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Characterization of Heat-Stable Enterotoxin from a Hypertoxigenic *Escherichia coli* Strain That Is Pathogenic for Cattle

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An enterotoxigenic *Escherichia coli* (ETEC) strain isolated from a calf with clinical scours was found to produce over 17- to 60-fold more heat-stable enterotoxin (STa) than four laboratory-adapted bovine ETEC strains. The purified STa of this strain was identical to those produced by other ETEC strains. A severe form of scours was induced in 5- to 15-day-old colostrum-fed calves and in 1- to 2-week-old piglets by oral administration of the purified STa. This study demonstrates that STa is a mediator of diarrhea in newborn calves and piglets and that under identical growth conditions diverse strains of bovine ETEC may produce variable amounts of homologous STa's.

The clinical significance of heat-stable enterotoxin (STa)-producing enterotoxigenic *Escherichia coli* (ETEC) as a cause of secretory diarrhea in humans and animals has been established by numerous investigators (1, 6, 7, 11). Experimentally, STa activation of intestinal mucosal guanylate cyclase and of intestinal fluid secretion in suckling mice has been found to be STa dose dependent (4, 5, 7, 8, 17). Therefore, it is possible that the severity of clinical STa-induced diarrhea could be related to the toxigenicity of the infective ETEC strains (i.e., ETEC strains may produce variable levels of STa's). To date, wild-type ETEC strains which are STa hyperproducers have not been reported.

In the present report, the biochemical characteristics of STa's from six ETEC strains are described. We examined whether the differences among these STa's are qualitative, as well as quantitative.

The following established laboratory strains of bovine ETEC were kindly furnished by C. Gyles, Ontario Veterinary College, Ontario, Canada: B41 (O101:K$^-$), B44 (O9:K30), M490 (O101:K30), and M524 (O8:K85). $WSUH_1$ is the hypertoxigenic strain of *E. coli* which was isolated from a clinical case of calf scours in Washington state. The strain has been found to be a stable STa producer and was kindly serotyped by Ida and Frits Ørskov of the International Collaborative *Escherichia* and *Klebsiella* Centre, Copenhagen, Denmark, to O20:KX106:H4 antigens. L_1 (untypable) is the low-STa-producing bovine *E. coli* which was also a clinical isolate from calf scours in Washington state. All six study strains were K99$^+$.

The nutrient medium, bacterial growth conditions, and STa purification procedures were identical for each of the six ETEC strains and followed a previously described protocol (20, 21). Amino acid composition and sequence analysis were performed as reported earlier (20, 21). For the suckling mouse assay, 1- to 2-day-old suckling Swiss Webster mice were used for detection and quantitation of STa's throughout the purification steps (4, 21).

Suckling Holstein dairy calves in three age groups (5, 10, and 15 days) were studied. The calves were selected from a farm in Washington state where calf scours had not been reported for the last 2 years. There were two animals in each age group. These colostrum-fed animals were healthy and were passing formed stools at the beginning of the experiment. A 2-mg amount of purified STa from the $WSUH_1$ strain was given with 100 ml of 10% glucose. Control calves were given glucose only. This was administered at 9 a.m., and both STa-challenged and control calves were monitored for onset and duration of diarrhea. Severity of diarrhea was measured as the percentage of fecal dry matter content at selected time intervals; the packed-blood-cell volume (PCV) was measured for the 5-day-old calves only. Signs of dehydration, such as eye recession, skin pliability, and the ability of the animal to stand, were noted.

Healthy, colostrum-fed, 5- to 15-day-old suckling piglets were used. Purified STa (200 μg/kg of body weight) was given orally with a syringe to each of four piglets in the challenge group. Two piglets of comparable age and weight were used as controls. Animals were monitored in a manner similar to that of the calf experiment.

