Detection of Toxin Genes in *Escherichia coli* Isolated from Normal Dogs and Dogs with Diarrhea

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ABSTRACT

The etiology of acute, nonviral diarrhea in dogs is poorly understood. Enterotoxigenic and verotoxigenic *Escherichia coli* are causal agents of diarrhea in humans, pigs, and cattle, but the association of these toxigenic *E. coli* with diarrhea in dogs has not been explored to a significant extent. In this study, DNA hybridization and PCR amplification were used to identify the frequency with which the genes for *E. coli* enterotoxins (STap, STb, and LTI) and verotoxins (VT1 and VT2) occur in association with diarrhea in dogs. Genes for VT1 (8.9%), VT2 (22.2%), STa (26.7%), and STb (4.4%) were identified in *E. coli* cultured from feces of 20 of 45 dogs (44.4%) with diarrhea. Genes for VT2, STa, and STb were not identified in feces from normal dogs. Genes for VT1 were observed in similar proportions in fecal samples from diarrheic (8.9%) and normal (12.3%) dogs. Heat labile enterotoxin (LTI) was not detected in fecal samples from either diarrheic or normal dogs. Our results suggest that heat stable enterotoxins and VT2 may be causally associated with diarrhea in dogs. Dogs appear to be able to carry VT1-producing *E. coli* without showing overt signs of disease.

RÉSUMÉ

Chez le chien, l'étiologie des cas de diarrhée aiguës d'origine nonvirale est peu connue. Chez les humains, les porcs et les bovins les isolats entérotoxinogéniques et vérotoxinogéniques d'*Escherichia coli* sont reconnus comme agent causal de diarrhée, par contre, chez le chien, l'association entre ces isolats toxinogéniques d'*E. coli* et la diarrhée n'a pas encore été élucidée ou confirmée. Des techniques d'hybridation d'ADN et d'amplification en chaîne par la polymérase ont été utilisées afin de déterminer la fréquence avec laquelle les gènes pour les entérotoxines STap, STb et LTI ainsi que pour les vérotoxines VT1 et VT2 sont retrouvés chez les isolats d'*E. coli* provenant de chiens avec de la diarrhée. À partir des isolats d'*E. coli* provenant des fèces de 20 des 45 chiens (44,4 %) avec de la diarrhée, il a été possible de mettre en évidence le gène pour VT1 chez 8,9 %, pour VT2 chez 22,2 %, pour STa chez 26,7 % et pour STb chez 4,4 % des isolats. Les gènes pour VT2, STa et STb n'ont pas été identifiés dans les fèces provenant d'animaux cliniquement normaux. Les gènes pour VT1 étaient présents à des fréquences similaires autant dans les matières fécales provenant d'animaux diarrhéiques (8,9 %) que dans celles d'animaux normaux (12,3 %). L'entérotoxine thermolabile n'a pas été détectée, que ce soit dans les matières fécales de chiens normaux ou diarrhéiques. Les résultats obtenus suggèrent que les entérotoxines thermostables et VT2 pourraient être associées à la diarrhée chez les chiens. De plus, les chiens semblent pouvoir être des porteurs asymptomatiques d'*E. coli* producteur de VT1.

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INTRODUCTION

Acute diarrhea in dogs is a commonly encountered disorder, but the etiology is poorly understood. Viral diarrhea associated with parvovirus and coronavirus has been studied extensively and is largely controlled through vaccination (1), but the role that bacteria play in the etiology of acute diarrhea in dogs is not well understood. Several bacteria, including *Campylobacter* spp. (2), *Salmonella* spp. (3), *Yersinia pseudotuberculosis*, *Y. enterocolitica* (4), *Clostridium perfringens* (5), and *Enterococcus durans* (6) have been associated with diarrheic illnesses in dogs. However, the frequency of isolation of these organisms is very low.

Toxin-producing *E. coli* strains are causal agents of diarrhea in humans, pigs, and cattle (7,8). Verotoxins (VTs) or Shiga-like toxins (SLTs) produced by verotoxigenic *E. coli* (VTEC) are implicated in diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans, diarrhea in calves and edema disease in pigs (7,8). Enterotoxigenic *E. coli* (ETEC) produce heat stable (STa, STb) and heat labile (LT) enterotoxins that are implicated in diarrhea in pigs (STap, STb, and LTI), calves (STap), and humans (STa and LTI) (8–10). Enterotoxins and VTs have also been detected in *E. coli* isolated from rabbits, goats, camels, poultry, cats, and dogs (11–15). However, there have been few reports on the association of toxigenic *E. coli* with diarrhea in dogs (12,14).

