Pooling Method for Screening Large Numbers of *Escherichia coli* for Production of Heat-Stable Enterotoxin, and Its Application in Field Studies

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A modified suckling mouse assay was developed for use in field studies whereby five *E. coli* isolates could be tested as a pool for heat-stable enterotoxin.

The suckling mouse assay (SM) for the heat-stable enterotoxin (ST) of *Escherichia coli* first described by Dean et al. (1) has been used by many investigators to screen *E. coli* isolates for the production of this toxin. It is often necessary to test five to fifteen isolates from a single fecal specimen to detect the presence of enterotoxigenic *E. coli*. In applying this technique to field studies, the cost and availability of a large supply of suckling mice made the assay unacceptable for many laboratories. In view of the recent reports in the literature of diarrheal disease caused by ST *E. coli* (strains of *E. coli* which produce only ST) (2, 5, 6), an inexpensive yet sensitive assay for ST is increasingly needed for a complete evaluation of diarrheal etiology. This study was undertaken to reduce the cost but retain the efficiency of the SM assay for ST.

Five strains of either ST⁺/LT⁻ or ST⁺/LT⁺ (where LT⁺ and LT⁻ indicate, respectively, that the strain does or does not produce heat-labile enterotoxin) were selected as ST⁺ organisms for the initial pooling experiment (Table 1). Non-enterotoxigenic *E. coli* were selected from previous studies in which they proved to be negative in the suckling mouse assay. M-431, an ST-only porcine strain of *E. coli*, was kindly supplied by H. Moon. Each of the five ST⁺ organisms was grown separately, and their supernatants were mixed with two different random combinations of four ST⁻. Each pool was tested in four separate experiments, with six mice per sample pool.

The second study consisted of pooling 3,900 clinical isolates of *E. coli* collected over a 10-month period from infants and children from Houston and Mexico who had been enrolled in studies designed to look at the etiology of acute diarrhea. Details of the patient populations have recently been published (4). Five colonies of *E. coli* from each diarrheal stool were pooled as outlined above and assayed within 4 months after isolation. It was planned to test individual

colonies from any of the 780 pools tested which were found to be positive (ratio of gut to remaining body ≥ 0.090), individual colonies from those pools found to be intermediate (ratio = 0.080 to 0.089), and then a representative number of negative pools (ratio < 0.080 would be tested as individual colonies.

Crude test preparations were obtained as follows. An 8- to 10-ml amount of 2.0% Casamino Acids (Difco Laboratories)-0.6% yeast extract (Difco)-salts (CYE) broth (3) in 50-ml Erlenmeyer flasks were loop inoculated from the peptone agar stab cultures. Flasks were incubated for 16 to 18 h at 37°C in a rotary shaker adjusted to 200 rpm. A 2-ml amount of each of the five cultures tested as a pool was mixed and centrifuged at 12,000 rpm at 5°C for 30 min, and the supernatant fluid was removed. Two drops of 2.0% Evans blue dye solution were added per ml of test sample immediately before injection. All samples included positive and negative controls and were assayed within 48 h of preparation.

Three- to five-day-old mice with milk-filled stomachs and no overall body hair were used. On the day of the assay, mice were mixed in one container to assure random distribution, and six mice were inoculated per test sample. Each mouse was injected directly into the stomach with 0.1 ml of sample containing the dye marker with a 1-ml syringe and a 27-gauge needle. Mice were kept at room temperature (28°C) for 4 h and then sacrificed by inhalation of chloroform vapors. Data were obtained by weighing the intestines of all mice containing the blue dye in one preweighed container and weighing the bodies, including stomachs, in another container. A ratio of intestinal weight to remaining body weight was computed for each sample in which at least four mice were successfully inoculated.

Table 1 demonstrates the lack of influence of four negative culture supernatants on a positive suckling mouse reaction induced by one ST⁺

TABLE 1. Suckling mouse assays performed to detect possible interference of ST⁻ *E. coli* preparations on ST⁺ samples assayed by the pooling technique

Experimental pools of <i>E. coli</i>	Statistical analysis of data pooling 1 ST ⁺ /4 ST ⁻ culture supernatants ^a		
	Mean ratio	SD	Mean $\pm$ 1 SD
ST ⁺ <i>E. coli</i> strain			
MP-51H (child, ST ⁺ LT ⁻)	0.123	0.021	0.102-0.144
PB-88-A3 (adult, ST ⁺ LT ⁻)	0.124	0.020	0.104-0.144
PB-122-B1 (adult, ST ⁺ LT ⁺)	0.124	0.022	0.102-0.146
NIH*111-A (child, ST ⁺ LT ⁺)	0.116	0.020	0.096-0.136
M-431 (porcine, ST ⁺ LT ⁻)	0.105	0.015	0.090-0.120
Control strain			
M-431, 1:5 in CYE	0.118	0.010	0.108-0.128
HS, 1:5 in CYE	0.060	0.003	0.057-0.063