The levels of STa production by the six ETEC strains in 10-liter batches were monitored by the suckling mouse assay. It was found that different ETEC strains produce different amounts of STa when grown under identical conditions. When STa in the culture filtrate was quantitated, it was found that the $WSUH_1$ strain produced 50-fold more STa than did the B41 and B44 strains, 17-fold more than did the M490 strain, 60-fold more than did the M254 strain, and

TABLE 1. Comparison of STa production by six strains of bovine ETEC

Strain	STa production in 10-liter batches (10^6 MU)[a]	Minimal effective dose (ng) of STa[b]
B41	20 ± 2	0.01 ± 0.0012
B44	18 ± 4	0.011 ± 0.0014
M490	60 ± 9	0.012 ± 0.002
M524	16 ± 2	0.015 ± 0.003
$WSUH_1$	1,000 ± 100	0.012 ± 0.002
L_1	1.0 ± 0.4	0.014 ± 0.001

[a] Numbers represent the mean values of five experiments ± 2 SD.
[b] Protein concentration of high-performance liquid chromatography-purified STa's as determined by the modified method of Lowry (16) in the suckling mouse assay. Numbers represent the mean values of five 2 experiments ± 2 SD.

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TABLE 2. Time course evaluation of the dry fecal matter content and PCV for the three age groups of calves challenged with STa

Time after toxin challenge	Results with calves aged:							
	5 Days[a]				10 Days (% fecal dry matter)		15 Days (% fecal dry matter)	
	% Fecal dry matter		% PCV[b]					
	STa[c]	Control	STa	Control	STa	Control	STa	Control
9 a.m.	46.9	53.0	41	26.0	18.0	23.0	21.0	18.3
11 a.m.	15.0	50.8	52	26.5	3.6	21.0	11.0	19.7
1 p.m.	1.67	48.8	53	27.0	1.38	19.0	1.78	20.2
2 p.m.	2.12	42.9	ND[d]	ND	2.7	24.0	2.3	20.0
3 p.m.	2.23	52.3	ND	ND	1.84	26.0	4.1	19.8
5 p.m.	1.38	40.0	52	25.0	7.48	25.0	3.8	20.3

[a] Newborn calves usually pass formed stool of high solids because of meconium content. However, when fed milk replacements, the 10- and 15-day-old calves were normally passing softer fecal matter.
[b] The PCV values in healthy cattle range between 25 and 45%.
[c] STa-challenged calves.
[d] ND, Not done.

1,000-fold more than did the L_1 strain (Table 1). Furthermore, the specific activity and recovery of STa also appeared to vary from strain to strain throughout purification.

The amino acid compositions of STa from the hypertoxigenic ETEC strain ($WSUH_1$) and of those STa's produced by the low-STa-producing strain (L_1) were identical to the amino acid compositions of other strains which were reported earlier (3, 20, 21). The amino acid sequence of STa purified from the hypertoxigenic strain was Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr. This sequence is identical to those of other STa's purified from bovine ETEC (20, 23). Tyrosine was the carboxy-terminal residue of the STa peptide, as determined by carboxypeptidase Y digestion. The biochemically characterized STa's of $WSUH_1$ and L_1 strains were identical to STa's characterized from bovine ETEC B41, B44, M420, and M524 (2, 20).

Signs of watery diarrhea were evident in STa-challenged calves in each of the three age groups as early as 2 h after STa administration. By 4 h postadministration, the calves manifested frequent episodes of profuse watery diarrhea. At that time, the fecal dry matter contents were at their lowest, usually less than 2% (Table 2). Clinical signs of dehydration, as indicated by obvious recession of the eyeball and decreased skin pliability, were also evident at that time in the STa-challenged calves. These findings, accompanied by the finding of general depression, seemed most severe in the 5-day-old calf. Also at that time (1 p.m.), the PCV values were indicative of dehydration and hemoconcentration. At 24 h postinoculation, the STa-challenged calves showed signs of recovery, and the consistency of the fecal matter had become thicker. It should be noted that there is normally considerable variation in the PCV values of neonatal calves and that the newborn calves pass formed stool of high solid content because the animal continues to pass meconium several days after birth. Throughout the experiment, the control calves in each of the three age groups passed fecal matter of normal consistency and showed normal appetite and activity.