Bioassays for *E. coli* enterotoxins and VTs have been available for some considerable time (16–20), but these

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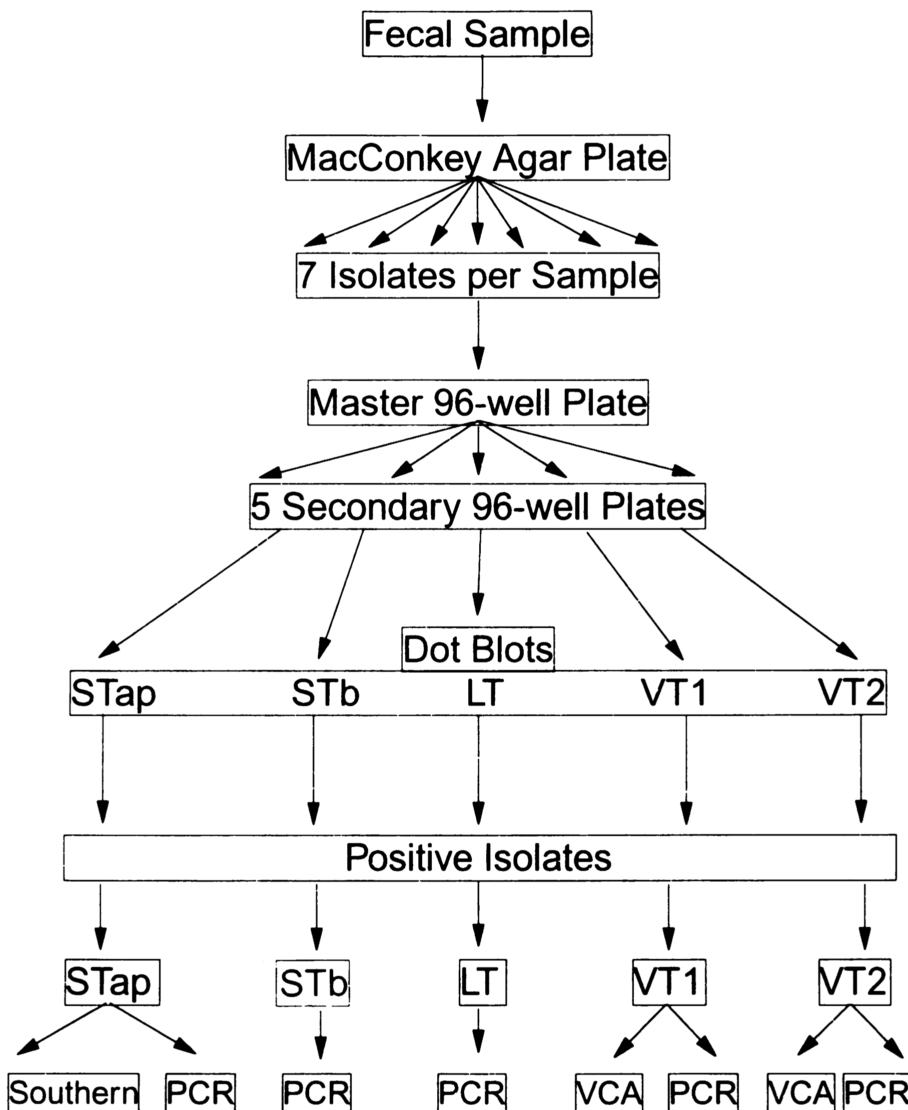

Fig. 1. Diagram of sample processing scheme.

are generally unsuitable for diagnostic laboratories and may involve the use of animals. More rapid and convenient immunological methods have been developed (21), but the unavailability of specific antisera and a lack of sensitivity frequently limit their use. Molecular biology techniques to screen for genes which encode these protein toxins are sensitive, rapid, accurate, and readily applied to large numbers of samples. Gene probes for LT, STap, STb, VT1, and VT2 have been used in hybridization procedures for the detection of toxin encoding genes in *E. coli* isolates (22). The polymerase chain reaction (PCR) method for amplifying specific target DNA sequences is an even more sensitive procedure for detecting specific genes. The availability of sequences for the genes encoding LT, STap, STb, VT1, and VT2 (22–26) permits the design of specific oligonucleotide primers and the development of protocols for the amplification and detection of these sequences by PCR.

