Vero cytotoxin-producing strains of *Escherichia coli* from children with haemolytic uraemic syndrome and their detection by specific DNA probes

S. M. SCOTLAND, B. ROWE*, H. R. SMITH, G. A. WILLSHAW and R. J. GROSS

Division of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

**Summary.** Faecal specimens from 66 children with haemolytic uraemic syndrome in the United Kingdom were examined for strains of *Escherichia coli* producing Vero cytotoxin (VT). Initially, conventional bacteriological methods were used to identify colonies of *E. coli* which were then tested for VT production. Subsequently, specific DNA probes for VT1 and VT2 were used in hybridisation tests to detect VT-producing *E. coli* (VTEC). VTEC strains were isolated from 19 cases and in 15 they belonged to serogroup 0157. Fourteen of these O157 strains possessed the flagellar antigen H7 and one was non-motile. The VTEC strains from the remaining four cases belonged to serotypes 026:H11, 0104:H2, 0153:H25, and 0163:H19 together with a rough VT+ strain with flagellar antigen H51. The O157 strains hybridised with either the VT2 probe or both VT1 and VT2 probes. The other VTEC strains hybridised with either the VT1 or VT2 probe. Confirmation of the production of VT1 and VT2 in *vivo* was obtained by the neutralisation of faecal VT with specific antisera raised against these two cytotoxins.

**Introduction**

Haemolytic uraemic syndrome (HUS) comprises micro-angiopathic haemolytic anaemia, thrombocytopenia and acute renal failure (von Gasser *et al.*, 1955). HUS occurs in all age groups but most commonly in infants and young children in whom it is recognised as an important cause of renal failure (Levin and Barratt, 1984). The disease may present in several forms (Levin and Barratt, 1984; Drummond, 1985). A well-recognised presentation with prodromal bloody diarrhoea occurs in the summer months in young children and infants. Intra-familial spread and geographically related clusters suggest an infective aetiology. Various bacteria, viruses and rickettsiae have been implicated but the relationship of these organisms with the disease is uncertain. Infection with *Shigella dysenteriae* type 1 can lead to HUS (Koster *et al.*, 1978) and, during an outbreak of dysentery in Bangladesh, HUS was a frequent complication (Rahaman *et al.*, 1975).

Certain strains of *Escherichia coli* produce a cytotoxin (VT) active on Vero cells that is distinct from the heat-labile enterotoxin (LT) and the heat-stable enterotoxin (ST) (Konowalchuk *et al.*, 1977).

Neutralisation studies indicate that there is more than one VT (Konowalchuk *et al.*, 1977, 1978b; Smith *et al.*, 1983; Scotland *et al.*, 1985; Karmali *et al.*, 1986). One VT is neutralised by a rabbit antiserum prepared against Shiga toxin (O’Brien *et al.*, 1982) and we proposed that this be termed VT1 and the VT produced by strain E32511, of serogroup O157, be termed VT2 (Scotland *et al.*, 1985). Some strains of serogroup O157, including strain E32511, produced only VT2, whereas others produced VT1 and VT2 (Scotland *et al.*, 1985). Production of VT has been shown to be phage-mediated (Scotland *et al.*, 1983; Smith *et al.*, 1983; O’Brien *et al.*, 1984; Smith *et al.*, 1984) and specific VT probes have been developed for use in DNA hybridisation tests (Newland *et al.*, 1985; Willshaw *et al.*, 1985, 1987; Huang *et al.*, 1986). Under stringent conditions, no cross hybridisation was detected between VT1 and VT2 probes developed from appropriate VT-producing strains (Willshaw *et al.*, 1987).

