SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Unlimited
AD-A224 107 S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: USA Med Comp,AFRIMS; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION: Walter Reed Army Institute of Research
6c. ADDRESS (City, State, and ZIP Code): APO SF 96346-5000	7b. ADDRESS (City, State, and ZIP Code): WRAMC, Washington, D.C. 20307
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: US Arny R&D Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code): Ft. Detrick Frederick, MD. 21701	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
Shiga-Like-Toxin-Producing Escherichia Coli in Retail Meats and Cattle in Thailand

12. PERSONAL AUTHOR(S)
Suthienkul O., Brown J.E.,Setiwatana J.,Tienthongdee S.,Sastravah S.,Echeverria P.

13a. TYPE OF REPORT	13b. TIME COVERED FROM ____ TO ____	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code) / 22c. OFFICE SYMBOL

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1990, p. 1135–1139 Vol. 56, No. 1
0099-2240/90/041135-05$02.00/0

Shiga-Like-Toxin-Producing *Escherichia coli* in Retail Meats and Cattle in Thailand

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Received 6 February 1989/Accepted 22 January 1990

Specific DNA probes were used to identify Shiga-like toxin I (SLT I)- and SLT II-producing *Escherichia coli* in vegetables, meats, cattle, and farm animals in Thailand. SLT-producing *E. coli* was isolated from 9% of market beef specimens, from 8 to 28% of fresh beef specimens at slaughterhouses, and from 11 to 84% of fecal specimens from cattle. Animals were frequently infected with several different SLT-producing *E. coli* types that hybridized with either the SLT I, SLT II, or both SLT probes. Of 119 SLT-producing *E. coli* isolates, 24% hybridized with the SLT I probe, 31% hybridized with the SLT II probe, and 44% hybridized with both SLT probes. The enterohemorrhagic *E. coli* plasmid probe hybridized with 64% (68 of 106) of SLT-producing *E. coli* isolates from food and cattle and with 8% (17 of 201) of *E. coli* isolates from pigs. No SLT-producing *E. coli* was detected in pigs. Seventy-six percent (26 of 34) of *E. coli* isolates that hybridized with the SLT II probe were cytotoxic to Vero but not to HeLa cells, suggesting that they produced the variant of SLT II. The high prevalence of SLT-producing *E. coli* in beef-producing animals suggests that exposure to animals and eating beef may pose a health risk for acquiring enterohemorrhagic *E. coli* infections in Thailand.

Some *Escherichia coli* strains produce cytotoxins similar to Shiga toxin of *Shigella dysenteriae* 1 (23) which are referred to as Shiga-like toxins (SLT) or Vero toxins. Toxin-producing strains associated with hemorrhagic colitis (30, 35) and hemolytic uremic syndrome (12, 30) have been referred to as enterohemorrhagic *E. coli* (EHEC) (14). In an outbreak of hemorrhagic colitis, *E. coli* O157:H7 was implicated (26) and was later shown to produce two cytotoxins, termed SLT I and SLT II (33). Hamburger was identified as the source of infection in that outbreak. Subsequently, SLT-producing *E. coli* was associated with unpasteurized milk (7; M. L. Martin et al., Letter, Lancet ii:1043, 1986) and retail beef (9). SLT-producing *E. coli* has been isolated from beef, from water buffalo calves with diarrhea (1, 2, 3, 20, 29; A. A. Borczyk et al., Letter, Lancet i:98, 1987; F. Orskov et al., Letter, Lancet ii:276, 1987), and from weanling pigs with edema disease (8, 11, 15, 32). *E. coli* associated with pig edema disease produce a variant of SLT II (SLT IIv) that is cytotoxic to Vero cells but not to HeLa cells (17).

DNA probes for genes coding for SLT I and SLT II have been constructed (22) and used to identify SLT-producing *E. coli* in children in Thailand (4). A third DNA probe is a cryptic fragment of a plasmid associated with adhesion of *E. coli* O157:H7 (14). This probe, referred to here as the EHEC plasmid probe, has been used to identify EHEC of a variety of serotypes isolated from patients with hemolytic uremic syndrome and hemorrhagic colitis.