The objective of this study was to determine whether *E. coli* carrying the genes for enterotoxins or verotoxins were associated with acute diarrhea in dogs. Hybridization and PCR techniques were used to identify the frequency of occurrence of genes for *E. coli* enterotoxins and verotoxins in association with diarrhea in dogs.

MATERIALS AND METHODS

FECAL SAMPLES

Fecal samples were collected from 58 dogs that were presented to the Veterinary Teaching Hospital (VTH) of the Ontario Veterinary College (OVC) with acute diarrhea, or had developed diarrhea in hospital following admission for medical or surgical problems not related to the gastrointestinal system. Feces from 95 normal dogs were collected from animals that had entered the VTH for outpatient procedures such as vaccinations, or the feces were collected by their owners at home. No dogs received antimicrobial drugs prior to sampling.

ISOLATION OF *E. coli*

Approximately 10 g of feces were collected in plastic containers and stored at −70°C until cultured on MacConkey agar (Difco, Detroit, Michigan) plates. In 9 diarrhea samples and 29 control samples, there was no growth on MacConkey agar plates. In 4 diarrhea and 9 control samples, only nonlactose fermenting colonies which were not *E. coli* were recovered. Seven well-isolated lactose fermenting colonies per fecal sample were selected from plate cultures of the remaining 45 diarrhea and 57 normal samples. These isolates were confirmed as *E. coli* by indole (+), H_2S (−), MR (+), urea (−), citrate (−) biochemical reactions, and were subcultured on Trypticase Soy Agar (Difco) slopes for storage and testing for toxin genes.

PREPARATION OF DNA PROBES

Probes for the various toxin genes were obtained from recombinant plasmids. The probe for VT1 was a 625 base pair (bp) *Hinf*I fragment from plasmid pJLB28 (25) and the probe for VT2 was a 900 bp *Acc*I-*Pst*I fragment of the plasmid pCG6 (26). The STap probe was a 157 bp *Hinf*I fragment of the recombinant plasmid pVWIST; the STb probe was a 460 bp *Eco*RI-*Bam*HI fragment of the plasmid pVWIIST; and the LT probe was a 750 bp *Hind*III fragment derived from pVWLT (22). The STap and STb probes were cloned from isolates of porcine origin (22). Following digestion of the plasmids with the appropriate restriction endonucleases, the DNA was subjected to electrophoresis through agarose (VT1, VT2, Stb, and LTI) or polyacrylamide (STap) gels (27). Probe DNA fragments of appropriate sizes were cut from the gels, electroeluted (Tyler Research Instruments, Edmonton,

Alberta) and labelled with ^{32}P (^{32}P dCTP from ICN, Montreal, Quebec) by the random primed method (Boehringer Mannheim, Mississaugua, Ontario).

SCREENING BY DOT-BLOT HYBRIDIZATION

Dot-blot DNA hybridization was used to screen for STap, STb, LTI, VT1, and VT2 genes in 7 bacterial isolates from each of the 45 diarrhea and 57 normal fecal samples (Fig. 1). The 714 *E. coli* isolates from 102 fecal samples were screened for the 5 different toxin genes by a modification of the dot-blot DNA hybridization procedure described by Hammermueller et al. (28). Individual wells of 96 well plates containing 200 μL of Luria-Bertani medium (LB) (27), or Brain Heart Infusion broth (BHI) (Difco), were inoculated from individual TSA slopes and incubated overnight at 37°C. Nontoxigenic *E. coli* K12 strain 711 (F^{-}(λ), *lac*-28, *his*-51, *trp*-30, *pro*C23, Phe, Nal) was included as a negative control, and *E. coli* strains H30 (VT1), 933W (VT2), P155 (LTI), B41 (Stap), and P3 (STb) were used as positive controls. These plates were designated as master plates and stored at 4°C until used, within 7 d, to generate secondary plates.

Five secondary 96 well plates, 1 for each of the 5 toxin genes, were prepared from each master plate (Fig. 1). A 100 μL volume of LB or BHI was dispensed into each well of the secondary plate, wells were inoculated with a 20 μL aliquot from the corresponding well of the master plate, incubated overnight at 37°C and stored at 4°C for no more than 24 h. Cells were lysed and DNA was solubilized and denatured by hot alkali treatment. Sodium hydroxide was added to each well of the secondary plates to a final concentration of 0.4 N. Plates were incubated in an 80°C oven for 30 min. The cell lysates were transferred to prewetted Hybond (Amersham, Oakville, Ontario) nylon membranes by a 96-well dot-blot vacuum filtration manifold (Bethesda Research Laboratories, Burlington, Ontario) under low vacuum, and blots were then washed, air dried, and baked at 80°C for 2 h in a vacuum oven. Membranes were then sealed in plastic bags and stored at 4°C.

