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Production of Shiga-Like Toxin among *Escherichia coli* Strains and Other Bacteria Isolated from Diarrhea in São Paulo, Brazil

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An elevated level of Shiga-like toxin I (SLT-I) production was found in 1 of 466 *Escherichia coli* strains studied. Among the 34 sonic lysates obtained from classical enteropathogenic *E. coli*, 5 produced SLT-I. The *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Klebsiella*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Yersinia*, and *Vibrio* strains also studied were not SLT producers, except for a *Shigella dysenteriae* type 1 strain. Although SLT-I-producing *E. coli* strains were isolated from diarrhea, they seem to be an uncommon cause of disease in children less than 1 year old in our community.

Production of Shiga-like toxin (SLT) or Verotoxin (VT) has been associated with *Escherichia coli* strains isolated from cases of hemorrhagic colitis and hemolytic uremic syndrome in different countries (12, 21). These strains belong mainly to serotype O157:H7 (10), and two antigenically distinct toxins, SLT-I and SLT-II, have been previously described (18). Marques et al. (15) studied several *E. coli* strains isolated from diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and other sources and, on the basis of the cytotoxic dose required to kill 50% of the HeLa cells tested per milliliter of sonic lysate (CD_{50}/ml), defined three levels of cell-associated cytotoxin production (low, moderate, and high). Only moderate- or high-level (elevated-level) producers had cytotoxic activity in culture supernatants (equal or 10- to 100-fold-lower CD_{50}/ml than that of sonic lysate). Other authors also reported elevated levels of cytotoxin production by *E. coli* strains isolated from diarrheal disease, some of them belonging to enteropathogenic *E. coli* (EPEC) serogroups, suggesting that SLTs could play a role in the pathogenesis of EPEC strains (3, 9, 22). However, the relationship of SLTs with *E. coli* and EPEC strains associated with diarrhea is somewhat controversial, since there are other reports in which no cytotoxin production was found (24–26). The purpose of this study was to verify the occurence of SLT production in *E. coli* strains isolated from infants with diarrheal disease in São Paulo. Other members of the family *Enterobacteriaceae*, as well as some *Vibrio*, *Aeromonas*, and *Pseudomonas* strains, were also studied.

A total of 466 *E. coli* strains isolated from the feces of 95 children less than 1 year old with diarrhea and 63 age-matched controls visiting an outpatient clinic in São Paulo were studied from May 1985 to July 1987. The strains were maintained in nutrient agar (Difco Laboratories) at room temperature and tested for toxin production no more than 2 months after isolation. Among the *E. coli* strains studied, 16 were identified as enterotoxigenic *E. coli* by the passive immune hemolysis test (6; adapted to microdilution plates) and the infant mice assay (4), 15 were considered enteroinvasive *E. coli* by the Serény test (23), 5 belonged to serogroup O157 serotypes O157:H^- (2 strains) and O157:H45 (3 strains), and 45 belonged to EPEC serogroups as assayed by serological tests (27). The remaining 385 *E. coli* isolates did not belong to any of the groups mentioned above. The serotypes found among the EPEC serogroups were O26:H^- (1 strain), O26:H10 (1 strain), O26:H11 (2 strains), O55:H6 (5 strains), O55:H7 (4 strains), O111:H^- (5 strains), O111:H2 (5 strains), O111:H9 (1 strain), O119:H2 (1 strain), O119:H6 (10 strains), O125:H9 (2 strains), O127:H10 (2 strains), O127:H21 (1 strain), O127:H40 (1 strain), and O128ab:H2 (4 strains). Additionally, 216 strains belonging to the genera *Aeromonas* (19 strains), *Citrobacter* (16 strains), *Edwardsiella* (10 strains), *Enterobacter* (14 strains), *Klebsiella* (18 strains), *Proteus* (20 strains), *Providencia* (4 strains), *Pseudomonas* (7 strains), *Salmonella* (20 strains), *Serratia* (4 strains), *Shigella* (29 strains), *Yersinia* (9 strains), and *Vibrio* (46 strains) were studied. These strains were identified by standard methods (5), and most of them were isolated from cases of diarrhea, except for *Vibrio cholerae* non-O1 and *Vibrio parahaemolyticus*, which were isolated from water and kindly sent by M. I. Sato, CETESB, São Paulo.

Cytotoxin production was detected by the HeLa and Vero cell culture assay (7). Briefly, strains were cultured in Penassay broth (antibiotic medium no. 3, Difco), and polymyxin extracts were obtained as previously described (11). Sonic lysates of 34 EPEC strains were prepared on the basis of the method of O'Brien and Laveck (19). Filtered samples of polymyxin extracts and sonic lysates diluted 1:5 were tested. When a cytotoxic effect was observed by microscopic examination of the HeLa and Vero cells, 10-fold serial dilutions of these samples were assayed and neutralization tests were carried out simultaneously with rabbit antiserum against Shiga toxin (final dilution, 1:100), SLT-II (1:50), and preimmune rabbit serum. Neutralization tests were performed as described by O'Brien et al. (20), except that the mixtures of toxin and antisera were incubated at 37°C for 2 h and then 0.1 ml was inoculated in the cell culture microplates. It was considered an elevated level of cytotoxin production when cytotoxin was detected in polymyxin extracts and was considered a low level when cytotoxin was observed only in sonic lysates. Shiga toxin and SLT antisera were kindly provided by Alison O'Brien, Department of Microbiology, Uniformed Services University of Health Sciences, Bethesda, Md. One hundred fifteen *E. coli* strains were tested only by the HeLa cell culture assay. *E. coli* H30 (13) was used as an elevated-level-producing control. In HeLa and Vero cells, the CD_{50}/ml of polymyxin extracts and sonic lysates were 10^4 to 10^6 and 10^5 to 10^7, respectively. *E. coli* K-12 substrain C600 was used as a negative or low-level