The purpose of this study was to determine whether meats and meat-producing animals in Thailand are potential sources of EHEC. Using the specific DNA probes for SLT I and SLT II and the EHEC plasmid, we surveyed market foods, dairy cows, beef cattle, water buffalo, pigs, and chickens for the prevalence of SLT-producing *E. coli*. These strains were tested for production of cytotoxin to Vero and HeLa cells.

MATERIALS AND METHODS

Samples. (i) Food. Four types of food (e.g., pork, beef, chicken, and vegetables consumed without being cooked [lettuce, radishes, etc.]) were collected from each of 10 local markets in Bangkok, Thailand, as described previously (25). All food samples were collected in sterile plastic bags and processed within 2 h of collection. One gram of each type of food was homogenized in 2 ml of brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) with a sterile glass mortar and pestle.

(ii) Farm animals. Rectal swabs were collected from beef cattle, dairy cows, water buffalo, young pigs, and chickens at farms outside of Bangkok. Rectal swabs were transported in Cary-Blair medium and processed within 24 h of collection.

(iii) Slaughterhouses. Fecal specimens from cows and buffalo at slaughter were inoculated into Cary-Blair medium. Surfaces (10 by 10 cm) of freshly butchered meat (e.g., hanging carcasses, livers, hearts, lungs, and stomachs) from cows or buffalo and environmental samples (e.g., hands, cutting blocks, doors of cold rooms, saws, hooks, trays, and floors) were swabbed with sterile cotton swabs. Swabs were placed in 4 ml of Trypticase soy broth (BBL), kept on ice, and processed within 24 h of collection.

Bacteriology. Homogenates, rectal swabs, and overnight surface cultures of butchered-meat and environmental samples were cultured directly onto MacConkey agar. For all specimens, *E. coli* isolates were selected from the primary culture of each specimen in a nonbiased manner and stored on nutrient agar slants for colony hybridization testing.

DNA hybridization assays. The DNA probe for the gene of SLT I was a *Bam*HI 1,142-base-pair fragment isolated from recombinant plasmid pJN 37-19, and the probe for the gene

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of SLT II was a *SmaI-PstI* 842-base-pair fragment isolated from recombinant plasmid pNN100-18 (22). The EHEC plasmid probe was a 3.4-kilobase cryptic fragment cloned from an adherence factor plasmid of *E. coli* O157:H7 (14). Fragments were labeled with α-^{32}P-labeled deoxynucleotide triphosphates (Dupont, NEN Research Products, Boston, Mass.) by nick translation (16). Five isolates from each specimen were spotted onto MacConkey agar and incubated at 37°C overnight, except for rectal swabs from chickens, of which one isolate per animal was tested. Occasionally, inoculated broths were 10-fold serially diluted in sterile phosphate-buffered saline, spread onto MacConkey agar plates, and incubated to produce a lawn of approximately 300 lactose-positive colonies (30). Whatman no. 541 paper (Whatman, Inc., Clifton, N.J.) was then pressed evenly over *E. coli* growth and processed as previously described (18). Hybridization was carried out under stringent conditions (21). *E. coli* C600(933J) (encoding SLT I), C600(933W) (encoding SLT II), and CDC933 O157:H7 (encoding both cytotoxins) were used as positive controls. *E. coli* C600 was used as a negative control. Groups were compared by using the Fisher exact test.

Cytotoxicity assays. *E. coli* isolates that hybridized with the SLT I, SLT II, or EHEC plasmid probes were tested for cytotoxicity to Vero cells and HeLa cells (5, 13) with the following modifications. *E. coli* isolates were grown stationary in 1 ml of sterile Penassay broth at 37°C for 6 h. Samples (0.1 ml) were added to 15 ml of Penassay broth in 50-ml Erlenmeyer flasks and incubated with shaking (200 rpm) at 37°C for 18 h. Clarified supernatants were sterile filtered (pore size, 0.45 μm; Millipore Corp, Bedford, Mass), and five-fold serial dilutions were assayed. Cytotoxicity at a dilution greater than 1:5 was considered positive. *E. coli* O157:H7 933 was used as a positive control.

Serology. SLT I- and SLT II-producing *E. coli* were serogrouped with commercially prepared OK antisera (O157, O145, O111 O26, and O25; Difco Laboratories, Detroit, Mich., and Denka Seiken, Tokyo, Japan) by the slide agglutination test. Agglutinating isolates were boiled for 1 h and tested by tube agglutination. Selected isolates were O and H serotyped by standard methods (24) at the Statens Seruminstitut, Copenhagen, Denmark.

