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AN EVALUATION OF THE POOLING METHOD FOR DETECTING ENTEROTOXIGENIC ESCHERICHIA COLI

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Abstract

The effectiveness of the common practice of using a mixture (pool) of bacterial colonies for detecting enterotoxigenic *Escherichia coli* (ETEC) was evaluated. Pools were constructed with *E. coli* whose phage lysotypes were known. After pooling and storage of isolates in the normal manner, attempts were made to recover and identify the original lysotypes in the pool. It was observed that bacteria from one or two colonies (lysotypes) almost always overgrew in the pool, thus reducing the number of recoverable individual strains. In general, enterotoxigenic *E. coli* (ETEC) did not show a survival advantage in the pools over non-ETEC strains. Therefore, the use of the pooling technique to enhance the likelihood of recovery of ETEC from faecal specimens is of doubtful value.

Key words: Bacteria; *Escherichia coli;* Diarrhoea; Microbial; *E. coli* Phage.

Introduction

The population of *Escherichia coli* in the gut is often a mixture of different strains (1,10-12). If enterotoxigenic *E. coli* (ETEC) are present in low numbers they may be missed unless a large number of isolates are tested. Considering the practical difficulties of testing several isolates, it has become common practice to stock and test a pool of 5 or 10 *E. coli* colonies in addition to 2 or 3 individual isolates, so as to increase the likelihood of detecting an ETEC strain, if present (4,8). This hypothesis is based on at least two assumptions:

1. ETECs have at least the same survival ability in a pool as non-ETECs.
2. Toxin can be detected in the culture supernatant from pooled isolates even when only few toxin producers are present.

Thus, testing a pool is expected to detect the presence of ETEC in the mixture by performing a single test, instead of testing the individual bacterial colonies which comprise the pool. As the strains which comprise the pool are drawn from the gut, the potential genetic exchange they may undergo in the pool may mimic that which occurs *in vivo*.

Component isolates of pooled bacterial growth cannot be easily distinguished. One way to identify them and to determine if isolates survive pooling in approximately equal proportions is to serotype colonies derived from the pool and to construct a serotypic "profile" of pool members. This method could be used to determine the survival of strains originally introduced into the pool if (a) the serotypes of the members of the pool were known and; (b) a large enough sample of isolates derived from the pool were tested. To date, no such study has been reported. We attempted to evaluate the pooling technique using a non-serological method.

Using 24 bacteriophages against *E. coli*, we could identify and differentiate bacterial isolates by their phage susceptibility pattern, or lysotype (5). By testing approximately 50 colonies from a pool and identifying each lysotype we were able to determine the number of strains recoverable from the pool. It was our intent to determine if a pool of 5 or 10 colonies of *E. coli* actually maintained

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the proportionate numbers of original lysotypes or, if by some mechanism inherent in the pooling or storage process, there occurred a reduction of the number of recoverable lysotypes from the pool.

Materials and methods

Six pooled bacterial cultures were made to contain either 5 or 10 *E. coli* isolates. All strains were initially grown as pure cultures and their lysotypes determined following methods described earlier (6). On testing it was found that in one pool of 10 colonies a pair of isolates had the same lysotype. Similarly, another pool of 10 colonies was found to contain only eight different lysotypes. Therefore, we actually constructed pools of 5, 8, 9 or 10 *E. coli* lysotypes.

Strains were initially grown as pure cultures on MacConkey agar (Gibco) and then collectively subcultured (pooled) to a trypticase soy agar (TSA, Difco) slant in 1-dram screw-capped vials. TSA slants containing the freshly made bacterial pools were incubated for 18h at 35°C ±1°C. After incubation, the caps were tightened and cultures were placed in the dark at room temperature for varying periods of time, ranging from two days to 43 days (Tables I and II).

After storage, growth from the TSA pool of organisms was inoculated into broth following standard techniques for toxin production (4). Specifically, 5 ml of trypticase soy broth (TSB; Difco) with yeast extract was inoculated with bacterial growth from the TSA slant and incubated for 18h at 35°C ±1°C on a rotary shaker (100 RPM). Following incubation, a loopfull of the TSB was streaked on to MacConkey agar for isolation purposes. Approximately 50 well-isolated colonies were then randomly selected from this plate for lysotype determination. The original pure cultures of colonies comprising the pools were likewise re-examined for their lysotypes following the same procedures as for the pools. Thus the lysotypes of the pure cultures used to construct the pools were reconfirmed and the lysotypes of approximately 50 colonies from the pools were determined. In this way we attempted to determine the numbers of lysotypes which were recoverable from those originally introduced into the pool.

In addition to these studies, five additional pools were constructed, each containing five *E. coli* strains, each having a known lysotype. A member of these pools was a known toxin-producing *E. coli*. This was done to see if we could detect any growth or survival advantage of the ETEC in the culture methods we used *in vitro*.

Statistical analysis of results was performed using the chi-square test for goodness of fit.

Results

In the first set of experiments, 20 different examinations were made on bacterial growth from the six *E. coli* pools. The pools contained 5, 8, 9 or 10 lysotypes of *E. coli* as explained earlier and were stored for varying periods of time ranging from 5 to 43 days. Table I records the results of the 20 retrieval experiments. Table II gives the results of five tests, examining a pool of five isolates, one of which was an ETEC, the remaining four being non-ETECs.

