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Enterotoxigenic Bacteria in Food and Water from an Ethiopian Community

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Food and water samples from an Ethiopian community were screened for the presence of enterotoxin-producing bacteria. Using the Chinese hamster ovary cell assay, 40 of 213 isolates (18.8%) produced heat-labile (LT) enterotoxin. These LT-producing isolates comprised 33 of 177 (18.6%) strains from 24 of 68 food samples (35.3%) and 7 of 36 (19.4%) isolates of 4 of 17 water samples (23.5%). One LT-producing strain each of *Salmonella emek* and of *Shigella dysenteriae* was found. Three pseudomonads, all LT producers, produced heat-stable enterotoxin as gauged by the suckling mouse test. Two strains of LT-enterotoxigenic *Escherichia coli* O68 were found in water samples. No enterotoxigenic *E. coli* were isolated from food samples, but 13 of the LT-producing strains were *Enterobacter, Klebsiella, Serratia,* and *Proteus* species, and 7 food samples yielded more than one species of enterotoxigenic bacterium. Of the enterotoxigenic isolates from food, 15 were oxidase-positive strains of the genera *Aeromonas, Pseudomonas, Achromobacter, Flavobacterium,* and *Vibrio.* LT-enterotoxigenic *Enterobacter, Acinetobacter, Klebsiella, Proteus, Providencia,* and *Serratia* species represented 20 of the food and water isolates. Culture supernatant fluids of representative strains of oxidase-positive and oxidase-negative species giving positive reactions in Chinese hamster ovary cell tests induced fluid accumulation in rabbit ileal loops. Eight of the food samples and two of the water samples contained more than one isolate or species of enterotoxigenic bacterium. The stability of the LT production by oxidase-positive bacteria and non-*E. coli* strains was assessed by the rabbit skin and adrenal cell tests after 9 months and 1 year of storage, respectively, in Trypticase soy broth with glycerol at −70°C. Only 33% of the oxidase-positive strains were still LT enterotoxigenic. Of the oxidase-negative strains, 50 and 33% were LT producing at 9 months and 1 year, respectively. None of the *E. coli* isolates, both enterotoxigenic and nonenterotoxigenic, possessed K88, K99, or colonization factor antigen. The survey demonstrates the presence in food and water of enterotoxigenic bacteria of the same species as those isolated from cases of infantile diarrhea in the same community, although a correlation between these sources and infantile diarrhea remains to be established.

Food and water are well recognized vehicles of diarrheal infections caused by bacteria, viruses, and protozoa (17, 26). Apart from the classical "food-poisoning" bacteria such as *Clostridium botulinum, Clostridium perfringens,* and *Staphylococcus aureus,* interest has centered on the presence of the well established enteric pathogens of the genera *Salmonella* and *Shigella.*

In the last decade, increasing attention has focused on the role of bacteria other than salmonellae and shigellae as causes of diarrhea, in particular on enterotoxin-producing *Escherichia coli.* Although the significance of members of the family *Pseudomonadaceae* and of other coliforms has been questioned consistently in the past (26), an increasing number of reports in recent years have implicated enterotoxigenic *Klebsiella, Citrobacter, Proteus, Enterobacter, Pseudomonas, Aeromonas, Plesiomonas,* and vibrios other than *Vibrio cholerae,* i.e., NAG vibrios and group F vibrios (2, 10, 23, 30, 31, 34, 46, 55, 56). Even *Salmonella* and *Shigella,* which have been regarded as causing diarrhea by their invasiveness, have been reported to produce enterotoxins (29, 51, 54).

The extent of indicator organisms and consequently of enteric pathogens in vegetables and natural waters has been assessed by various workers (11, 12, 35, 52). Moreover, surveys for the presence of enterotoxigenic bacteria in food products have been performed by two groups (9,

41). In the United States, Sack et al. (41) found that 8% of 240 *E. coli* isolates produced heat-labile (LT) or heat-stable (ST) enterotoxin(s). They also showed that these enterotoxigenic isolates were of various serotypes not included among the so-called classical enteropathogenic serotypes of *E. coli* (37). In the Swedish study, only one ST-producing *E. coli* was found among 323 isolates from routine bacteriological analyses of food products but LT-producing *E. coli* and LT-producing *Klebsiella* were reported from three different food-poisoning outbreaks (9).

Attempts have also been made to correlate travelers' diarrhea in Mexico with food consumption (33, 36, 53). There was a significant association of illness with the eating of raw vegetables (36) and the eating of food from street vendors and from public restaurants (53). Moreover, outbreaks of food-borne illness in the United States and Canada have been associated with imported French cheese from which enterotoxigenic or enteroinvasive *E. coli* was isolated (16, 18).

