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Virulence Markers of Shiga-Like Toxin-Producing *Escherichia coli* Strains Originating from Healthy Domestic Animals of Different Species

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Shiga-like toxin (verotoxin)-producing strains of *Escherichia coli* (SLTEC) originating from healthy cattle, sheep, goats, pigs, cats, and dogs were investigated for properties which are related to virulence of *E. coli* for humans. The *slt*-II (Shiga-like toxin II) and *slt*-IIc genes were frequent in SLTEC from healthy cattle and dogs but were rarely found in SLTEC from other animals. The *slt*-IIe gene was detected only in porcine SLTEC. SLTEC from goats and SLTEC from sheep were found to carry different SLT-II determinants which were not further characterized genetically. Sixty (28.8%) of 208 SLTEC from healthy animals showed diffuse adherence to HEp-2 cells. However, none of the strains was positive for genes specific for the local adherence (*eaf*), diffuse adherence (*daa*), or enteroaggregative (EAggEC) *E. coli* type. Only 3 (1.4%) of the 208 SLTEC were positive for attaching and effacing *E. coli* (*eae*) sequences. The enterohemolytic phenotype was present in 128 of the 208 SLTEC. Almost all enterohemolytic animal SLTEC were found to carry DNA sequences specific for the plasmid-encoded enterohemorrhagic *E. coli* hemolysin of *E. coli* O157. Bacteriophage-associated enterohemolysin (Ehly1 and Ehly2)-specific sequences were detected only in 14.4% of the 208 SLTEC and were linked with certain serotypes. The SLTEC from healthy animals constitute a very heterogeneous group of *E. coli*, and many of these strains appeared to be specific for their hosts. The absence of *eae* sequences in most animal SLTEC could indicate that these strains are less virulent for humans than the classical *eae*-positive enterohemorrhagic *E. coli* types.

Shiga-like toxin-producing strains of *Escherichia coli* (SLTEC) occur in the fecal floras of healthy domestic animals and are most frequently found in ruminants (5, 10, 22). Certain types of animal SLTEC, which were designated enterohemorrhagic *E. coli* (EHEC) (18), were shown to behave as pathogens in humans who became infected by consumption of SLTEC-contaminated food or by direct transmission of SLTEC from animals (11, 13, 17, 28). SLTEC infection in humans can cause severe diseases such as hemorrhagic colitis and hemolytic uremic syndrome (17).

It is not known if all SLTEC occurring in animals are equally pathogenic for humans (1, 17, 26). Most of the SLTEC which were isolated from ill humans have properties in common which are known or supposed to be related to their virulence. One of these is the production of SLTs; among these are the SLT-I, SLT-II, and SLT-IIc toxin types (25, 26, 29, 38). Besides production of SLTs, production of a new type of hemolysin (called EHEC hemolysin) causing an enterohemolytic phenotype (Ehly phenotype), possession of large EHEC plasmids, and induction of attaching and effacing *E. coli* lesions in host intestinal epithelial cells are closely associated with human pathogenic SLTEC (3, 15, 18, 30, 31).

In a previous study, we had investigated the prevalence of SLTEC in seven different species of healthy domestic animals, and 208 animal SLTEC were isolated and examined for their serotypes and for production of SLTs and hemolysins (5). None of these strains belonged to serogroup O157, O26, or O111, which are associated with human pathogenic SLTEC (3, 11, 17). In the present work, we have extended our studies of animal SLTEC by analyzing these for their SLT genotypes, EHEC hemolysin, and adhesins which are related to virulence of human SLTEC and other enteral pathogenic *E. coli* types.

The aim of this study was to characterize in more detail a group of serologically heterogeneous SLTEC from domestic animals and to compare these with human intestinal pathogenic *E. coli* for their virulence markers. The relatedness of animal SLTEC to SLTEC from diseased humans might be taken as an indicator for the pathogenic potential of SLTEC types which naturally occur in healthy animals.

MATERIALS AND METHODS

Bacteria. The origin, serotypes, and SLT and hemolysin production of the SLTEC strains from domestic animals were previously described (5).

Origin and specificity of DNA probes. The DNA probes used for detection of different virulence factors in animal SLTEC are described in Table 1. The following *E. coli* strains served as positive controls in DNA hybridization: 17-2 for enteroaggregative *E. coli* (EAggEC)-specific sequences and EDL933 for EHEC plasmid- and *eae*-specific sequences. Strain HS served as a negative control in hybridizations with these DNA probes. The control strains for *eaf* sequences were previously described (7), as were the reference strains for plasmid-encoded EHEC hemolysin (30, 31) and for bacteriophage-associated enterohemolysins 1 and 2 (Ehly1 and Ehly2) (8, 34).

