INFECTION AND IMMUNITY, May 1988, p. 1158–1161
0019-9567/88/051158-04$02.00/0
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Vol. 56, No. 5

Type II Heat-Labile Enterotoxin-Producing *Escherichia coli* Isolated from Animals and Humans

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Received 10 November 1987/Accepted 12 January 1988

Heat-labile enterotoxin (LT)-producing *Escherichia coli* strains, as identified by the Y1 adrenal cell assay, were examined with a DNA probe coding for type I and type II LTs. Of 236 LT-producing *E. coli* isolates, 60% hybridized with LT-I, 17% hybridized with LT-II, and 23% did not hybridize with either probe and no longer produced LT as determined by the Y1 adrenal cell assay. These isolates presumably lost plasmids coding for LT-I during storage. A total of 75% of LT-producing *E. coli* isolates (27 of 36) from cows, 64% of LT-producing *E. coli* isolates (7 of 11) from buffalo, 31% of LT-producing *E. coli* isolates (4 of 13) from beef obtained in markets, and 2% of LT-producing *E. coli* isolates (3 of 168) from humans contained genes coding for LT-II. Genes coding for LT-II were not found in 50 LT-I-producing and heat-stable enterotoxin-producing *E. coli* isolates from 11 children with diarrhea and 44 LT-nonproducing and heat-stable enterotoxin-producing *E. coli* isolates from 12 other children with diarrhea. A total of 9% of LT-II-producing *E. coli* isolates (3 of 34) from cows and buffalo hybridized with DNA probes for genes coding for verocytotoxin 2 (VT2), and 18% (6 of 34) hybridized with a DNA probe coding for enterohemorrhagic *E. coli* (EHEC) adhesin fimbriae. *E. coli* SA-53, the original isolate in which LT-II was found, contained genes coding for VT2 and EHEC adhesin fimbriae. Five VT-producing, LT-II-producing *E. coli* isolates that hybridized with the EHEC probe did not contain DNA sequences coding for VT1 or VT2. LT-II-producing *E. coli* strains were frequently isolated from cattle and buffalo but were rarely isolated from humans.

Vibrio cholerae enterotoxin and a group of *Escherichia coli* heat-labile enterotoxins (LTs) are closely related in structure and mechanisms of action (8). LTs produced by strains of *E. coli* from humans (LT-H) and pigs (LT-P) have common and unique antigenic determinants (1, 2, 5, 9, 15, 18); LT-H and LT-P are neutralized by antibodies to *V. cholerae* enterotoxin and have been designated LT-I (25). *E. coli* SA-53, isolated from a water buffalo in Thailand, produced a toxin that resembled LT-I with respect to several of its biological properties but was not neutralized by antibodies to *V. cholerae* enterotoxin, LT-H, or LT-P (10, 11, 14, 15). Crude or partially purified toxin from this isolate increased vascular permeability in intracutaneous tests in rabbits, increased fluid accumulation in ligated intestinal segments in rabbits, and killed mice injected intraperitoneally (10, 17). Purified LT-II stimulated adenylate cyclase in human fibroblasts by ADP ribosylation (4). This strain did not hybridize with genes coding for LT-I under hybridization conditions which allowed for up to a 20% base-pair mismatch (10). The gene coding for this new toxin was not plasmid mediated and was presumed to be chromosomally encoded (10). This toxin, originally called LT-like toxin, has been proposed as a prototype of a second group of toxins designated LT-II (25). Two different subgroups of LT-II have been described: LT-IIa from *E. coli* SA-53 and LT-IIb from *E. coli* 41, isolated from beef in Brazil (12, 13).

The genes for LT-II have been cloned from *E. coli* SA-53 (25). Subcloning and minicell experiments demonstrated that LT-II is composed of two polypeptide subunits. The two LT-II subunits had mobilities on a polyacrylamide gel that were similar to those of *V. cholerae* enterotoxin and LT-I subunits. A 0.8-kilobase (kb) DNA probe for LT-II did not, however, hybridize with the structural genes for LT-I under stringent hybridization conditions.

DNA probes for genes coding for LT-I and LT-II were used to determine the prevalence of DNA sequences coding for LT-II among strains of *E. coli* that caused rounding of Y1 adrenal cells in tissue culture (26). To further characterize these isolates, endonuclease digests of whole-cell DNA from LT-II-producing *E. coli* strains were examined by the Southern technique with the LT-II DNA probe (28). LT-II-producing *E. coli* strains were also examined for hybridization with DNA probes for verocytotoxin 1 (VT1) and VT2 (24) and plasmid-encoded genes for adherence fimbriae of enterohemorrhagic *E. coli* (EHEC) (20). Isolates that hybridized with the VT1, VT2, or EHEC probe were tested for VT production (19).

