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Enterotoxin-producing *Escherichia coli* and Diarrheal Disease in Adult Travelers: A Prospective Study

Eleanor G. Shore, Andrew G. Dean, Kathleen J. Holik, and Betty R. Davis

From the Department of Microbiology, Harvard University School of Public Health, Boston, Massachusetts; and Enterobacteriology Section, Center for Disease Control, Atlanta, Georgia

Twenty-eight United States adult travelers to developing countries participated in a prospective study designed to determine whether there was an association between diarrheal illness and the acquisition of strains of enteropathogenic *Escherichia coli*. Stool specimens were collected and stored in transport media before, during, and after travel and at the onset of any diarrheal illness. From each specimen, five *E. coli* colonies were screened for enterotoxin production with the suckling mouse test, and three *E. coli* colonies were tested for penetration of epithelial cells (Serény test). Positive colonies were serotyped. Eleven of 28 travelers experienced diarrheal illness. Of these, four (36%) acquired enterotoxin-producing *E. coli* at the time of illness. None of the 17 well travelers had identifiable enterotoxin-producing *E. coli* before, during, or after travel. No strains of *E. coli* that penetrated epithelial cells were found. Three of the four travelers with enterotoxin-producing *E. coli* acquired serotype O27:K·:H20, a serotype not previously recorded at the Center for Disease Control. This study suggests that enterotoxin-producing *E. coli*, as detected by the suckling mouse test, may be a significant cause of traveler's diarrhea in adults.

Diarrheal disease is a frequent occurrence during international travel, and in most cases the cause is unknown [1, 2]. Serotypes of "enteropathogenic" *Escherichia coli* known to have caused outbreaks of infant diarrheal disease in the past have been identified in the stools of some infants and children with diarrhea, but such "classical" serotypes have not often been found to be associated with diarrheal disease in adults [3, 4].

Recent studies, however, have suggested that *E. coli* other than the classical enteropathogenic serotypes may play a significant role in human adult as well as infant diarrheal disease. Using rabbit intestinal loops to test for enterotoxin production [5–7]. Gorbach et al. found enterotoxin-producing *E. coli* in isolates from the small and large bowel in eight of 17 Indian adults with acute cholera-like diarrheal illness where no cholera vibrios were present [8]. Du Pont et al. demonstrated that a diarrheal illness was produced in seven of 10 human adult volunteers fed either of two strains of enterotoxin-producing *E. coli* (O6:H16 and O148:H28) isolated from American soldiers in Vietnam [9]. Du Pont's group was also able to produce a severe diarrheal illness in seven of 13 adult volunteers fed either of two strains of *E. coli* that had been shown to be capable of invading the epithelium of intestinal mucosa.

Dean et al. have recently demonstrated that the enterotoxin produced by *E. coli* can be assessed in a suckling mouse test with results comparable to those of the rabbit ileal loop test [10]. This development has permitted screening of large numbers of strains with less expense and less difficulty.

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Please address requests for reprints to Dr. Eleanor G. Shore, Department of Microbiology, Harvard University School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115.

To determine whether or not *E. coli* strains capable of causing adult diarrheal disease were acquired by adult travelers from the United States and whether there was any correlation between the acquisition of these strains and the appearance of a diarrheal illness, a study of adults traveling to developing countries for short trips was initiated. The factors studied were (*1*) acquisition of enterotoxin-producing *E. coli,* (*2*) acquisition of strains capable of penetrating epithelial cells and causing a shigellalike illness, and (*3*) shifts in *E. coli* serotypes during travel.

Materials and Methods

Subjects. Adult travelers obtaining their immunizations at the Harvard University Health Services for short trips to developing countries were offered the opportunity to participate in this study before their departure. Blood and stool specimens were collected before and after travel. In addition, stool specimens were collected with charcoal-treated cotton swabs and preserved in Cary and Blair's transport media [11] at the onset of any diarrhea and one week before return, whether the traveler was well or ill. The time between collection in transport media and plating varied from a few hours to eight days.

