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Characterization of *Escherichia coli* Strains Producing Heat-Stable Enterotoxin b (STb) Isolated from Humans with Diarrhea

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Two of 49 cytolethal distending toxin-producing strains of *Escherichia coli* isolated from human stools contained the gene coding for heat-stable enterotoxin b (STb), as detected by a colony hybridization assay. The STb gene was found to be on a 70-kb plasmid also coding for heat-labile enterotoxin (pLT-I). Restriction endonuclease analysis showed the STb gene from human isolates to be similar to the STb gene found in porcine strains. Moreover, by enzymatic amplification based on oligonucleotide primers designed from a porcine STb sequence, the expected portion of the STb gene was amplified for the human *E. coli* strains. The STb enterotoxin from these strains was bioactive in rat jejunal loops and was detected with an enzyme-linked immunosorbent assay by using polyclonal antiserum raised against purified porcine STb toxin.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of infant diarrhea in less-developed countries and is also the agent most frequently responsible for traveler's diarrhea (16). Two classes of enterotoxins, heat-labile toxin (LT) and heat-stable toxin (ST), can be implicated in the pathogenesis (21). The STb gene codes for a 71-amino-acid peptide, containing a signal peptide of 23 amino acids and a mature protein of 48 amino acids (~5,000 Da) (14, 15, 20). The nucleotide sequence of the gene is totally different from those coding for LT and STa but is also encoded on a plasmid (2, 15, 20). It has also been demonstrated that the gene could be associated with a transposable element of 9 kb (2).

STb has been primarily associated with ETEC isolated from swine with diarrhea and is the most prevalent toxin associated with diarrheagenic isolates of porcine origin (2, 19). The toxin was demonstrated to be sensitive to trypsin degradation (23). Using a trypsin inhibitor to block intestinal protease activity caused an intestinal response to STb in mice, rats, rabbits, and calves (24). In studies of the prevalence of STb genes in human ETEC strains, the genes were rarely detected (6, 7). The expression of STb in the previous studies (6, 7) was not evaluated, and the strains examined were not associated with diarrhea. This report describes the characterization of STb-positive strains isolated from humans with diarrhea, by using an STb probe to detect the toxin-encoding gene and an immunoassay to detect STb-producing isolates.

We examined 49 cytolethal distending toxin-positive *E. coli* strains isolated from stools of individuals with diarrhea, gastroenteritis, or hemolytic uremic syndrome (National Enteric Reference Center, Laboratory Center for Disease Control, Ottawa, Ontario, Canada) and one porcine ETEC strain isolated from a pig with diarrhea (Faculty of Veterinary Medicine, St-Hyacinthe, Québec, Canada). Results of this study are summarized in Table 1. The STb probe and the LT probe were prepared as previously described and used for the colony hybridization assay and/or the Southern blot hybridization of the plasmids (3). By colony hybridization, the STb probe hybridized with two human strains (H15 and H46), with the positive control pRAS1 (a recombinant plasmid carrying the STb gene [15, 17]), and with the porcine strain P7169. The method used to extract plasmid DNA was derived from Broes et al. (3). The plasmids were separated by electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane (Zeta-probe; Bio-Rad Laboratories, Richmond, Calif.) (18), and finally hybridized with the STb probe as previously described (3). The strains H15 and H46 both possessed the STb gene on a 70-kb plasmid, and the porcine strain P7169 carried STb on a 110-kb plasmid (Fig. 1). Mainil et al. have demonstrated that the STb gene is often associated with LT genes in porcine ETEC strains (17). The 70-kb plasmids from human strains and the 110-kb plasmid from the porcine strain also hybridized with the LT probe (Table 1). By using the primers described by Furrer et al. (11), we have enzymatically amplified a 275-bp DNA fragment which was sensitive to restriction endonuclease *Sma*I but resistant to *Cfo*I, indicating the presence of genes coding for pLT-I.

The 70-kb plasmids from the human strains were digested with restriction endonucleases (Pharmacia LKB Biotechnology, Baie d'Urfé, Québec, Canada) by the manufacturer's instructions, and the fragments were separated by electrophoresis in a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with the STb probe as previously described (3).

Restriction endonuclease analysis of the STb genes carried by the 70-kb plasmids from human strains and by the plasmid pRAS1 produced a 1.2-kb DNA fragment hybridizing with the STb probe when digestion was with endonuclease *Hin*dIII, a 400-bp DNA fragment hybridizing with the STb probe when digestion was with *Hin*fI and *Pst*I, and two DNA fragments of 150- and 325-bp hybridizing with the STb probe when digestion was with *Hin*fI and *Bgl*II (data not shown). Therefore, the STb gene found in the human strains had restriction sites similar to those of the STb gene isolated from porcine strains.