MATERIALS AND METHODS

Bacteria. The *E. coli* strains examined in this study were isolated during studies of diarrheal disease in children in Thailand, the Philippines, India, Indonesia, and Somalia; from travelers with diarrhea in Nepal; from cows, buffalo, and pigs in Sri Lanka and Thailand; and from beef obtained in markets in Bangkok, Thailand. Isolates were initially tested for LT and heat-stable enterotoxin (ST) production by the Y1 adrenal and suckling mouse assays (7, 26). Enterotoxin-producing isolates were frozen in skimmed milk at –70°C or lyophilized. LT^+ST^- *E. coli* 357900 was isolated from a patient with diarrhea in Bangladesh.

DNA probes. Plasmid DNA was isolated from *E. coli* C600 (pEWD299) (LT-I), *E. coli* HB101(pCP2725) (LT-II), *E. coli* C600(pCVD419) (EHEC), *E. coli* C600(pJN37-19) (VT1), and *E. coli* C600(pNN110-18) (VT2) as described by So et al. (27), and the DNA was then digested with the appropriate restriction endonuclease (Bethesda Research Laboratories,

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TABLE 1. Identification of genes coding for LT-II in 236 LT-producing ETEC isolates

Origin of isolate	No. of LT-producing isolates[a]	No. (%) of LT-II-producing isolates	Source
Philippines, children	38	0	C. Moe
Nepal, travelers	3	0	AFRIMS[b]
Thailand			
Children	84	1 (1)	AFRIMS
Buffalo	8	6 (75)	AFRIMS
Cow	1	1 (100)	AFRIMS
Pigs	5	0	AFRIMS
Beef	13	4 (31)	AFRIMS
India, children	12	0	R. Sarkar
Sri Lanka			
Cows[c]	35	26 (74)	S. Peiris
Buffalo[c]	3	1 (33)	S. Peiris
Pigs	3	0	S. Peiris
Somalia, children	15	2 (12)	A. L. Bourgeois
Indonesia, children	16	0	S. Hoffman

[a] One isolate per person, animal, or food as identified by testing culture supernatants by the Y1 adrenal cell assay (26).
[b] Armed Forces Research Institute of Medical Sciences.
[c] Calves.

Gaithersburg, Md.) under conditions specified by the manufacturer. The LT-I DNA probe consisted of an 850-base-pair *Hin*cII digestion fragment of pEWD299 encoding predominantly the A and part of the B subunit of the LT-I molecule (6). The LT-II DNA probe consisted of an 800-base-pair *Hin*dIII-*Pst*I digestion fragment of pCP2725 (25). The EHEC probe consisted of a 3.4-kb *Hin*dIII fragment of pCVD419 (20). DNA probes for VT1 and VT2 were derived from phages 933W and 933J, originally isolated from *E. coli* 933 serotype O157:H7. The VT1 probe consisted of a 1,140-base-pair *Taq*I-*Hin*cII fragment derived from phage 933J cloned into pUC19, and the VT2 DNA probe was an 842-base-pair *Sma*I-*Pst*I fragment derived from 933W cloned into pUC18 (24; J. Newland, personal communication). The appropriate DNA fragments were separated by polyacrylamide gel electrophoresis, removed from the gel, and labeled in vitro with deoxynucleotide [α-^{32}P]triphosphates (New England Nuclear Corp., Boston, Mass.) by nick translation (21).

Colony hybridizations. Colonies were retested for LT by the Y1 adrenal cell assay (26) and then inoculated onto nitrocellulose filters (pore size, 0.45 μm), placed on MacConkey agar, and incubated at 37°C overnight. Filters were processed as previously described (22, 23) and were heated at 80°C under vacuum for 2 h.

Filters were incubated in hybridization solution: 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, 1 mM EDTA, and Denhardt solution (0.02% Ficoll [molecular weight, 400,000; Pharmacia Fine Chemicals, Piscataway, N.J.], 0.02% polyvinylpyrrolidone [molecular weight, 360,000], and 0.02% bovine serum albumin). The filters were then transferred to fresh hybridization solution containing 10^6 cpm of heat-denatured DNA probe per ml and 75 μg of sheared, heat-denatured calf thymus DNA per ml and were incubated at 37°C overnight. The filters were then washed in 5× SSC with 0.1% sodium dodecyl sulfate for 45 min at 65°C, rinsed in 2× SSC at 22°C, and air dried. The filters were exposed to X-Omat-R X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a single Cronex Lightening-Plus intensification screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 24 h at −70°C. The film was developed according to the instructions of the manufacturer.

