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Characteristics of Vero cytotoxin producing *Escherichia coli* associated with intestinal colonization and diarrhea in calves

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ABSTRACT

Isolates of *Escherichia coli* which produce Vero cytotoxin (VTEC) were obtained during 1983–1989 from calves raised in 5 north-central states of the USA. All of the calves experienced intestinal epithelial colonization by VTEC, diarrhea or both; twelve of the calves had bloody diarrhea. Twenty one isolates were serogroup O111 and the others were O103, O69, O45, 026, O5, or non-typable (4 isolates). All but one of the isolates hybridized with the CVD419 probe which identifies most VTEC strains. Thirty two isolates hybridized with the VT1 probe, 3 with both the VT1 and VT2 probes, and one with neither probe. The culture filtrate of the VT probe negative isolate was partially neutralized by SLT I monoclonal antibody. For the other isolates, the results of toxin neutralization by anti-SLT I and anti-SLT II monoclonal antibodies corresponded exactly with the VT1 and VT2 probe hybridization results. Three of the strains adhered in a localized manner to HEp-2 cells and Intestine 407 cells.

INTRODUCTION

Konowalchuk et al. (1977) first reported that certain *E. coli* isolated from human diarrhea produced cytotoxins which acted in vitro on Vero cells. Two serologically distinct Vero cytotoxins have been described: VT1 and VT2, also called SLT I and SLT II because of their similarity to the toxin produced by *Shigella dysenteriae* 1 (O'Brien and Holmes, 1987). *E. coli* producing Vero cytotoxin (VTEC) have been isolated from cattle, beef, other meats, milk

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and milk products (Karmali, 1989; Giffin and Tauxe, 1991). Many of the bovine VTEC have been isolated from cattle with diarrhea. Pathological examination of the intestinal tracts of some of these animals revealed characteristic epithelial lesions described as attaching and effacing with loss of microvilli and pedestal and cup formations of the cell membrane (Hall et al., 1985; Moxley and Francis, 1986).

VTEC of animal and human origin did not hybridize with the enteropathogenic *E. coli* (EPEC) adherence factor (EAF) DNA probe (Dorn et al., 1988). Many of the VTEC of bovine origin have characteristics in common with those proposed for the enterohemorrhagic *E. coli* (EHEC) (Levine, 1987). These *E. coli* of human origin produce Vero cytotoxin; they are associated with hemorrhagic colitis; they lack production of enterotoxins LT and ST characteristic of enterotoxigenic *E. coli* (ETEC); and they are not invasive. A DNA probe, designated CVD419, has been developed which hybridizes with most *E. coli* O157:H7 and other serotypes that fit the above criteria (Levine et al., 1987).

The source of the CVD419 probe was serotype O157:H7 which is associated with human outbreaks and sporadic illnesses. This serotype is a serious human pathogen capable of producing hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (Karmali, 1989). The sources of infection and associated human illnesses have been traced to undercooked beef and unpasteurized milk; serotype O157:H7 *E. coli* have been isolated from cattle (Karmali, 1989; Giffin and Tauxe, 1991). The isolates have been from healthy cattle, except for four reports of serotype O157:H7 from diarrheic calves (Kashiwazaki et al., 1981; Orskov et al., 1987; Blanco et al., 1988; Yano et al., 1988). None of these calves was reported to have blood present in the feces. The purpose of this study was to characterize the VTEC isolates from cattle obtained by two veterinary diagnostic laboratories. This information may be used in the diagnosis and epidemiologic investigation of VTEC infections of cattle, and in understanding their possible role in human infections.

MATERIAL STUDIED AND METHODS

Bacterial strains and tissue specimens

VTEC isolates from calves with diarrhea were collected by the South Dakota Animal Disease Research and Diagnostic Laboratory and the Department of Diagnostic Investigation at the University of Minnesota. Entire calves and tissues collected at field necropsies were routinely submitted to the laboratories. Historical and clinical information about each animal was obtained from submission forms and interviews with producers and veterinarians. Intestines were processed for histopathological examination using routine methods. Colonization was based upon observations under light microscopy

of short bacilli which were intimately adherent to intestinal epithelial cells exhibiting irregular erosive lesions typical of attaching and effacing infections (Moxley and Francis, 1986; Janke et al., 1990).

