b, lane 7). The results from PCR-RFLP analyses are presented in the table. DNA sequences specific for VT2 only were found in nine strains, and for VT2c only in one. Three *E. coli* O157:H7 strains had both VT2 and VT2c genotypes. Genotyping was in agreement with phenotyping for those strains harbouring a single type of VT2 gene. Surprisingly, the three O157:H7 isolates carrying both VT2 and VT2c genotypes had the VT2 phenotype, instead of the expected VT2c phenotype. When the same PCR-RFLP procedure was applied directly to 20 of the stool specimens (table), VT2 or VT2c-specific DNA sequences, or both, were identified.
in 11 samples, three of which had not yielded a VTEC isolate. Conversely, PCR was negative in four samples positive for VTEC isolation. When both the stool sample and the VTEC isolate from the same patient were available for PCR analysis, the same toxin genotype was observed.

**Discussion**

In this report, VT2-positive stool samples from Italian and German patients with HC or HUS, or both, together with the corresponding VTEC isolates, were studied by VT-sero-neutralisation assays and...
PCR-RFLP analysis of VT genes. Both typing procedures sought to define the subtype of VT2 produced by the infecting *E. coli* strain.

For 21 of the 22 patients, the toxin produced *in vivo* and detected in the stools was serotyped as the classical VT2; VT2c was found in only one sample. For 13 of the 14 VTEC strains isolated from the stools, the toxin produced *in vitro* corresponded to that found free in the stools. A good correlation was found also between the results of neutralisation assays and PCR-RFLP analysis for those strains carrying only one type of VT2 gene. Conversely, a discrepancy was observed in the results of neutralisation assays and PCR-RFLP analysis for those strains carrying only one type of VT2c genotype. According to Hii *et al.*, isolates with such a genotype should exhibit the VT2c phenotype, but the VT produced *in vitro* and that found in the corresponding stool sample were both typed as VT2. A similar finding was reported by Hii and co-workers, who described an O157 isolate that produced VT2 but showed both VT2 and VT2c genes. This was taken as a false positive result of the VT2c-specific gene probe (AB157) used in that work. In light of the present observation of three O157 strains with a similar behaviour, the possibility that some isolates may possess the VT2c B-subunit gene but be unable to produce active cytotoxin, should not be ruled out.

The hypothesis that the toxin genotype of the infecting *E. coli* O157:H7 strain may influence the risk of developing micro-angiopathic sequelae was first proposed by Ostroff *et al.*, who found that strains with the SLTII (group) gene alone were more likely to cause HUS than other genotypes (SLTI and SLTI-SLTII). This strengthens the importance of subclassifying the VT2 produced by O157:H7 strains, that was not performed in their study.

The present VT2-phenotyping results seem to confirm those of Hii and co-workers regarding production of the classical VT2 by *E. coli* isolates of serogroup O157. Indeed, all but one of the 10 O157 strains examined in this study produced VT2, and the same type of toxin was found in the stool from four other cases with serological evidence of infection by *E. coli* belonging to that serogroup. On the other hand, VT2c-specific DNA sequences were present in four of the O157 strains, confirming that VT2c genes are frequent among isolates belonging to this serogroup. Further studies considering both genotype and phenotype are needed to define the prevalence and expression of the VT2 group genes among VTEC.

Recently, direct PCR amplification of VT genes in the stools of patients with HUS has been proposed for rapid diagnosis of VTEC infection. In our hands, PCR gave positive results in only 11 of 20 VT-positive stool samples. This discrepancy could be due either to the presence of inhibitors in the stools or to the long storage at −20°C and to the thawings to which the specimens were subjected. However, these procedures did not hamper the phenotypic detection of active VT. This suggests that the Vero cell cytotoxicity assay is still more sensitive than PCR in diagnosing VTEC infection, at least for stool samples kept frozen.

The combined use of different diagnostic techniques indicated that a mixed infection with different VTEC strains could have occurred in at least two of our patients. For HUS/58, the *E. coli* O111 isolate produced only VT1, but the patient's stool contained both VT1 and VT2. For HUS/68, the patient had *E. coli* O120 in the stool, but developed antibodies against the LPS of serogroup O157. Concomitant infections with VTEC belonging to different serogroups in HUS patients have been reported in other studies and it has been suggested that the isolation of a non-O157 VTEC may be a marker for exposure to *E. coli* O157:H7 acquired from the same food.

This study represents the first attempt at immunological and genetic subtyping of VT2 performed directly on stool samples. Because verotoxins are still detectable in the faeces of many HUS patients when the infecting VTEC can no longer be isolated, this could be a useful complementary approach to the understanding of the role played by the different types of VT in this syndrome.

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References


