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Detection and Characterization of Fecal Verotoxin-Producing *Escherichia coli* from Healthy Cattle

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Verotoxin-producing *Escherichia coli* isolates from feces of healthy cattle were identified by DNA hybridization with verotoxin 1- and verotoxin 2-specific gene probes. Among 259 animals investigated, 28 (10.8%) were found to carry verotoxin-producing *E. coli* strains. Characterization of the verotoxin-producing isolates revealed a heterogeneous population in terms of serotype and toxin type. Nearly 40% of the strains belonged to serogroups known to be pathogenic for humans, i.e., O22, O39, O82, O91, O113, O116, O126, and O136. Two isolates from different bulls were identified as serotype O157:H7. Results obtained in this study indicate that cattle may be an important source of verotoxigenic *E. coli* involved in human disease.

Verotoxin-producing *Escherichia coli* (VTEC) strains of different serotypes are increasingly isolated from cases of human and animal diseases (1, 2, 7, 10, 11, 33). Most of them belong to the serotype O157:H7, which can cause hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura in humans (11). Several outbreaks have occurred in the last 6 years. In 1983, Riley et al. (22) reported an outbreak in the United States which was due to the consumption of hamburgers, involving 26 patients with HC. In Canada, another infection, also due to the consumption of hamburgers, led to 73 cases of HC, with 17 fatalities (5). Raw milk was the source of another outbreak in Canada, where 48 persons were treated for either HC or HUS (4). Consequently, cattle are regarded as a possible reservoir of these highly virulent strains (4, 14, 18; R. Clark, S. McEwen, N. Harnett, H. Lior, and C. Gyles, Abstr., Annu. Meet. Am. Soc., Microbiol. 1988, P48, p. 282). In 1988, Morgan et al. (16) reported the first community outbreak of HC in the United Kingdom; 24 persons were affected, 1 of whom died. In this outbreak, vegetables were thought to have been the source of infection. In a recent publication, Bockemühl et al. (2) reported the first foodborne infection due to VTEC in the Federal Republic of Germany.

As a contribution to the epidemiology of VTEC in Germany, we focused our work on cattle as a possible reservoir of these strains. This was achieved by analyzing *E. coli* isolates originating from fecal samples of animals which are used for milk and meat production in West Berlin. Fecal *E. coli* isolates from bulls and dairy cows were collected and analyzed by DNA hybridization with specific gene probes for verotoxin 1 (VT1) and VT2. Positive strains were serotyped and confirmed for toxin production by the Vero cell assay.

MATERIALS AND METHODS

Isolation of *E. coli* strains. Fecal samples of 212 bulls and 47 dairy cows were investigated (Table 1). Male animals came from different regions of the German Democratic Republic. The female animals were derived from different sites in the Federal Republic of Germany, but they were housed together in West Berlin for various periods (1 to 7 months) before sample collection.

The fecal samples were homogenized (10 g of feces in 90 ml of NaCl-peptone water) not more than 2 h after having been taken aseptically from the colons of the slaughtered bulls and from the rectums of the dairy cows. Serial dilutions (0.1 ml) were plated on plate count Monensin KCl medium (19), supplemented with 50 μg of 4-methyl-umbelliferyl-β-D-glucuronid (Sigma Chemical Co.) per ml or HC medium as described by Szabo et al. (30). Presumptive *E. coli* colonies were biochemically confirmed by Gram staining, the cytochrome oxidase test, and a modified IMViC procedure (20). The resulting 1,387 confirmed *E. coli* isolates were stored in microdilution plates at −70°C in L broth (Difco Laboratories) containing 20% glycerol (15).

Serotyping. Serological analyses were performed by slide and tube agglutination with adsorbed O and H antisera against 170 O and 56 H antigens by following the scheme of Orskov and Orskov (17).

VT assay. Isolates were tested for VT production by the method of Konowalchuk et al. (12). Strains were grown overnight at 37°C in L broth. Supernatants were obtained by centrifuging the cultures at 10,000 × *g* for 15 min and were evaluated for toxic activity immediately.

DNA probes. The VT1-specific gene probe was a 750-base-pair *Hin*cII fragment obtained from the recombinant plasmid NTP705 (32), and the VT2 probe was an 850-base-pair *Ava*I-*Pst*I fragment from the recombinant plasmid NTP707 (31). These fragments were purified from agarose gels by electroelution. The gene probes were labeled by nick translation either with ^{32}P or with biotin by using nick translation kits from Amersham Corp. or GIBCO Bethesda Research Laboratories, respectively.