Isolation of plasmid DNA, preparation of labeled DNA fragments, and colony blot hybridization. Plasmid DNA was isolated from 25-ml overnight cultures of bacteria by using the Qiagen kit according to the procedure described by the supplier (Diagen GmbH, Hilden, Germany). Probe DNA was isolated after digestion of plasmids with the appropriate restriction endonucleases followed by gel electrophoresis for separation of DNA fragments (20). Probe-specific DNA fragments were isolated from agarose gels with a JETSORB gel extraction kit by following the procedure provided by the supplier (Genomed GmbH, Bad Oeynhausen, Germany). Labeling of DNA probes with digoxigenin-11-dUTP and

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TABLE 1. Gene probes used for detection of different virulence markers in animal SLTEC by DNA-DNA hybridization

Gene probe (reference[s])	Probe fragment used for DNA hybridization	Virulence marker detected with gene probe[a]	Phenotype investigated[a]
pJPN16 (24)	1-kb *Bam*HI-*Sal*I fragment	EPEC adherence factor (*eaf*)	Local adherence to HEp-2 cells
pCVD434 (15)	1-kb *Sal*I-*Kpn*I fragment	Attaching and effacing *E. coli* (*eae*)	None
pCVD432 (2)	0.7-kb *Eco*RI-*Pst*I fragment	EAggEC plasmids	Enteroaggregative adherence to HEp-2 cells
pSLM852 (9)	0.5-kb *Pst*I fragment	*daa* and *daa*-related DNA sequences	Diffuse adherence to HEp-2 cells
pCVD419 (18)	3.4-kb *Hin*dIII fragment	EHEC plasmids	Enterohemolysis (5), maintenance of large plasmids
pEO40 (30, 31)	11.9-kb *Bam*HI fragment	Plasmid-encoded EHEC hemolysin	Enterohemolysis (5)
pEO19 (34)	464-bp *Bam*HI-*Eco*RV fragment	Bacteriophage-encoded Ehly1	Enterohemolysis (5)
pEO39 (8)	187-bp *Hin*dIII-*Ava*II fragment	Bacteriophage-encoded Ehly2	Enterohemolysis (5)
NTP705 (39)	0.75-kb *Hin*cII fragment	Bacteriophage-encoded SLT-I (VT1) (5)	Vero cell toxicity (5)
NTP707 (39)	0.85-kb *Ava*I-*Pst*I fragment	Bacteriophage-encoded SLT-II (VT2) (5)	Vero cell toxicity (5)

[a] Where no reference number is given, the examination was performed in this study.

colony blot DNA hybridizations under conditions of high stringency were performed as previously described (5).

Detection of SLT sequences by PCR. The oligonucleotides used as primers were purchased from Roth, Karlsruhe, Germany. The primers KS7 (5′-CCC GGA TCC ATG AAA AAA ACA TTA TTA ATA GC-3′) and KS8 (5′-CCC GAA TCC AGC TAT TCT GAG TCA ACG-3′) were used for amplification of *slt*-IB sequences (32), and the primer pair GK5 (5′-ATG AAG AAG ATG TTT ATG GCG-3′) and GK6 (5′-TCA GTC ATT ATT AAA CTG CAC-3′) was used for amplification of *slt*-IIB genes (29). Amplification of SLT-specific sequences and agarose gel electrophoresis of PCR products were performed as described previously (29). *slt*-II and *slt*-IIc were distinguished by separate digestions of *slt*-IIB PCR products with *Fok*I and *Hae*III followed by separation of restriction fragments on 2% agarose gels (29). The *E. coli* O157:H$^-$ strain E32511 and the *E. coli* K-12 strains C600(H19J) and C600(933W) were used as reference strains for *slt*-IIc, *slt*-I, and *slt*-II, respectively (25, 32). *slt*-IIe (synonymous with *slt*-IIv) sequences were detected by PCR with the primers BB1 (5′-CCC GGA TCC AAG AAG ATG TTT ATA G-3′) and BB2 (5′-CCC GAA TTC TCA GTT AAA CTT CAC C-3′) (36). Strain E57 (21) was taken as a standard for *slt*-IIe. The primers KS7 and BB1 contain a *Bam*HI restriction site (GGA TCC), and the primers KS8 and BB2 contain an *Eco*RI restriction site (GAA TTC). The 5′-CCC triplet was added to increase the distance between the blunt end of the amplification product and the beginning of the restriction enzyme-specific sequence. Endonuclease recognition sites were added to primer pairs, allowing subcloning of PCR products in pUC vectors for control of specificity of PCR products by *Taq* cycle sequencing.