RESULTS

Isolation of SLT-producing *E. coli* from retail food. A total of 2,205 *E. coli* isolates were screened from 93 beef, 107 chicken, 111 pork, and 130 vegetable samples by colony hybridization with the SLT I, SLT II, and EHEC plasmid DNA probes (Table 1). The number of *E. coli* isolates from beef specimens that hybridized with the SLT probes and produced cytotoxin was significantly higher than that from chicken ($P = 0.013$), pork ($P = 0.012$), or vegetable ($P = 0.00076$) specimens. Of 10 specimens containing SLT-producing *E. coli*, 5 contained *E. coli* that hybridized with the SLT I probe, 3 contained *E. coli* that hybridized with the SLT II probe, and 2 contained *E. coli* that hybridized with both probes. Of the 10 strains from these 10 specimens, 6 hybridized with the EHEC plasmid probe. Of the beef and pork specimens, 3% (7 of 203) contained *E. coli* that hybridized with the EHEC plasmid probe but not with the SLT gene probes. One specimen contained both SLT-producing *E. coli* and *E. coli* which hybridized only with the EHEC plasmid probe. Six beef and pork specimens contained *E. coli* that hybridized only with the EHEC plasmid probe. The serotypes of several *E. coli* that hybridized with the SLT I, SLT II, or EHEC plasmid DNA probes are shown in Table 2.

TABLE 1. Market foods containing SLT-I- and SLT-II-producing *E. coli*

Food	No. of specimens[a]	No. (%) of specimens containing *E. coli* detected by probe(s) for:				
		Any SLT	SLT with EHEC plasmid	SLT I	SLT II	Both SLT I and II
Beef	93	8 (9)	5[b]	5	2	1
Chicken	107	1 (1)	1	0	0	1
Pork	111	1 (1)	0	0	1	0
Vegetables	130	0	0	0	0	0

[a] Five isolates were tested per specimen.
[b] Three specimens contained isolates which did not hybridize with EHEC plasmid probe.

Isolation of SLT-producing *E. coli* from farm animals. *E. coli* isolates from fecal specimens from a total of 145 beef cattle, water buffalo, and dairy cows at six locations were screened by colony hybridization with the SLT I, SLT II, and EHEC plasmid DNA probes (Table 3). SLT-producing *E. coli* was found in 11 to 60% of animals tested. The highest frequency of isolation occurred for cattle in holding pens at slaughterhouses, where animals were usually kept for less than 24 h. Often animals were infected with multiple *E. coli* strains of several SLT genotypes. For example, an animal might be infected with *E. coli* that hybridized with the SLT II probe and with other strains that hybridized with both SLT DNA probes. In addition, 2% (3 of 145) of the animals were infected with *E. coli* that hybridized with the EHEC plasmid DNA probe but did not hybridize with the SLT gene probes.

TABLE 2. *E. coli* serotypes isolated from food and animals that hybridized with the SLT I and II and EHEC plasmid DNA probes[a]

Toxin	EHEC plasmid	Serotype[b] in:	
		Food (type)	Cows
SLT I	+	O117:H8 (beef)	O11:H8[c]
	−	O22:H8 (beef)	Ont:H8[c]
SLT II	+	O149:H45 (beef)	Ont:H19
	−	Ont:H45 (pork)	
SLT I and II	+	O4:H21 (beef)	O113:H21
		O54:H21 (chicken)[c]	O116:H10
		O110:H16 (chicken)[c]	O25, O26, O68:H14
None	+	Ont:H7 (beef)[d]	O112ac(O149):H21
		O159:H7 (beef)[d]	
		O22,O101:H7 (pork)	
		O76:H7 (pork)	
		O11:H2 (pork)	
		O146:H10 (pork)	
		OC70/86:H49[e] (beef)	

[a] The genotypes of these isolates were determined by colony hybridization with cloned DNA probes for SLT I or II or for the EHEC adherence plasmid.
[b] From one to three isolates were serotyped per specimen. Fifteen isolates from six beef, five pork, and one chicken sample and 17 isolates from fecal specimens of six cows were analyzed. Ont, Untypeable.
[c] Same animal.
[d] Different specimens.
[e] OC70/86 is a provisional, not-yet-numbered O group.