DNA hybridization experiments. *E. coli* strains were screened for the presence of VT genes by colony hybridization (8). Isolates were grown in L broth in microdilution plates at 37°C overnight and transferred to lactose agar plates with a multipoint inoculator. After overnight incuba-

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TABLE 1. Frequency of VT genes in fecal *E. coli* isolates from bovines

Group (no. of animals)	No. (%) of VT^+ animals	No. of isolates	No. (%) of VT^+ isolates
Dairy cows (47)	8 (17.0)	181	21 (11.6)
Bulls (212)	20 (9.4)	1,206	36 (3.0)
Total (259)	28 (10.8)	1,387	57 (4.1)

tion at 37°C, grown colonies were further transferred to Schwarzband filters (no. 586; Schleicher & Schuell, Inc.). The filters were processed by the method of Maas (13). Hybridizations with ^{32}P-labeled probes were performed as described previously (15), except that the hybridization temperature was 42°C. For hybridizations with biotin-labeled DNA probes, processed filters were previously treated with proteinase K (Bethesda Research Laboratories; 1 mg/ml) by the method of Sethabutr et al. (25). Hybridization experiments with biotinylated probes and detection of positive colonies were carried out as recommended by the instructions of the manufacturer (BluGene; Bethesda Research Laboratories).

For preparation of chromosomal DNA, bacteria were lysed by the method of Hull et al. (9). DNA was purified by phenol extraction and ethanol precipitation and dissolved in 10 mM Tris hydrochloride, pH 7.6–1mM EDTA. Digestion with the restriction endonuclease *Hin*dIII (Bethesda Research Laboratories) was performed as recommended by the supplier. Southern blots (27) were prepared as described previously (15). Hybridization experiments using biotinylated probes were carried out as described above.

RESULTS

Distribution of VT-related sequences in fecal *E. coli* isolates from healthy cattle. A total of 1,387 *E. coli* isolates obtained from 259 animals were analyzed by colony hybridization with VT1- and VT2-specific gene probes. Table 1 shows the numbers and frequencies of gene probe-positive isolates, distributed according to the sex of the animals. A total of 21 (11.6%) of the 181 isolates from dairy cows and 36 (3.0%) of the 1,206 isolates from bulls hybridized with one or both probes. In all, 57 strains (4.1%) carrying one or both VT genes could be identified in 28 animals (10.8%).

The distribution of VT genes according to serotype is shown in Table 2. Twenty-six strains (45.6%) were found to carry both toxin genes. Hybridization to the VT1 probe alone was observed in only nine isolates (15.8%). Homology to the VT2 gene probe alone was found in 22 isolates (38.6%).

Production of VT by bovine *E. coli* strains carrying VT genes. Culture supernatants of the 57 strains that hybridized with the VT probes were tested for cytotoxicity in the Vero cell assay. All of them elicited the typical cytopathic effect described for VTs. In contrast, none of 24 arbitrarily chosen, probe-negative isolates produced VT. Toxin titers were determined as the highest dilution giving a cytotoxic effect on Vero cells after 24 h of incubation. No differences in toxin production could be associated with a particular toxin type; toxin titers ranged from 10^2 to $>10^5$. It can be concluded that all isolates that hybridized to the VT probes were VTEC strains.

Southern blot analysis of VTEC strains. In order to determine the location of VT genes, total DNA was extracted from some strains producing VT1 or both toxins, digested with the restriction endonuclease *Hin*dIII, and separated on an agarose gel. The DNA fragments were blotted onto nitrocellulose and probed with VT1 and VT2 (Fig. 1). VT1 hybridized with two *Hin*dIII fragments in each case (Fig. 1A). Since strains belonging to the same serotype showed a common hybridization pattern, apart from sharing other properties, such as plasmid profiles, biovars (not shown), and toxin genes, we conclude that they were related or identical isolates. In contrast to that, we could observe differences in the locations of VT1 genes when strains belonging to different serotypes were analyzed. Strains belonging to serotypes O116:H21 and Orough:H18 shared a common *Hin*dIII fragment of 3.2 kilobases, while strains of serotype O3:H^- had totally different hybridizing fragments of 10.7 and 6.7 kilobases (Fig. 1A). Hybridization to VT2 sequences always occurred to large *Hin*dIII fragments of about 20 kilobases (Fig. 1B). This was also found in strains that only produced VT2 (data not shown).