In 1983, Karmali *et al.* reported the isolation of VT-producing (VT+) strains of *E. coli* from cases of HUS in Canada. Also in 1983, an outbreak of HUS occurred in the West Midland area of England. There was no evidence of infection by salmonellae shigellae, campylobacters or viruses (Gully, 1984); however, studies in this laboratory showed that VT+ strains of *E. coli* O157:H7 were isolated from faeces in several cases (Taylor *et al.*, 1983, 1985).
A prospective study was subsequently carried out to investigate the possible association between VTEC infection and HUS in the United Kingdom and the bacteriological findings are reported here.

Materials and methods

Subjects

From August 1983 to May 1985, 66 patients, aged between 3 weeks and 11 years, with HUS were admitted to the study. The patients were from many areas of the United Kingdom; five were children from the West Midlands outbreak of 1983 (Gully, 1984; Taylor et al., 1986).

Identification of VTEC in faeces

In phase 1 (August 1983 to December 1983), specimens from 23 patients were studied by culture on MacConkey Agar (Oxoid; CM115). Representative lactose-fermenting and non-fermenting colonies were selected for further examination and were identified biochemically (Cowan, 1974). Up to 40 colonies identified as E. coli were serotyped (Gross and Rowe, 1985) and screened for the production of VT; any production of VT was confirmed by tests with sterile culture supernates as described below.

In phase 2 (January 1984 to November 1984), 32 patients were studied. Up to 40 colonies of E. coli were tested for VT production, but only those which proved to be VT' were serotyped. In addition, the faecal specimens were plated out to give colonies which were examined with DNA probes specific for VT genes.

In phase 3 (December 1984 to May 1985), 11 faecal specimens were examined for the presence of VTEC with DNA probes only. Probe-positive colonies were confirmed as E. coli by biochemical tests and their serotype was determined; sterile culture supernates were also tested for VT production.

The strains of E. coli isolated in phase 1 were tested retrospectively with the two DNA probes for VT1 and VT2.

Tests for resistance to antibacterial drugs

Strains were tested by the method of Anderson and Threlfall (1974) for resistance to amikacin, ampicillin, ceftazidime, cephaloridine, cephalaxin, chloramphenicol, colistin, furazolidone, gentamicin, kanamycin, mexitilin, nalidixic acid, netilmicin, spectinomycin, streptomycin, sulphasoxazole, tetracycline, tobramycin and trimethoprim.

Testing for free cytotoxin in faeces

In all three phases, the presence of free cytotoxin in the faecal specimens was investigated as described by Karmali et al. (1983). Dilutions in tissue-culture medium (see below) of a faecal filtrate were tested for cytotoxic activity on Vero cells and Y1 mouse adrenal tumour cells growing as monolayers in 96-well tissue culture microtitration plates. Cytotoxic activity was expressed as the highest dilution which had a detectable cytotoxic effect on a monolayer of cells after incubation for 4 days at 37°C. The cytotoxic effect was considered to be specific for Vero cells, that is VT, if it had no effect on Y1 cells. Vero cells were grown on E199 medium (Flow Laboratories) supplemented with fetal calf serum 10%. Y1 cells were grown on F10 medium (Flow Laboratories) supplemented with horse serum 12.5% and fetal calf serum 2.5%.

Testing bacterial cultures for VT and other enterotoxins

Large numbers of colonies were screened for VT production by a rapid method (Willshaw et al., 1985). To confirm VT production, sterile culture supernates of bacteria grown in Trypticase Soy Broth (BBL) were tested as described previously (Scotland et al., 1980). All VT+ strains, and, in phase 1, a colony representative of each serotype isolated from a patient, were tested for the production of ST and LT (Gross et al., 1976). All VT+ strains were also tested for invasiveness by a tissue-culture method (Day et al., 1981).