The most studied outbreak of water borne diarrheal disease due to enterotoxigenic *E. coli* (O6:K15:H16) which produced both LT and ST occurred at Crater Lake National Park, Ore., in 1975. More than 1,000 cases of diarrhea were reported when park water became contaminated with effluent sewage (39). Differences in the survival times of *E. coli* and other enteric bacteria have been shown to depend upon the water source (e.g., well, river, pond) and upon ambient temperature (15, 17, 35). Such differences may be of particular significance in African countries (15, 17, 19, 28).

Diarrheal disease is one of the main killer diseases in early childhood in most developing countries, with attack rates of one to three episodes per year per child not being uncommon (19, 33). Studies from Ethiopia (56), Zaire (55), and from South Africa (45) on pediatric diarrhea have shown that enterotoxigenic coliforms such as *Klebsiella, Enterobacter, Citrobacter,* and *Proteus* are putative causal organisms in addition to enterotoxin-producing *E. coli.*

At the present time more information is needed concerning the vehicles of transmission and the survival of such pathogens outside the body. This is particularly so in many countries where the food industry does not operate under adequate regulation of quality and hygiene control (28, 53). The high numbers of enterotoxigenic bacteria, including members of the vibrio family such as *Aeromonas hydrophila,* in pediatric diarrhea in Ethiopia (56) and the high incidence of diarrhea among newly arrived Swedes (3) prompted a study of the occurrence of enterotoxigenic bacteria in food and water in Addis Ababa in 1977, concurrently with a second epidemiological study of pediatric diarrhea (50).

MATERIALS AND METHODS

Source of food samples. Of the surveyed vegetables, fish, and meat products, 70% were bought from stall-owners at the Mercato market, Addis Ababa. The history of these foods before purchase was unknown. The remainder of the food samples were purchased from city hawkers from March through December 1977. Individual food products were transported and stored in separate plastic bags, and the samples were cultured within 2 h of purchase.

Processing of food samples. Surface bacterial examination of all samples was done by the balloon print isolation technique described by Rusch and Leben (40), and culture was performed on MacConkey agar (Oxoid Ltd., Basingstoke, England) and salmonella-shigella agar (SS agar; Difco Laboratories, Detroit, Mich.). Each food sample was also homogenized or blended in a Vortex mixer in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C for 24 h, and at 4°C for 3 weeks for cold enrichment of *Yersinia enterocolitica* (44). Each homogenized sample was also incubated in alkaline peptone water at 4°C for 8 to 12 h to enrich vibrios and subcultured onto thiosulfate citrate bile salts sucrose medium (BBL). Each sample was also grown on selenite broth (Difco) at 37°C for 24 h to enrich *Salmonella* (6).

Identification procedures. Three to six isolates of different colony morphology from each food sample were subcultured onto cysteine-lactose-electrolyte-deficient agar (Oxoid). The isolates were then inoculated in agar deeps in Durham tubes for transport by courier to Sweden without refrigeration.

Upon arrival in Sweden, all isolates were recultured to check purity on heart infusion agar (Difco) containing 1% lactose and 0.003% bromocresol purple. Samples growing *Proteus* were grown on cysteine-lactose-electrolyte-deficient agar. All isolates were frozen at −70°C in TSB containing 15% (wt/vol) glycerol.

A total of 213 isolates were identified biochemically by the API-20E system (API System S.A., La Balme les Grottes, France). An oxidase test was performed on each isolate (6). Oxidase-positive isolates which were not identifiable on API-20E were examined using the Oxi-Ferm system (Hoffman-La Roche, Basel, Switzerland) which is designed to identify oxidase-positive bacteria (27). In addition, conventional biochemical tests were performed on some strains. Suspected *Salmonella* and *Shigella* were identified at the National Bacteriological Laboratory, Stockholm. *Klebsiella* species producing LT enterotoxin were phage-typed at the National Bacteriological Laboratory by L. Sjöberg (38).

Serological tests for K88, K99, and colonization factor antigens. All *E. coli* isolates were screened for possession of K88, K99, or colonization factor antigen. Preparation of specific immunoglobulins against K88 antigen and colonization factor antigen have been described previously (for details see reference 48). K99 antiserum was prepared using the

same immunization schedule (48). The immunogen comprised a laboratory strain K-12 carrying the K99 plasmid transferred from strain B41 (a bovine K99-positive *E. coli*) kindly supplied by C. Gyles, University of Guelph, Canada. The strain was grown on Minca-IsoVitaleX (BBL) medium (24). Serum pools from two rabbits were extensively absorbed with live and heat-treated K-12 ($K99^-$) (C. J. Smyth, personal communication).