Signs of watery diarrhea in the piglets were seen as early as 2 h after STa administration. Data from five piglets showed that before administration dry fecal matter content was 29 ± 2%. At 2, 4, and 10 h postadministration of STa, the dry matter fecal content was 12 ± 4%, 4 ± 1%, and 14%, respectively. The stool consistency had returned to control values by 24 h postadministration.

Characterization and comparison of the STa's purified from the low (leaky mutant [22])- and high-STa-producing strains with the STa's purified from four laboratory-established bovine ETEC strains revealed similarities, which included similar isoelectric points (4.0 to 4.3), identical chromatograms by analytical reverse-phase high-performance liquid chromatography, and identical amino acid composition. The STa amino acid sequence of the hypertoxigenic ETEC is identical to the amino acid sequence of STa's purified from M490 and B44 strains, as reported earlier (20). The amino acid sequence of the STa from the hypertoxigenic bovine ETEC also matches the sequence reported for the STa of other bovine ETEC described by other investigators, as well as those sequences of STa's from porcine and human ETEC strains (3, 10, 18, 20, 24).

Our data also show that the amounts of STa produced by different strains of bovine ETEC can be quantitatively different. The genetic factors that control the STa production are not yet known. Copy numbers of plasmids in various ETEC strains may differ; alternatively, an uncharacterized regulatory mechanism involving a promoter or attenuator may be involved.

The window of susceptibility for reliable experimental induction of *E. coli* diarrhea in calves is limited to the first 20 h of life (9, 14, 19, 25). However, spontaneous or naturally occurring enteric colibacillosis is not limited to this time period, even in cases that appear to be solely associated with bovine ETEC infections mediated by K99 antigen attachment (14, 15). Unlike diarrhea caused by *E. coli* through an unknown mechanism (12, 13), cases of scours were produced by oral administration of the purified STa to 5- to 15-day-old calves and piglets. The STa-challenged animals manifested the typical signs of clinical scours for up to 12 h after STa administration. Recovery from these symptoms was complete by 24 h postadministration. Hence, STa exerted its effect for approximately 12 h before it was eliminated with fecal matter, inactivated, or lost its effect through an unknown mechanism.

LITERATURE CITED

1. **Acres, S. D., J. R. Saunders, and O. M. Radostits.** 1977. Acute undifferentiated neonatal diarrhea of beef calves: the prevalence of enterotoxigenic *Escherichia coli*-reo-like (rota) virus and other enteropathogens in cow-calf herds. Can. Vet. J. **18**:113–121.
2. **Dreyfus, L. A., J. C. Frantz, and D. C. Robertson.** 1983. Chemical properties of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* of different host origins. Infect. Immun. **42**:539–548.
3. **Gerady, C., M. Herman, J. Olivy, N. G. Otthiers, D. Art, E. Jaquemin, A. Kaeckenbeeck, and J. V. Beeumen.** 1984. Isolation and characterization of the heat-stable enterotoxin from a pathogenic bovine strain of *Escherichia coli*. Vet. Microbiol. **9**:399–414.
4. **Giannella, R. A.** 1976. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. Infect. Immun. **14**:95–99.
5. **Giannella, R. A., M. Luttrel, and M. Thompson.** 1983. Binding of *Escherichia coli* heat-stable enterotoxin to receptors on rat intestinal cells. Am. J. Physiol. **245**:G492–G498.
6. **Greenberg, R. N., and R. L. Guerrant.** 1981. *Escherichia coli* heat-stable enterotoxin. Pharmacol. Ther. **11**:507–531.
7. **Guerrant, R. L., J. M. Hughes, B. Chang, D. C. Robertson, and F. Murad.** 1980. Activation of intestinal guanylate cyclase by heat-stable enterotoxin of *Escherichia coli*: studies of tissue specificity potential receptors and intermediates. J. Infect. Dis.