Bacteriologic and parasitologic exams. Isolation and preliminary identification were done according to the methods of Edwards and Ewing [12]. Each stool specimen was plated on blood, MacConkey's bismuth sulfite, xylose-lysine-deoxycholate, and thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates. Tetrathionate broth was inoculated and incubated overnight before the cells were plated on additional bismuth sulfite, hektoen enteric, xylose-lysine-deoxycholate, and eosin-methylene blue plates. All specimens obtained before and after travel were also examined for ova and parasites in the Clinical Laboratory at the New England Deaconess Hospital.

From the blood agar and MacConkey plates, 10 presumed *E. coli* colonies were isolated from each specimen and stored on beef heart infusion agar slants at 24 C for subsequent biochemical confirmation.

Identification of the Enterobacteriaceae was accomplished according to the biochemical methods of Edwards and Ewing [12]. Further identification was performed by the Massachusetts State Diagnostic Laboratories when necessary.

Suckling mouse test for enterotoxin. Five biochemically confirmed *E. coli* colonies (in a few cases less than five were available) from each specimen were tested for evidence of enterotoxin production by intragastric inoculation of suckling mice [10]. Trypticase soy broth (4 ml) was inoculated with a test culture and shaken for 16–20 hr at 200 rpm at 37 C in a 25- or 50-ml flask. After centrifugation at 3,000 *g* for 30 min, the supernatant liquid was combined with two drops of 1.25% Evans Blue as a marker, and 0.1 ml was injected with a syringe and a no. 27 needle through the abdominal wall into the milk-filled stomach of each of four one- to four-day-old mice. Four hours later, the mice were killed and the intestinal tract inspected, removed, and weighed. The presence or absence of a gut that appeared turgid, swollen, and liquid-filled (as occurs when a known enterotoxin has been injected) was noted. The ratio of the combined weight of the intestines of the four mice to the combined weight of the bodies was determined. An intestine-to-body weight ratio of over 0.09 was regarded as indicative of the presence of enterotoxin [10]. Each positive test was repeated with the supernatant liquid of a centrifuged overnight shake culture passed through a Millipore filter (pore size, 0.22 or 0.45 μm). In each series of tests, a known positive strain (B2C, serotype O6:H16, obtained from Samuel B. Formal, Walter Reed Army Institute of Research) was used as a control. Each strain identified as a toxin-producer was stored both on beef heart infusion agar in a sealed glass ampule at 24 C and in trypticase soy broth with glycerol at −70 C.

Guinea pig eye test. Three colonies from each stool specimen collected at the onset of an episode of diarrhea were tested for evidence of epithelial penetration (Serény Test) [13]. In each case, the bacteria from an agar slant inoculated and incubated for 24 hr were suspended in trypticase soy broth, and one to two drops were placed in one eye of each of two guinea pigs. The eyes were examined daily for four days for signs of keratitis or conjunctivitis.

Serotyping. After preliminary serotyping in our laboratory, three *E. coli* colonies from each of the specimens of the four patients who acquired enterotoxin-producing *E. coli* during illness were

serotyped in the Enteric Bacteriology Laboratories at the Center for Disease Control, Atlanta, Georgia.

Results

Twenty-eight travelers, aged 19 to 55, completed the study. The duration of travel ranged from 19 to 38 days; the areas visited included Asia, Africa, and South and Central America. Eleven travelers (39%) reported having a diarrheal episode with two or more watery stools in a single day during or immediately after the trip.

No salmonellae or shigellae were found in the stools of any of the travelers in this study. One patient acquired *Giardia lamblia* during the trip; parasitologic findings in the others did not change.

As shown in table 1, four of the 11 travelers with diarrhea (36%) had strains of enterotoxin-producing *E. coli* detected after onset of diarrhea but not before. Each of these strains produced a characteristic distended fluid-filled gut in the test mouse and a gut-to-body weight ratio of more than 0.09. Of the 17 travelers who remained free of diarrhea, no enterotoxin-producing *E. coli* was found in any specimen before, during, or after travel.

The interval between initial isolation of *E. coli* strains and testing in the mouse system was five to 10 months. Over the following 14–17-month period, repeated tests were performed on stored colonies of each strain identified initially as enterotoxin-producing *E. coli*. Two of the 18 strains apparently lost their enterotoxic activity on this further storage. The other 16 enterotoxic strains showed varying activity on repeated testing; nine were positive in every test, and seven were positive in the majority of tests. All 16 gave a positive mouse test after filtration of the supernate through filters with a pore size of 0.22 or 0.45 μm. The cause of the loss of activity during storage has not been determined. Strains stored at —70 C retained their enterotoxic activity somewhat better than those in sealed ampules at 24 C.

