Vero Cytotoxin Production and Presence of VT Genes in *Escherichia coli* Strains of Animal Origin

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Vero cytotoxin (VT) production has been studied in 34 *Escherichia coli* strains isolated from animals with enteric diseases. The strains were tested by DNA hybridization with probes for VT1 and VT2 sequences and also in toxin neutralization experiments with specific antisera. Twenty bovine strains, belonging to nine different O serogroups, produced VT1 or VT2 but not both toxins. In contrast, all 14 porcine strains of four different O serogroups produced VT2 only. Six of these porcine strains, belonging to serogroups O138, O139 and O141, were isolated from cases of oedema disease. In general, the porcine isolates produced toxin at a lower level than the bovine strains.

INTRODUCTION

Production of a cytotoxin active on Vero cells, termed Vero cytotoxin (VT), has been detected in strains of *Escherichia coli* from cases of human and animal disease (Konowalchuk *et al.*, 1977). The Vero-cytotoxin-producing *E. coli* (VTEC) of human origin belong to a number of different O serogroups, particularly O157. The VT⁺ O157 strains have been associated with cases of diarrhoea, haemolytic uraemic syndrome (HUS) and haemorrhagic colitis (HC) in Canada, USA and Britain (Karmali *et al.*, 1983; Riley *et al.*, 1983; Scotland *et al.*, 1988; Smith *et al.*, 1987). VTEC have also been isolated from calves and pigs with enteric diseases. The strains of bovine origin belong to a large number of O serogroups and have been isolated from cases of diarrhoea and also from healthy animals (Sherwood *et al.*, 1985; Mohammad *et al.*, 1985, 1986). VT⁺ strains of serogroups O138, O139 and O141 have been associated with post-weaning diarrhoea and oedema disease in pigs (Kashiwazaki *et al.*, 1980, 1981; Dobrescu, 1983; Smith *et al.*, 1983; Blanco *et al.*, 1983; Gonzalez & Blanco, 1985).

Attempts to neutralize VT with specific antisera have shown that there is more than one VT (Konowalchuk *et al.*, 1977; Smith *et al.*, 1983; Scotland *et al.*, 1985; Karmali *et al.*, 1986). VT produced by strains of human origin such as H19 and H30, of serotype O26.H11, could be neutralized by a rabbit antiserum prepared against purified Shiga toxin (O'Brien *et al.*, 1982); this toxin has been termed VT1 (Scotland *et al.*, 1985). A second VT, designated VT2, was first shown in strains of serogroup O157 isolated from cases of HUS and HC (Scotland *et al.*, 1985). Some strains of serogroup O157 produced both VT1 and VT2 while others produced either VT1 or VT2 (Scotland *et al.*, 1985, 1988; Smith *et al.*, 1987). The terms Shiga-like toxins I and II (SLTI and SLTII) have also been used to describe the toxins VT1 and VT2 respectively (Strockbine *et al.*, 1986). A number of strains isolated from calves were reported to produce a cytotoxin neutralized by an antiserum to Shiga toxin (Sherwood *et al.*, 1985). However, a

Abbreviations: VT, Vero cytotoxin; VTEC, Vero-cytotoxin-producing *Escherichia coli*; SLT, Shiga-like toxin; ST_A, heat-stable enterotoxin; LT, heat-labile enterotoxin; HUS, haemolytic uraemic syndrome; HC, haemorrhagic colitis; DEP, Division of Enteric Pathogens; CVL, Central Veterinary Laboratory.

subsequent study failed to confirm the neutralization by anti-Shiga toxin of the VT produced by some of these bovine strains (Marques *et al.*, 1986) and showed that the VT produced by strains belonging to serogroups O4, O8 and O149 was neutralized by antiserum to SLTII (= VT2). VT produced by strains of porcine origin that belonged to serogroups O138, O139 and O141 could not be neutralized by antisera against VT of O26 strains of human origin which produce VT1 (Konowalchuk *et al.*, 1977; Smith *et al.*, 1983). However, VT produced by these porcine strains was not tested with an antiserum raised against VT2 as defined with the O157 strains of human origin.