Bacterial adherence tests. The HEp-2 adherence test for detection of cell-adhering and nonadhering *E. coli* strains was performed as described previously (7). The following *E. coli* strains served as positive controls for different adhesion types: E20513 (O111:H2) for localized adherence (7), DH5α(pSSS1) for diffuse adherence (9), and strain 17-2 for the EAggEC phenotype (2). Strain E20518 (O128:H2) served as a negative control for adherence to HEp-2 cells (7).

RESULTS

Detection of different SLT-II genotypes in SLTEC from animals. Representative strains of the different serotypes were selected from 208 SLTEC which were isolated from animals (5) and investigated for SLT genes by amplification of SLT-specific sequences. Forty SLTEC were investigated for *slt*-I, and 54 were investigated for *slt*-II. Thirty-eight (95.0%) of the 40 SLTEC which had hybridized with the SLT-I (VT1)-specific gene probe (5) were positive for *slt*-I. Two strains from cats (O6:K13:H1) which were formerly VT1 probe positive had lost the ability to produce SLT upon subculture and became negative for VT1 DNA hybridization and *slt*-I PCR.

The 54 SLTEC which had hybridized with the SLT-II (VT2) gene probe (5) were heterogeneous with regard to their *slt*-II genes (Table 2). Only three (8.8%) of the 34 VT2 probe-positive strains from goats and sheep were positive for *slt*-II, but the others were negative for all: *slt*-II, *slt*-IIc, and *slt*-IIe. These findings were in contrast to those obtained with VT2-hybridizing strains from cattle. With cattle, 13 (86.7%) of 15 VT2 strains were positive for *slt*-II, *slt*-IIc, or both and only 2 VT2 probe-positive strains were negative for all of the SLT-II types tested. The porcine SLT-II variant (*slt*-IIe) was detected only in three VT2 hybridization-positive strains from pigs but was not found in SLTEC originating from other animal species.

Adherence of animal SLTEC to HEp-2 cells and detection of genes associated with different patterns of adherence. Sixty (28.8%) of the 208 SLTEC showed diffuse adherence when tested with cultivated HEp-2 cells. The other 148 SLTEC did not adhere to HEp-2 cells. No patterns indicating local adherence to HEp-2 cells were observed with any of the strains. This observation corresponded to the finding that none of the 208 SLTEC carried DNA sequences specific for the enteropathogenic *E. coli* (EPEC) adherence factor (EAF), which was tested in colony blot DNA hybridizations with the pJPN16-derived *eaf*-specific gene probe. The 60 SLTEC showing HEp-2 cell adherence were additionally tested for the presence of *daa* sequences specific for diffuse adherence (pSLM852) and for EAggEC types (pCVD432). However, none of these strains reacted positively with the *daa*- or EAggEC-specific gene probe in colony blot DNA hybridizations.

Attaching and effacing *E. coli* (*eae*) sequences. Only 3 (1.4%) of the 208 SLTEC from animals were positive for *eae* sequences when tested by DNA-DNA hybridization with the *eae*-specific gene probe derived from plasmid pCVD434. The three *eae*-positive strains were fecal isolates from 3 sheep which were kept together in one flock of 20 animals. The *eae*-positive strains all belonged to serotype O119:H25 and were positive for *slt*-I but negative for *slt*-II, *slt*-IIc, and *slt*-IIe. All 205 other SLTEC were negative for *eae* DNA sequences.

TABLE 2. Detection of SLT-II and different SLT genotypes in animal SLTEC which are positive for DNA hybridization with a VT2 DNA probe

Animal species	No. of SLTEC isolates:				
	Positive with the VT2 DNA probe[a]	Positive for *slt*-II	Positive for *slt*-IIc	Positive for *slt*-IIe	Negative for *slt*-II, *slt*-IIc and *slt*-IIe
Cattle[b]	15	9	6	0	2
Sheep	21	1	0	0	20
Goats	12	2	0	0	10
Pigs	3	0	0	3	0
Dogs	3	2	0	0	1
Total	54	14	6	3	33

[a] From reference 5.

[b] Some SLTEC from cattle were found to carry both the *slt*-II and *slt*-IIc genes.