142:220–228.
8. **Hughes, J. M., J. D. Rouse, F. A. Barada, and R. L. Guerrant.** 1980. Etiology of summer diarrhea among the Navajo. Am. J. Trop. Med. Hyg. **29**:613–619.
9. **Koritzer, I., R. Tamarin, Y. Breene, A. Cohen, and Z. Trainin.** 1980. Protection of calves against experimental enterotoxigenic *Escherichia coli* infection by immunization of dams. Refu. Vet. **37**:71–75.
10. **Lazure, C., N. G. Seidah, M. Chrtien, R. Lallier, and S. St-Pierre.** 1983. Primary structure determination of *Escherichia coli* heat-stable enterotoxin of porcine origin. Can. J. Biochem. Cell Biol. **61**:287–292.
11. **Levine, M. M., E. S. Caplan, D. Waterman, R. A. Cash, R. B. Hornick, and M. J. Snyder.** 1977. Diarrhea caused by *Escherichia coli* that produce only heat-stable enterotoxin. Infect. Immun. **17**:78–82.
12. **Levine, M. M., D. R. Nailin, R. B. Hornick, E. J. Berquist, D. H. Waterman, C. R. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet **i**:1119–1122.
13. **Long-Krug, S. A., C. S. Weikel, K. T. Tiemen, E. L. Hewlett, M. M. Levine, and R. L. Guerrant.** 1984. Does enteropathogenic *Escherichia coli* produce heat-labile enterotoxin, enterotoxins a or b, or cholera toxin A subunits? Infect. Immun. **46**:612–614.
14. **Moon, H. W., A. W. McClurkin, R. E. Isaacson, J. Pohlenz, S. M. Habil, S. M. Skartvedt, K. G. Gillette, and A. L. Baetz.** 1978. Pathogenic relationships of rotavirus, *Escherichia coli* and other agents in mixed infections in calves. J. Am. Vet. Med. Assoc. **173**:577–583.
15. **Morin, M., S. Larieviere, and R. Lallier.** 1976. Pathological and microbiological observations made on spontaneous cases of acute neonatal calf diarrhea. Can. J. Comp. Med. **40**:228–240.
16. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
17. **Rao, M. C., S. Guandalini, P. L. Smith, and M. Field.** 1980. Mode of action of heat-stable *Escherichia coli* enterotoxin: tissue and subcellular specificities and role of cyclic GMP. Biochim. Biophys. Acta **632**:35–46.
18. **Ronnberg, B., T. Wadstrom, and H. Jernvall.** 1983. Structure of heat-stable enterotoxin produced by a human strain of *Escherichia coli*. FEBS Lett. **155**:183–186.
19. **Runnels, P. L., H. W. Moon, and R. A. Schneider.** 1980. Development of resistance with host age to adhesion of K99$^+$ *Escherichia coli* to isolated epithelial cells. Infect. Immun. **28**:298–300.
20. **Saeed, A. M. K., N. S. Magnuson, N. Sriranganathan, D. Burger, and W. Cosand.** 1984. Molecular homogeneity of heat-stable enterotoxins produced by bovine enterotoxigenic *Escherichia coli*. Infect. Immun. **45**:242–247.
21. **Saeed, A. M. K., N. Sriranganathan, W. Cosand, and D. Burger.** 1983. Purification and characterization of heat-stable enterotoxin from bovine enterotoxigenic *Escherichia coli*. Infect. Immun. **40**:701–707.
22. **Silva, M. M., W. K. Maas, and C. L. Gyles.** 1978. Isolation and characterization of enterotoxin-deficient mutants of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75**:1384–1388.
23. **So, M. R., R. Atchinson, S. Falkow, S. Mosely, and B. J. McCarthy.** 1980. A study of the dissemination of Tn1681: a bacterial transposon encoding a heat-stable toxin among *Escherichia coli* isolates. Cold Spring Harbor Symp. Quant. Biol. **45**:53–58.
24. **Thompson, M. R., and R. A. Giannella.** 1985. Revised amino acid sequence for a heat-stable enterotoxin produced by an *Escherichia coli* strain (18D) that is pathogenic for humans. Infect. Immun. **47**:834–836.
25. **Trainin, Z., J. Brenner, I. Kornitzer, R. Tamarin, A. Cohen, and R. Meirom.** 1981. Oral passive immunization of newborn calves against enterotoxigenic *Escherichia coli*. Refu. Vet. **38**: 1–6.