The genes controlling VT production are phage-encoded in some strains (Scotland *et al.*, 1983; Smith *et al.*, 1983; O'Brien *et al.*, 1984; Smith *et al.*, 1984), and VT sequences have been cloned in *E. coli* K12 from phage DNA originating in strains producing VT1 or VT2 (Newland *et al.*, 1985; Willshaw *et al.*, 1985, 1987; Huang *et al.*, 1986). Mapping of the cloned VT sequences led to the identification of specific DNA probes for VT1 and VT2 (Willshaw *et al.*, 1985, 1987).

In this paper we have examined the types of VT produced by strains of bovine and porcine origin. VT production was detected using culture supernatants on Vero cells and neutralization experiments were done with rabbit antisera against VT1 and VT2. The strains were tested by colony hybridization with the DNA probes for VT1 and VT2. The sources of the VT1 and VT2 toxins and toxin genes, for the production of antisera and development of DNA probes, were strains of human origin.

METHODS

Bacterial strains. Twenty VTEC of bovine origin and 14 VT⁺ strains of *E. coli* isolated from pigs were examined (Table 1). The organisms were serotyped by the methods of Gross & Rowe (1985). The bovine strains were from calves with diarrhoea or dysentery: strains E40867, E40868 and E40870 to E40879 were reported as VT⁺ by Sherwood *et al.* (1985) and strain S102-9 was described by Hall *et al.* (1985). The remaining bovine strains were from the Division of Enteric Pathogens (DEP) collection. E26817 and E26818 were isolated in Thailand, E37445, E37446 and E37450 were received from Sri Lanka and E7688 and E10394 were isolated in Britain. Five porcine strains, isolated from cases of oedema disease, were from the Central Veterinary Laboratory (CVL) collection: E57, E68II, E145 and G1108E (Sojka, 1965) and P3 (Smith *et al.*, 1983). The remaining strains were received by the CVL between 1981 and 1984 and were from pigs or piglets with diarrhoea with the exception of E40888, serotype O139.H1, which was isolated from a fatal case of bowel oedema. Two strains of human origin were included as controls: strain H19, serotype O26.H11 (VT1) and strain E32511, serotype O157.H⁻ (VT2) (Scotland *et al.*, 1985).

Tests for VT production and neutralization experiments. The method for detecting VT in culture supernatants has been described previously (Scotland *et al.*, 1980). Bacterial cultures were grown with shaking in 10 ml trypticase soy broth at 37 °C for 18 h. After centrifugation and filtration, sterile supernatants were added to monolayers of Vero cells grown in microtitre plates. The plates were incubated at 37 °C for 4 d, stained and examined for any cytotoxic effect. Toxin titres were the highest dilution giving a cytotoxic effect on Vero cells after 4 d incubation. Antisera were prepared against partially purified VT1 from strain H19 and VT2 from strain E32511 by the method of Konowalchuk *et al.* (1978): these antisera do not cross-react (Scotland *et al.*, 1985). The VT1 antiserum gave the same results as those obtained with an antiserum raised to purified Shiga toxin kindly provided by Dr A. D. O'Brien (Uniformed Services University, Bethesda, Md., USA). Neutralization experiments were done as described by Scotland *et al.* (1988).

Tests for production of heat-stable enterotoxin (ST_A) and heat-labile enterotoxin (LT). All VT⁺ strains were tested for production of ST_A, detectable in the infant mouse assay, and LT (Gross *et al.*, 1976).

DNA hybridization experiments. Strains were prepared for colony hybridization as described by Willshaw *et al.* (1985) on nylon membranes (Hybond-N, Amersham). The cloning of VT1 and VT2 sequences and the development of specific gene probes has been described previously (Willshaw *et al.*, 1985, 1987). The VT1 probe was a 0.75 kb *HincII* fragment derived from the VT-encoding phage isolated from strain H19. The VT2 probe was a 0.85 kb *AvaI*-*PstI* fragment present in VT2 sequences cloned from the VT2-encoding phage in strain E32511. The DNA fragments were purified from vector DNA, labelled and used in hybridization experiments under 'stringent' conditions as described previously (Willshaw *et al.*, 1985).

RESULTS

DNA hybridization experiments

All the VT⁺ strains of bovine or porcine origin hybridized with either the VT1 or the VT2 probe (Table 1). Fourteen of the twenty calf strains were detected with the VT1 probe including

Table 1. *Properties of VT⁺ E. coli of animal origin*

The toxin titrations were done in at least two separate experiments. NT, Not tested.