All *E. coli* were selected for the pellicle phase by repeated passage in TSB broth tubes after incubation at 37°C for 48 h. Pellicle growth was subcultured onto Minca-IsoVitaleX agar, 5% (vol/vol) horse blood agar (heart infusion agar base), and improved colonization factor antigen agar (48) for tests for K99, K88, and colonization factor antigens, respectively. Slide agglutination tests were performed with known positive and negative controls using suspensions in 0.15 M NaCl.

Growth of strains for enterotoxin production. TSB-stored isolates were cultured overnight on bromocresol-lactose agar. Loopfuls of growth were seeded into 50-ml portions of TY-1 medium containing 10 g of glucose per liter (25, 57). All oxidase-positive strains were grown at 22°C, and oxidase-negative strains were grown at 37°C as shake cultures (150 rpm). In addition, LT-positive strains were also grown in a Casamino Acids-yeast extract medium (pH 7.5) (1, 14) for confirmatory LT and/or ST tests. Supernatant fluids (three times, 250 × *g*, 30 min, 4°C) were divided into two portions. The portion for LT tests was stored frozen at −70°C and tested within 1 week of preparation in the cell tests or the rabbit skin test. The other portion was heat treated (80°C, 30 min) before storing at −70°C. ST tests were usually performed within 1 week of preparation.

CHO and Y1 adrenal cell tests for LT. The Chinese hamster ovary (CHO) cell test was performed by the method of Guerrant et al. (22). All culture supernatant fluids were tested undiluted and at dilutions of 1 in 10 and 1 in 100. All tests were read by two observers independently. Strains which were LT positive in this screening test were regrown and retested. Tests were performed for all isolates before biochemical identification other than as oxidase-positive or oxidase-negative bacteria. Some of the supernatant fluids (CHO positive and CHO negative) were run in parallel in the Y1 adrenal cell test in a miniculture modification (49).

One year after initial testing, the strains were retested twice by Y1 adrenal cell test with and without antibodies against *A. hydrophila* cytotoxic factor protein (34) to neutralize cytotoxic factors produced by oxidase-positive isolates (Å. Ljungh, unpublished data).

Suckling mouse test. For detection of ST, the suckling mouse test described by Giannella (21) was used. Three 2- to 3-day-old mice (NMRI strain) were injected intragastrically with 100 μl each of heat-treated TY-1 or Casamino Acids-yeast extract culture supernatant fluid containing one drop of 0.2% (wt/vol) Evans blue per ml of test material added as a marker just before testing. After 4 h at 20 to 22°C, the mice were killed by cervical dislocation, and the pooled intestinal-weight-to-remaining-body-weight ratio of the three mice was determined. Samples yielding ratios ≥0.085 were considered positive.

Rabbit skin test. Fresh culture supernatant fluids from all CHO test-positive isolates were tested in rabbit skin about 8 months after the strains had first been identified as LT positive. This test was performed as described by Sandefur and Peterson (42) in 2- to 2.5-kg New Zealand White rabbits. Evans blue (0.5%) was injected intravenously 18 h after intradermal injection of test samples, and the tests were read 2 h later.

Rabbit ileal loop test. Fresh culture supernatant fluids from representative CHO test-positive isolates were tested in rabbit ileal loops (2). Fluid accumulation was measured after 18 h. A volume-to-length ratio of 0.5 ml/cm of intestine was regarded as positive. Positive and negative controls were always included.

Controls. Crude cholera toxin kindly provided by J. Craig, The National Institutes of Allergy and Infectious Diseases, Bethesda, Md., and 0.15 M NaCl and phosphate-buffered saline were used as positive and negative controls, respectively, in all rabbit skin, rabbit loop, and cell tests. Supernatant fluids from known ST-positive and -negative strains of *E. coli* were included with ST tests (21, 56).

RESULTS

Incidence of enterotoxigenic bacteria. A total of 213 isolates, 177 from vegetables, raw fish, and raw meat products, and 36 from water, were screened for LT production by the CHO cell test. Food and water yielded 33 and 7 enterotoxigenic isolates, respectively (18.6% and 19.4% of isolates, respectively). Four of 17 (23.5%) water samples yielded LT-producing bacteria, including one strain of *Salmonella emek*. LT-producing bacteria including one strain of *Shigella dysenteriae* were found in 24 of 68 food samples (35.3%).