Microbiological examination

Specimens of intestines were cultured aerobically on blood agar, tergitol-7 agar (Difco Laboratories, Detroit, MI) and E agar (Francis et al., 1982) and on Brilliant green agar plates (Flow Laboratories, McLean, VA). The following biotyping tests were performed: indole, methyl red, Voges-Proskauer, citrate, H_2S, urease, phenylalanine deaminase, lysine decarboxylase, arginine decarboxylase, ornithine decarboxylase, motility, gelatin hydrolysis, salicin, malonate, esculin hydrolysis, nitrate, oxidase, and 11 carbohydrate and 6 sugar alcohol fermentations. Isolates from the intestines identified as *E. coli* were examined for production of enterotoxin by methods previously described (Francis, 1983) and serotyped by the *E. coli* Reference Center, Pennsylvania State University, University Park, Penn.

DNA probe hybridization

The VT1 probe was a *Hin*cII fragment of 0.75 kb specific for VT1 sequences cloned from a VT1 encoding phage carried by H19, *E. coli* O26:H11 (Willshaw et al., 1985). The VT2 probe was a 0.85 kb *Ava*I-*Pst*I fragment specific for VT2 sequences cloned from a VT2-encoding phage carried by *E. coli* strain E32511 of serotype O157:NM (Willshaw et al., 1987). The CVD419 probe was a 3.4 kb *Hin*dIII fragment cloned from strain 933, *E. coli* O157:H7 (Levine et al., 1987). The probe fragments were cut from low melting point agarose gels and labelled with deoxyadenosine 5′-α[^{35}S] thiotriphosphate (Amersham, Arlington Heights, IL) using random primers (Feinberg and Vogelstein, 1984). Hybridization with the ^{35}S-labelled probes was performed according to Maniatis et al. (1982). In all hybridizations, the following three controls were used: E3787, *E. coli* O26:H11, VT1 positive, VT2 negative and CVD419 positive; E32511, *E. coli* O157:NM, VT1 negative, VT2 positive and CVD419 positive; and 14R519, *E. coli* K12, negative with all of these probes.

Vero cytotoxin assay

The Vero cytotoxin assay used was a modification of the method of Scotland et al. (1985). Five-fold dilutions of filtrates was tested on Vero cell monolayers. The plates were resealed and incubated at 37°C, 5% CO_2 for 2 days. A filtrate was considered positive if a 1:10 or greater dilution produced a cytotoxic effect on the monolayer and the corresponding heat-inactivated preparation was negative.

Neutralization tests were performed using two mouse ascitic fluid reagents containing monoclonal antibody (mAb) 13C4 and mAb BC5-BB12, respec-

tively, obtained from Dr. N. A. Strockbine, Centers for Disease Control, Atlanta, Georgia. mAb 13C4 has approximately a 1:1000 neutralizing titer against 25 CD_{50} of SLT I. mAb BC5-BB12 has approximately a 1:50000 neutralizing titer against 25 CD_{50} of SLT II. The stock ascitic fluid preparations were diluted 1:1000 and 1:100 for use in the neutralization tests. The respective ascitic fluid dilutions (0.025 ml) were mixed with an equal amount of filtrate in 0.2 ml supplemented MEM and incubated in a 96 well plate at 37°C for 3 hrs. The neutralization mixtures were then transferred to 96 well plates containing Vero cell monolayers and incubated at 37°C, 5% $C0_2$ for 2 days. Neutralization of the original filtrate was recorded when the addition of anti-SLT specific ascitic fluid resulted in a decrease in the cytotoxic titer by one or more dilutions from the cytotoxic titer of the original filtrate.