TABLE 3. Association of the Ehly phenotype in animal SLTEC with DNA hybridization to the EHEC plasmid probe (pCVD419)

Animal species	Total no. of SLTEC	No. of SLTEC hybridizing to pCVD419 (no. showing the Ehly phenotype)	No. of SLTEC showing the Ehly phenotype (no. pCVD419 positive)
Cattle	33	20 (19)	19 (19)
Sheep	113	70 (69)	71 (70)
Goats	40	35 (35)	36 (35)
Pigs	9	3 (0)	0 (0)
Cats	10	0 (0)	0 (0)
Dogs	3	2 (2)	2 (2)
Total	208	130 (125)	128 (126)

Association of hemolytic activity of SLTEC with hybridization to gene probes derived from pCVD419 (EHEC plasmid) and pEO40 (EHEC hemolysin). The Ehly phenotype was observed in 128 of the 208 animal SLTEC strains (5). The Ehly phenotype was closely associated with hybridization to the EHEC plasmid gene probe derived from pCVD419 (Table 3). This gene probe consists of a 3.4-kb *Hin*dIII DNA fragment cloned from the 90-kb plasmid of the O157:H7 strain EDL933. By DNA hybridization, the *Hin*dIII pCVD419 DNA fragment was shown to be identical to the 3.4-kb *Hin*dIII fragment of the EHEC hemolysin recombinant plasmid pEO40 and was found to encode EHEC hemolysin-specific sequences (30, 31; also this work). Consequently, all strains which were positive with the pCVD419 DNA probe also reacted with the 11.9-kb *Bam*HI fragment of pEO40 which was used as an EHEC hemolysin-specific DNA probe. These findings indicated that 98.4% of all Ehly-positive SLTEC from animals encode EHEC hemolysin, which appears to be responsible for the Ehly phenotype elicited by these strains.

Only 30 (14.4%) of the 208 SLTEC reacted with bacteriophage-derived DNA probes specific for Ehly1 and Ehly2 (7, 34) (Table 4). Of these, 27 (90.0%) were positive for the EHEC hemolysin, indicating that these strains might encode more than one type of hemolysin.

DISCUSSION

We have previously shown that SLTEC originating from different species of healthy animals are very heterogeneous with regard to their serotypes. Moreover, most SLTEC serotypes were found to be associated with only one animal species, indicating that they might be specific for their hosts (5). In this work, we have examined serologically diverse animal SLTEC strains for their SLT genotypes and for other markers which are related to the virulence of *E. coli* for humans. The aim of this study was to characterize SLTEC from healthy animals for their virulence markers and thereby for possible relationships with known human pathogenic types of *E. coli*.

TABLE 4. DNA hybridization of animal SLTEC with Ehly1- and Ehly2-specific DNA probes

Animal species	No. of strains positive:				
	For SLT	With the Ehly1 DNA probe	With the Ehly2 DNA probe	With probes for Ehly1, Ehly2, or both	With Ehly probes and pCVD419
Cattle	33	2	0	2	2
Sheep	113	15	15	24	21
Goats	40	1	1	2	2
Pigs	9	1	0	1	1
Cats	10	0	0	0	0
Dogs	3	1	1	1	1
Total	208	20	17	30	27

For *slt*-I, full correspondence between the results from DNA hybridization with the VT1 gene probe (750-bp *Hin*cII DNA fragment of phage H19) (39) and those from the *slt*-IB-specific PCR was found. This indicates that *slt*-I genes are homogeneous in these *E. coli* strains independent of their origin or serotype. Similar findings were made for *slt*-I in SLTEC which were from human clinical isolates (16, 27). In contrast to that, there was less association between hybridization with the VT2 probe (850-bp *Ava*I-*Pst*I DNA fragment of phage 32511) (39) and *slt*-IIB PCR-positive strains in animal SLTEC. The VT2 gene probe was shown to react with a variety of different SLT-II genotypes, including *slt*-II, *slt*-IIc, *slt*-IIe, and others (5, 33; also this work). Interestingly, the distribution of SLT-II genotypes in animal SLTEC could be directly associated with the hosts. *slt*-II and *slt*-IIc were associated with SLTEC from cattle (86.7%) and dogs (66.6%) but not with SLTEC from sheep (5%), goats (16.6%), or pigs (<33.3%). *slt*-IIe was detected only in SLTEC from pigs. In contrast, one or more types of SLT-II genetically different from *slt*-II, *slt*-IIc, and *slt*-IIe appear to be present in most VT2 hybridization-positive strains from sheep and goats. Comparison of SLT-II genotypes shows that SLTEC from cattle and dogs were most similar to SLTEC O157 strains which are important human pathogens (1, 26, 29, 35).