Serotype*	DEP no.	Hybridization with DNA probes		Toxin titre		
		VT1	VT2	Without antiserum	With antiserum to VT1	With antiserum to VT2
<i>Bovine strains</i>						
O2.H7	E26817†	—	+	1 250	NT	NT
O2.H7	E26818†	—	+	250	NT	NT
O5.H ⁻	S102-9	+	—	6 250	NT	NT
O8.H9	E40873	—	+	250	250	0
O26.H11	E7688	+	—	6 250	NT	NT
O26.H11	E10394	+	—	31 250	NT	NT
O26.H11	E40877	+	—	1 250	0	1 250
O26.H11	E40878	+	—	156 250	1 250	156 250
O29.H34	E37445	+	—	31 250	50	31 250
O55.H17	E37446	+	—	250	NT	NT
O111ac.H ⁻	E40867	+	—	1 250	10	1 250
O111ac.H ⁻	E40875	+	—	6 250	50	6 250
O111ac.H ⁻	E40876	+	—	6 250	250	6 250
O149.H?	E40879	—	+	160	160	10
O153.H12	E37450	+	—	250	NT	NT
O rough	E40868	—	+	31 250	31 250	0
O? H25	E40874	—	+	156 250	156 250	250
O? H ⁻	E40870	+	—	1 250	50	1 250
O? H ⁻	E40871	+	—	1 250	50	1 250
O? H ⁻	E40872	+	—	250	10	250
<i>Porcine strains</i>						
O65.H9	E40896	—	+	10	10	0
O65.H9	E40897	—	+	40	40	0
O65.H9	E40898	—	+	10	10	0
O65.H9	E40899	—	+	20	20	0
O65.H9	E40900	—	+	160	160	0
O138.H14	E40893	—	+	20	20	0
O138.H ⁻	E57†	—	+	160	160	0
O138.H ⁻	E40901†	—	+	40	40	0
O139.H1	P3	—	+	20	20	0
O139.H1	E40888	—	+	40	40	0
O141.H4	E145	—	+	80	80	0
O141.H4	E68II	—	+	10	10	0
O141.H4	E40891	—	+	80	80	0
O? H4	G1108E†‡	—	+	80	80	0

* ? indicates O or H antigens unidentifiable with the antisera used.

† These strains produced ST_A detectable in the infant mouse assay.

‡ Strain G1108E also produced LT.

the four strains of serotype O26.H11 and three belonging to serogroup O111. Four bovine strains hybridizing with the VT2 probe belonged to serogroups O2, O8 and O149 and the remaining two could not be serotyped.

The VTEC isolated from pigs belonged to four different O serogroups (65, 138, 139 and 141) and one was unidentifiable; all strains hybridized with the VT2 probe only. Six of the strains of serogroups O138, O139 and O141 were isolated from cases of oedema disease whereas the other strains were from piglets or weaned pigs with diarrhoea.

Toxin titres and neutralization experiments

Culture supernatants of all the strains were examined for the level of VT production using Vero cells (see Methods). The VT titres of the calf strains ranged from 160 to 156 250 (Table 1) but the level of VT production did not correlate with the presence of either VT1 or VT2; for

example, strains E40878 and E40874 gave the highest titres of 156250 and produced VT1 and VT2 respectively. Neutralization experiments using antisera to VT1 or VT2 were in agreement with the results of the DNA hybridization in all cases tested. In some tests with the bovine strains there was incomplete neutralization of toxin activity that may have occurred because there was insufficient antibody in these experiments.

The VT titres of the porcine strains were much lower than those of the VTEC from calves; the titres ranged from 10 to 160. All these strains hybridized with the VT2 probe and toxin activity was neutralized in all cases with the antiserum to VT2 but not with the VT1 antiserum. No relationship was observed between the amount of VT produced and the strength of the hybridization signal.

Two bovine strains and two porcine strains produced ST_A in addition to VT and strain G1108E, of porcine origin, produced both ST_A and LT as well as VT (Table 1).

