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THE JOURNAL OF INFECTIOUS DISEASES · VOL. 125, NO. 4 · APRIL 1972

Test for *Escherichia coli* Enterotoxin Using Infant Mice: Application in a Study of Diarrhea in Children in Honolulu

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In a new test for detection of *Escherichia coli* enterotoxin, supernates of broth cultures were injected into the stomachs of infant mice and fluid accumulation in the intestine was measured after 4 hr by weighing. Results with known positive and negative strains were comparable to those obtained with the rabbit-loop test, and the mouse test was easier to perform. Cholera toxin, unlike *E. coli* enterotoxin, did not dilate infant mouse intestine significantly, even in high concentrations. Use of the infant-mouse test in a study of 37 children with diarrhea in Honolulu revealed no enterotoxin-producing coliform bacteria in the stools. This is in contrast to studies reported from India, where such strains were found in a large proportion of undifferentiated cases of acute diarrhea in adults. None of 15 stock strains of *E. coli* serotypes generally thought to be enteropathogenic produced significant amounts of enterotoxin as measured by the test in mice.

Certain strains of *Escherichia coli* have been found to produce an enterotoxin which causes fluid accumulation in ligated ileal segments of adult rabbits and other animals [1–3]. There is evidence from experiments in volunteers [4] and epidemiologic studies [5] that such strains may cause diarrheal disease in humans.

Assay of enterotoxin in rabbits is expensive, time consuming, and sometimes gives variable results. In our laboratory for example, of 34 adult rabbits in which ligated loops at the anterior and posterior ends of the ileum were inoculated with an enterotoxic culture filtrate, 23 gave a positive response in both loops, six had a negative result in the anterior loop, and five were negative in the posterior loop. Other investigators also have referred to the rather wide variability of the rabbit-loop test [3].

In searching for a more convenient and reliable test system, it was thought that newborn mice, because of the initial absence of bacteria in their intestines, their high degree of uniformity, and their small size, might offer an alternative to rabbit tests. Intragastric inoculation of infant mice has indeed proved to be a useful test system with a number of enterotoxic and negative control strains and offers a much cheaper, faster, and, in our laboratory, more uniform test than those using rabbits.

The epidemiology of diarrhea caused by enterotoxin-producing *E. coli,* in contrast to that caused by enteropathogenic serotypes of *E. coli,* is not yet well described. Gorbach et al. [5] studied cases of diarrhea of unknown cause in adults in India and found enterotoxin-producing *E. coli* in the intestinal tract of eight of 17 patients. A similar study of diarrheal disease in a U.S. population has not been reported. Using the infant-mouse test, we examined the stools from 37 cases of diarrhea in children in Honolulu for enterotoxin-

Received for publication October 15, 1971, and in revised form December 28, 1971.

We thank Miss Sandra Arakawa and Rita Holland for their technical assistance, and the staff of the clinical laboratories of the Kaiser Medical Center and the U.S. Army Tripler General Hospital for their cooperation.

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producing coliform bacteria. To explore the relation between serotype (the indicator of enteropathogenicity now in general clinical use) and production of enterotoxin, culture supernates of enteropathogenic serotypes of *E. coli* from the American Type Culture Collection were tested in the mouse system. A standard preparation of cholera toxin was also tried to evaluate the usefulness of the mouse test in work with cholera toxin.

Materials and Methods

Strains of *E. coli* known to dilate intestinal loops of adult rabbits were obtained from Drs. Sherwood Gorbach of Chicago, Ill., and Herbert L. DuPont and Craig Wallace of Baltimore, Md. Strains giving negative reactions were obtained from Drs. DuPont and Gorbach and from normal laboratory personnel at Pacific Research Section, Honolulu, Hawaii. Serotypes of *E. coli* enteropathogenic for humans were obtained from the American Type Culture Collection.[1] Crude cholera toxin (Merck Sharp & Dohme, lot no. 4493G) was obtained in lyophilized form from Dr. John Seal, chairman, National Institutes of Health Cholera Advisory Committee.

