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Prevalence and Some Properties of Verotoxin (Shiga-Like Toxin)-Producing *Escherichia coli* in Seven Different Species of Healthy Domestic Animals

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Fecal samples from 720 healthy, domestic animals representing seven different species (cattle, sheep, goats, pigs, chickens, dogs, and cats) were investigated for verotoxin (VT [Shiga-like toxin])-producing *Escherichia coli* (VTEC). VTEC were isolated from 208 animals (28.9%), most frequently from sheep (66.6% VTEC carriers), goats (56.1%), and cattle (21.1%). VTEC were isolated less frequently from pigs (7.5%), cats (13.8%), and dogs (4.8%) and were not found in chickens (<0.7%). Forty-one different O:H serotypes and 23 untypeable O-groups were isolated. Five serotypes (O5:H$^-$, O91:H$^-$, O146:H21, O87:H16, and O82:H8) occurred in more than one animal species. Serotypes O5:H$^-$, O91:H$^-$, O146:H21, O128:H2, and OX3:H8 represented 54.8% of the VTEC strains. Nearly 60% of all VTEC O:H serotypes isolated in this study have been implicated as human pathogens, indicating that healthy, domestic animals may serve as a reservoir of human pathogens. All VTEC, except nine feline strains, hybridized with one or both of the VT1 and VT2 specific DNA probes. VT production and enterohemolysin (E-Hly$^+$) production were associated in *E. coli* from goats, sheep, and cattle but not in *E. coli* from chickens, pigs, dogs, and cats. A close association of VT with E-Hly$^+$ was found in O5:H$^-$, O146:H21, O128:H2, O77:H4, O119:H25, and O123:(H10) strains. Thirty of 240 (12.5%) E-Hly$^+$ strains hybridized with an E-Hly$^+$ specific DNA probe, indicating heterogeneity of regulatory or structural E-Hly$^+$ genes in strains of *E. coli*.

Verotoxin (VT [Shiga-like toxin])-producing strains of *Escherichia coli* (VTEC) may cause severe diseases in humans, such as hemorrhagic colitis and hemolytic uremic syndrome (14). Human infections with these pathogens are often caused by consumption of contaminated foodstuffs like meat and unpasteurized milk (7, 14, 25). Epidemiological investigations revealed that cattle frequently harbor VTEC in their feces and thus may represent an important source of infection (20, 22, 34).

VTEC from different sources and geographical areas were found to belong to a large number of different serotypes (10, 14, 20, 36). However, most of the documented outbreaks of hemorrhagic colitis in humans were attributed to only a few serotypes of VTEC which have been designated enterohemorrhagic *E. coli* (EHEC) (15, 16, 32). It is not known whether all variants of VTEC which have been identified are equally pathogenic for humans (2, 14, 23, 32).

We have previously investigated the association of VT (Shiga-like toxin) production with enterohemolysin (E-Hly) production in *E. coli* strains which were isolated from humans, cattle, and pigs (5). Many of the human isolates were typical EHEC belonging to serogroups O157, O111, and O26. In these strains, VT production was found to be closely associated with E-Hly production, an association which potentially might be a useful epidemiological marker for rapid and easy detection of EHEC strains (5, 28, 37).

In the present work, we have investigated VTEC which were isolated from feces of healthy domestic animals. One aim of this study was to determine the frequency of VTEC carriers in populations of healthy animals belonging to different species. Animal VTEC were further characterized by their serotypes and by DNA hybridization with VT specific DNA probes. The association of VT production with E-Hly production was investigated in order to evaluate E-Hly as an epidemiological marker for detection of serologically diverse strains of animal VTEC.

MATERIALS AND METHODS

Animals. A total of 720 domestic animals belonging to seven different species (cattle, sheep, goats, pigs, chickens, dogs, and cats) from the Berlin area were included in the study. Four to seven separate sources of cattle, sheep, goats, pigs, and chickens were investigated (Table 1). The animals were either from private farms, university institutes, the Berlin Zoological Garden, or from the central slaughterhouse of Berlin. Dogs and cats were from private owners and from the animal house of the Berlin Society for Protection of Animals. Only animals without any signs of disease were taken into the study. Aliments fed to animals were free of antibiotics or food additives which are used as growth promoters in agriculture.