All LT-producing strains plus strains identified as *E. coli, Klebsiella pneumoniae,* and *Pseudomonas* spp. were tested for ST production. None of the *E. coli* and *K. pneumoniae* produced demonstrable ST when grown in Casamino Acids-yeast extract medium. Three pseudomonads (Table 1), all of which were LT producers, were ST positive. In addition, one strain each of *Flavobacterium odoratum, Pseudomonas aeruginosa,* and *Achromobacter calcoaceticus* var. *anitratus* gave borderline results.

Rabbit ileal loop test. Culture supernatant fluids of 12 representative isolates of CHO test-positive species were tested in ligated ileal loops (Fig. 1). Positive ileal loops were obtained for each tested isolate on two or three separate occasions. Volume-to-length ratios ranged from 0.5 to 3.1 ml/cm of gut. The test species were *E. coli, Enterobacter agglomerans, K. pneumoniae, Proteus vulgaris, Serratia liquefaciens, Serratia rubidaea, S. dysenteriae, F. odoratum, P. aeruginosa, Pseudomonus fluorescens, Pseudomonas maltophila* and *Pseudomonas putida.*

Enterotoxigenic bacteria in food. Of the 33 enterotoxigenic isolates, 15 (45.5%) were oxi-

TABLE 1. *Enterotoxigenic bacteria isolated from Ethiopian food and water*

Bacterium	No. of strains according to enterotoxin type[a]	
	LT only	LT-ST
Oxidase-positive		
A. hydrophila	3	
Achromobacter spp.	1	
CDC Group II K2	1	
F. odoratum	1[b,c]	
P. aeruginosa	1[b,d]	
P. fluorescens	1	2[e]
P. maltophila	1	
P. putida	1	1[f]
P. stutzeri	1	
V. alginolyticus	1	
Oxidase-negative		
A. calcoaceticus var. *lwoffi*	2[c]	
A. calcoaceticus var. *anitratus*	3[g]	
E. cloacae[h]	4[c]	
E. agglomerans	2[c]	
E. coli[h]	2[i]	
K. pneumoniae	4[c]	
P. vulgaris	1[c]	
P. alcalifaciens	1	
S. rubidaea	2[c]	
S. emek	1	
S. dysenteriae	1	
Serratia liquefaciens	2	

[a] LT-producing = positive CHO cell test; ST-producing = ratio of intestine to remaining body weight = 0.085 in suckling mouse test, using heat-treated supernatant fluids from TY-1 medium.

[b] Reaction in suckling mouse test gave indeterminate values between 0.075 to 0.084 according to Giannella (21).

[c] Strains tested in parallel in adrenal Y1 cell test and found to be positive: *A. calcoaceticus* var. *lwoffi* (1), *E. cloacae* (1), *E. agglomerans* (2), *K. pneumoniae* (1), *P. vulgaris* (1), *S. rubidaea* (2), *F. odoratum* (1); in addition five strains which were negative in CHO cell tests were also tested and found to be negative in the adrenal Y1 cell test.

[d] Strain was negative in suckling mouse test upon retesting 1 year later, heat-treated supernatant fluid from Casamino Acids-yeast extract medium.

[e] Both strains still positive in suckling mouse test when retested 1 year later, heat-treated supernatant fluid from Casamino Acids-yeast extract medium.

[f] Strain negative for ST 1 year later, heat-treated supernatant fluid from Casamino Acids-yeast extract medium.

[g] One strain gave an indeterminate value for ST (see footnote *b*).

[h] Isolated from water samples—*E. coli* (2), *E. cloacae* (2).

[i] Includes one strain which was only tested on Y1 adrenal cells for LT and found to be positive.

dase positive (Table 1). *Pseudomonas* spp. represented 50% of these. The oxidase-positive isolates were from 12 food samples. One of the enterotoxigenic *A. hydrophila* was from fish.

No enterotoxigenic *E. coli* were isolated from the food samples. In contrast, 13 of the LT-producing isolates were *Enterobacter, Klebsiella, Serratia,* and *Proteus* species. These were isolated from 7 of the 12 food samples yielding oxidase-negative enterotoxigenic bacteria.

A total of seven food samples yielded more than one species of enterotoxigenic bacterium. Two food samples yielded oxidase-positive and oxidase-negative LT-producing strains (Table 2). One sample yielded one strain each of two LT-producing *Pseudomonas,* and one sample yielded two LT-producing isolates of *K. pneumoniae.* Other combinations are shown in Table 2.