Enterotoxic material was prepared by inoculating trypticase-soy broth (TSB) (Baltimore Biological Laboratories) with a culture of the organism grown overnight in broth. Usually 10 ml of TSB in a 250-ml Erlenmeyer flask was inoculated with 2 ml of culture, but for larger quantities 10 ml of culture was placed in 200 ml of TSB in a 2,000-ml flask. The flasks were then shaken overnight at 37 C at 200 rpm on a New Brunswick model G2 rotary shaker. The culture was centrifuged at 1,200–1,700 g for 20–60 min (depending upon the volume of the centrifugate) and the supernate was used for inoculation of mice. In some experiments, the supernate was filtered through Millipore filters with a pore size of 0.45 μ.

Mice were obtained from the Charles River Breeding Laboratories, Wilmington, Massachusetts, and from the colony at National Institutes of Health. One to four days after birth, infant mice were inoculated with 0.1 ml of test material through the body wall directly into the milk-filled stomach with a no. 30 hypodermic needle. Mice used before suckling gave erratic results. Experiments showed that pontamine sky blue 6BX (DuPont Chemical Co.) did not interfere with the tests, and two drops of a 2% solution of this dye were added to each 1 ml of inoculum. Results from mice with no dye in the intestinal tract at autopsy were discarded.

The mice were separated from their mothers shortly before use and divided randomly into groups of four. After inoculation, they were kept at 28 C for 4 hr and then decapitated or killed with chloroform. The abdomen was opened, and the small intestine was examined for distention and then removed with forceps. The intestines from four mice were weighed together and the ratio of gut weight to remaining body weight calculated. Ratios of less than 0.070 were considered negative. Those in the range of 0.070–0.090 were considered questionably positive, and those over 0.090 strongly positive.

Rectal swabs were collected from children with diarrhea coming to the pediatric outpatient departments at Kaiser Hospital, U.S. Army Tripler General Hospital, and Schofield Barracks Pediatric Clinic on Oahu from January to July, 1971. Specimens were sent to the Tripler or Kaiser Hospital Laboratories where routine cultures were made for *Salmonella, Shigella,* and enteropathogenic serotypes of *E. coli.* A separate swab was placed in buffered glycerol-saline solution and within 24 hr it was streaked on eosin-methylene blue and on veal-infusion agar (VIA) and incubated overnight. Sixteen colonies resembling coliform bacteria were picked from the VIA plate and, after purification on trypticase-soy agar and preliminary biochemical tests, 10 of these were selected as representative of the stool flora and were tested for production of enterotoxin as described above. Identification was based on the methods of Ewing and Davis [6]. The following tests were used on all strains tested for enterotoxin: gram stain, indophenol oxidase, triple sugar-iron agar, indol, methyl red, Voges-Proskauer, Simmon's citrate, and phenylalanine deaminase. Strains shown not be *E. coli* by the above reactions were identified by further tests such as motility, urease, lactose fermentation, lysine and ornithine decarboxylases, and arginine dehydrolase.

[1] The types tested were: 026:B6; 055:B5:H; 086:B7; 086a,086b:B9; 0111:B4:H; 0112a,112b:B13; 0112a,112c:B11:NM; 0119:B14; 0124:B17:H; 0125a,125b:B15:H; 0125a,125c:B15:H; 0126:B16:H; 0127:B8; 0128a,128b:B12:H; and 0128a,128c:B12:H.

The media used were dehydrated products from Difco or Baltimore Biological Laboratories.

Efforts were made to test the strains for enterotoxin within a few days of isolation. When this was not possible, the culture was grown in TSB and then frozen at —70 C for testing in mice within a few weeks. The enterotoxic activity was found to be stable for at least this period at —70 C.

Results

The results of testing known enterotoxic strains of *E. coli,* six from human sources and one originally from a pig, are shown in table 1. The strains that produced positive rabbit-loop tests also gave strongly positive results in mice. Strains negative in rabbits gave negative reactions in mice. A typical (not maximally) positive reaction is shown in figure 1, along with a negative control. The results were easily interpreted visually, although weighing the intestine provided more objective data.