Isolation of *E. coli* from animal feces. Fecal samples were collected as rectal swabs and immediately transported to the laboratory for further processing. Samples from slaughtered animals were taken directly from the colon. Aliquots of samples were inoculated on Luria-Bertani agar (19), Endo agar (Merck, Darmstadt, Germany), and blood-agar plates (Difco Laboratories, Detroit, Mich.) containing 5% washed sheep blood (Unipath, Wesel, Germany) (5), and the plates were incubated for 24 h at 37°C. Colonies resembling *E. coli*

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TABLE 1. Frequency of VTEC carriers in different species of healthy, domestic animals in Berlin

Animal group[a]	No. of animals with VTEC/no. examined (% VTEC carriers)					
	Cattle	Sheep	Pigs	Goats	Cats	Dogs
1	0/30 (<3.3)	11/20 (55.0)	0/20 (<5.0)	21/25 (84.0)	0/26 (<4.0)[b]	1/35 (2.9)[b]
2	9/20 (45.0)	19/20 (95.0)	1/20 (5.0)	3/9 (33.3)	9/39 (23.1)[c]	2/28 (7.1)[c]
3	2/20 (10.0)	17/20 (85.0)	0/24 (<4.2)	12/17 (70.6)		
4	8/20 (40.0)	12/20 (60.0)	3/20 (15.0)			
5[d]	2/20 (10.0)	10/20 (50.0)	5/20 (25.0)			
6[e]	8/12 (66.6)	11/20 (55.0)	0/16 (<6.3)	1/15 (6.7)		
7	1/20 (5.0)					
Total	30/142 (21.1)	80/120 (66.6)	9/120 (7.5)	37/66 (56.1)	9/65 (13.8)	3/63 (4.8)

[a] Animal group indicates discrete populations which were not in contact with each other.
[b] Animals from private households.
[c] Animals from the Berlin Society for Protection of Animals.
[d] Animals collected for slaughtering.
[e] Animals housed in the Berlin Zoo.

were examined for hemolysis, fermentation of lactose, and morphological differences. For storage, pure cultures of bacteria were kept frozen in a 1:1 mixture of 87% sterile glycerol (Merck) and 1.5% Bacto Peptone (Difco) at −70°C. The bacterial isolates were differentiated by biochemical reactions as described previously (9).

Serotyping of *E. coli*. *E. coli* O, K, and H antigens were examined according to standard procedures (21). The serogroup OX3 is a provisional designation for a new O antigen.

Detection of *E. coli* Hly. E-Hly and α-Hly were phenotypically discerned on blood-agar plates as described previously (5). α-Hly specific DNA-sequences were detected in colony blot hybridizations with the *Ava*I-A fragment of the α-Hly plasmid pSF4000 as DNA probe (33). The ~450-bp *Bam*HI-*Eco*RV fragment of plasmid pEO19 was used as a DNA probe for the *E. coli* O26 bacteriophage C3888-associated E-Hly (E-Hly1) (4). The *E. coli* strains U4-41 (O4:K3:H5 α-Hly$^+$ VT$^-$), C4170 (O78:H$^-$ α-Hly$^+$ VT$^-$), and C3888 (O26:H$^-$ E-Hly1$^+$ VT$^-$) served as reference strains for discrimination between different Hly phenotypes as described previously (4, 5, 24).

Detection of VTs and DNA sequences specific for VT1 and VT2. All isolates were investigated for production of VTs by the Vero cell toxicity test as described previously (5). Verotoxigenic isolates were tested for the presence of VT1 and VT2 specific DNA sequences in colony blot hybridizations with the 750-bp *Hin*cII fragment of plasmid NTP705 and the 850-bp *Ava*I-*Pst*I fragment of plasmid NTP707 as gene probes (38, 39).

Colony blot DNA hybridizations. Colony blots on 0.45-μm nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany) and preparation of DNA probes specific for α-Hly, E-Hly, and VTs were performed as described previously (5, 18). DNA was labeled with digoxigenin-11-dUTP with the Boehringer DIG-DNA labeling and detection system (Boehringer Mannheim, Mannheim, Germany) according to the instructions of the supplier. Hybridization reactions and subsequent washing steps were performed at conditions of high stringency. The hybridization reaction with the labeled DNA probes was performed for 20 h at 68°C in 5× SSC (1× SSC is 0.3 M NaCl plus 0.03 M Na-citrate [pH 7.0])–0.1% *N*-laurylsarcosine (Sigma, Deisenhofen, Germany)–0.02% sodium dodecyl sulfate (SDS) with 1% blocking reagent (Boehringer Mannheim). Washing of membranes was performed twice for 5 min in 2× SSC–0.1% SDS at room temperature and then twice for 15 min in 0.1× SSC–0.1% SDS at 68°C. Labeled DNA was detected by an enzyme-linked color reaction (anti-digoxigenin enzyme-linked immunosorbent assay) on the nitrocellulose filters.