The four enterotoxigenic *K. pneumoniae* (Table 1) were phage typed (38) as I/II/III, VII, I/II, and nontypable (100 × routine test dose). The latter two were from the same food sample.

Enterotoxigenic bacteria in water. The only enterotoxigenic *E. coli* isolate (O-group 68) came from a water sample (Tables 1 and 3), interestingly from the same sample yielding the enterotoxigenic strain of *S. emek* (Table 2). Two of the four water samples yielded more than one enterotoxigenic isolate (Table 2). The other two samples each yielded one isolate of LT-producing *Enterobacter cloacae.*

Salmonella, Shigella, Yersinia, and *Vibrio*. No salmonellae or *Y. enterocolitica* were isolated from the food samples, but one enterotoxigenic strain of *S. dysenteriae* was found together with an enterotoxigenic strain of *A. calcoaceticus* var. *lwoffi.* No vibrios other than one strain of *Vibrio alginolyticus* were found. No salmonellae other than *S. emek,* shigellae, yersiniae, or vibrious were isolated from water samples.

Stability of LT production by food and water isolates. All LT-positive isolates were maintained frozen at −70°C in TSB-glycerol. The stability of the LT characteristic in most strains was assessed twice, after 9 months of storage using the rabbit skin test and after 1 year using the Y1 adrenal cell test. Not all strains were tested on both occasions. The results of the skin tests are shown in Table 4, and the results of the adrenal cell tests are shown in Table 5.

Only 4 of the 15 LT-producing oxidase-positive strains were positive in the rabbit skin test after 9 months of storage. Three of these four strains were positive in the adrenal cell test after 1 year. In addition, two strains giving negative skin findings at 9 months were found to be LT-positive by the Y1 adrenal cell test after 1 year of storage.

Regarding the oxidase-negative LT-producing isolates, 11 of 21 strains gave positive rabbit skin tests. At 1 year, 5 of 14 tested strains were still