Cholera toxin diluted 1:100 gave negative results in mice, and even a 1:10 dilution gave borderline ratios averaging 0.081 (in 12 mice). Extending the observation period in mice to overnight did not affect the results. The same toxic material produced positive tests in rabbit loops in our laboratory when diluted 1:20,000.

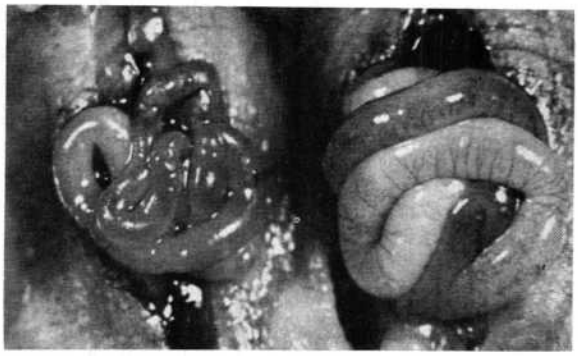

Figure 1. Intestines of infant mice inoculated 4 hr previously with toxin-containing B2C culture supernate (right) and nontoxic (HS strain) supernate (left). The mouse given enterotoxic material has a small intestine distended with clear fluid. The darker color of the intestine in the control is due to the normal content of bile.

Titrations of positive supernates from the first five strains listed in table 1 gave end points at dilutions of 1:5–1:40. Supernates of strain B2C had a titer of 1:40 in mice and also in the rabbit-loop test. Storage at —70 C for 18–20 days did not change the titer of filtered supernates in the mouse test. The filtrates were boiled for 15 min; four of them still produced positive tests after boiling. The activity of supernates from strain 263, originally from a pig, was destroyed by this treatment. Autoclaving for 15 min at 121 C destroyed the activity of all five filtrates.

Table 1. Enterotoxin-producing *Escherichia coli* and negative controls tested in infant mice.

Strain (source)	Rabbit loop reaction [Reference]	No. of tests (4 mice each)	Gut weight:body weight (average)	Range
B2C (DuPont)	Positive [4]	6	0.120	0.109–0.129
1105F (Gorbach)	Positive [*]	4	0.107	0.087–0.140
410G (Gorbach)	Positive [*]	3	0.113	0.104–0.131
339T5† (Gorbach)	Positive [*]	5	0.126	0.099–0.160
263 (DuPont)	Positive [4, 10]	8	0.119	0.101–0.136
334A† (Wallace)	Positive [‡]	3	0.118	0.101–0.152
B7A (DuPont)	Positive [4]	1	0.186	
HS (DuPont)	Negative [4]	5	0.062	0.058–0.069
111A (Gorbach)	Negative [*]	5	0.062	0.048–0.086
18 strains (Pacific Research Section) from 6 normal adults in Honolulu		18	0.058	0.040–0.079

* S. L. Gorbach, Department of Infectious Diseases, Cook County Hospital, Chicago, Illinois.

† Strains 339T5 and 334A came originally from the same culture, isolated in Calcutta [5].

‡ C. K. Wallace, Division of Infectious Diseases, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

Strains 263, B7A, and 334A gave variable results during the course of the experiments, and it was discovered that cultures passed several times on TSA slants became inactive. By reculturing from the original slants, strains giving consistently positive results were obtained. Only the latter are shown in table 1.

Of the 15 enteropathogenic serotypes from the American Type Culture Collection, only two gave positive results in the mouse test: types 0128a, 128b:B12:H and 0128a,128c:B12:H. The former gave a ratio of gut-to-body weight of 0.95 on initial testing but was negative in four subsequent tests. The 0128a,128c strain gave ratios averaging 0.088 in four tests (range, 0.082–0.093).