RESULTS

Prevalence of VTEC in healthy domestic animals in Berlin. *E. coli* was isolated from a total of 720 animals examined. Carriers of VTEC were found in six species of domestic animals (Table 1). VTEC were most frequently detected in feces from sheep, followed by goats and cattle, but were less frequent in pigs, cats, and dogs (Table 1). VTEC were not found in fecal samples of 144 (<0.7%) chickens. The frequency of VTEC carriers varied considerably between different groups of animals (Table 1).

Serological characterization of animal VTEC. A total of 208 strains that were positive in the Vero cell toxicity test were serotyped for their O and H antigens. Forty-one different O:H serotypes were identified. Twenty-three strains expressed untypeable O antigens, and two strains expressed untypeable H antigens. Only five serotypes of VTEC (O5:H$^-$, O91:H$^-$, O146:H21, O87:H16, and O82:H8) occurred in more than one animal species (Table 2). Serotypes O5:H$^-$, O91:H$^-$, O146:H21, O128:H2, and OX3:H8 were most frequent, representing 114 (54.8%) of the 208 animal VTEC. VTEC from cattle were most heterogeneous, with 15 different O groups and eight O-untypeable strains among 33 isolates. With the exception of O113:H21 strains, the serotypes of bovine VTEC differed from one group to the other. VTEC from sheep and goats were serologically more homogeneous. In both species, VTEC O5:H$^-$ strains predominated and were present in all flocks of animals. Besides O5:H$^-$, four other VTEC types (O91:H$^-$, O128:H2, O77:H4, and OX3:H8) were frequent in sheep (Table 2). Individual sheep which carried VTEC often harbored more than one serotype of these strains in their feces (Table 3). This was less frequently found in cattle and goats and was not observed in pigs, cats, and dogs.

Detection of VT1 and VT2 specific DNA sequences. With the exception of nine strains which were from cats, all other VTEC hybridized with one or both of the VT specific DNA probes used (Table 2). Among these, strains carrying both VT1 and VT2 sequences were most frequent (55.3%), followed by VT2$^+$ strains with 27.1% and VT1$^+$ strains with 17.6%.

Production of Hly. A total of 240 *E. coli* strains showing the E-Hly$^+$ phenotype were isolated. None of these strains were positive for α-Hly specific DNA sequences, and only

TABLE 2. Frequency and occurrence of different VTEC serotypes in animals

Serotype	Animals	No. of isolates	No. of strains with VT genotype:		
			VT1	VT2	VT1 + VT2
O5:H$^-$	Sheep	26	0	13	13
	Goats	26	0	1	25
O91:H$^-$	Sheep	25	4	0	21
	Pigs	1	1	0	0
O146:H21	Cattle	5	5	0	0
	Sheep	8	0	5	3
O128:H2	Sheep	12	1	5	6
OX3:H8[a]	Sheep	11	0	2	9
O21:K5:H14	Cats	8	0	0	0
O77:H4	Sheep	8	7	0	1
O123:(H10)[b]	Sheep	5	1	4	0
O20:K38:H19	Cattle	3	0	0	3
O100:H$^-$	Pigs	3	0	3	0
O113:H21	Cattle	3	0	3	0
O119:H25	Sheep	3	3	0	0
O146:H8	Sheep	3	3	0	0
O9:H$^-$	Pigs	2	0	2	0
O71:H12	Sheep	2	2	0	0
O76:H21	Cattle	2	0	0	2
O82:H8	Goats	1	0	1	0
	Cattle	1	0	1	0
O87:(H21)	Goats	2	0	0	2
O136:H20	Sheep	2	2	0	0
O141:H4	Pigs	2	0	2	0
O156:H21	Cattle	2	1	0	1
O:H[c]	Cattle	9	1	4	4
	Dogs	2	0	1	1
	Sheep	4	0	1	3
	Goats	2	0	0	2
	Cats	1	1	0	0
	Pigs	1	1	0	0
Ont:Hdiv.[d]	Cattle	8	0	1	7
	Sheep	4	1	0	3
	Goats	9	1	4	4
	Dogs	1	0	1	0
	Cats	1	0	0	0
Total		208	35	54	110

[a] OX3 is a provisional designation for a new O antigen.
[b] Four of these were O123:H10, and one was an O123:H$^-$ isolate.
[c] Single typeable strains from the following: cattle, O2:H25, O8:H2, O8:H19, O8:H25, O22:H8, O87:H16, O88:H25, O103:H42, and O152:H$^-$; sheep, O6:H10, O87:H16, O120:H$^-$, and O153:H$^-$; goats, O5:H10 and O74:H$^-$; pigs, O6:K53:H7; dogs, O116:H$^-$ and O17:H$^-$; and cats, O6:K13:H1.
[d] O-untypeable strains with different H antigens.