TABLE 3. Cows and buffaloes infected with SLT-producing *E. coli*

Site[a]	Animal[b]	No. tested	No. (%) of animals infected with *E. coli* detected by probe(s) for:				
			Any SLT	SLT with EHEC plasmid	SLT I	SLT II	Both SLT I and II
1	C	45	5 (11)	5	1	1	3
2	C	21	5 (24)[c]	2	1	2	3
3	C and B	24	6 (25)	4	1	4	1
4	D	20	3 (15)[c]	2	2	1	1
5	C	10	4 (40)	3	0	1	3
6	B	25	15 (60)[c]	11[c]	1	6	11

[a] Sites 1 through 4 were farms. Sites 5 and 6 were holding pens at slaughterhouses.
[b] B, Water buffalo; C, beef cattle; D, dairy cows.
[c] Animals infected with more than one type of SLT- or EHEC-probe-positive *E. coli*.

Forty-three SLT-producing *E. coli* strains were isolated from these animals. Of these strains, 86% (37 of 43) hybridized with the DNA probe for SLT II and 51% (22 of 43) carried both genes. Isolates that hybridized with the SLT I gene probe alone accounted for 14% (6 of 43) of the SLT-producing *E. coli* isolates. Sixty-three percent (27 of 43) of the SLT-producing *E. coli* isolates hybridized with the EHEC plasmid probe. The serotypes of 17 *E. coli* isolates from six cows are shown in Table 2. A wide variety of serotypes was encountered, and no further isolates were serotyped.

SLT-producing *E. coli* was not detected in fecal specimens from either pigs or chickens. In 201 weanling pigs, none of the 1,005 isolates tested hybridized with either SLT gene probe. Isolates from 8% (17 of 201) of the animals hybridized with the EHEC plasmid probe. In one litter of 10 pigs in which edema disease had been observed 2 weeks earlier, SLT-producing *E. coli* was not detected even when 300 colonies were tested per animal. *E. coli* isolates from 134 chickens did not hybridize with either the SLT or the EHEC plasmid DNA probes.

SLT-producing *E. coli* in cattle at slaughter. To determine whether the introduction of SLT-producing *E. coli* into market meats occurred during the slaughtering process, two slaughterhouses were surveyed for the presence and distribution of SLT-producing *E. coli*. Fecal specimens from 80% of animals at slaughter contained SLT-producing *E. coli* at both slaughterhouses examined (Table 4). Again, multiple genotypes of SLT *E. coli* could be recovered from some animals and specimens. SLT-producing *E. coli* isolates were isolated from the surfaces of both hanging carcasses and internal organs. In addition, SLT-producing *E. coli* was isolated from the utensils, benches, and other sites. In each slaughterhouse, SLT-producing *E. coli* isolates were recovered from workers' hands. No cases of bloody diarrhea or hemorrhagic colitis were reported for these workers. Seventeen percent (7 of 40) of the animal fecal specimens and 1 of 25 carcasses sampled contained *E. coli* that hybridized with the EHEC plasmid probe but did not hybridized with the SLT gene probes.

TABLE 5. Cytotoxicity of *E. coli* isolates that hybridized with the SLT or EHEC plasmid DNA probes

DNA genotype[a]	No. of isolates tested	No. of isolates cytotoxic for:	
		Vero cells	HeLa cells
SLT I	21	21	21
SLT II	35	34	8
SLT I and II	50	50	50
EHEC plasmid (SLT negative)	34	1	1

[a] The genotypes of these isolates were determined by colony hybridization with the cloned DNA probes for SLT I or II or the EHEC plasmid probe. SLT I and II isolates hybridized with both SLT gene probes.

Sixty-six SLT-producing *E. coli* isolates were recovered from specimens at the slaughterhouses. Seventy-three percent (48 of 66) of the isolates hybridized with the SLT II gene probe. Of these, 44% (29 of 66) hybridized with both gene probes. Twenty-seven percent (18 of 66) hybridized only with the SLT I gene probe. Of 53 SLT-producing *E. coli* isolates examined with the EHEC plasmid probe, 40 (75%) hybridized with the probe. None of the SLT-producing *E. coli* isolated from either meats, animals, or slaughterhouse sources agglutinated with antisera specific for serogroups O157, O145, O111, O26, or O25.