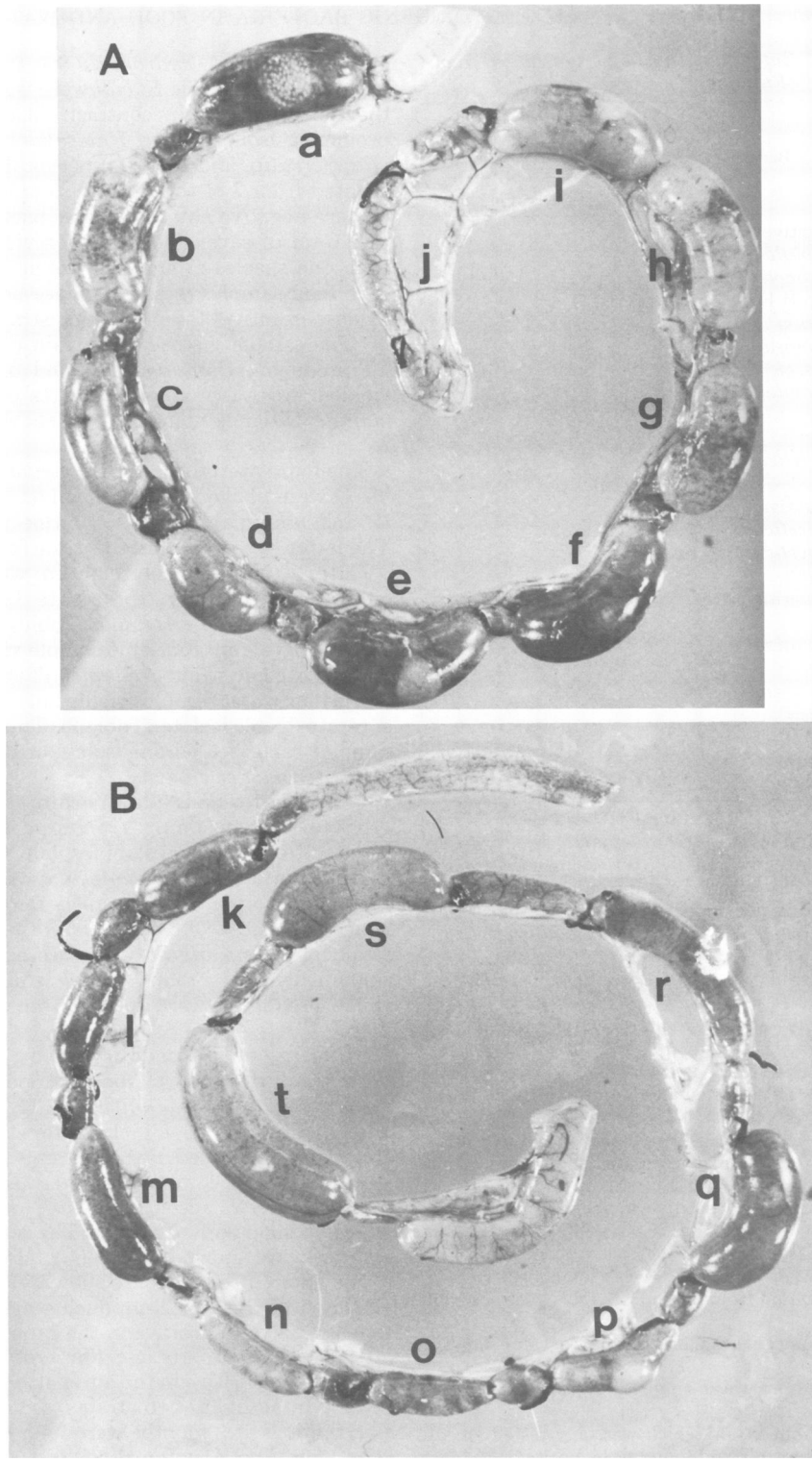

FIG. 1. *Rabbit intestinal loop tests with culture supernatant fluids from representative isolates of oxidase-positive and oxidase-negative bacteria isolated from Ethiopian food and water samples. (A) V. cholerae crude enterotoxin, positive control (a); A. hydrophila F2 (b); V. alginolyticus F42-1 (c); F. odoratum F63-5 (d); P. fluorescens F74-4 (e); P. putida F92-6 (f); P. aeruginosa F108-5 (g); P. maltophila F3-2 (h); A. hydrophila F31 (i); physiological saline, negative control (j). (B) S. dysenteriae F101-4 (k); E. coli O68 strain FW82-5 (l); S. emek FW82-1 (m); physiological saline, negative control (n); S. liquefaciens FW81-6 (o); P. vulgaris F61-6 (p); K. pneumoniae F64-4 (q); S. rubidaea (r); E. agglomerans F42-1 (s); V. cholerae crude enterotoxin, positive control (t).*

TABLE 2. *Food and water samples yielding more than one enterotoxigenic isolate*

Sample no.	Source	Enterotoxigenic bacteria
F42	Meat	*E. agglomerans, S. rubidaea* (2)[a]
F63	Fish	*F. odoratum, A. calcoaceticus* var. *lwoffi*
F64	Tomato	*E. agglomerans, K. pneumoniae*
F65	Lettuce	*P. alcalifaciens, A. calcoaceticus* var. *anitratus*
F68	Meat	*K. pneumoniae* (2)[a]
F74	Fish	*K. pneumoniae, P. fluorescens*
F101	Lettuce	*S. dysenteriae, A. calcoaceticus* var. *lwoffi*
F108	Lettuce	*P. putida, P. aeruginosa*
FW81	Water	*S. liquefaciens* (2)[a]
FW82	Water	*S. emek, E. coli* (2)[a]

[a] Two LT-producing isolates of the indicated species.

TABLE 3. *Data on water isolates tested for enterotoxigenicity*[a]

Bacterium	No. of isolates	No. of enterotoxigenic strains
E. coli	10	2
C. freundii	3	0
E. cloacae	3	2
S. liquefaciens	3	2
S. odorifera	1	0
K. pneumoniae	3	0
P. morganii	1	0
S. emek	1	1
Enteric group 8	1	0
Unidentified	10	0

[a] Seventeen water samples were tested.

LT producing. The findings for strains of *E. cloacae, S. liquefaciens,* and *S. dysenteriae* (5 strains in all) agreed on both test occasions. The strains of *P. vulgaris, E. coli, E. agglomerans, Providencia alcalifaciens, S. rubidaea,* and *S. emek,* plus one strain each of *K. pneumoniae* and *A. calcoaceticus* var. *lwoffi* (11 in all) were only retested on one of the occasions. Whereas three of the four *K. pneumoniae* were positive in skin tests at 9 months, none was positive in the adrenal cell test at 1 year.

Examination of *E. coli* isolates for K88 antigen, K99 antigen, and colonization factor antigen. None of the isolates of *E. coli* from water possessed K88 antigen, K99 antigen, or colonization factor antigen as gauged by direct culture on appropriate media and after selection for the pellicle phase of the bacteria by passage in TSB before culture on appropriate media.