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TABLE 1. Cytotoxin titers and neutralization assay of polymyxin extracts from *E. coli*, *Aeromonas* spp., and *S. dysenteriae* type 1 strains

Strain	CD_{50}/ml[a] in:		Neutralization with anti-SLT antiserum type[b]:	
	HeLa cells	Vero cells	I	II
E. coli 0651-1	1×10^4	1×10^4	+	NT
Aeromonas spp.				
1	5×10	1×10^2	−	−
2	5×10	2×10^2	−	−
3	1×10^2	2×10^2	−	−
4	1×10^2	2×10^2	−	−
5	1×10^2	2×10^2	−	−
S. dysenteriae type 1	1×10^3	1×10^3	+	NT

[a] Reference 20.
[b] +, Totally neutralized; −, negative; NT, not tested.

producer, and the CD_{50}/ml varied from 0 to 10^2 in both HeLa and Vero cells.

Cytotoxin production was found in 1 of 466 *E. coli* strains studied. Cytotoxin was detected in the polymyxin extract and was completely neutralized by antibody to Shiga toxin (Table 1). This strain was isolated from diarrhea, and although its serogroup was not determined, it did not belong to EPEC, enteroinvasive *E. coli*, or enterohemorrhagic *E. coli* serotypes. Our data are similar to reports by Smith et al. (26) in the United Kingdom that they found a low frequency of VT-producing strains among tested *E. coli* isolates from humans. Brown et al. (2), studying the role of cytotoxins in diarrheal disease in Thailand, observed that a small number of *E. coli* strains hybridized with the specific probes employed for detection of SLT-I and SLT-II.

Elevated levels of cytotoxin production were not found among the 45 EPEC polymyxin extracts we studied. A lack of cytotoxin production in EPEC strains isolated in São Paulo had been previously reported (24), and Smith et al. (25) also verified that most EPEC strains they studied did not produce VT in culture filtrates and did not hybridize with VT-1 and VT-2 probes. In order to determine whether the EPEC isolates could produce at least low levels of cytotoxin, 34 sonic lysates were tested. Cytotoxin activity neutralized by antibody to Shiga toxin was found in 5 of 34 EPEC lysates (Table 2). Most of them were detected in Vero cells, and only one also produced cytotoxic effect in HeLa cells. These strains belonged to serotypes O111:H$^-$, O111:H2, and O119:H6, which have been mentioned in the literature as elevated-level-cytotoxin producers (9, 15).

There are no data about the occurrence of hemolytic uremic syndrome and hemorrhagic colitis in our community.

TABLE 2. Cytotoxin titers of EPEC strains detected in sonic lysates

Serotype (no. of strains)	CD_{50}/ml in:	
	HeLa cells	Vero cells
O111:H$^-$ (1)	0	5×10–1×10^3
O111:H$^-$ (1)	5×10–1×10^2	1×10^3
O111:H2 (2)	0	1×10^2
O119:H6 (1)	0	1×10^2

E. coli O157:H7 was not isolated in the present survey, and the detected serotypes, O157:H$^-$ and O157:H45, did not produce cytotoxin. These results are similar to those reported by Levine et al. (14), who found enterohemorrhagic *E. coli* to be an uncommon cause of diarrhea in infants and young children in Chile.

Some papers described production of SLTs by some strains of *Salmonella typhimurium* (20), *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii* (1), *V. cholerae*, and *V. parahaemolyticus* (17). Nevertheless, among the several strains from different genera that we studied, SLT was found only in *Shigella dysenteriae* type 1. Cytotoxin production not neutralized by SLT antisera was found in 5 of 19 *Aeromonas* strains (Table 1). Although the incidence of cytotoxin production among *Aeromonas* isolates was lower than that noted by others, our results confirm the observations previously reported that the *Aeromonas* cytotoxin seems not to belong to the Shiga toxin family (8, 16).

Thus, the results obtained in the present study suggest that production of SLTs is not a common characteristic of *E. coli* strains and other bacteria isolated from children in São Paulo. Although several serotypes of *E. coli* have been described as SLT or VT producers (10), they probably do not have a universal distribution and are restricted to some geographical areas. Besides that, production of SLT was rarely found among the EPEC strains we studied, and the levels produced were low. Cytotoxin was detected only in strains isolated from gastrointestinal disorders, but their importance as agents of diarrhea in our community could not be established.