HEp-2 Cell and Intestine 407 Cell Adherence Tests

The method of Scotland et al. (1985) was used to test each strain for ability to adhere to HEp-2 cells and Intestine 407 cells, in the presence of D-mannose, during a 6 h incubation period. Only localized adherence as described by Nataro et al. (1987) was observed and it was scored as the percentage (mean of at least 2 tests) of HEp-2 or Intestine 407 cells with 10 or more adherent bacteria. Strains which adhered to less than 10% of the HEp-2 or Intestine 407 cells were considered non-adhesive for that cell line.

RESULTS

General properties

A total of 36 VTEC isolates, each from a different calf between 1983 and 1989, were identified for study (Table 1). They were isolated predominantly from calves designated as dairy or dairy-crossbred; only 4 of the isolates were from beef or beef-crossbred calves. One calf was 3 months old and all the other calves of known age were less than 3 weeks of age. Sixteen of the calves were from Minnesota, 14 from South Dakota, 4 from Iowa, one from North Dakota and one from Nebraska. Thirty two of the 36 calves had bacteria colonizing the epithelium of their intestinal tract and diarrhea, 3 had colonization with no observed diarrhea, and one had diarrhea but colonization was obscured by post-mortem autolysis. Thirteen of these were observed by histopathology to have lesions of the colon. Twelve of the calves were observed to have bloody diarrhea.

All 36 isolates fermented sorbitol within 24 hours. The four O5:NM and two other isolates did not ferment raffinose. None of the isolates were positive for the K99 antigen.

Twenty one of the isolates were serogroup O111, 4 isolates were non-typable, 4 isolates were O5 and the other 7 isolates were distributed among 6 other

TABLE 1

Properties of Vero cytotoxic producing *E. coli*[1]

Serotype/ strain	Probe hybridization[2]			SLT titers		
	CVC419	VT1	VT2	Without anti-SLT ascites fluid	With anti-SLT I	With anti-SLT II
O111:NM						
83-16193	+	+	–	6250	250	6250
86-10049	+	+	–	1250	250	1250
D87-3784	+	+	–	6250	250	6250
87-9751	+	+	–	1250	250	1250
D87-25045-2	+	+	–	250	50	250
D87-25181	+	+	–	50	10	50
88-1430	+	+	–	6250	1250	6250
D88-6881	+	+	–	50	10	50
88-1551-S	+	+	–	50	0	50
88-2437	+	+	–	10	0	10
88-15863	+	+	–	50	10	50
88-19274	+	+	–	50	0	50
88-19539	+	+	–	50	0	50
D88-21606	+	+	–	6250	250	6250
89-329-A	+	+	–	50	10	50
89-947	+	+	–	50	10	50
89-2036-B	+	+	+	250	50	0
89-3143	+	+	+	6250	250	1250
89-13880	+	+	–	6250	1250	6250
D89-21487	+	+	–	1250	50	1250
O111:H11						
88-4110-NH	+	+	–	50	10	50
O103:NM						
89-118	+	+	–	250	10	250
O103:H2,12						
89-7321	+	+	–	1250	50	1250
O69:NM						
89-5636	+	+	–	1250	250	1250
O45:NM						
D88-28058	+	+	–	50	10	50
O45:H2,6,12						
88-4110-H	+	+	–	50	0	50
O26:H11						
88-1577	+	+	–	50	0	50
89-4911	+	+	–	1250	0	1250
O5:NM						
84-5406	–	–	–	250	50	250
87-5680	+	+	+	6250	250	1250
D87-6627-3	+	+	–	250	10	250
D87-24061	+	+	–	50	10	50
O?:NM						
D87-214	+	+	–	250	10	50
D87-4377	+	+	–	6250	1250	6250
88-2278	+	+	–	50	10	50
89-7496-1	+	+	–	31250	6250	31250

[1]Intestinal colonization and diarrhea were observed in all calves, except for strain 89-7321 from a calf with diarrhea only and strains 88-4110-NH, D88-28058 and 88-4110-H with colonization only. The following calves had bloody diarrhea: 83-16193, 86-10049, D87-25045-2, D87-25181, 88-1430, 88-15863, D89-21487, 89-7321, D87-24061, D87-214, D87-4377 and 88-2278. Adherence to HEp-2 and Intestine 407 cells was less than 10% for all strains, except strains D87-25045-2, 88-19539 and 89-7321. [2]VT1 and VT2 correspond to SLT I and SLT II, respectively.

serotypes (Table 1). None of the isolates was serotype O157:H7, the VT serotype most often isolated from cases of human illness.