DISCUSSION

Little information on the epidemiology of different species of enterotoxigenic bacteria is as yet available (28, 33, 36, 39, 52, 53). One aim of the present investigation was to identify possible environmental sources of such bacteria in an Ethiopian community. Enterotoxigenic strains of *E. coli, Enterobacter, Serratia, Klebsiella, Aeromonas, Pseudomonas, Citrobacter,* and *Acinetobacter* have been isolated from cases of infantile diarrhea in Addis Ababa during surveys in 1974 and 1977 (4, 50, 56). Such isolates were found in this survey of food and water from the principal market of this city and from the traditional street vendors. However, it is unclear whether these food and water isolates are pathogenic for infants and/or adults and represent etiological agents of diarrhea (with the exception of the well established enteropathogens *S. emek* and *S. dysenteriae*). Although neither of the two enterotoxigenic *E. coli* strains from water possessed any of the three well characterized adhesins on human and animal enterotoxigenic *E. coli,* they possessed O antigen 68, which has

TABLE 4. *Skin test reactivity of CHO cell test-positive strains after 9 months of storage*[a]

Bacterium	No. of strains tested[b]	No. of strains reactive in rabbit skin test
Oxidase-positive		
A. hydrophila	3	1
Achromobacter spp.	1	0
CDC Group II K2	1	0
F. odoratum	1	0
P. aeruginosa	1	1
P. fluorescens	3	1
P. maltophila	1	1
P. putida	2	0
P. stutzeri	1	0
V. alginolyticus	1	0
Oxidase-negative		
A. calcoaceticus var. *lwoffi*	2	2
A. calcoaceticus var. *anitratus*	3	0
E. cloacae	2	1
E. agglomerans	2	0
K. pneumoniae	4	3
P. vulgaris	1	0
P. alcalifaciens	1	1
S. liquefaciens	2	1
S. rubidaea	2	2
S. emek	1	0
S. dysenteriae	1	1

[a] The skin test had advantages over cell tests at this time because neutralizing antiserum for cytotoxic protein was not available.

[b] All strains had been LT-producing at 3 months after isolation.

TABLE 5. *Long-term stability of LT production by food and water isolates of enterotoxigenic bacteria from Ethiopia (cell test data)*

Bacterium	No. of strains tested[a]	No. of strains LT^+ on retesting after 1 yr[b]
Oxidase-positive		
A. hydrophila	2	1[c]
Achromobacter spp.	1	1
CDC Group II K2	1	0
P. fluorescens	3	1
P. maltophila	1	1
P. putida	2	0
P. stutzeri	1	0
V. alginolyticus	1	1
Oxidase-negative		
A. calcoaceticus var. *lwoffi*	1	0
A. calcoaceticus var. *anitratus*	3	2[c]
E. cloacae	2	1
E. coli	2	0
K. pneumoniae	3	0
S. liquefaciens	2	1[c]
S. dysenteriae	1	1

[a] Positive for LT in CHO cell tests (and adrenal cell tests, Table 2) at 3 months after isolation.
[b] Positive for LT in adrenal Y1 cell test.
[c] Strong positive results.

been found on isolates from infantile diarrhea in Addis Ababa (4).

Phage typing of enterotoxigenic *K. pneumoniae*, in conjunction with biotyping and serotyping and biotyping of enterotoxigenic *A. hydrophila* (34) and *Achromobacter* spp. (5), respectively, may provide clues as to the environmental sources of strains involved in the etiology of diarrhea (38).

Characteristic changes in the morphology of CHO and Y1 adrenal cells form the basis for determining whether or not culture supernatant fluids or filtrates of strains of *E. coli* and *V. cholerae* contain LT enterotoxin and cholera toxin, respectively (22, 49). Two questions are therefore relevant to the present survey. (i) Can factors other than enterotoxins cause such changes in morphology? (ii) Is a typical morphological change prima facie evidence of enterotoxin production? LT from *K. pneumoniae* has been shown to cause typical morphological changes in CHO cells (23). Indeed, the LT toxins of *Klebsiella* and *E. cloacae* appear to be immunologically related to *E. coli* LT (30) and can reasonably be assumed to have a similar mechanism of action. Thus, although direct evidence of adenylate cyclase activation is lacking still for the LTs produced by coliforms other than *E. coli*, (20, 22), the evidence to date (30) strongly suggests that the morphological changes observed in this study with culture supernatants from coliforms in both the CHO and adrenal cell tests represent effects of LT toxins. Moreover, the positive rabbit ileal loop tests obtained with culture supernatant fluids of representative isolates of CHO test-positive, oxidase-positive, and oxidase-negative species support the view that the actve principle(s) causing positive cell tests are enterotoxic.