Neutralisation of faecal cytotoxin

An antiserum to Shiga toxin to neutralise VT1 and an antiserum to VT2, produced by strain E32511 (E. coli O157:H–) were used to test for neutralisation of faecal cytotoxins. The antiserum to purified Shiga toxin was prepared in rabbits and was received from Dr A. D. O’Brien. The antiserum raised against partially purified VT2 (Scotland et al., 1985) was prepared in rabbits according to the method of Konowalchuk et al. (1978a). Dilutions of the faecal cytotoxin (25 µl) were added to 0·2 ml of the Vero-cell tissue-culture medium alone or with 20 units of each antiserum separately or with 20 units of both antisera together. After 3 h at 37°C and 18 h at 4°C these preparations were added to a monolayer of Vero cells in microtitration plate wells. After incubation for 4 days at 37°C the monolayers were examined to determine whether the cytotoxic titre was decreased by the presence of one or both antisera. A unit of antibody was defined as the amount present in the highest dilution of antiserum which neutralised 1–5 units of VT. A unit of VT activity was defined as the amount present in the highest dilution of a toxin preparation which caused any cytotoxic effect in the otherwise completely confluent monolayer of Vero cells.

Preparation of faecal specimens for DNA hybridisation

Faecal specimens were re-suspended in an equal volume of PBS and diluted to 10^{-3}-fold. Samples (0·1 ml)
of each dilution were spread on MacConkey agar and the plates were incubated overnight at 37°C. For each sample a plate with several hundred well-separated colonies was replicated on to nitrocellulose (Schleicher and Schuell BA85) or nylon (Amersham Hybond-N) membranes placed on the surface of MacConkey agar plates. The master plates and the replicas were incubated at 37°C for at least 6 h. The membranes were processed for hybridisation by placing on a series of 3MM papers saturated with the following solutions: SDS 10%, lysis solution containing 0.5 M NaOH and 1.5 M NaCl (5–10 min), neutralising solution containing 1.5 M NaCl and 0.5 M Tris-HCl, pH 8.0 (5 min), and finally 2 × SSPE (5 min). SSPE contained 0.3 M NaCl, 17.5 mM NaH₂PO₄ and 2–2 mM EDTA. The membranes were left to dry at room temperature. Nitrocellulose membranes were baked for 2 h at 80°C and nylon membranes were wrapped in cling film and placed on a UV transilluminator (wavelength 365 nm) for 4 min.

**Preparation of DNA probes**

A HincII fragment of 0.75 kb was used as a probe specific for VT1 sequences. It was obtained by cloning from a VT-encoding phage carried by strain H19, *E. coli* O26:H11 (Willshaw et al., 1985). The colonies from faecal platings were tested for VT2-specific sequences by hybridisation with a 2 kb HincII fragment that was identified in cloning experiments from a phage carried by strain E32511, *E. coli* O157:H-- (Smith et al., 1984; Willshaw et al., 1985). VTEC that hybridised with the 2 kb HincII probe were tested again with a more specific VT2 probe comprising a 0.85 kb AvaI-PstI fragment developed from the VT phage in E32511 (Willshaw et al., 1987). Fragments for use as probes were prepared by electro-elution from agarose or acrylamide-agarose gels, purified with Elutip minicolumns (Schleicher and Schull) and labelled by nick translation with deoxyadenosine 5'-α-[³⁵S]thiotriphosphate (Amersham).

**Hybridisation experiments**

Hybridisation with ³⁵S-labelled VT probes was performed under stringent conditions and filters were prepared for autoradiography as described previously (Willshaw et al., 1985). The autoradiographs were examined together with the original plates, and colonies that hybridised with the VT probe or probes were identified. These were tested for VT production and identified biochemically and serologically. Up to ten probe-positive colonies were tested from each faecal sample. On some plates it was difficult to identify an individual probe-positive colony, so a number were picked and re-tested by hybridisation until a separate probe-positive colony was obtained.

