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Use of Colony Pools for Diagnosis of Enterotoxigenic *Escherichia coli* Diarrhea

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Diagnosis of enterotoxigenic *Escherichia coli* diarrhea was made in 109 adult males with an acute dehydrating cholera-like syndrome in Dacca, Bangladesh, by testing 10 colonies isolated from admission stool specimens for production of heat-labile and heat-stable toxins. Toxin testing of one colony yielded a diagnosis in 92% of the cases, testing of two colonies yielded a diagnosis in 95% of the cases, testing of a pool of 5 colonies yielded a diagnosis in 95% of the cases, and testing of a pool of 10 colonies yielded a diagnosis in 96% of the cases. From stool cultures obtained on subsequent days, toxin testing of individual colonies and pools revealed diminished efficacy of pooling with decreasing numbers of enterotoxin-positive isolates in the pool. To detect the presence of enterotoxigenic *E. coli* in stools, toxin testing of 5 individual isolates and a pool of 10 colonies was found to be almost as effective as the testing of 10 individual isolates.

Enterotoxigenic *Escherichia coli* is now a well-recognized cause of dehydrating diarrhea (11). Unlike the classical bacterial pathogens such as *Shigella*, *Salmonella*, and *Vibrio*, which are readily isolated in the laboratory by the use of selective enrichment and plating media, enterotoxigenic *E. coli* cannot be differentiated from non-enterotoxin-producing *E. coli* by conventional enteric isolation procedures. Identifying enterotoxigenic *E. coli* strains usually involves selecting colonies with typical *E. coli* morphology from MacConkey agar plates and testing these colonies for production of heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST).

To limit the number of enterotoxin tests required for each patient, some investigators have tested a "pool" of colonies (1, 8, 9) or supernatant fluids (3). As a part of a study of the effect of tetracycline therapy on the course of severe enterotoxigenic *E. coli* cholera-like diarrhea in Dacca, Bangladesh, we had the opportunity to evaluate the use of colony pools in diagnosing severe enterotoxigenic *E. coli* disease and in detecting enterotoxigenic *E. coli* in stools.

MATERIALS AND METHODS

Isolation of *E. coli*. From October to December 1976 we studied the etiology of acute, cholera-like diarrhea in 176 adult males admitted to the Cholera Research Laboratory Hospital in Dacca, Bangladesh. These patients had the onset of their illness at an average of 12 h before admission and had lost an average of 7% of their body weight at the time of admission. Stools were obtained on admission by rectal catheter and were promptly plated on *Salmonella-Shigella*, Trypticase-tellurite-gelatin, thiosulfate-citrate-bile salt-sucrose, and MacConkey agar plates. The plates were incubated at 37°C for 18 to 24 h. From each MacConkey plate 10 lactose-fermenting colonies with typical *E. coli* morphology were identified and streaked onto individual nutrient agar slants. The first five colonies were then repicked consecutively with a single sterile toothpick, and this pool (designated "5 pool") was inoculated on a single nutrient agar slant. All 10 colonies were then repicked in the same manner to form another pool (designated "10 pool"), which was also inoculated on a single nutrient agar slant. All slants were incubated overnight at 37°C and then stored at room temperature until testing.

An objective of this study was to determine the effect of tetracycline on enterotoxigenic *E. coli* diarrhea, including the duration of excretion of enterotoxigenic *E. coli*. Because it was not known at the time of therapy which cases had enterotoxigenic *E. coli* diarrhea, convalescent stool cultures were obtained by rectal swabs from all 176 cases for 4 consecutive days after admission and at 7 and 14 days thereafter. The swabs were processed in the manner described for the admission stool specimens, and 10 individual *E. coli* colonies and 5 and 10 pools were similarly isolated and stored on nutrient agar slants until testing.