A polymerase chain reaction (PCR) using a forward primer (5′-GCAATAAGGTTGAGGTGAT-3′) covering the 5′ end of the gene and a reverse primer (5′-GCCTGCAGTGAGA AATGGAC-3′) flanking the 3′ extremity of the STb gene (15) was performed. Briefly, for each sample, three bacterial

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TABLE 1. Characterization of two STb-positive *E. coli* strains isolated from humans with diarrhea and comparison with an STb-positive *E. coli* strain isolated from a pig with diarrhea

Strain	Origin	Serotype	Characteristics[a]	Hybridization to probe	STb enterotoxin		Plasmids (kbp)[b]
					Rat loop assay[c]	ELISA[d]	
H15	Human	O7:H24	Ap^r Sp^r Tc^r LT^+ $CLDT^+$	+	+ (0.12 ± 0.02)	+	150, **70,** 3.3, 3.0
H46	Human	O7:H24	Ap^r Sp^r Tc^r LT^+ $CLDT^+$	+	+ (0.15 ± 0.02)	+	150, **70,** 3.3, 3.0
P7169	Porcine	O149	Sp^r STb^+ LT^+ $CLDT^+$	+	+ (0.17 ± 0.01)	+	**110,** 90, 11, 6.0, 5.2, 4.1, 3.3

[a] Ap^r, ampicillin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant; LT^+, porcine heat-labile type I toxin producer; STb^+, heat-stable toxin b producer; $CLDT^+$, cytolethal distending toxin producer.
[b] Plasmid in boldface type carried the STb gene.
[c] Numbers shown are the mean value ± standard deviation (n = 2); positive, >0.05.
[d] Positivity was deduced by comparison with negative [HB101, P3922(STb^-), culture media] and positive [pRAS1, P4247(STb^+)] controls as previously described (5).

colonies were transferred with a sterile toothpick into 100 μl of sterile distilled water. Each sample was boiled for 1 min, and then 67.5 μl was withdrawn and added to 10 μl of 10× PCR buffer (25)–16 μl of 1.25 mM deoxynucleotide triphosphates–1 μg of forward primer–1 μg of reverse primer for a final volume of 99.5 μl. The samples were heated to 94°C for 5 min, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added, and the samples were subjected to PCR in a thermal cycler. The program used was 15 s at 94°C, 15 s at 40°C, and 60 s at 72°C for 30 cycles, followed by a final 7-min extension at 72°C. A volume containing 5 μl of each reaction mixture was subjected to electrophoresis on a 1.2% agarose gel stained with ethidium bromide, transferred onto a nylon membrane, and hybridized with the STb probe as for the plasmid preparations. The expected fragment of 377 bp was produced by PCR amplification for strains H15, H46, P7169, and pRAS1 (Fig. 2). These results demonstrate that the STb-encoding gene can be found in human *E. coli* strains associated with diarrhea and that this gene is similar to the STb gene isolated from a porcine *E. coli* strain described by Lee et al. (15) and Picken et al. (20). The PCR amplification method that we have described here could be used to detect STb-positive *E. coli* strains and can be considered as an alternative method of detection to the colony hybridization assay.

The presence of STb toxin was assayed by an enzyme-linked immunosorbent assay (ELISA) procedure as previously described (5). The immunoassay demonstrated that for human strains H15 and H46, the gene coding for STb is translated into a protein, as for the wild-type porcine strain P7169. In addition, the STb produced by recombinant strain pRAS1, by strains H15, H46, and P7169, and by two control strains described elsewhere [P3922(STb^-) and P4247(STb^+)] (5) were tested for bioactivity in a rat loop assay (5). Supernatants were heated to 65°C for 30 min to inactivate LTs. Each sample was tested in duplicate. The mean value and the standard deviation were calculated. A ratio of volume (milliliters) to length times diameter (centimeters) greater than 0.05 was considered positive. The two human strains H15 and H46 produced bioactive STb enterotoxin, as did the porcine strains P7169 and P4247 and the recombinant strain pRAS1. Thus, the STb gene contained in those human *E. coli* strains codes for a mature bioactive protein.