^a Each ST⁺ organism was assayed with two random combinations of ST⁻ strains repeated in four separate experiments. Thus, 48 mice were tested for each ST-positive strain tested by the pooling technique. SD, Standard deviation. See text for explanation of LT⁻ and LT⁺.

strain in each pool. Only 1:40 ratio values were below the 0.090 ratio considered to be positive by our laboratory for the two random combinations of ST⁻ strains which were run in four separate experiments, with six mice per sample pool. One pool of M-431 with four ST⁻ strains in one experimental run was in the 0.080-0.089 range (0.082). This represents one intermediate ratio with one ST⁺ strain pool from a total of 40 pools tested.

We tested 780 culture supernatant pools of five colonies of *E. coli*; each set of five colonies had been obtained from the same stool from each of 305 patients with diarrhea (Fig. 1). The majority of pools (764, or 98%) revealed ratio values less than 0.090, with a mean ratio of 0.063 ± 0.006 (standard deviation) and a 95% confidence interval of 0.052 to 0.075. Nine of these pools were within the questionably positive range of 0.080 to 0.089. When each of the individual isolates from these intermediate pools was tested, all revealed ratios less than 0.080. In the same study, 16 (2%) of the sample pools tested were ST⁺, with a ratio value greater than or equal to 0.090 with a range of values from 0.101 to 0.147. The mean ratio value for the 16 ST⁺ pools was 0.129 ± 0.013 (standard deviation), with a 95% confidence interval of 0.103 to

FIG. 1. Results of the suckling mouse assay for ST production by 3,900 clinical isolates of *E. coli* tested as pools of five culture supernatants combined in equal proportion.

0.155. When individual culture supernatants from each of these 16 ST⁺ pools were tested, each pool contained at least one positive isolate with a ratio of 0.102 or greater. Three positive pools contained a single positive isolate, five had two positive isolates, one possessed three positive isolates, and seven pools contained four or five positive individual isolates. Individual supernatants were examined from 16 negative pools obtained at random, and each of the 80 individual isolates was negative for ST, showing a ratio less than 0.075 in each case.

As has been found by other groups, we have established that a ratio of 0.090 of gut weight to remaining body weight constitutes as positive reaction for ST. We examined a method of pooling five colonies of *E. coli* as a means of improving the efficiency of the assay for screening large numbers of *E. coli* in field studies designed to characterize the causes of diarrhea. Assays of samples from five ST⁺ strains mixed in two random combinations of four ST⁻ strains showed no interference by the ST⁻ strains. Since one pool containing an ST⁺ strain and four ST⁻ isolates among the 40 pools tested did show an intermediate ratio value (0.082), we would advise that any sample pool with an intermediate ratio value (0.080 to 0.089) be repeated by growing the five isolates and retesting them as individual colonies. In a second experiment, 780 pools of five *E. coli* supernatants obtained from single

fecal specimens of 305 patients with diarrhea were tested for ST in the SM assay. The 16 positive pools (ratio ≥ 0.090) contained at least one ST⁺ isolate when tested individually, whereas nine pools giving intermediate results (0.080 to 0.089) and 16 randomly selected negative pools (< 0.080) failed to contain a positive isolate upon retesting of individual colonies.

These observations need to be extended in future experiments to determine how many isolates may be pooled without loss of assay sensitivity and accuracy and to determine whether weakly positive strains might reliably be detected after pooling. A dilution experiment needs to be performed to determine a dose response for ST⁺ strains. It is likely that a pooling procedure will occasionally be associated with a dilution near the brink of a false-negative reaction. We feel that this preliminary study along with our previous experience that, in acute diarrhea cases due to enterotoxigenic *E. coli*, the causative agent is present in heavy numbers, justify a pooling method to be employed in looking for ST⁺ strains in stool samples. We have estimated a greater than 50% savings in cost by adoption of a pooling method. It is suggested that five colonies from a single diarrheal stool be combined in assay pools to minimize the dilution of ST⁺ isolates in cases of diarrheal illness.

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