Toxin testing. Within 1 month after collection, the individual isolates and pools obtained from the admission cultures of the 176 cases were each inoculated into Erlenmeyer flasks containing 25 ml of Trypticase

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soy broth with 0.6% yeast extract (surface-to-volume ratio, 1.9 cm/m^2). The flasks were placed on a shaker set at a rate of 50 to 70 shakes per min for 18 to 24 h at 37°C. The broths were then centrifuged at 6,000 rpm, and the supernatants were tested for LT by the Chinese hamster ovary cell assay (5) and for ST by the infant mouse assay (7). One enterotoxigenic isolate from each patient was confirmed as *E. coli* by conventional methods (2).

Approximately 6 to 8 months after collection, isolates and pools from convalescent stool cultures of 62 cases that had enterotoxigenic *E. coli* that produced LT and ST (LT-ST strains) and of 2 cases that had enterotoxigenic *E. coli* that produced only LT (LT strains) in their admission stools were tested for LT production in the adrenal cell assay by the miniplate method as modified by Sack and Sack (10), except that before testing the organisms were grown in a resting culture in miniculture plates containing syncase broth, rather than in 8-ml vials as originally described. This method was used because it was more practical for screening a large number of specimens. Colonies and pools from convalescent stool cultures of 28 cases that had enterotoxigenic *E. coli* which produced only ST (ST strains) in their admission stools were tested only for ST production. These strains were inoculated into tubes (13 by 100 mm) containing 3 ml of Trypticase soy broth with 0.6% yeast extract, and the tubes were placed on a roller drum (22 rpm) for 18 to 24 h at 37°C. The broths were then spun at 6,000 rpm for 30 min, and the supernatants were tested for ST by the infant mouse assay (7).

RESULTS

Admission stool specimens. A total of 109 cases had one or more enterotoxigenic *E. coli* colonies in their admission stool cultures; 69 had LT-ST *E. coli* strains, 34 had ST *E. coli* strains, and 6 had LT *E. coli* strains. In 104 cases (95%) there were six or more colonies, and in 100 cases (92%) there were 9 or 10 enterotoxigenic colonies isolated. All of the cases with six or more colonies had a positive 10 pool. For five persons only one colony was enterotoxigenic (three had LT-ST *E. coli*, one had ST *E. coli*, and one had LT *E. coli*), and one of these cases (LT-ST *E. coli*) had a positive 10 pool. The 10 pool was enterotoxigenic in 105 cases (96%), the first colony selected was enterotoxigenic in 100 cases (92%), and the first or second colony selected was enterotoxigenic in 104 cases (95%) (Table 1).

Only one fewer case was identified with the 5 pool than was identified with the 10 pool (Table 2). None of the cases with a negative 10 pool had a positive 5 pool. Of the three cases in which there was no enterotoxin-positive isolate among the first five organisms isolated, each had only one toxin-positive isolate in its stool, and this isolate was not among the first five selected; one of the three had a positive 10 pool. As was found for the 10 pools, the efficacy of testing one isolate, two isolates, and a 5 pool was almost identical.

TABLE 1. *Toxin positivity of individual colonies and 10 pools in admission stool specimens of enterotoxigenic E. coli cases*[a]

No. of toxin-positive colonies (of 10)	No. of cases	No. showing positive result with:[b]		
		10 Pool	Isolate 1	Isolate 1 or 2
10	95	95	95	95
9	5	5	4	5
8	1	1	0	1
7	1	1	1	1
6	2	2[c]	0	2
1	5	1	0	0

[a] Test conditions were as follows: shake culture; Trypticase soy broth with 0.6% yeast extract; Chinese hamster ovary cell assay for LT testing; 69 LT-ST, 34 ST, and 6 LT strains.

[b] Total percentages of positive results were 96, 92, and 95% for the tests with the 10 pool, isolate 1, and isolate 1 or 2, respectively.

[c] One LT-ST case had a 10 pool that was only ST positive.