TABLE 2. Distribution of VT1 and VT2 genes in VTEC strains from bovines according to serotype

Serotype	No. of:		Hybridization with probe for:	
	Strains	Animals	VT1	VT2
O3:H^-	2	1	+	−
O10:H21[a]	2	1	+	−
O22:H8	2	1	−	+
O39:H40[b]	1	1	−	+
O75:H8	1	1	−	+
O82:H8[c]	6	4	+	+
O82:H8[c]	1	1	−	+
O82:H40[c]	1	1	+	+
O91:H10	1	1	−	+
O104:H21	1	1	+	+
O105:H18	1	1	+	−
O113:H21	6	4	−	+
O116:H21[c]	15	10	+	+
O116:H21	1	1	−	+
O126:H20	2	1	−	+
O126:H21	1	1	−	+
O136:H12[a]	4	2	+	−
O139:H8[b]	1	1	−	+
O156:H21[b]	1	1	+	+
O157:H7	2	2	−	+
Orough:H18	2	1	+	+
O?:H16[d]	1	1	−	+
O?:H29	2	1	−	+

[a] Five strains with these characteristics were isolated from one bull.
[b] Strains isolated from one bull.
[c] Five strains with these characteristics were isolated from one cow.
[d] This serovar possesses a new O antigen, which is currently under investigation.

Serotyping of VTEC isolates. Fifty-four VTEC strains could be classified into 19 defined serotypes (Table 2). For the other three isolates, the O antigen was not typeable. Almost 58% of all the strains belonged to only four serotypes, namely O116:H21 (n = 16), O82:H8 (n = 7), O113:H21 (n = 6), and O136:H12 (n = 4). The rest of the strains were distributed among 19 other serotypes. With two exceptions, strains of serotypes O82:H8 and O116:H21 produced both VT1 and VT2. Isolates that belonged to serotype O113:H21 carried only VT2 genes, whereas only VT1 sequences were found in all four strains of serotype O136:H12. VTEC belonging to serotypes O82:H8 and O113:H21 (n = 12) have been associated with human infections (11). Other potentially pathogenic strains of serogroups O22, O39, O91, O126, and O157 (n = 9) were found in small numbers. All of

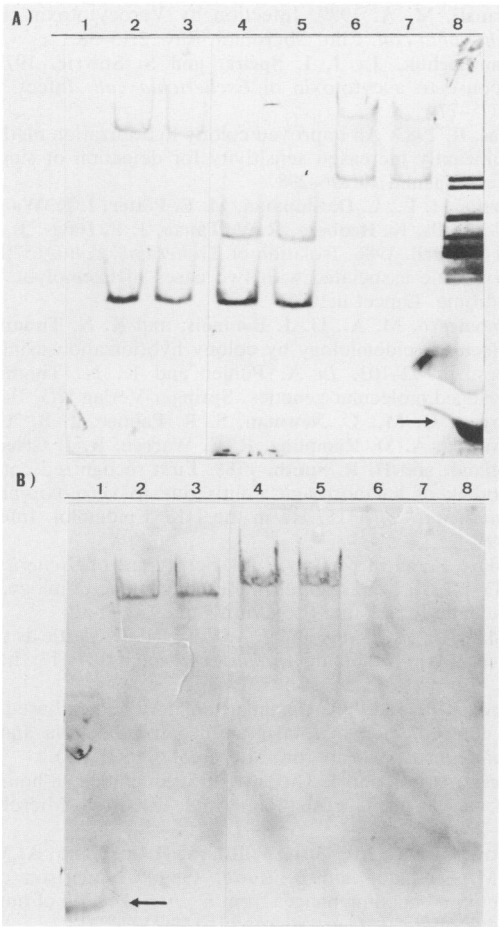

FIG. 1. Southern blot analysis of VTEC belonging to three different serotypes. (A) Chromosomal blot probed with a biotinylated VT1 probe. (B) The same blot as in panel A probed with a biotinylated VT2 probe. Lanes 1, NTP707 cut with *Ava*I and *Pst*I (arrow indicates fragment used as the probe); 2 through 7, *Hin*dIII-digested chromosomal DNA from *E. coli* 2478 (Orough:H18) (lane 2), *E. coli* 2477 (Orough:H18) (lane 3), *E. coli* 2431 (O116:H21) (lane 4), *E. coli* 2430 (O116:H21) (lane 5), *E. coli* 2405 (O3:H$^-$) (lane 6), *E. coli* 2403 (O3:H$^-$) (lane 7); 8, NTP705 cut with *Hin*cII (probe fragment is indicated by the arrow; other fragments are partial products of digestion).

them produced VT2 only. Among the latter strains, two isolates from two different bulls were identified as belonging to the high-virulence serotype O157:H7. In all, 22 (38.6%) isolates belonged to *E. coli* serogroups known to be pathogenic.

Some animals were found to carry more than one VTEC strain. The simultaneously occurring strains were unrelated to each other. Strains of serotypes O82:H8, O82:H40, and O116:H21 were isolated from the same animal (Table 2). Strains of serotypes O39:H40, O139:H8, and O156:H21 were isolated from another animal. Also, O10:H21 and O136:H12 isolates were found together in a third bovine.