Cytotoxicity assays. Culture supernatants or sonic lysates from 99% (105 of 106) of the isolates that hybridized with the SLT gene probes were cytotoxic to Vero cell monolayers (Table 5). One of thirty-four isolates that hybridized with the EHEC plasmid probe but did not hybridize with the SLT gene probes produced cytotoxic activity at a 1:5 dilution.

To determine whether any isolates produced the variant of SLT II (SLT IIv), culture supernatants and sonic lysates

TABLE 4. Specimens with SLT-producing *E. coli* at slaughterhouses

Slaughterhouse	Specimen source	Total no. of specimens	No. (%) of specimens containing *E. coli* detected by probe(s) for:				
			Any SLT	SLT with EHEC plasmid	SLT I	SLT II	Both SLT I and II
A	Stool	15	12 (80)[a]	9	3	5	8
	Hanging carcass	25	7 (28)[a]	4	1	5	2
	Internal organs	25	2 (8)[a]	1	1	1	1
	Environmental sources	12	1 (8)	1	0	1[b]	0
B	Stool	25	21 (84)[a]	20[a]	5	5	15
	Hanging carcass	25	4 (16)	NT[c]	3	0	1
	Internal organs	25	4 (16)	NT	0	2	2
	Environmental sources	16	5 (31)	NT	5[d]	0	0

[a] Specimens contained more than one genotype of SLT-producing *E. coli*.
[b] Hand of meat worker.
[c] NT, Not tested.
[d] Table, cold room door, and hands of workers.

were tested for cytotoxicity to HeLa cells. Seventy-six percent (26 of 34) of the *E. coli* isolates that hybridized with the SLT II gene probe alone were cytotoxic to Vero cells but not cytotoxic to HeLa cells. This result suggests that the isolates which were not cytotoxic to HeLa cells produced SLT IIv.

DISCUSSION

This study identified significantly more SLT-producing *E. coli* in beef than in other foods sampled in street markets in Bangkok. SLT-producing *E. coli* was recovered from a high proportion of fecal specimens from healthy cattle at farms and was isolated from freshly butchered meat as well as from fecal specimens from animals in slaughterhouses. Isolates which hybridized with the gene probes for SLT I, SLT II, or both were identified in these samples. These results suggest that the meat was contaminated with SLT-producing *E. coli* from fecal material during the slaughtering process.

Fifty-seven percent of the total SLT-producing *E. coli* isolates appeared to produce SLT type IIv. *E. coli* producing SLT type IIv has been associated with pig edema disease (17). The presence of cytotoxicity to Vero cells in extracts from strains associated with pig edema disease has been noted by others (8, 11, 15, 32). More recently, we have used DNA oligonucleotide probes to confirm these isolates as encoding an SLT II variant (6). No SLT-producing *E. coli* isolates were found among 200 weanling pigs. In contrast, *E. coli* producing SLT IIv was common in cattle and market beef samples in Thailand.

Cytotoxicity to Vero cells of bacterial extracts of *E. coli* isolated from calves has previously been noted both in the United Kingdom (29) and in Sri Lanka (20). In the United Kingdom, SLT-producing *E. coli* was isolated from 3% of calves with diarrhea. In Sri Lanka, SLT-producing *E. coli* was found in 28% of calves with diarrhea but in only 4% of healthy calves. SLT-producing *E. coli* isolates from cattle or water buffalo can also encode the type II heat-labile enterotoxin (28). In Thailand, 11 to 60% of the cattle and water buffalo were infected with SLT-producing *E. coli*. In south Asia, both animals serve as common meat sources.

Doyle and Schoeni (9) isolated *E. coli* O157:H7 from 4% of beef, 1.5% of pork, and 1.5% of poultry samples in Wisconsin and Alberta, Canada. However, 31% of beef specimens from Calgary, Alberta, contained *E. coli* O157:H7. In that study, toxin-producing isolates were present in low numbers, requiring the screening of 10 to 500 colonies per sample. The approach used in our study was much less sensitive, testing only five isolates per specimen. No attempt was made to determine the number of SLT-producing *E. coli* per gram of specimen. Nevertheless, in samples containing SLT-producing *E. coli*, all five colonies tested per specimen were often positive. Therefore, in Thailand, both the percentage of contaminated meat specimens and the number of SLT-producing *E. coli* in a specimen relative to the total coliforms present were much higher than in North America.