Prehybridization was performed by incubating membranes (8 cm × 12 cm) in 15 mL each of prehybridization buffer (6× SSPE (52.59 g/L NaCl, 8.82 g/L NaH_2PO_4, 2.22 g/L ethylene diamine tetraacetate, pH7.4), 10× Denhardt's solution, 1% SDS (sodium dodecyl sulfate), and 50 μg/ mL fragmented denatured salmon testes DNA) in sealed polyethylene bags for 12 h at 42°C with shaking. The prehybridization buffer was removed and membranes were incubated with 15 mL each of hybridization buffer (6× SSPE, 1% SDS, 50% formamide (pH 7.0), and 50 μg/mL fragmented denatured salmon testes DNA), and with the appropriate ^{32}P labelled, denatured probe DNA, for 12 h at 42°C with shaking. After hybridization, blots were washed twice for 15 min each in 6× SSPE, 0.5% SDS and twice for 15 min each in 1× SSPE, 1% SDS at 42°C. Blots were then exposed to Kodak X-Omat-AR X-ray film (M & S Xray Services, Guelph, Ontario).

TABLE I. Sequences of oligonucleotides used as primers in polymerase chain reaction amplifications

Target gene	Primer sequence 5′ > 3′	Product size	Source
STap	TCTTTCCCCTCTTTTAGTCAG ACAGGCAGGATTACAACAAAG	166 bp	Woodward (1992)
STb	AAGATCTGTGTGAACATTATAG AAATAATGGTTGCAGCAA	116 bp	This study
LTI	ATTTACGGCGTTACTATCCTC TTTTGGTCTCGGTCAGATATG	280 bp	Woodward (1992)
VT1	ACCCTGTAACGAAGTTTGCG ATCTCATGCGACTACTTGAC	140 bp	Pollard (1990b)
VT2	TTAACCACACCCACGGCAGT GCTCTGGATGCATCTCTGGT	346 bp	Johnson (1990)
VT-Generic	GAGCGAAATAATTTATATGT CGAAATCCCCTCTGTATTTGCC	322 bp	Read (1990)

CONFIRMATORY PROCEDURES

Both strongly and weakly dot-blot positive isolates, as well as at least 10 dot-blot negative isolates per toxin, were subjected to confirmatory procedures. Approximately half the isolates died during storage, and confirmatory tests for those isolates were restricted to PCR amplification of DNA from dead cultures.

Cytotoxicity assay for verotoxins

The Vero cell cytotoxicity assay (VCA) was used as a confirmatory procedure for the presence of VT1 and VT2 genes (Fig. 1) in viable *E. coli* isolates. VCAs were performed essentially as described by MacLeod and Gyles (29). Bacteria were incubated overnight in 3 mL of LB at 37°C with shaking. Cultures were centrifuged at 12 000 × *g* for 5 min and two-fold serial dilutions of the supernates were assayed. Supernates with greater than 50% cytotoxicity for Vero cells in the one-in-four dilution were considered positive.

Southern hybridization

Southern hybridization was used as an inexpensive rapid confirmatory procedure for the presence of genes coding for STap in viable *E. coli* isolates (Fig. 1). Plasmid DNA for Southern hybridization was purified by the alkaline lysis miniprep method (27), electrophoresed through agarose gels (1%) and transferred to Hybond nylon membranes by the procedure for capillary transfer under neutral conditions detailed by Sambrook et al. (27). Hybridization with the relevant ^{32}P labelled, denatured probe was performed using the conditions previously described for dot-blots.

Polymerase chain reaction amplification

Polymerase chain reaction amplification (PCR) was used as a confirmatory procedure for the presence of STb and LT genes (Fig. 1). PCR was also used to confirm the presence of verotoxin and STap genes in isolates that did not survive storage. The oligonucleotide primers for PCR are shown in Table I. Primers for VT1, VT2, and generic primers (VTG) which amplify a conserved region of the VT genes were used with VT dot blot positive samples. The generic primers were used to eliminate false negatives and reduce the number of samples for the VT1 and VT2 primers. Primers for VT1, VT2, VTG LT, and STap were synthesized according to published PCR oligonucleotide primer