Specimens were obtained from 37 children with acute diarrhea, from 24 patients during the first three days of illness, but from the others as long as 12 days after onset of illness. The age of the patients ranged from six weeks to six years, with an average of 19 months. Twelve had rectal temperatures higher than 100 F when seen in the clinic. Eight had associated respiratory-tract symptoms. Pathogenic Enterobacteriaceae were found in the stools of three. These were *Shigella flexneri, Salmonella weltevreden,* and *E. coli,* type 0111: B4. Two others had a predominance of coagulase-positive *Staphylococcus* in their stools.

Ten or more strains of bacteria from the stool of each patient (in four cases, only nine strains) were tested in mice for gut-dilating properties. In all cases except three with staphylococci, two with single colonies of *Pseudomonas aeruginosa,* and one with a *Bacillus* species, the strains tested were Enterobacteriaceae. There were 381 strains tested: 242 *E. coli,* 79 *Klebsiella,* 23 *Citrobacter,* 21 *Staphylococcus,* nine *Proteus,* and seven miscellaneous or unidentified organisms. The stool specimens fell into two rather clearly defined groups, those with predominantly *E. coli* in the aerobic flora and those with other coliforms or staphylococci. The latter usually had several different organisms among the 10 chosen for testing. Twenty-three (62%) of the specimens had predominantly *E. coli,* and fourteen had a miscellaneous flora.

Culture supernates from the 381 strains were tested in mice. None gave gut-to-body weight ratios above 0.090, and only seven, (four *E. coli,* two *Klebsiella,* and one *Citrobacter,* had ratios above 0.080.

Discussion

The results from assays of *E. coli* enterotoxin in infant mice appear to parallel those in the rabbit. Six human and one porcine strain of *E. coli* known to produce positive results in rabbit loops also strongly dilated the intestine of the infant mouse. Titers obtained with one of the strains were similar in mice and rabbits.

The mouse test is less expensive and time-consuming than that using the ileal loop of the rabbit. For this reason it may facilitate epidemiologic, biochemical, or immunologic studies in which large numbers of tests are required. It is not difficult for two people to do 90 tests, using 360 mice, in a day. The same tests would require 36–60 rabbits, depending on the number of loops made per rabbit, and would take four to eight days in our laboratory.

The difference in response of the mouse to enterotoxins of *Vibrio cholerae* and *E. coli* is a most interesting phenomenon. Since bacteria-free supernates were used, the difference suggests that species specificity of *V. cholerae* and *E. coli* toxins is determined by properties of the toxins, not merely by differences in growth of the organisms in the gut. An explanation of the different effects of *E. coli* and *V. cholerae* toxins in the mouse might well clarify many details about the two toxins and their mechanism of action.

The heat stability of the toxic preparations suggests that the mouse test measures mainly the heat-stable extracellular enterotoxin of *E. coli* [7] rather than the heat-labile cell-associated type of enterotoxin recently described [8]. However, preparations of the heat-labile type, containing whole-cell lysates, were not made, and it is possible that the mouse may respond to heat-labile toxins as well. It should be noted that cholera enterotoxin is heat labile; it is destroyed by heating at 60 C for 30 min [9].

Our failure to find toxigenic strains of *E. coli* in 37 children with diarrhea, 23 of whom had predominantly *E. coli* in their stools, suggests that the findings of Gorbach et al. in Calcutta may not apply to the population of children in the United States. In any case, the mouse test should make it economically feasible to search for enterotoxin-producing organisms in various populations and areas of the world, and should assist in clarifying their role in the causation of diarrheal disease.

The negative results obtained with stock strains of enteropathogenic serotypes may mean that the strains have lost their pathogenicity after prolonged storage in the laboratory, or it may be that mechanisms other than production of enterotoxin are responsible for their pathogenic activity. The moderately positive result obtained with type 0128a, 0128c may indicate the production of small amounts of enterotoxin by this strain. The relation of the classical enteropathogenic serotypes of *E. coli* to the newly discovered mechanisms of enteropathogenicity—enterotoxin production and epithelial penetration [4]—needs to be investigated.

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