Of the four patients with enterotoxin-producing *E. coli* at the time of illness, all had abdominal pain, but none had fever or blood in their stools. Only one was incapacitated (for two days). Illnesses lasted for two to 16 days, with two to 10 diarrheal stools per day. The patient with the longest illness had acquired *G. lamblia* as well as enterotoxin-producing *E. coli*. No antibiotics were used.

Because the number of subjects was small, Fisher's exact test was used to assess the association between acquisition of enterotoxin-producing *E. coli* and diarrheal illness. The exact P value (one tail) was 0.016 and suggests that this association is quite unlikely to be due to chance. The fact that enterotoxin-producing *E. coli* were found during or after the illness and never before lends added significance to the findings.

The guinea pig eye tests for penetrating strains of *E. coli* were negative in all cases, although known penetrating strains obtained from Samuel B. Formal, Walter Reed Army Institute of Research, gave positive results with each lot of guinea pigs used.

The serotypes of *E. coli* isolated from the stools of the four patients with enterotoxin-producing *E. coli* are shown in table 1. In each patient, there was a change in serotypes from the strains isolated before illness to those isolated after illness. Three of the patients (no. 7, 12, and 13) acquired type O27:K·:H20 at the time of illness.

The fourth patient (no. 14) had five different serotypes detected after the onset of illness. The three strains of rel O80:K·:H9 consistently demonstrated toxin production, but the other two strains were those which lost activity upon storage.

The MICs of ampicillin, chloramphenicol, and tetracycline were determined by serial broth dilution. Seven of the enterotoxin-producing *E. coli* strains were sensitive to all three drugs, while six were found to be resistant to chloramphenicol (MIC, 200–400 μg/ml) and tetracycline (MIC, 800–1600 μg/ml). An additional two strains were resistant to tetracycline (MIC, 800 μg/ml) only. The resistant strains were recovered from three persons traveling in Central and South America but not from the person traveling in Africa. The resistance pattern was not identical in enterotoxin-producing *E. coli* strains of identical serotype, even when recovered from the same specimen.

Discussion

The occurrence of diarrhea in 39% of 28 travelers taking short trips to underdeveloped areas emphasizes the frequency of traveler's diarrhea and suggests that the amount of time and effi-

Table 1. Enterotoxin production and serotypes of *Escherichia coli* isolated from stools of travelers who acquired enterotoxin-producing *E. coli*.

Patient no.	Area of travel	Days before (−) or after(+) onset of diarrhea	Serotypes* identified	Mouse test (gut:body weight ratio)	Enterotoxin production	Antibiotic resistance†
7	Kenya	−17	O(und):H21	0.063	No	NT
			O73:K−:H45	0.063	No	NT
		−3	O128ad:K−:H12	0.066	No	NT
			O(und):H10	0.063	No	NT
			O128ad:K−:H12	0.076	No	NT
		+1	O21:K·:NM	0.065	No	NT
			O27:K·:H20	0.099	Yes	0
		+6	O27:K·:H20	0.132	Yes	0
			O27:K·:H20	0.121	Yes	0
			O27:K·:H20	0.125	Yes	0
12	Colombia, Brazil	−9	O75ab:K−:H5	0.053	No	NT
			O75ab:K−:H5	0.071	No	NT
			O75ab:K−:H5	0.065	No	NT
		−6	O45:K·:H19	0.062	No	NT
			O45:K·:H19	0.066	No	NT
			O78:K80:H2	0.053	No	NT
		−3	O78:K80:H2	0.052	No	NT
			O78:K80:H2	0.065	No	NT
			sl rel OX13: rel K22:NM	0.057	No	NT
		+2	O27:K·:H20	0.144	Yes	CT
			O27:K·:H20	0.113	Yes	CT
			O27:K·:H20	0.121	Yes	CT
13	Colombia, Brazil	−37	O(und):H19	0.062	No	NT
			O(und):H19	0.070	No	NT
			O(und):H19	0.059	No	NT
		−6	O64:	0.069	No	NT
			Rough:NM	0.067	No	NT
			O54:K−:H2	0.069	No	NT
		0	O27:K·:H20	0.103	Yes	CT
			O27:K·:H20	0.099	Yes	T
			O27:K·:H20	0.104	Yes	T
		+2	O27:K·:H20	0.106	Yes	CT
			O27:K·:H20	0.109	Yes	T
			O27:K·:H20	0.095	Yes	CT
14	Central America	−30	Rough:H30	0.063	No	NT
			O29:K·:H10	0.068	No	NT
			O27:K·:H12	0.065	No	NT
		0	O(und):K·:H40	0.120	Yes‡	NT
			O91:K·:H10	0.059	No	NT
		+13	O102:K−:H36	0.054	No	NT
			O(und):H9	0.104	Yes‡	NT
		+19	rel O80:K·:H9	0.127	Yes	0
			rel O80:K·:H9	0.139	Yes	0
			rel O80:K·:H9	0.102	Yes	0