TABLE II. Conditions[a] used for polymerase chain reaction amplificafions

Target Gene	Cycles	$MgCl_2$ mM	Denature		Anneal		Extend	
			Temp.[b]	Time[c]	Temp.[b]	Time[c]	Temp.[b]	Time[c]
STap	30	1.5	94	1.5	59	1.5	72	2.0
STb	35	3.0	94	1.0	49	1.0	72	1.5
LTI	30	1.5	94	1.5	59	1.5	72	2.0
VT1	35	1.5	94	1.0	55	1.0	72	1.0
VT2	35	1.5	94	1.0	49	1.0	72	1.0
VT-G	35	1.5	94	1.0	49	1.0	72	1.0

[a] All 25 μl reaction mixtures contained 50 mM KCl, 10 mM TrisHCl (pH 9), 0.1 % Triton X-100, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 5 μL sample DNA, and 1.25 U of thermostable DNA polymerase (Promega). The reaction mixtures were overlaid with an equal volume of mineral oil. The reaction was started by the addition of 1.25 U of Taq DNA polymerase to samples that were preheated to 94°C for 2 min. A final extension of 5 min at 72°C followed the last cycle

[b] Temperature in °C

[c] Time in min

sequences (30–33), or designed from published gene sequences for the STb primers (23,24). PCR reaction conditions and cycling parameters (Table II) were those previously published (30–34) or were determined by optimization experiments.

Samples were prepared for PCR from 1 mL BHI broth cultures for viable cells. If the slant stored cultures were no longer viable, they were rinsed with 5 mL BHI and the washed cells were used for PCR. Sample preparation was performed with the Insta-gene kit according to the manufacturer's instructions (BioRad, Mississauga, Ontario).

EXPERIMENTAL DESIGN

Dot-blot DNA hybridization was used to screen for VT1, VT2, STap, STb, and LTI genes in 7 *E. coli* isolates from each of 102 fecal samples. Confirmatory procedures were undertaken with both strongly and weakly dot-blot positive isolates, as well as with at least 10 dot-blot negative isolates per toxin. Vero cell cytotoxicity assays were used as confirmatory procedures for viable isolates. PCR amplification was used as a confirmatory test for isolates that died during storage and for all isolates that were positive for STb or LT genes. Southern hybridization of purified plasmid DNA was used as a confirmatory procedure for STap.

RESULTS

Vero cell assay results corresponded with PCR results in each case in which both techniques were used on the same culture. Southern hybridization results corresponded with PCR results in each case where both techniques were used on the same isolate. No sample that was negative on dot blot hybridization tested positive with any of the confirmatory procedures.

Of 64 fecal samples with dot-blot positive isolates, 35 were confirmed as having *E. coli* carrying toxin genes. Of the 29 false positives, 20 were only weakly positive on dot-blot hybridization. In 9 of the 35 fecal samples with confirmed positive isolates, only 1 of the *E. coli* isolates from the same sample carried a toxin gene. The other 26 fecal samples resulted in 2 or more toxin positive *E. coli* isolates per fecal sample. In no fecal sample in which the STb gene was detected, was there more than 1 positive isolate per fecal sample.

Genes for VTs or heat stable enterotoxins were identified in *E. coli* cultured from the feces of 20 of 45 dogs (44.4%) with diarrhea. Specifically, genes for VT1, VT2, STap, and STb were present in 4 (8.9%), 10 (22.2%), 12 (26.7%), and 2 (4.4%) diarrhea samples, respectively (Table III). Toxin genes for VT2, Stap, and STb were not detected in any of the *E. coli* cultured from normal dogs. Genes for VT1 were observed in similar proportions in fecal samples from dogs with diarrhea (8.9%) and normal dogs (12.3%). Interestingly, in each of the fecal samples from dogs with diarrhea where VT1 positive cultures were isolated, either the VT1 positive isolate itself, or other isolates from the same fecal sample, also contained genes for VT2, STap, or STb. VT1 positive isolates from normal dogs did not carry genes for these other toxins, nor did other isolates from the same fecal sample. Six *E. coli* isolates (13%) from diarrhea samples had genes for more than 1 toxin. Toxin gene combinations occurred as follows: VT1 + VT2 (2 isolates), VT1 + VT2 + STap (1 isolate), VT1 + STap (1 isolate), VT2 + STb (1 isolate) and VT2 + STap + STb (1 isolate). Heat labile enterotoxin (LT) genes were not detected in any isolate.