Cultures filtrates of *A. hydrophila* also induce typical changes in the morphology of Y1 adrenal cells (34) and cause positive rabbit intestinal loop tests (2, 34). However, it has recently been reported that *A. hydrophila* enterotoxin is cytotoxic (7). This finding is at variance with the observations of Ljungh et al. (34). Anti-cytotoxic protein as used in this study with adrenal cell tests neutralizes the cytotoxic factor produced by *A. hydrophila* without neutralizing enterotoxic activity in crude culture supernatants (Å. Ljungh, personal communication). These observations substantiate our belief that the changes in cellular morphology interpreted as indicative of LT production by *A. hydrophila* and other oxidase-positive bacteria herein are caused by enterotoxins with properties common to cholera toxin.

A toxin produced by *S. dysenteriae* has also been reported to cause identical changes in the morphology of CHO cells to that observed with *E. coli* LT (51) and to give typical permeability reactions in rabbit skin (29, 51). The present findings confirm these observations with one strain of *S. dysenteriae* from water. However, it is unclear at present whether or not the factor causing morphological changes in CHO cells is a true enterotoxin and what its role in shigellosis is.

Enterotoxin produced by strains of *Salmonella* species has been assayed mainly in rabbit intestinal loops or the rabbit skin test (42, 54). Two skin permeability factors have been distinguished by Sandefur and Peterson (42). One of these, the delayed skin permeability factor, has been shown to elicit morphological changes in CHO cells that are indistinguishable from those produced by cholera toxin and are neutralized by monospecific cholera antitoxin (43). The morphological changes in CHO cells reported herein with culture supernatant fluids of *S. emek* support this, and similar findings have been obtained with other salmonellae in this laboratory (S. Jiwa, unpublished data).

It is recognized that enterotoxigenic *E. coli* exhibit to varying degrees a tendency to lose the capacity to produce LT and/or ST (13). The pattern of loss of enterotoxigenicity has been

correlated to O-antigen (13). Non-*E. coli* enterotoxigenic bacteria isolated from the stools of infants with diarrhea in Addis Ababa in a parallel study (4) were shown to lose the property of LT enterotoxigenicity more rapidly than enterotoxigenic *E. coli*. In the latter survey, strains were all stored on deep agar and subcultured approximately every 3 months. In contrast, herein all enterotoxigenic strains were preserved in TSB with glycerol as a cryoprotective agent at −70°C. However, despite this, enterotoxigenicity was not stabilized markedly in non-*E. coli*.

That LT and ST production are plasmid mediated in *E. coli* is well established (8, 47). The occurrence of multiple species of enterotoxigenic bacteria in food and water samples suggests that, under certain environmental conditions, *ent* plasmid transfer may occur between species, although its stability in certain species may not be high (Tables 4 and 5).

LT and ST production by isolates in this report was assessed using media previously shown to be very satisfactory for enterotoxin production by *E. coli* strains, i.e., TY-1 and Casamino Acids-yeast extract media (1, 14). However, no attempts were made to optimize cultural conditions for production of LT and ST by other coliforms or the oxidase-positive bacteria. Moreover, Kunkel and Robertson (32) have demonstrated that cultural conditions can affect synthesis and/or release of LT from *E. coli*. In addition, the production of cytotoxins or hemolysins may interfere with cell test assay systems (34). The rabbit skin test for permeability appears to circumvent some of the problems with cytotoxic effects and has been used for demonstration of enterotoxins by *A. hydrophila* and salmonellae (10, 42, 43).

Enterotoxin production by isolates was assessed before identification to the species level. Such a screening procedure removes preselection and bias from an epidemiological survey for enterotoxigenic bacteria. Escheverria et al. (11) screened environmental sources for heat-labile enterotoxigenic *E. coli* in a community in the Philippines. Although *E. coli* was present in all food samples, no enterotoxigenic *E. coli* was found among the isolates from vegetables, beef, and pork samples. Moreover, all water samples were contaminated with *E. coli*, but no strain produced LT. No attempts were made to screen for other potential enterotoxigenic bacteria, although Asian studies have reported the occurrence of such bacteria in the environment (e.g., 46).

Our approach for surveying a community in a developing country for possible sources of enterotoxigenic bacteria might be usefully applied elsewhere. Only in reports from the African continent has the multifactorial nature of infantile diarrhea of bacterial etiology been emphasized (4, 45, 50, 56). Epidemiological surveillance may usefully contribute to control of infantile diarrhea by identifying vectors and sources.

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