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LITERATURE CITED

1. **Bartlett, A. V., III, D. Prado, T. G. Cleary, and L. K. Pickering.** 1986. Production of Shiga toxin and other cytotoxins by serogroups of *Shigella*. J. Infect. Dis. **154**:996–1002.
2. **Brown, J. E., P. Echeverria, D. N. Taylor, J. Seriwatana, V. Vanapruks, U. Lexomboon, R. N. Neill, and J. W. Newland.** 1989. Determination by DNA hybridization of Shiga-like-toxin-producing *Escherichia coli* in children with diarrhea in Thailand. J. Clin. Microbiol. **27**:291–294.
3. **Cleary, T. G., J. J. Mathewson, E. Faris, and L. K. Pickering.** 1985. Shiga-like cytotoxin production by enteropathogenic *Escherichia coli* serogroups. Infect. Immun. **47**:335–337.
4. **Dean, A. G., Y. C. Ching, R. G. Williams, and L. B. Harden.** 1972. Test for *Escherichia coli* enterotoxins using infant mice: application in a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407–411.
5. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of Enterobacteriaceae, 3rd ed. Burgess Publishing Co., Minneapolis.
6. **Evans, D. J., Jr., and D. G. Evans.** 1977. Direct serological assay for the heat-labile enterotoxin of *Escherichia coli* using passive immune hemolysis. Infect. Immun. **16**:604–609.
7. **Gentry, M. K., and J. M. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J. Clin. Microbiol. **12**:361–366.
8. **Johnson, W. M., and H. Lior.** 1981. Cytotoxicity and suckling mouse reactivity of *Aeromonas hydrophila* isolated from human sources. Can. J. Microbiol. **27**:1019–1027.
9. **Karch, H., J. Heesemann, and R. Laufs.** 1987. Phage-associated cytotoxin production by an enteroadhesiveness of enteropathogenic *Escherichia coli* isolated from infants with diarrhea in West Germany. J. Infect. Dis. **155**:707–715.
10. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2**:15–38.
11. **Karmali, M. A., M. Petric, C. Lim, R. Cheung, and G. S. Arbus.**

1985. Sensitive method for detecting low numbers of Verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of Verotoxin. J. Clin. Microbiol. **22**:614–619.

12. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by Verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151**:775–782.
13. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18**:775–779.
14. **Levine, M. M., J. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. P. Nataro, H. Karch, and K. Wachsmuth.** 1987. A DNA to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect. Dis. **156**:175–182.
15. **Marques, L. R. M., M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien.** 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154**:338–341.
16. **Millership, S. E., M. R. Barer, and S. Tabaqchali.** 1986. Toxin production by *Aeromonas* spp. from different sources. J. Med. Microbiol. **22**:311–314.
17. **O'Brien, A. D., M. E. Chen, R. K. Holmes, J. Kaper, and M. M. Levine.** 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce *Shigella dysenteriae* I (Shiga)-like cytotoxin. Lancet **i**:77–78.
18. **O'Brien, A. D., and R. K. Holmes.** 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51**:206–220.
19. **O'Brien, A. D., and G. D. Laveck.** 1982. Immunochemical and cytotoxic activities of *Shigella dysenteriae* 1 (Shiga) and Shiga-like toxins. Infect. Immun. **35**:1151–1154.
20. **O'Brien, A. D., G. D. Laveck, M. R. Thompson, and S. B. Formal.** 1982. Production of *Shigella dysenteriae* type I-like cytotoxin by *Escherichia coli*. J. Infect. Dis. **146**:763–769.
21. **Riley, W. L.** 1987. The epidemiologic, clinical, and microbiologic features of hemorrhagic colitis. Annu. Rev. Microbiol. **41**:383–407.
22. **Scotland, S. M., N. P. Day, and B. Rowe.** 1980. Production of a cytotoxin affecting Vero cells by strains of *Escherichia coli* belonging to traditional enteropathogenic serogroups. FEMS Microbiol. Lett. **7**:15–17.
23. **Serény, B.** 1957. Experimental keratoconjunctivitis shigellosa. Acta Microbiol. Acad. Sci. Hung. **4**:367–376.
24. **Silva, M. L. M., I. C. A. Scaletsky, and L. H. Viotto.** 1983. Non production of cytotoxin among enteropathogenic strains of *Escherichia coli* isolated in São Paulo, Brasil. Rev. Microbiol. **14**:161–162.
25. **Smith, H. R., S. M. Scotland, H. Chart, and B. Rowe.** 1987. Vero cytotoxin production and presence of VT genes in strains of *Escherichia coli* and *Shigella*. FEMS Microbiol. Lett. **42**:173–177.
26. **Smith, H. W., P. Green, and Z. Parsell.** 1983. Vero cell toxin in *Escherichia coli* and related bacteria: transfer by phage conjugation and toxic action in laboratory animals, chickens and pigs. J. Gen. Microbiol. **129**:3121–3137.
27. **Toledo, M. R. F., M. do C. B. Alvariza, J. Murahovschi, S. R. T. S. Ramos, and L. R. Trabulsi.** 1983. Enteropathogenic *Escherichia coli* serotypes and endemic diarrhea in infants. Infect. Immun. **39**:586–589.