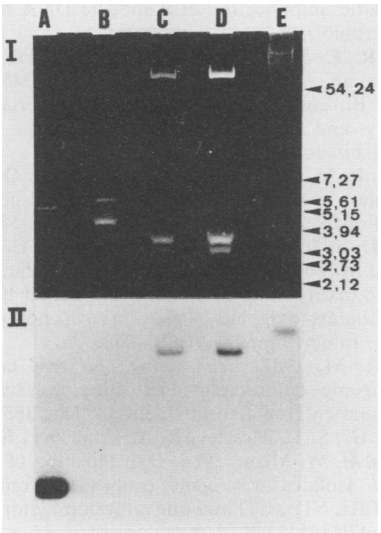

FIG. 1. (I) Agarose gel electrophoresis of plasmid DNA extracted from recombinant strain pRAS1 (lane A), a human STb-negative *E. coli* strain (lane B), two human STb-positive *E. coli* strains (lane C, H15; lane D, H46), and a porcine STb-positive *E. coli* strain (lane E, P7169). (II) Autoradiograph from hybridization of plasmid DNA with the STb probe. Plasmid pRAS1 (lane A) hybridized with the probe as the 70-kb plasmid of strains H15 (lane C) and H46 (lane D) and the 110-kb plasmid of strain P7169 (lane E). Sizes of molecular DNA standards are expressed in kilobase pairs.

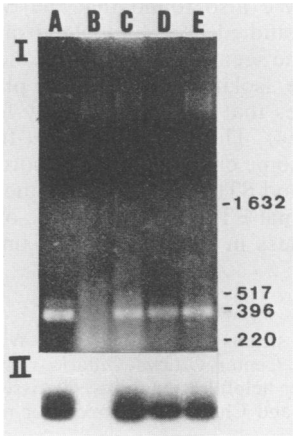

FIG. 2. (I) Agarose gel analysis of PCR products of the plasmid pRAS1 (lane A), a human STb-negative *E. coli* strain (lane B), two human STb-positive *E. coli* strains (lane C, H15; lane D, H46), and a porcine STb-positive *E. coli* strain (lane E, P7169). (II) Autoradiograph from hybridization of amplified DNA with the STb probe. The 377-bp amplified fragment hybridized with the probe. Sizes of molecular DNA standards are expressed in base pairs.

Susceptibility to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, neomycin, nitrofurantoin, spectinomycin, and tetracycline was tested by the Kirby-Bauer method (1). The human strains were resistant to ampicillin, spectinomycin, and tetracycline, and the porcine strain was resistant to spectinomycin only. We do not know whether the genes coding for resistance to these antimicrobial agents are located on the 70-kb plasmid carrying the STb gene, but if this is the case, the use of antimicrobial agents could increase the distribution of genes coding for STb and LT (8). The human STb-positive *E. coli* isolates identified by Echeverria et al. also contained plasmids coding for tetracycline resistance and carried the STb gene and sometimes STb and LT genes, but these isolates were not from humans with diarrhea (8).

The two human strains producing active STb belong to serogroup O7, which is not classically related to diarrheal diseases (13). The two human strains did not produce the classical animal colonization factors F4, F5, F6, F41, and F165 or either of the human colonization factor antigens (CFA) I and II when tested by indirect immunofluorescence (10). Moreover, these two human strains did not hemagglutinate the bovine and the human group A erythrocytes, indicating the absence of CFA/I and CFA/II (12). There is no evidence to relate cytolethal distending toxin to STb or to diarrheal disease. In our study, the diarrhea caused by strains H15 and H46 was probably attributable to the production of LT. The role of STb, also produced by these strains, is not known, but it has been shown that some strains producing only STb are responsible for diarrheal diseases in swine (9, 20). This enterotoxin may also play a role in human diarrheal diseases. Recently, Whipp has proposed to change the concept of species specificity of the STb response by inferring that failure to demonstrate a response to STb in other species could reflect the presence of one or more intraluminal factors, such as protease activity, that would preclude a response (23).

In conclusion, we found that the gene coding for STb enterotoxin can be detected in *E. coli* strains isolated from humans with diarrhea. Our study demonstrates that in human strains, the gene coding for STb can be correctly expressed and that produced enterotoxin is active in rat jejunal loops. We also found that the STb gene, for the human strains studied here, is located on a 70-kb plasmid and that this gene seems to be similar to the STb gene cloned from a porcine isolate. These 70-kb plasmids contained pLT-I sequences that had been initially found in ETEC of porcine origin (4). Thus, these isolates from humans with diarrhea show some characteristics of porcine ETEC strains such as pLT-I and STb but are lacking the classical fimbriae involved in animal ETEC (12). The role of STb enterotoxin in diarrheal illness in humans remains unclear for the moment.