* K− = K antigen lacking; K· = K antigen present but not typable with available antiserum; rel = related to; sl rel = slightly related to; und = undetermined (i.e., not typable with available antiserum); NM = nonmotile.

† A = resistance to ampicillin (MIC ⩾ 12 μg/ml); C = resistance to chloramphenicol (MIC ⩾ 200 μg/ml); T = resistance to tetracycline (MIC ⩾ 800 μg/ml); 0 = no resistance to ampicillin, chloramphenicol, or tetracycline; NT = not tested.

‡ Subsequent tests failed to produce a positive result.

ciency lost to illness on such trips must be appreciable. In agreement with other studies, we could not find a likely cause for the diarrhea by routine bacteriologic and parasitologic methods, except in the one case where *G. lamblia* was found after travel.

The appearance of enterotoxin-producing *E. coli* in the stools after, but not before, the occurrence of diarrhea and the absence of enterotoxin-producing *E. coli* in all the travelers who did not become ill suggests a causal relationship. To test this relationship further, studies of a larger group will be necessary. A more rapid and specific test for enterotoxin-producing *E. coli* would expedite such studies, but development of such a test awaits purification and more exact characterization of the enterotoxin. In the meantime, the suckling mouse test serves as a valuable research tool.

Penetrating strains of *E. coli* were not associated with diarrheal illness in our adult study population, but these strains too may lose activity with storage. Our tests were performed approximately one year after collection of the specimens.

The strain O27:K·:H20 which was acquired by three of the travelers, is a serotype not previously detected at the Center for Disease Control. Although patients no. 12 and 13 were husband and wife and may have acquired their organisms from the same source while traveling in Colombia and Brazil, patient no. 7 traveled to Kenya and had no contact with the other two. The fact that patient no. 14 had three different serotypes that were positive for enterotoxin production on initial screening but only one serotype (rel O80:K·:H9) that was a consistent producer of enterotoxin might be due to genetic transfer of the toxin-producing property among different strains [14], resulting in a factor that was unstable on storage.

Neither serotypes O27:K·:H20 or rel O80:K·:H9 have been regarded in the past as "enteropathogenic." This study suggests that screening of *E. coli* strains from adult patients with acute diarrheal illness with antisera to enteropathogenic *E. coli* will be less fruitful than testing for enterotoxin production.

It is interesting to note that a traveler to Indonesia who became ill after completion of this study had a strain of enterotoxin-producing *E. coli* at the time of illness (but not before the trip) that was identified as O27:K?:H20 at the Center for Disease Control. This is the fourth traveler in our experience to have enterotoxin-producing *E. coli* of this OH serotype. Three different continents are represented in the travels of these four individuals.

Screening of *E. coli* strains from Ugandan and Saudi Arabian infants and children with diarrheal illness with this same test has been attempted (authors' unpublished data). To date, our experience confirms the finding of Gorbach et al. [15] that the animal model, which is satisfactory for studying enterotoxin-producing *E. coli* from adults, is not satisfactory for screening strains from children. An understanding of the differences between the enterotoxin of *E. coli* strains from children and those from adults is essential but will probably be dependent on more accurate biochemical and immunologic characterization of the enterotoxins.

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