DISCUSSION

The cloning and sequencing of the genes for *E. coli* verotoxins and enterotoxins (STa, STb, LT) has made it possible to detect toxin-producing strains by DNA hybridization and PCR techniques, rather than by elaborate and lengthy bioassays. The 96-well dot-blot DNA hybridization technique used in this study was effective as a preliminary screening tool for *E. coli* carrying genes for enterotoxins or VTs. False-positives, but not false-negatives were obtained. Lysis of the cultures in stationary phase was expected to minimize the impact of cell numbers on dot blot results; however, false-positives results did occur. Due to the variability in cell numbers, background hybridization was inconsistent. Since dot blot hybridization was used as a screening procedure to minimize the numbers of samples for the more expensive and time consuming confirmatory procedures, any sample which showed even slightly more hybridization than the negative control was subjected to further testing. This approach was intended to minimize the risk of false-negatives, but did lead to a number of false-positives, likely resulting from variable cell, DNA and plasmid concentrations.

The association between *E. coli* enterotoxins and diarrheal disease has been well established in adult humans, pigs, and calves (8,40,41) and it has been suggested that enterotoxigenic *E. coli* may also be involved in the etiology of diarrhea in dogs (12,14). Olson et al. (12) found LT and/or ST in *E. coli* from 4.1% of dogs with diarrhea. Drolet et al. reported the presence of STap and STb genes in 1 *E. coli* isolate associated with diarrhea in a dog. In the study reported here, we detected the genes

for STap and STb in 26.7% and 4.4% respectively, of the *E. coli* recovered from stools of dogs with diarrhea, but not in *E. coli* recovered from stools of normal dogs. Wasteson et al. (14) also isolated strains of ST-producing *E. coli* from stools of diarrheic dogs. In both the present study and the investigation by Wasteson et al. (14), *E. coli* carrying the genes for LT were not detected in samples from either diarrheic or normal dogs.

Verotoxin-producing *E. coli* have been convincingly linked to a group of illnesses encompassing watery diarrhea, bloody diarrhea, and hemolytic uremic syndrome in humans (VT1 and VT2) (7), edema disease in pigs (VTe) (42–44), and diarrhea in calves (VT1 and VT2) (13,22). Abaas et al. (15) observed that *E. coli* isolated from diarrheic cats produced VT more frequently and at higher titers than did strains isolated from healthy cats.

Our study suggests that a possible association may also exist for VT2 with diarrhea in dogs. We observed that *E. coli* producing VT2 were present in feces from diarrheic (22%), but not normal dogs, whereas VT1-producing *E. coli* were isolated in similar proportions from diarrheal (8.9%), and normal (12.3%) feces. Interestingly, in diarrheic dogs in which VT1-producing *E. coli* were isolated, genes for STap, STb, or VT2 were also present, either in the same isolate, or in different isolates, from the same fecal sample. No other toxin genes were detected in isolates from feces of normal dogs from which VT1-producing *E. coli* colonies were isolated. It would be interesting to use a larger population of dogs from various geographical areas to determine whether similar observations could be made.

Other researchers have shown that VT-producing *E. coli* may be present in cattle and pigs without causing overt disease (43,45–48). There is evidence that, in farm animals, predisposing factors such as intensive husbandry, change of temperature, feed composition and consistency, immune status, the presence of other infectious agents, and susceptibility of the animal, may determine whether or not infection is followed by disease (43,46,49).

In view of previous associations of VTs and *E. coli* enterotoxins with

TABLE III. Frequency of occurrence of toxin genes in *E. coli* isolates from diarrheal and normal dog fecal samples

Toxin Gene	% Positive	
	Diarrheal $n = 45$	Normal $n = 57$
VT1	8.9	12.3
VT2	22.2	0
STap	26.7	0
STb	4.4	0
LTI	0	0

diarrhea in other animals, it is likely that *E. coli* which produce these protein toxins may be involved in diarrhea in dogs. It is interesting that a group of investigators reported a drop in morbidity from diarrheic illness from 83.5% to 6.5% in a kennel in which autologous *E. coli* bacterins were used (50).

Human infection with VT-producing *E. coli* has frequently been associated with consumption of contaminated beef or dairy products, which suggests that cattle might act as a reservoir for human infections (51). There have also been reports of the isolation of these organisms from retail pork and lamb (52). It would be useful to determine if dogs are exposed to VT-producing *E. coli* through the feeding of table scraps or uncooked meat. In view of the known occurrence of VTEC transmission to household contacts of infected children (53), the potential for dog to human transmission is an issue that may well merit investigation.

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