DISCUSSION

The results show that the VTs and VT genes of the 34 strains of animal origin could be identified with antisera and DNA probes developed using strains producing VT1 or VT2 of human origin. Most of the VT⁺ animal strains described in this paper belong to serogroups not usually associated with VTEC of human origin, the exceptions being strains of serogroups O26, O111 and O153 that have been isolated from both human and bovine infections (Scotland *et al.*, 1980, 1988; Karmali *et al.*, 1983; Sherwood *et al.*, 1985).

Preliminary studies at the CVL on strains from animals with diarrhoea showed that VTEC were detected in 22% of 237 porcine strains and 8% of 85 *E. coli* from cattle. (C. Wray & I. M. McLaren, unpublished). By comparing isolation rates of VTEC in animals with and without diarrhoea in Sri Lanka, Mohammad *et al.* (1985) showed a significant association between VT production and diarrhoea in cattle and buffalo calves. The VT probes could be used in further studies to investigate the role of VTEC in animal disease. The bovine strain S102-9, serotype O5.H⁻, has been used in studies with experimental animals. This strain, which produces VT1 and was isolated from calves with dysentery (Hall *et al.*, 1985), caused a disease in experimental animals that was indistinguishable from the natural infection. The VTEC most commonly associated with human disease belong to serogroup O157 and such strains have been isolated from milk and healthy cattle associated with O157.H7 human infection in North America (Martin *et al.*, 1986; Borczyk *et al.*, 1987). Recently, it has been reported that O157.H7 VTEC isolated in Argentina in 1977 were found in a diseased calf (Orskov *et al.*, 1987). It was suggested that cattle may be primary reservoirs of VTEC serotype O157.H7 that are associated with human disease.

The production of VT by the porcine strains was very low so the use of DNA probes provided a useful method for examining such strains for the presence of genes for VT1 or VT2. Previous studies (Konowalchuk *et al.*, 1977; Smith *et al.*, 1983) showed that porcine strains produce a VT immunologically unrelated to the VT of some human strains examined by these workers, and now known to be VT1. Our finding that porcine strains produce VT2 confirm these early observations. We have found VT2 production also in human strains belonging to several serogroups but none was in common with those of the porcine strains (Scotland *et al.*, 1985, 1988; Smith *et al.*, 1987). Animal studies have suggested that VT may be important in oedema disease of pigs. Fourteen pigs were injected with extracts of *E. coli* K12 strains producing VT and ten of the animals developed clinical signs of oedema disease (Smith *et al.*, 1983). Dobrescu (1983) showed that 'oedema disease principle' extracted from porcine strains was cytotoxic for Vero cells but it was not neutralized by an antiserum to VT1. In the present study, we have shown that two of the strains used by Dobrescu, E57 and E68II, produce VT2. Further studies with VT2 toxin should be useful in showing whether this toxin is an aetiological factor in oedema disease.

It appears that VTEC of animal origin are associated with different clinical symptoms: diarrhoea, dysentery and oedema disease. VTEC of human origin, particularly strains of serogroup O157, are also associated with a spectrum of clinical manifestations from mild

diarrhoea to serious conditions such as HUS. Even within an outbreak of O157.H7 infection in a nursing home there were patients with asymptomatic infection, non-bloody diarrhoea, HC and HUS (Ryan *et al.*, 1986). In some studies of HUS it has been observed there are patients suffering from disorientation and seizures and also peripheral oedema (Karmali *et al.*, 1985). This suggests that there are similarities between HUS and oedema disease in pigs in which ataxia and mental confusion are prominent features (Sojka, 1965; Smith *et al.*, 1983). Studies on the role of Shiga toxin in disease have demonstrated that cytotoxic, enterotoxic and paralytic lethal effects are all due to a single purified toxin (review, O'Brien & Holmes, 1987). Both VT1 and VT2 also have these three properties (O'Brien *et al.*, 1982; Strockbine *et al.*, 1986; O'Brien & Holmes, 1987) and this may explain why VTEC are associated with several different clinical conditions. In addition production of ST_A or LT by some of the animal VTEC is also likely to be important in the observed clinical symptoms.

Results of this and previous studies suggest that VT1 and VT2 are important in both animal and human infections. Further studies are required to trace the sources of infection and also to examine VT production of the animal strains in more detail.

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