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REFERENCES

1. **Bauer, A. W., W. W. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotics susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. **45**:493–496.
2. **Betley, M. J., V. L. Miller, and J. J. Mekalanos.** 1986. Genetics of bacterial enterotoxins. Annu. Rev. Microbiol. **40**:577–605.
3. **Broes, A., J. M. Fairbrother, J. Mainil, J. Harel, and S. Larivière.** 1988. Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* serotype O8: K"2829" strains isolated from piglets with diarrhea. J. Clin. Microbiol. **26**:2402–2409.
4. **Dallas, W. S., D. M. Gill, and S. Falkow.** 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. **139**: 850–858.
5. **Dubreuil, J. D., J. M. Fairbrother, R. Lallier, and S. Larivière.** 1991. Production and purification of heat-stable enterotoxin b from a porcine *Escherichia coli* strain. Infect. Immun. **59**:198–203.
6. **Echeverria, P., J. Seriwatana, U. Patamaroj, S. L. Moseley, A. McFarland, O. Chityothin, and W. Chaicumpa.** 1984. Prevalence of heat-stable II enterotoxigenic *Escherichia coli* in pigs, water, and people at farms in Thailand as determined by DNA hybridization. J. Clin. Microbiol. **19**:489–491.
7. **Echeverria, P., J. Seriwatana, D. N. Taylor, C. Tirapat, W. Chaicumpa, and B. Rowe.** 1985. Identification by DNA hybridization of enterotoxigenic *Escherichia coli* in a longitudinal study of villages in Thailand. J. Infect. Dis. **151**:124–129.
8. **Echeverria, P., J. Seriwatana, D. N. Taylor, C. Tirapat, and B. Rowe.** 1985. *Escherichia coli* contains plasmids coding for heat-stable b, other enterotoxins, and antibiotic resistance. Infect. Immun. **48**:843–846.
9. **Fairbrother, J. M., A. Broes, M. Jacques, and S. Larivière.** 1989. Pathogenicity of *Escherichia coli* O115: K"V165" strains isolated from piglets with diarrhea. Am. J. Vet. Res. **50**:1029–1036.
10. **Fairbrother, J. M., S. Larivière, and W. Johnson.** 1988. Prevalence of fimbrial antigens and enterotoxins in nonclassical serogroups of *Escherichia coli* isolated from newborn pigs with diarrhea. Am. J. Vet. Res. **49**:1325–1328.
11. **Furrer, B., U. Candrian, and J. Luthy.** 1990. Detection and identification of *E. coli* producing heat-labile enterotoxin type I by enzymatic amplification of a specific DNA fragment. Lett. Appl. Microbiol. **10**:31–34.
12. **Holland, R. E.** 1990. Some infectious causes of diarrhea in young farm animals. Clin. Microbiol. Rev. **3**:345–375.
13. **Kaper, J. B., and C. O. Tacket.** 1988. Bacterial infections of respiratory and gastrointestinal mucosae, p. 113–127. IRL Press, Washington, D.C.
14. **Lawrence, R. M., P.-T. Huang, J. Glick, J. D. Oppenheim, and W. K. Maas.** 1990. Expression of the cloned gene for enterotoxin STb of *Escherichia coli*. Infect. Immun. **58**:970–977.
15. **Lee, C. H., S. L. Moseley, H. W. Moon, S. C. Whipp, C. L. Gyles, and M. So.** 1983. Characterization of the gene encoding heat-stable toxin II and preliminary molecular epidemiological studies of enterotoxigenic *Escherichia coli* heat-stable toxin II producers. Infect. Immun. **42**:264–268.
16. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155**:377–389.
17. **Mainil, J. G., S. L. Moseley, R. A. Schneider, K. Sutch, T. A. Casey, and H. W. Moon.** 1986. Hybridization of bovine *Escherichia coli* isolates with gene probes for four enterotoxins (STaP, STaH, STb, LT) and one adhesion factor (K99). Am. J. Vet. Res. **47**:1145–1148.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Moon, H. W., R. A. Schneider, and S. L. Moseley.** 1986. Comparative prevalence of four enterotoxin genes among *Escherichia coli* isolated from swine. Am. J. Vet. Res. **47**:210–212.
20. **Picken, R. N., A. J. Mazaitis, W. K. Maas, M. Rey, and H. Heyneker.** 1983. Nucleotide sequence of the gene for heat-stable

enterotoxin II of *Escherichia coli*. Infect. Immun. **42**:269–275.
21. **Sack, R. B.** 1975. Human diarrheal disease caused by ETEC. Annu. Rev. Microbiol. **29**:333–353.
22. **Weikel, C. S., H. N. Nellans, and R. L. Guerrant.** 1986. *In vivo* and *in vitro* effects of a novel enterotoxin, STb, produced by *Escherichia coli*. J. Infect. Dis. **153**:893–901.
23. **Whipp, S. C.** 1987. Protease degradation of *Escherichia coli* heat-stable, mouse-negative, pig-positive enterotoxin. Infect. Immun. **55**:2057–2060.
24. **Whipp, S. C.** 1990. Assay for enterotoxigenic *Escherichia coli* heat-stable toxin b in rats and mice. Infect. Immun. **58**:930–934.
25. **Zon, L. I., D. M. Dorfman, and S. H. Orkin.** 1989. The polymerase chain reaction colony miniprep. Biotechniques **7**:696–698.