About one-third of the 208 SLTEC from animals showed diffuse adherence to HEp-2 cells. The HEp-2 cell adherence assay has been shown to be suitable for detection of adhering human EPEC and EAggEC types (12). However, none of the HEp-2 cell-adhering SLTEC from animals carried *eaf* sequences (coding for localized adherence in human EPEC) (24) or *daa* sequences (coding for diffuse adherence in diarrheagenic *E. coli*) (9). These results indicate that the adherence factors of animal SLTEC are different from those found in human enteral pathogenic *E. coli*. The HEp-2 cell-adhering animal SLTEC were also found to be unrelated to human EAggEC by their adhesion patterns and by lack of hybridization with the EAggEC-specific gene probe (2).

Intimate adherence to intestinal epithelial cells and effacing of microvilli are major virulence determinants of human pathogenic SLTEC and EPEC strains (15, 23). The *eae* genes were shown to be essential for intimate adherence and are present in most human SLTEC and EPEC strains (1, 3, 15, 37). In contrast to that, only 3 of 208 (1.4%) animal SLTEC investigated in this study were positive for *eae*, and all 3 were closely related. The very low frequency of *eae*-positive SLTEC that we have found in healthy animals corresponds to recently published data (1, 19). *eae* was found to be frequent in SLTEC from diarrheagenic cattle and humans, whereas *eae*-negative SLTEC were more frequent in healthy cattle (1, 19). On the basis of our findings, we can extend the latter observation to SLTEC which were from healthy animals of different species such as cattle, sheep, goats, pigs, and dogs.

The Ehly phenotype was found in 128 of the 208 animal SLTEC (5). The Ehly phenotype is detectable only on washed (serum-free) erythrocytes, and erythrocyte lysis becomes visible only after overnight incubation of bacteria on blood agar plates (5, 6). We have recently reported that the Ehly phenotype in SLTEC O157 strains is generated by a new type of hemolysin called EHEC hemolysin (30, 31). EHEC hemolysin is plasmid encoded in *E. coli* O157, and the pCVD419 EHEC plasmid DNA probe was shown to encode a part of the EHEC hemolysin-specific sequence (30). In this work we could dem-

onstrate that almost all animal SLTEC showing the Ehly phenotype were positive for EHEC hemolysin-specific DNA sequences. This indicates that the EHEC hemolysin is present in practically all SLTEC with an Ehly phenotype independent of their origins or serological classifications. In contrast to that, only a smaller part of the Ehly-positive SLTEC were positive with gene probes specific for the bacteriophage-associated Ehly1 and Ehly2. Ehly1 and Ehly2 sequences derive from DNA of two different temperate bacteriophages present in *E. coli* O26 strains (4, 7, 34). In human *E. coli*, Ehly1 and Ehly2 sequences were associated with serogroup O26 strains (7, 34). In animal SLTEC, Ehly1 and Ehly2 sequences were frequent in only some serotypes, such as O119:H25, O128:H2, and O146:H21 (data not shown). These results indicate that some of the Ehly-positive SLTEC may encode more than one type of hemolysin.

By analysis of a large number of SLTEC from healthy animals for their serotypes, phenotypical traits, and virulence markers, a very heterogeneous group of *E. coli* was revealed (5; also this work). Despite many differences between individual strains, the absence of *eae* genes was found to be characteristic for this group of SLTEC. It was previously reported that *eae* may be required for the expression of full virulence of SLTEC for humans and calves (1, 19). Considering this, the *eae*-negative SLTEC types would present a more minor health hazard for humans than the classical EHEC types. This idea is supported by the fact that despite the high incidence of SLTEC-positive animals, human infections due to contact and transmission are rare.

Many SLTEC from healthy animals appear to be limited in their host range (5, 14, 33, 36; also this work). Most of these strains might not be able to colonize the human host efficiently, since no colonization factors which play a role in human infections with SLTEC, EPEC, or EAggEC were found in these strains. At present, the exact cause of the pathology of SLTEC infections in humans is not completely understood. Apart from SLTs and *eae* determinants, other factors might contribute to the virulence of SLTEC for humans. An analysis of *E. coli* from animals exclusively for SLTs has a poor positive predictive value for human pathogenicity.

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