**Results**

**Identification of VTEC or specific cytotoxin (VT) in faecal specimens**

Twenty-three patients were studied in phase 1 and VTEC strains were isolated from seven (table I), including two from the West Midlands outbreak (patients 2 and 3). Only VT- strains of *E. coli* were isolated from 12 patients and *E. coli* could not be isolated from the remaining four. Thirty-two patients were studied in phase 2 and VTEC strains were isolated from nine (table II). Only VT- strains of *E. coli* were isolated from 20 patients and *E. coli* could not be isolated from the remaining three. In this second phase, two methods were used for the detection of VTEC and in all but two cases they agreed; for patients 28 and 40, VTEC were detected only with the specific DNA probes which hybridised respectively with two of 270 and one of 1200 colonies growing on MacConkey-agar plates. Eleven patients were studied in phase 3; VTEC

**Table I.** Examination of faecal specimens in phase 1

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>Geographical location</th>
<th>VT* E. coli isolated by picking colonies (%)</th>
<th>Titre of VT in faecal specimens</th>
<th>Serotype of VT* E. coli</th>
<th>Results of VT* E. coli with DNA probe* for</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>F</td>
<td>15 mo</td>
<td>Wolverhampton</td>
<td>8</td>
<td>NA</td>
<td>O157:H7</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>17 mo</td>
<td>Wolverhampton</td>
<td>100</td>
<td>NA</td>
<td>O157:H7</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>16 mo</td>
<td>Salisbury</td>
<td>22</td>
<td>NA</td>
<td>O157:H7</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>6 yr</td>
<td>Peterborough</td>
<td>95</td>
<td>40</td>
<td>O157:H7</td>
<td>–</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>4 mo</td>
<td>Southampton</td>
<td>80</td>
<td>5120</td>
<td>O163:H19 and</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>5 mo</td>
<td>Belfast</td>
<td>85</td>
<td>640</td>
<td>O157:H25</td>
<td>–</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>16 mo</td>
<td>Birmingham</td>
<td>18</td>
<td>40</td>
<td>O157:H-</td>
<td>–</td>
</tr>
</tbody>
</table>

* VT1, 0.75 kb HincII fragment, VT2, 0.85 kb AvaI-PstI fragment (see Methods).
NA = Not available for test.
strains were isolated from three (table III) and  VT−
E. coli from eight.

Fourteen of the 19 faecal specimens from which
VTEC strains were isolated were also tested for the
presence of free faecal VT (tables I–III). In ten
specimens, VT was detected, at titres in the range
20–780 000. In four, VT was not found but a non-
specific cytotoxin was present in three which may
have masked low levels of VT. Forty two faecal
specimens from which VTEC had not been isolated
were also tested for free faecal VT. VT was detected
in only three at titres of 80, 100 and 500. A non-
specific cytotoxin was observed at a low titre of 20
in another seven faecal specimens but it was not
investigated further.

Table IV summarises the results from all three
phases in which a total of 66 patients was studied.
VTEC strains were isolated from 19 patients and
free faecal VT was detected in 13. By combining
the results of both tests, evidence of infection by
VTEC was obtained for 22 of the 66 patients (33%).

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laboratories and these pathogens were reported to be absent except for patient 41 whose faeces yielded a *Salmonella* sp.

**Properties of *E. coli* isolated from faecal specimens**

VTEC strains were isolated from 19 of the 66 patients with HUS and in 15 cases these belonged to serogroup O157; 14 possessed the flagellar antigen H7 and one strain (E32511) was non-motile. The *E. coli* O157 strains were of two groups with respect to hybridisation with the DNA probes (tables I–III). Ten strains (including E32511 from which the probe was developed) hybridised only with the VT2 probe, and five strains hybridised with both VT1 and VT2 probes. The two O157 strains isolated from the West Midlands outbreak belonged to the class hybridising only with the VT2 probe.

From the faeces of patient 19, VTEC of two serotypes, O163:H19 and O rough:H51, were isolated; both hybridised with the VT2 probe only (table I). The remaining three patients from whom VTEC strains were isolated yielded strains of *E. coli* of serotypes O26:H11, O104:H2 and O153:H25, respectively; the first of these hybridised with the VT1 probe only and the second and third hybridised with the VT2 probe only (tables I and II).