DNA probe hybridization

Except for isolate 84–5406, all of the isolates hybridized with the CVD419 probe (Table 1). Thirty two of the 36 isolates hybridized with only the VT1 probe; two of the serotype O111:NM isolates and one of the O5:NM isolates hybridized with both VT1 and VT2 probes. None of the isolates hybridized with only the VT2 probe.

Vero cytotoxin assay

The highest titer obtained for these VTEC isolates was 31250. Filtrates of the other isolates had lower titers: 8 were 6250 and the other 27 were less than 6250 (Table 1). For the VT1 and VT2 positive isolates, the results of neutralization of their filtrates by anti-SLT I and anti-SLT II ascites fluid matched exactly the VT probe hybridization results. The filtrate of isolate 84–5406, which did not hybridize with either VT1 or VT2, was partially neutralized by anti-SLT I monoclonal antibody. The anti-SLT I ascites fluid completely neutralized the filtrates of 7 of the 36 SLT I positive isolates. Incomplete neutralization of SLT I was observed with filtrates from the remaining 29 SLT I positive isolates. Anti-SLT II ascites fluid completely neutralized one of the 3 filtrates of SLT II positive isolates.

HEp-2 cell and intestine 407 cell adherence tests

Three isolates possessed the ability to adhere in a localized manner to greater than 10% of both HEp-2 and Intestine 407 cells; two were serotype O111:NM and one was serotype O103:H2,12. The adherence test results for a particular isolate were similar for both HEp-2 and Intestine 407 cells.

Other pathogens

Other pathogenic agents were isolated from 24 of the 36 calves; 8 were infected with two other pathogens besides VTEC. Fifteen co-infections involved cryptosporidia, 7 coronavirus, 9 rotavirus, one enterotoxigenic *E. coli* (ETEC) and one bredavirus.

DISCUSSION

The absence of serotype O157:H7 in this series of VTEC isolates from calves is consistent with previous reports that O157:H7 is not a frequent cause of illness in cattle. There are only 4 reports of this serotype being isolated from ill cattle (Kashiwazaki et al., 1981; Orskov et al., 1987; Blanco et al., 1988; Yano et al., 1988) and in none of these was O157:H7 confirmed as the cause of illness.

The most common serogroup in this series, O111, has been recorded and also identified as verotoxigenic in other studies of bovine isolates (Sherwood et al., 1985; Marques et al., 1986; Mohammad et al., 1986; Mainil et al., 1987; Schoonderwoerd et al., 1988; Smith et al., 1988; Dorn et al., 1989). Other serogroups represented in this series have also been previously isolated from cattle and shown to be verotoxigenic: O103 (Mohammad et al., 1986; Dorn et al., 1989; Gonzalez et al., 1989): O26 (Kashiwazaki et al., 1981; Sherwood et al., 1985; Marques et al., 1986; Mainil et al., 1987; Smith et al., 1988; Rietra et al., 1989; Scotland et al., 1990; Woodward et al., 1990): and O5 (Chanter et al., 1986; Hall et al., 1985; Dorn et al., 1989). No previous published reports of verotoxigenic isolates belonging to serogroups O69 and O45 were found. None of the VTEC isolates showed delayed sorbitol fermentation, a unique characteristic of serotype O157:H7 (Farmer and Davis, 1985).