Whole-cell DNA. Whole-cell DNA was isolated from strains that hybridized with the LT-II DNA probe by the method of Brenner et al. (3), digested with *Hin*dIII and *Pst*I, and separated by electrophoresis on a 0.7% agarose gel. Digestion fragments were transferred to nitrocellulose paper and examined with the LT-II DNA probe under stringent hybridization conditions by the Southern technique (28).

VT production. LT-II-producing *E. coli* that hybridized with the VT1, VT2, or EHEC DNA probe was inoculated into 10 ml of Penassay broth (Difco Laboratories, Detroit, Mich.) in a 125-ml Erlenmeyer flask and incubated at 37°C for 24 h. Sterile culture supernatants (10-fold dilutions) were tested for cytotoxicity to Vero cells after 3 days (19).

RESULTS

LT^+ST^- *E. coli* isolates, identified by the Y1 adrenal cell and suckling mouse assays, were screened by colony hybridization with the LT-I and LT-II DNA probes. Of 236 LT^+ST^- *E. coli* isolates, 60% hybridized with the LT-I probe, 17% hybridized with the LT-II probe, and 23% did not hybridize with either the LT-I or LT-II DNA probe and no longer produced LT as determined by the Y1 adrenal cell assay. These isolates presumably lost plasmids coding for LT-I during storage. None of the *E. coli* examined contained DNA sequences coding for both LT-I and LT-II.

A total of 75% of LT^+ST^- *E. coli* isolates (27 of 36) from cows, 64% of LT^+ST^- *E. coli* isolates (7 of 11) from buffalo, 31% of LT^+ST^- *E. coli* isolates (4 of 13) from beef, and 2% of LT^+ST^- *E. coli* isolates (3 of 168) from humans contained genes coding for LT-II (Table 1). LT-II-producing *E. coli* was detected in 2 of 15 LT^+ST^- *E. coli* isolates from children (one with diarrhea and one without diarrhea) in Somalia and 1 of 84 LT^+ST^- *E. coli* isolates from children with diarrhea in Thailand but in none of LT^+ST^- *E. coli* isolates from 38 children with diarrhea in the Philippines, 12 children with diarrhea in India, 16 children with diarrhea in Indonesia, or three travelers with diarrhea in Nepal.

In Bangkok, enterotoxigenic *E. coli* (ETEC) was isolated form 37 (9%) of 410 children with diarrhea and 15 (4%) of 410 age-matched children without diarrhea. LT-I^+ST^+ ETEC and LT-I^-ST^+ ETEC isolated from 23 children with diarrhea and 3 children without diarrhea were isolated significantly more often from children with diarrhea (P = 0.0002). LT-I^+ST^- ETEC isolated from 14 children with diarrhea and 12 children without diarrhea were not isolated significantly more often from children with diarrhea. LT-II^+ST^- *E. coli* was isolated from a 5-year-old boy with diarrhea who was also infected with *Shigella flexneri*. Genes coding for LT-II were not found in 50 LT-I^+ST^+ ETEC isolates from 11 children with diarrhea and 44 LT-I^-ST^+ ETEC isolates from 12 other children with diarrhea.

Whole-cell DNA isolated from 44 LT-II^+ST^- *E. coli* isolates, including *E. coli* 357900, was digested with *Hin*dIII and *Pst*I and examined by the Southern technique with the LT-II DNA probe. In each isolate, only a single *Hin*dIII-*Pst*I digestion fragment contained sequences that hybridized with the LT-II DNA probe. The sizes of these fragments that contained sequences encoding LT-II varied from 0.55 to 10.2 kb. The DNA fragments of the five LT-II^+ST^- *E. coli* isolates from a child with diarrhea in Bangkok were the same size (1.5 kb). The DNA fragment coding for LT-II in *E. coli* 357900 was 3.6 kb, and the fragments of DNA from LT-II^+ST^- *E. coli* isolates from two children in Somalia were 4.3 kb. The sizes of the DNA fragments from cows and buffalo in Sri Lanka and Thailand ranged from 0.55 to 10.2

kb and were different in size from fragments of DNA encoding LT-II in isolates from patients in Thailand, Somalia, and Bangladesh (Table 2).

A total of 9% of LT-II-producing *E. coli* isolates from cows and buffalo hybridized with a DNA probe for VT2, and 18% (6 of 34) hybridized with a DNA probe coding for EHEC adhesin fimbriae. *E. coli* SA-53 isolated from a buffalo in Thailand hybridized with both the EHEC adherence probe and the VT2 probe (Table 3). Five EHEC probe-positive, VT- and LT-II-producing *E. coli* isolates did not contain genes coding for VT1 or VT2.