30 strains (most of them O6, O8, O26, O83, and O128) were positive with the E-Hly1 specific DNA probe. E-Hly$^+$ strains were most frequent in sheep (65.8% of the animals), followed by goats (54.5%), pigs (29.2%), cattle (23.9%), chicken (13.2%), dogs (7.9%), and cats (6.2%). The α-Hly$^+$ phenotype was observed in 119 strains which all reacted with the α-Hly specific DNA probe in colony blot hybridizations. Forty-four of the 119 α-Hly$^+$ strains were also positive with the E-Hly1 specific DNA probe. α-Hly production was frequent in isolates from cats (43.1%), followed by pigs (35.0%), dogs (28.6%), cattle (12.0%), goats (4.5%), and sheep (4.2%). E-Hly1 was not found in chicken strains (<0.7%).

TABLE 3. Occurrence of multiple VTEC serotypes in single animals

Animals	No. of animals with given no. of serologically different VTEC			
	1	2	3	4
Cattle	27	3	0	0
Sheep	53	22	4	1
Goats	34	3	0	0

Association of origin, serotype, and Hly and VT production in *E. coli* from animals. The association of VT production with Hly production in *E. coli* from animals is presented in Table 4. E-Hly and VT production was not associated in *E. coli* from chickens, pigs, or cats. However, a high association between VT and E-Hly production was found in *E. coli* isolated from goats, sheep, and cattle. It is not certain whether both markers are associated in *E. coli* from dogs, because only a few canine VT$^+$ and E-Hly$^+$ strains were isolated.

A clearer picture was obtained by comparing serotypes and E-Hly and VT production. E-Hly and VT were fully associated in five serotypes, which are represented by 87 strains (Table 5). O146:H21 isolates were different according to their origin. E-Hly and VT were only associated in ovine O146:H21 strains. Bovine O146:H21 and feline O21:K5:H14 strains were characterized by an association of VT with α-Hly production.

DISCUSSION

Many of the first reported outbreaks of O157:H7 VTEC infections in humans were associated with ingestion of contaminated foodstuffs of bovine origin (14). The isolation of VTEC from cattle all over the world has since identified cattle as a principal reservoir of *E. coli* O157:H7 and other VTEC (20, 30, 34). Besides cattle, pigs have occasionally been shown to harbor specific types of VTEC which were associated with bloody diarrhea and edema disease in piglets (10, 17). Very little is known about the occurrence of VTEC in other species of domestic animals. In this study, we detected VTEC in fecal samples of animals belonging to six different species. VTEC were found to be particularly frequent in the three ruminant species (cattle, sheep, and goats) which were examined, but in contrast, VTEC were much more sporadically isolated from nonruminants (chicken, pigs, dogs, and cats).

The high frequency of VTEC excreters found among sheep and goats indicates that these, together with cattle, represent an important natural reservoir of VTEC. The conclusion that goats and sheep often carry VTEC is supported by the high number of animals found to carry multiple VTEC serotypes and by the occurrence of some VTEC serotypes like O5:H$^-$ and O91:H$^-$ in animals which were not in contact with each other. Moreover, VTEC appear to be present in high numbers in feces from animals without any signs of disease, because they were detected by examination of a relatively small number of coliform colonies per animal. We have only collected samples from a relatively small geographical area, but we have no reason to believe that our findings cannot be generalized. This view is supported by the few reports on isolation of VTEC from sheep, cats, and goat milk (1, 8, 26).

The 208 VTEC we had isolated from animals were serologically very diverse. Multiple serotypes were found to be typical for VTEC which were from humans, animals, or other sources (14, 20, 34, 36). Interestingly, none of the 208 VTEC we have isolated were identified as typical EHEC like O4:H$^-$, O26:(H11), O45:H2, O145:H$^-$, O111:H$^-$, and O157:

TABLE 4. Association of VT with E-Hly production in *E. coli* strains from animals

Animals	Hly VTEC phenotype (no.):			Total no. of strains:		Association (%) with[a]:	
	E-Hly$^+$	α-Hly$^+$	Hly$^-$	VT$^+$	E-Hly$^+$	VT/E-Hly	E-Hly/VT
Cattle	19	5	9	33	40	57.6	47.5
Sheep	71	2	40	113	93	62.8	76.3
Goats	36	0	4	40	39	90.0	92.3
Cats	0	9	1	10	4	<10.0	<25.0
Dogs	2	0	1	3	6	66.6	50.0
Pigs	0	3	6	9	39	<11.0	<2.6
Chickens	0	0	0	0	19		<5.3

[a] VT/E-Hly, VTEC producing E-Hly; E-Hly/VT, E-Hly$^+$ strains producing VTs.