The VTEC strains isolated in this study did not produce ST or LT, and they gave negative results in the test for invasiveness, in agreement with previous reports (Johnson *et al.*, 1983; Riley *et al.*, 1983; Wells *et al.*, 1983; Karmali *et al.*, 1985). The VT+ strains were lactose-fermenting with the exception of the strain of serotype O163:H19. The VT+ strains of serogroup O157 failed to ferment sorbitol after overnight incubation, which is in agreement with previous reports (Johnson *et al.*, 1983; Wells *et al.*, 1983); the VT+ strains of other serogroups did ferment sorbitol. The VT+ strains of serogroup O157 were sensitive to all the antimicrobial agents tested. The rough VT+ strain of H-type 51 was resistant to streptomycin, sulphanthiazole and tetracycline; the VT+ O153:H25 strain was resistant to ampicillin, streptomycin and sulphanthiazole. The remaining three VT+ strains were fully sensitive.

In phase 1, all the colonies of *E. coli* tested from 12 specimens were VT− in the tissue-culture test. These strains belonged to 27 different serogroups and none produced ST or LT. They included three strains belonging to the enteropathogenic (EPEC) serogroups O18ac, O86 and O128. None of these strains hybridised with either of the DNA probes for VT. However in phase 2 colonies from the faeces of patient 24 that hybridised with the VT1 probe failed to produce VT in the Vero-cell test (table II).

The 2 kb probe for VT2 was used in hybridisation tests with colonies on primary plating. In addition to the VT2 sequences this probe contains flanking phage DNA that is shared by some VT1-encoding phages (Willshaw *et al.*, 1987). Subsequently the more specific 0.85 kb *Ava*I-*Pst*I probe for VT2 became available and the VT+ strains were retested. Twenty strains had hybridised with the 2 kb probe and 19 of these hybridised with the 0.85 kb probe also. The strain that failed to hybridise with the 0.85 kb probe was the O26:H11 strain from patient 45. This strain therefore hybridised with the VT1 probe only (table II).

**Examination of successive faecal specimens**

For four patients more than one faecal specimen was available for study. Patient 11 suffered from diarrhoea and the first specimen was taken 3 days after the onset of diarrhoea. This specimen (shown in table I) contained free VT at a titre of 40, and 38 of the 40 *E. coli* colonies tested were VT+. In two subsequent specimens taken on days 6 and 7, VTEC colonies were not found (0 out of 40 colonies for each specimen) and VT was not detected. Patient 23 had also been admitted to hospital with bloody diarrhoea and 7 of 40 colonies from the first faecal specimen were VTEC and VT was present at a titre of 40 (shown in table I). However, all of 30 colonies were VT− from a second specimen taken one day later, although VT at a titre of 40 was still present. Patient 41 had also suffered from bloody diarrhoea and the first specimen (shown in table II) had yielded VTEC (3 of 17 colonies) and VT at a titre of 780 000. Three other specimens from patient 41 were studied, taken 1, 2 and 9 days after the first. VTEC strains were not found in any of these either by picking colonies (none of 40, 25 and 35 colonies respectively) or by testing with the probes. It was also observed that the VT titre had decreased to 20, 20 and 0 respectively. Five of 40 colonies tested from the first faecal specimen from patient 45 were VT+ and VT was detected at a titre of 300 000 (table II); in a second specimen taken two days later, VTEC colonies were not detected by probes or by direct picking but VT was still present at the high titre of 300 000.