Twelve of the 36 calves had bloody diarrhea. Seven of these calves yielded cultures of serotype O111:NM, 3 were non-typeable, one was O103:H2,12 and one was O5:NM. Serotypes which were isolated from calves in this study and which have been reported from HC in humans include O111:NM, O45:H2, O26:H11 and O5:NM (Bopp et al., 1987)

Other O5:NM strains of bovine origin have been associated with hemorrhagic enteritis in cattle. A serotype O5:NM *E. coli* was isolated from an outbreak of dysentery among 8 to 21 day old calves in England (Chanter et al., 1986). In both natural and experimentally reproduced disease with this strain, the calves passed copious bright red blood in the feces and developed diarrhea (Hall et al., 1985). This O5:NM strain produced a toxin active on Vero cells which was neutralized by anti VT1 and hybridized with the VT1 and CVD419 probes (Dorn et al., 1989). One of the serotype O5:NM *E. coli* in the series (84–5406 in Table 1) was previously reported by Moxley and Francis (1986) to produce bloody diarrhea in calves and lambs. In the present study, isolate 84–5406 produced a toxin active on Vero cells which was incompletely neutralized by anti SLT I ascites fluid. However, it failed to hybridize with the VT1, VT2 and CVD419 probes. It apparently produces another toxin active on Vero cells, neutralizable by anti-SLT I ascites fluid, but not mediated by the phage genes encoding SLT I and SLT II. A similar observation has been made with porcine edema disease strains which produce a variant of SLT II that is chromosomally mediated and active on Vero cells, but not on HeLa cells (Marques et al., 1987; Weinstein et al., 1988).

Our observation that 32 of 36 VTEC isolates from calves in north-central states hybridized with only the VT1 probe is consistent with reports that most of the non-O157:H7 bovine VTEC isolated by laboratories located in England were positive for only VT1 (Smith et al., 1988; Dorn et al., 1989; Scotland et al., 1990). However, few of the non-O157:H7 VTEC isolated from healthy cattle (usually mature) hybridized with only the VT1 probe in Germany and in Thailand, 5% and 14%, respectively (Montenegro et al., 1990;

Suthienkul et al., 1990). Serotype O157:H7 and other serotypes isolated from human HC and HUS cases usually produce SLT II alone or both SLT I and II, but much less frequently SLT I alone (Scotland et al., 1987; Dickie et al., 1989; Ostroff et al., 1989). The SLT production pattern of VTEC isolates from human cases appears to be similar to that of VTEC isolates from healthy cattle, but dissimilar to that of calves with diarrhea. It is possible that SLT II strains are more pathogenic for humans while SLT I strains are more pathogenic for calves resulting in higher frequencies of these respective toxins among isolates from humans and from calves. It is also possible that SLT I producing *E. coli* are more common in calves and SLT II *E. coli* infections are more common among mature cattle. The higher prevalence of SLT II *E. coli* than SLT I *E. coli* infections among mature cattle (Montenegro et al., 1990; Suthienkul et al., 1990) might result in more passive transfer of SLT II antibody than SLT I antibody, resulting in lower incidence of clinical illness due to SLT II producing VTEC strains in calves. This hypotheses should be explored in future studies.

The VTEC isolates of this study were from midwestern states with a substantial beef production industry, yet 28 of 32 VTEC isolates came from dairy or dairy cross calves. The predominance of dairy calves as the source of VTEC strains suggests that these animals may be more susceptible to life threatening illness by VTEC than are beef calves. Dairy calves are usually fed a milk replacement starter and many may either not receive or poorly absorb colostrum: they are thus more at risk than beef calves which are usually nursed. This is consistent with the observation of higher incidence of gastroenteritis in breast-fed children infected with *E. coli* serogroup O111 compared to that in formula-fed infants, similarly infected (Belnap and O'Donnell, 1955).

All but one of the 36 VTEC isolates in this study hybridized with the CVD419 probe (Table 1). In another study of VTEC isolated from healthy cattle, only 71% hybridized with this probe. It is possible that the EHEC probe is more specific for pathogenic VTEC than for VTEC strains associated with subclinical infections; however, additional studies of cattle with different ages and from different geographic areas are needed.

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