DISCUSSION

The present study has shown that VTEC, the causative agent of HC, HUS, and thrombotic thrombocytopenic purpura, could be identified in fecal *E. coli* isolates from healthy cattle in the Federal Republic of Germany and the German Democratic Republic. To our knowledge, this is the first epidemiological survey on the occurrence of these pathogenic strains in healthy cattle in Germany. Workers in other countries, namely in Canada, the United States, the United Kingdom, and Sri Lanka, have postulated cattle to be a reservoir of VTEC strains (4, 11, 18, 26; Clark et al., Abstr. Annu. Meet. Am. Soc. Microbiol.). Only a few sporadic cases of HC, HUS, and thrombotic thrombocytopenic purpura have been reported in the Federal Republic of Germany (2, 10, 28, 33). One of these cases (2) involved serotype O22:H8, which we also found in our investigation.

The high incidence of VTEC strains found in cattle poses the question of whether humans are at a risk of acquiring these infections. Fecal contamination of meat during the process of slaughter cannot be totally ruled out. Therefore, the presence of VTEC in food is likely to occur. Investigations by Doyle and Schoeni (6) have shown a detection rate of VTEC in 3.7% of meat samples of bovine origin. Furthermore, a lower rate of contamination (between 1.5 and 2%) has been described by the same authors for food of porcine, avian, or sheep origin. Their data, however, referred only to *E. coli* serotype O157:H7. Our recent data on the serological detection of this serotype in more than 1,000 strains derived from over 100 food samples failed to show any positive strain (data not shown). To what extent other VTEC strains are present in food samples is the subject of our present work.

The rates of VTEC isolation from cattle described here are very similar to those found by Clark et al. (Abstr. Annu. Meet. Am. Soc. Microbiol.) in Canada, the country where the most food-borne infections due to VTEC have been documented. They found VTEC strains in 19.5% of the investigated dairy cows, the mean value for the total cattle population being 11.6%. These workers detected serotype O157:H7 in only one cow and three bulls from a total of 600 animals. Together with our data, these findings support the notion that cattle are an important reservoir of VTEC.

Nearly 40% of our isolates belonged to serogroups or serotypes recognized as being pathogenic for humans (2, 11). Most of them, i.e., O39:H40, O82:H8, O91:H10, O113:H21, O126:H20, O126:H21, and O157:H7, were isolated from cattle for the first time in this country. The lack of epidemiological data on VTEC other than O157:H7 probably results from the widespread use of sorbitol-supplemented media, on which only strains from serotype O157:H7 can be presumptively detected.

The risk of infection is also supported by the finding that all probe-positive isolates were able to express VT, as measured in the Vero cell test. Strains expressing either VT1 or VT2 are known to be pathogenic for humans (24). None of the animals carrying VTEC strains presented any obvious clinical symptoms. Smith et al. (26) were able to detect VTEC strains in cattle and swine with enteric diseases. These strains, however, belong to serogroups other than the ones reported here. These authors identified several O26:H11 strains in cattle, a serotype that has been also isolated from a sporadic case of HC (3). More recently, Appel et al. (1) also identified (in diseased pigs) VTEC strains of serogroups similar to the ones reported by Smith et al. (26). It seems likely that some VTEC serogroups are associated with infections of humans and animals, whereas others are found in infections of either humans or animals.

In some cases, different animals were found to carry identical isolates; others, in contrast, were found to harbor several different VTEC strains. For this reason, we assume that the detection of potential VTEC strains can only be successful if a sufficient number of *E. coli* isolates from each

fecal sample is examined. A mean of four to six isolates from each sample was investigated in this study, which in our opinion seems to be the lowest acceptable limit. Ideally, 10 isolates from each sample should be analyzed in order to increase the probability of identifying VTEC strains among the far more frequently occurring commensal strains.

Two chromosomal *Hin*dIII fragments were found to hybridize to the VT1 gene probe in Southern blot analyses of six arbitrarily chosen strains from three different serotypes (Fig. 1A). This was to be expected, since the *Hin*cII fragment used as a probe contains a *Hin*dIII recognition site (31, 32). However, when chromosomal blots from strains belonging to different serotypes were compared, different pairs of *Hin*dIII fragments hybridized in each case. This might reflect the presence of three different bacteriophages in the serotypes analyzed. Different phages carrying VT1 genes have already been demonstrated in serotypes O157:H7 and O26:H11 (23, 29). More recently, Rietra et al. (21) demonstrated the existence of diverse phages in VTEC strains from different serogroups.

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