**Neutralisation of faecal cytotoxin**

Faecal filtrates which were positive for VT were
stored at $-10^\circ$C. When specific antisera to Shiga toxin (VT1) and VT2 became available these filtrates were tested but only four, from patients 20, 41, 45 and 48, were still cytotoxic. The activity of filtrates from patients 20 and 41 was neutralised by anti-VT2 serum but not by serum against Shiga toxin; the faecal specimens from these patients had yielded VTEC, of serogroups O153 and O157 respectively, hybridising only with the VT2 probe. The activity of the filtrate from patient 45 was neutralised by the serum against Shiga toxin but not by the anti-VT2 serum; the faecal specimen of this patient had yielded a strain of O26 hybridising only with the VT1 probe. The activity of the filtrate from patient 48 was not neutralised by either antiserum alone but was neutralised when both antisera were used together; a strain of O157 hybridising with both VT1 and VT2 probes had been isolated from this faecal specimen.

Discussion

Karmali et al. (1983) first reported an association between HUS and infection with VT+ strains of E. coli. In a confirmatory study (Karmali et al., 1985) of 40 children who had developed HUS between September 1980 and September 1983, they isolated VTEC from 12 faecal specimens and free faecal VT without VTEC in a further 12 specimens. The VT+ strains belonged to seven serotypes—O26:H11, O111:H8, O111:H−, O113:H21, O121:H19, O145:H− and O157:H7—and one strain was rough and non-motile. We have now shown a significant association between VTEC infection and HUS in many areas of the United Kingdom. VT+ strains were isolated from the faeces of 19 of the 66 children studied and free faecal VT was detected in another three in the absence of VTEC. VTEC strains of several serotypes were isolated—O26:H11, O104:H2, O153:H25, O157:H7, O157:H−, O163:H19 and O rough:H51—but with the exception of O157:H7 and O26:H11, they were not the same as those identified in the Canadian study (Karmali et al., 1985). E. coli O157:H7 was the most common serotype, being found in 14 cases.

Strains of E. coli O157 producing either VT2 only or producing both VT1 and VT2 were detected previously (Scotland et al., 1985); additional strains belonging to these groups were identified in the present study. Four strains belonging to serogroups other than O157 hybridised with the VT2 probe and one with the VT1 probe.

One strain hybridised with the VT1 probe but did not produce VT (table II). However, when this strain was tested with another VT1 probe comprising a different restriction fragment of the cloned VT1 sequences from H19 (Willshaw et al., 1987), no homology was detected. This suggests that the strain had incomplete VT sequences. For all other strains there was complete agreement between the two tests used for VT and VT production was detected reliably in culture supernates without the use of a special iron-depleted medium or sonication as used in some studies (O’Brien et al., 1982; Cleary et al., 1985).

Several methods have been proposed for improving the detection of VTEC in faeces. Our results show that if a preliminary screening is based on sorbitol agar (Farmer and Davis, 1985), VT+ bacteria belonging to serogroups other than O157 would be missed. By use of the specific DNA probes it was possible to detect colonies of VTEC that were present in numbers as small as one in 1200 colonies. The sensitivity of the method probably accounts for the detection of VT+ bacteria in the absence of free VT, in contrast to the findings of Karmali et al. (1985). However since free VT was sometimes found in the absence of VT+ bacteria it seems preferable to use both techniques when possible.

No direct relationship was found between the titre of VT in faeces and the proportion of VT+ colonies present. Possible explanations for this include problems arising from the handling or storing of the samples before they are examined. Importantly the examination of more than one specimen from some patients showed that significant changes in the proportion of VT+ colonies occurred within days. This was also noted in cases of haemorrhagic colitis by Riley et al. (1983), and the observation needs further investigation.

Neutralisation tests with specific antisera showed that VT1 or VT2 could be present alone or together in the faeces. The toxins found correlated with the probe results on the VTEC isolated from each specimen. This is the first report which specifically identifies these two particular Vero cytotoxins in vivo.

This study has confirmed an association between some cases of HUS in infants and young children and infection with VT+ E. coli. A prospective case control study is now under way in order to identify specific risk factors and to elucidate the epidemiology of the disease.

We thank colleagues who sent specimens for this study.
REFERENCES