(H7), strains which were established as a subgroup of VTEC with proven human pathogenicity (15, 32). Nevertheless, several O:H serotypes isolated in this study have been implicated as human pathogens (i.e., O5:H$^-$, O91:H$^-$, O146:H21, O113:H21, O128:H2, O82:H8, O22:H8, Ont:H2, Ont:H8, and Ont:H16 (2, 3, 14, 40). Taken together, these serotypes represented nearly 60% of all VTEC isolated in this study. Four of these serotypes, i.e., O5:H$^-$, O91:H$^-$, O146:H21, and O82:H8, which accounted for 44.7% of all VTEC, were isolated from more than one of the animal species investigated. These strains were isolated from sheep, goats, cattle, and pigs, which might indicate that healthy, domestic animals serve as a reservoir of human pathogens. Typical EHEC strains were sporadically isolated from feces of cattle but appear to occur more frequently in animals with diarrhea than in healthy animals (20, 29, 34, 36).

Surprisingly, only one other VTEC serotype (O87:H16) was found to occur in more than one of the animal species investigated. This and other findings might indicate that many VTEC types are either very rare, are strongly restricted to their host, or both and might thus be rarely isolated from humans or from other host species (10, 17, 29, 35). Another possibility is that VT production in itself does not render an *E. coli* strain pathogenic to humans. Two other factors that may also affect virulence of VTEC are a plasmid of ~60 MDa (16) and the ability to cause attaching and effacing lesions of the intestinal mucosa in experimental animals (31).

It was previously reported that VT production is closely associated with E-Hly production in certain EHEC serotypes (5, 28, 37). The results of this study indicate that E-Hly production and VT production are frequently associated in most of the VTEC which were from cattle, sheep, and goats. The close association of VT and E-Hly production with some *E. coli* serotypes could indicate that these are genetically very closely related strains, as has been shown for O157:H7 isolates (35). We do not know whether the absence of hemolytic activity in some VTEC serotypes corresponds to a characteristic phenotype because it has been shown that VT and E-Hly phenotypes are not always stably inherited in some strains of *E. coli* (13, 28, and unpublished observations). Taken together, the results of this and other studies indicate that the E-Hly$^+$ phenotype is a suitable epidemiological marker for detection of some frequently occurring EHEC and VTEC serotypes in humans and animals. Other epidemiological markers used for detection of VTEC were found to be limited to the O157:(H7) group (6 and unpublished results).

Only some of the E-Hly-producing strains investigated here reacted with the DNA probe specific for an O26 bacteriophage-associated E-Hly called E-Hly1. Because there is no direct evidence that the E-Hly1 structural gene is

TABLE 5. Association of serotype, origin, Hly, and VT in *E. coli* strains from animals

Serotype	Animals	Total no. of strains	No. of strains:			Close association of VT + E-Hly
			VT$^+$	E-Hly$^+$	α-Hly$^+$	
O5:H$^-$	Sheep	26	26	26	0	Yes
	Goats	26	26	26	0	Yes
O91:H$^-$	Sheep	25	25	3	0	No
	Pigs	1	1	0	0	No
O146:H21	Cattle	5	5	0	5	No
	Sheep	10	8	8	2	Yes
O113:H21	Cattle	13	3	11	0	No
O17,77:H18	Chickens	12	0	12	0	No
O128:H2	Sheep	12	12	12	0	Yes
OX3:H8	Sheep	11	11	4	0	No
O21:K5:H14	Cats	8	8	0	8	No
O77:H4	Sheep	8	8	8	0	Yes
O108:H9	Pigs	6	0	6	0	No
O123:(H10)	Sheep	5	5	5	0	Yes
O70:H11	Sheep	5	0	5	0	No
O119:H25	Sheep	4	4	3	0	Yes
O156:H21	Cattle	4	2	4	0	No
O26:H$^-$	Cattle	3	0	0	3	No
	Sheep	5	0	4	1	No
O26:H11	Chickens	2	0	2	0	No

encoded by the bacteriophage, this finding could indicate the presence of regulatory or structural E-Hly genes different from the genes encoding E-Hly in the *E. coli* O26 strain C3888 (4). This needs to be investigated further.

With the exception of nine strains which were all from cats, the *E. coli* strains with verotoxigenic activity hybridized with one or both of the VT1 and VT2 specific DNA probes. At the present time, we do not know whether these feline VTEC produce a VT variant similar to these described for the Shiga-like toxin II family (11, 12, 27) or a different type of toxin which is similarly active on Vero cells.

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