TABLE 2. *Toxin positivity of individual colonies and 5 pools in admission stool specimens of enterotoxigenic E. coli cases*[a]

No. of toxin-positive colonies (of 10)	No. of cases	No. showing positive result with:[b]		
		5 Pool	Isolate 1	Isolate 1 or 2
5	95	95	95	95
4	7	6[c]	4	7
3	2	2	1	2
2	0	0	0	0
1	2[d]	1	0	0
0	3	0	0	0

[a] Test conditions were as follows: shake culture; Trypticase soy broth with 0.6% yeast extract; Chinese hamster ovary cell assay for LT testing; 69 LT-ST, 34 ST, and 6 LT strains.

[b] Total percentages of positive results were 95, 92, and 95% for the tests with the 5 pool, isolate 1, and isolate 1 or 2, respectively.

[c] One LT-ST case had a 5 pool that was only ST positive.

[d] Both cases were LT-ST strains; both had positive 10 pools.

Convalescent cultures. There were 88 convalescent cultures in which at least one LT-positive colony was found. There were no cultures in which a 5 or 10 pool was positive and an enterotoxigenic isolate was not found. As the number of LT-producing isolates in the 10 pool or 5 pool decreased, the frequency of toxin-positive stools decreased (Tables 3 and 4). Both 10 pools and 5 pools with three or more LT-positive isolates were positive significantly more often

TABLE 3. *Comparison of results of toxin testing of 10 individual colonies, 10 pools, and 5 pools from convalescent stool specimens for LT production*[a]

No. of toxin-positive colonies (of 10)	No. of cultures tested	No. with 10 pool positive	No. with 5 pool positive
10	24	24	24
9	7	7	7
8	7	7	6
7	6	6	6
	$n = 44$	$n = 44$ (100%)	$n = 43$ (98%)
		$n = 58^b$ (92%)	$n = 58$ (92%)
6	7	5	5
5	2	1	2
4	6	5	5
3	4	3	3
	$n = 19$	$n = 14$ (74%)	$n = 15$ (79%)
2	9	6	4
1	16	9	2
	$n = 25$	$n = 15^{b,c}$ (60%)	$n = 6^c$ (24%)

[a] Test conditions were as follows: resting culture; syncase broth; adrenal cell assay; 36 LT-ST and 2 LT *E. coli* cases.
[b] $\chi^2 = 10.8$; $P < 0.01$.
[c] $\chi^2 = 5.3$; $P < 0.05$.

TABLE 4. *Comparison of results of toxin testing of five individual colonies and 5 pools from convalescent stool specimens for LT production*[a]

No. of toxin-positive colonies (of 5)	No. of cultures tested	No. with 5 pool positive
5	32	32
4	14	12
3	7	6
	$n = 53$	$n = 50^b$ (94%)
2	12	9
1	9	5
0	14	0
	$n = 21$	$n = 14^b$ (66%)

[a] Test conditions were as follows: resting culture; syncase broth; adrenal cell assay; 36 ST-LT and 2 LT *E. coli* cases.
[b] $\chi^2 = 7.6$; $P < 0.01$.

than those with less than three. When less than three enterotoxigenic organisms were present, 10 pools were positive (60%) significantly more often than 5 pools (24%) (Table 3). Of the 88 cultures in which an LT-producing organism was detected by toxin testing of 10 individual colonies, 74 (84%) were detected by toxin testing of five individual colonies, 73 (83%) were detected by toxin testing of a 10 pool, 64 (73%) were detected by toxin testing of a 5 pool, and 83 (94%) were detected by toxin testing of five individual colonies and a 10 pool.

There were 37 convalescent cultures in which at least one ST-producing *E. coli* strain was found. There were no cultures in which a 5 or 10 pool was positive and an enterotoxigenic isolate was not found. Although a gradual decrease in the frequency of positive 10 pools with decreasing number of enterotoxigenic *E. coli* isolates was not observed (perhaps because of the small number of cultures tested), 10 pools with three or more toxin-positive organisms were more often positive than were those with less than three (Table 5). There was no difference between the frequency of toxin-positive 5 pools containing three or more enterotoxigenic *E. coli* colonies and that of 5 pools containing less than three such colonies. Of 36 cultures in which an ST-producing enterotoxigenic *E. coli* strain was detected by the testing of 10 individual colonies, 35 (97%) were detected by toxin testing of five individual colonies, 31 (86%) were detected by toxin testing of a 10 pool, 30 (83%) were detected by toxin testing of a 5 pool, and 35 (97%) were detected by toxin testing of five individual colonies and a 10 pool.