In this study, the EHEC plasmid probe, derived from the adherence plasmid of *E. coli* O157:H7, was not specific in detecting *E. coli* which produce SLT. From 2 to 17% of specimens contained isolates that hybridized with the EHEC plasmid probe but not with the SLT gene probes (data not shown). Conversely, 36% of the SLT-producing *E. coli* isolates tested did not hybridize with the EHEC plasmid probe. In previous studies, it has not been uncommon to detect *E. coli* isolates that hybridize with this probe but not with the SLT gene probes (2, 10, 27). In this report, the probe was referred to as the EHEC plasmid probe because of uncertainty about the specificity of this probe for detection of EHEC. While the probe hybridized with 80% of SLT-producing *E. coli* of various serotypes isolated from patients with hemorrhagic colitis and hemolytic uremic syndrome (14), use of the SLT gene probes to identify EHEC was more specific and allowed differentiation of SLT I-producing strains from SLT II-producing strains. Recently, oligonucleotide probes have been developed to differentiate SLT II-producing *E. coli* from those producing SLT IIv (6). The primary criterion in the definition of EHEC should remain the presence of SLT.

SLT production has most often been associated with serotypes O157:H7 and O26:H11 (14, 26, 30). However, a great amount of serological diversity has been observed among SLT-producing *E. coli* (1, 2, 3, 11, 19, 31). Most of the serotypes isolated in this study have not been implicated in enterohemorrhagic colitis or hemolytic uremic syndrome. *E. coli* O113:H21 has been associated with hemolytic uremic syndrome (12), and *E. coli* O4:H? was associated with hemorrhagic colitis (14). *E. coli* O113:H21 was isolated from bovine fecal specimens in Germany (2), *E. coli* O116:H? was isolated from calves in Sri Lanka (19), and *E. coli* O149:H? has been reported from calves with diarrhea in England (1, 31). SLT-producing *E. coli* O2:H7 has also been isolated from animals in Thailand (31). Because of serotypic diversity, serotyping appears to be of limited usefulness in the detection of SLT-producing *E. coli*.

The finding of relatively large numbers of SLT-producing *E. coli* was surprising since the percent isolation of these isolates from children with bloody diarrhea in Bangkok is low (4). SLT-producing *E. coli* isolates were identified in 4 of 54 children with bloody diarrhea from whom other enteric pathogens were not identified and from 3 of 50 children without diarrhea. In positive specimens, SLT-producing *E. coli* constituted only 0.3 to 4% of the 100 to 300 colonies on replica blots. EHEC is usually found in outbreaks of hemorrhagic colitis and hemolytic uremic syndrome. Although SLT-producing *E. coli* have been isolated from animals (1, 3, 11, 20, 29, 31), these *E. coli* occur infrequently as a cause of endemic diarrhea in both North America and Thailand. In areas with relatively high incidences of childhood diarrhea and dysentery, such as Thailand, outbreaks of EHEC diarrhea may be difficult to recognize.

The low prevalence of SLT-producing *E. coli*-associated diarrhea in the presence of high numbers of SLT-producing *E. coli* in meats and animals is not well understood. Perhaps people in Thailand acquire protective neutralizing antibodies at an early age or use cooking and hygiene practices that effectively eliminate the SLT-producing *E. coli*. Conversely, the pathogenicity of the SLT-producing *E. coli* isolated in this study for humans or animals has not been demonstrated. It has been recognized that EHEC possess other virulence determinants besides the production of high levels of SLT (34). Perhaps the *E. coli* isolated here lacked attachment factors or other surface components necessary for virulence. Comparison of these strains with isolates from patients with enterohemorrhagic colitis may help to better define the virulence of EHEC. Nevertheless, the presence of a large and heterogeneous gene pool of SLT genes in *E. coli* of food-producing animals may pose a public health risk in Thailand.

ACKNOWLEDGMENTS

We thank F. Orskov for serotyping of *E. coli* isolates. We thank M. Chulasiri, C. Saichua, and C. Siriphanich for assistance with

obtaining *E. coli* isolates and H. Dewey for statistical assistance. O. Chivavatanond, S. Changchawalit, N. Wongkkamhaeng, K. Boonnak, N. Siripraivan, and T. Juanvorachai provided excellent technical assistance.

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