DISCUSSION

This study suggests that LT-II-producing *E. coli* is primarily isolated from cows, buffalo, and beef but is less frequently isolated from children. Initial studies demonstrated that the crude extracts or partially purified LT-II from *E. coli* SA-53 caused rounding of cultured Y1 adrenal cells and death of mice inoculated intraperitoneally (10). Crude extracts of *E. coli* HB101(pCB2725) containing the cloned gene coding for LT-II had much higher specific activity by the Y1 adrenal cell assay than did extracts of SA-53, but the extracts were not lethal for mice (14). Since this isolate hybridized with the VT2 DNA probe and produced VT, the mouse lethality of the SA-53 extract was presumably caused by VT2. Genes coding for VT2 and plasmid-encoded adherence fimbriae were not found in LT-II-producing *E. coli* isolated from four humans.

Genes coding for LT-II were divergent at least in terms of digestion sites for *Hin*dIII and *Pst*I, suggesting that these isolates were not originally derived from a single clone, although LT-II-producing *E. coli* isolates from the same child or animal were similar. Divergence of endonuclease digestion sites around DNA sequences coding for LT-I have been found in LT-I isolated from human and animal sources (30). LT-II-producing *E. coli* was frequently isolated from cows, buffalo, and beef in Thailand and Brazil. It is curious that LT-II-producing *E. coli* which is isolated from foods of animal origin is rarely isolated from humans.

LT-II-producing *E. coli* from animals also contained genes coding for other enteropathogenic determinants. Five EHEC probe-positive, LT-II-producing *E. coli* did not contain DNA sequences coding for VT1 and VT2 but did produce VT. The significance of the VT probe-negative, EHEC probe-positive, LT-II-producing *E. coli* that produces VT is not known. *E. coli* isolates that produce cytotoxins but do not contain genes coding for VT1 or VT2 have been identified (J. Seriwatana, personal communication). A study with children with diarrhea and age-matched controls is under way to determine the isolation rate of cytotoxic *E. coli* that does not hybridize with VT probes. If cytotoxic *E. coli* is associated with disease, genes coding for the cytotoxin(s) should be cloned and specific probes should be used to determine the frequency of these potential enterovirulent determinants in *E. coli* isolated from different populations. Plasmid-encoded fimbriae were originally identified in VT probe-positive *E. coli* O157:H7; it is possible that other forms of cytotoxins are also associated with *E. coli* strains that contain these adherence fimbriae (20). The 60-megadalton plasmid encoding adherence fimbriae, however, is not essential for virulence of *E. coli* O157:H7, at least in gnotobiotic piglets (29).

TABLE 2. Restriction site heterogeneity of DNA sequences coding for LT-II[a]

Source of isolate	Size of fragment (kb)	No. of isolates
Humans		
Thailand	1.5	1
Somalia	4.3	2
Bangladesh	3.6	1
Animals		
Thailand	0.55	1[b]
	4.4	2[b]
Sri Lanka	10.2	1
	8.0	2
	4.5	8
	4.4	3 (1)[b]
	4.0	1
	3.8	1
	3.1	1
	3.0	3
	1.4	6
	0.7	2
	0.65	1
Beef	1.6	2
	1.1	1

[a] Whole-cell DNA from each isolate was digested with *Hin*dIII and *Pst*I and examined by the Southern technique using the LT-II DNA probe under stringent hybridization conditions.

[b] One isolate per source.

TABLE 3. LT-II-producing *E. coli* that hybridized with DNA probes for VT and EHEC adhesins

Isolate	Hybridization with DNA probe for[a]:		Origin[b]	Clinical status
	VT2	EHEC		
94	+	−	Buffalo	Diarrhea
259	+	−	Calf	No diarrhea
3210C11	−	+	Calf	No diarrhea
3222C11	−	+	Calf	No diarrhea
3209a2	−	+	Calf	No diarrhea
3216c11	−	+	Calf	No diarrhea
3218d4	−	+	Calf	No diarrhea
SA-53-7	+	+	Buffalo	No diarrhea

[a] None of the isolates hybridized with a DNA probe for VT1. Also, all isolates were negative for cytotoxin, as determined by testing sterile cultures by the Vero cell cytotoxicity assay (19).

[b] All animals were from Sri Lanka, except for the buffalo from which isolate SA-53-7 was obtained, which was from Thailand.

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