DISCUSSION

Results of admission stool cultures revealed that a diagnosis of enterotoxigenic *E. coli* diarrhea can be made in adult patients with moderate to severe dehydration with almost equal frequency by toxin testing of a 10 pool (96%), a 5 pool (95%), two isolates (95%), or a single isolate (92%). This was presumably because enterotoxigenic *E. coli* strains accounted for most of the *E. coli* fecal flora in these cases at the time of culture. Although we did not perform quantitative *E. coli* counts from the MacConkey plates on which the admission stools were streaked, in most instances only colonies with typical *E. coli* morphology were observed.

This high diagnostic yield from testing of a single pool or a single isolate was noted in a

TABLE 5. *Comparison of results of toxin testing of 10 individual colonies, 5 pools, and 10 pools from convalescent stool specimens for ST production*[a]

No. of toxin-positive colonies (of 10)	No. of cultures tested	No. with 10 pool positive	No. with 5 pool positive
10	6	6	6
9	2	2	2
8	6	6	5
7	1	0	0
	$n = 15$	$n = 14$ (93%)	$n = 13$ (87%)
	$n = 22$	$n = 21$[b] (95%)	$n = 18$ (82%)
6	2	2	0
5	2	2	2
4	1	1	1
3	2	2	2
	$n = 7$	$n = 7$ (100%)	$n = 5$ (71%)
2	9	8	9
1	5	2	3
	$n = 14$	$n = 10$[b] (71%)	$n = 12$ (86%)

[a] Test conditions were as follows: roller drum culture; Trypticase soy broth with 0.6% yeast extract; 28 ST *E. coli* cases.

[b] Difference significant at $P = 0.06$ by the Fisher two-tailed exact test.

smaller study at our hospital (9) but has not been observed in other studies of enterotoxigenic *E. coli* diarrhea. In one study of travellers' diarrhea in adult North Americans visiting Mexico City, toxin testing of a single isolate yielded a diagnosis in only about one-half of those diagnosed by testing of five individual isolates (7); each enterotoxigenic *E. coli* diarrhea case had an average of 2.3 toxin-positive colonies per 5 colonies tested. In another study of travellers' diarrhea in Peace Corps volunteers in Kenya, an average of 2.7 enterotoxigenic isolates per 5 colonies was reported (8). Donta et al. reported eight hospitalized cases of acute diarrhea in Mexican children, half of whom had 4 or 5 enterotoxigenic colonies isolated from their stools per 10 colonies tested; the remainder had no positive isolate, and the diagnoses were made only by toxin testing of a colony pool (1). Sack et al. reported an average of 4.9 toxin-positive colonies per 10 tested from children living on the Apache Reservation in Whiteriver, Ariz., and hospitalized with acute diarrhea (13). In a study of children treated for mild diarrhea at the outpatient department of our hospital, Black et al. reported an average of 3.5 enterotoxigenic isolates per 10 tested (R. Black, personal communication). The smaller number of enterotoxigenic *E. coli* isolates found in these studies is consistent with the less severe illness reported, as compared with the cholera-like disease in our patients and in those reported in Calcutta, where a high frequency of toxin-positive *E. coli* isolates was also found (4, 12). It is not known why this severe cholera-like syndrome caused by enterotoxigenic *E. coli* has so far been observed primarily on the Indian subcontinent.

Our data for LT testing of isolates and pools from convalescent stool specimens revealed the not surprising result that the frequency of positive 5 and 10 pools decreased with decreasing numbers of toxin-positive isolates in the pool. Over 90% of 5 and 10 pools with at least three enterotoxigenic isolates were toxin positive. When less than three enterotoxigenic isolates were present, only 60% of the 10 pools and 66% of the 5 pools were positive. This finding is similar to that reported by Sack et al. (9). Although the isolates and pools from the convalescent cultures were grown in resting culture, it is unlikely that this resulted in less detectable LT in the supernatants than would have been produced in a shake culture (6). Pooling data for ST testing were more limited, but they also demonstrated that 10 pools with more than three enterotoxigenic isolates were more often toxin positive. We suspect that the apparent high efficacy of 5 pools for detecting ST-producing strains was an artifact related to the smaller number of pools studied. The pooling results for LT and ST taken together suggested that toxin testing of five individual isolates and a 10 pool was almost as effective as the testing of 10 individual isolates.

Results of this study do not indicate the ideal number of colonies and types of pools that should be tested to diagnose enterotoxigenic *E. coli* diarrhea in all settings. The results suggest, however, that the diagnosis of enterotoxigenic *E. coli* disease can be readily made in persons with relatively severe illness. They also support previous data that indicate that in mildly ill

persons testing of a pool in addition to individual isolates increases the diagnostic yield (1, 3). Additional information is needed to explain the lack of toxin positivity found in this and other studies (1, 3) of pools that have only a few toxin-positive isolates.

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LITERATURE CITED

1. **Donta, S. T., R. B. Wallace, S. C. Whipp, and J. Olarte.** 1977. Enterotoxigenic *Escherichia coli* and diarrheal disease in Mexican children. J. Infect. Dis. **135**:482–485.
2. **Edwards, P. R., and W. Ewing.** 1972. Identification of *Enterobacteriaceae*, 3rd ed. Burgess Publishing Co., Minneapolis.
3. **Evans, D. G., J. Olarte, H. L. Dupont, D. J. Evans, E. Galindo, B. L. Portnoy, and R. H. Conklin.** 1977. Enteropathogens associated with pediatric diarrhea in Mexico City. J. Pediatr. **91**:65–68.
4. **Gorbach, S. L., J. G. Banwell, B. D. Chatterjee, B. Jacobs, and R. B. Sack.** 1971. Acute undifferentiated diarrhea in the tropics. I. Alteration in intestinal microflora. J. Clin. Invest. **50**:881–889.
5. **Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10**:320–327.
6. **Gurwith, M.** 1977. Rapid screening method for enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **6**:314–316.
7. **Morris, G. K., M. H. Merson, D. A. Sack, J. G. Wells, W. T. Martin, W. E. Dewitt, J. C. Feeley, R. B. Sack, and D. M. Bessudo.** 1976. Laboratory investigation of diarrhea in travellers to Mexico: evaluation of methods for detecting enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **3**:486–495.
8. **Sack, D. A., D. C. Kaminsky, R. B. Sack, I. A. Wamola, F. Orskov, I. Orskov, R. R. Arthur, and A. Z. Kapikian.** 1977. Enterotoxigenic *Escherichia coli* diarrhea of travellers: a prospective study of American Peace Corps volunteers. Johns Hopkins Med. J. **141**: 63–70.
9. **Sack, D.A., J. C. McLaughlin, R. B. Sack, F. Orskov, and I. Orskov.** 1975. Enterotoxigenic *Escherichia coli* isolated from patients at a hospital in Dacca. J. Infect. Dis. **135**:275–280.
10. **Sack, D. A., and R. B. Sack.** 1975. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. Infect. Immun. **11**:334–336.
11. **Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. **29**:333–353.
12. **Sack, R. B., S. L. Gorbach, J. G. Banwell, B. Jacobs, B. D. Chatterjee, and R. C. Mitra.** 1971. Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like diarrhea. J. Infect. Dis. **123**:378–385.
13. **Sack, R. B., N. E. Hirschhorn, I. Brownlee, R. A. Cash, W. E. Woodward, and D. A. Sack.** 1975. Enterotoxigenic *Escherichia coli*-associated diarrheal disease in Apache children. N. Engl. J. Med. **292**:1041–1045.