Both tables show that in each of the tests the number of lysotypes recovered from the pools far exceeded the expected range of random variation (nearly always $p < 0.001$). The last three columns of the tables show that in each of the tests one or two lysotypes predominated, at times totally eliminating the others. Of the 878 lysotypes tested (Table I), the one predominant in each experiment accounted for a total of 552 or 62.9% of the total colonies tested. The results from the 5 ETEC experiments are of the same order (117 or 55% of colonies isolated). The overall contribution of the second most frequent lysotype was 19.5% (Table I) and 26% (Table II). Most of the other lysotypes contributed few colonies, while a substantial number were not recovered at all (76/150 in Table I and 5/25 in Table II).

Discussion

These results indicate that in the examination of a pool of *E. coli*, an ETEC probably will be detected only if the strain is able to survive in sufficient numbers so as to predominate. When recovery of all original members was attempted we consistently observed that one strain (here identified as a lysotype) overgrew the pool and predominated. The majority of lysotypes which we recovered in our experiments were present in low numbers, and a full 50% could not be recovered at all. The results of these experiments do not support the use of colony pools as a suitable method for detecting ETEC from stool specimens.

The five experiments summarised in Table II

TABLE I — NUMBER OF COLONIES OF ORIGINAL STRAINS RECOVERED FROM POOLS USING PHAGE PATTERNS AS MARKERS

Pool Designation*	Lysotypes Pooled	Days after Pooling	Total colonies tested	Number of colonies recovered by lysotype										Colonies contributed by		Lysotopes recovered/pooled
				1**	2	3	4	5	6	7	8	9	10	best grower	2nd best grower	
1.	5	6	43	43	0	0	0	0						43	0	1/5
1.R_1	5	7	51	51	0	0	0	0						51	0	1/5
2.	5	5	40	15	10	6	4	5						15	10	5/5
2.R_1	5	7	40	13	22	1	4	0						22	13	4/5
2.R_2	5	20	50	19	17	3	9	2						19	17	5/5
2.R_3	5	21	48	24	17	4	3	0						24	17	4/5
3.	8	9	50	49	1	0	0	0	0	0	0			49	1	2/8
3.R_1	8	10	47	34	13	0	0	0	0	0	0			34	13	2/8
3.R_2	8	21	32	3	29	0	0	0	0	0	0			29	3	2/8
3.R_3	8	22	50	0	50	0	0	0	0	0	0			50	0	1/8
4.	9	9	51	28	14	3	5	1	0	0	0	0		28	14	5/9
4.R_1	9	10	52	27	10	3	5	4	1	2	0	0		27	10	7/9
5.	10	22	50	1	3	4	4	0	0	13	5	10	10	13	10	8/10
5.R_1	10	26	47	0	0	6	12	0	0	11	4	9	5	12	11	6/10
5.R_2	10	42	35	0	0	0	0	0	1	6	15	5	8	15	8	5/10
5.R_3	10	43	55	0	0	0	1	0	0	1	11	9	33	33	11	5/10
6.	10	7	40	19	12	6	2	1	0	0	0	0	0	19	12	5/10
6.R_1	10	8	47	35	5	0	2	5	0	0	0	0	0	35	5	4/10
6.R_2	10	10	50	16	34	0	0	0	0	0	0	0	0	34	16	2/10
Total			878											552 62.9%	171 19.5%	74/150

* Each pool (1-6) was made up of a different set of bacterial isolates R_1, R_2 and R_3 are repeats.
** 1-10 are arbitrary designations for phage lysotypes.

were designed to investigate if ETECs have a growth advantage over other *E. coli*. In none of them could ETECs emerge as the most numerous lysotype. In one experiment, ETECs could not be detected at all. Thus, it appears that ETECs do not have a growth or survival advantage over non-

TABLE II — NUMBER OF ETEC COLONIES RECOVERED FROM A POOL OF FIVE USING PHAGE PATTERNS AS A MARKER

Pool	Number of colonies tested	Number of colonies recovered by lysotype					Number of colonies From		Lysotypes recovered/ pooled
		1*	2	3	4	5	best grower	2nd best grower	
1	37	4**	1	0	23	(9)	23	9	4/5
2	43	(7)	3	4	15	14	15	14	5/5
3	48	42	0	2	4	(0)	42	4	3/5
4	43	0	0	(7)	19	17	19	17	3/5
5	43	8	4	(2)	18	11	18	11	5/5
Totals	214						117 (55.0%)	55 (26.0%)	20/25

*Lysotypes
**number of colonies recovered
() = ETEC strain

ETECs in a pool of isolates.

In view of these observations, the justification for using a pool of colonies to detect cases of ETEC diarrhoea is questionable. Although a few studies have shown ETEC to comprise only 50% of the *E. coli* in a symptomatic patient (7, 8), a majority have shown that the toxigenic strain is present as the predominant *E. coli* population in the gut. Sack *et al.* (9) observed that all 10 isolates from 18 of 20 patients with diarrhoea due to ETEC were toxigenic. In another study, Sack *et al.* (8) compared the toxin assay results of stocks of single colony *E. coli* with that of pools of 10 colonies. The pool was only more likely to be positive than individual isolates when the majority of pool members were toxin-positive ($p=0.05$). Likewise, studies carried out by us (5) confirmed that in most cases of diarrhoea due to ETEC, almost all isolated strains of *E. coli* were toxigenic.

There are some experimental results which question the efficacy of pools in exhibiting toxin expression. Donta *et al.* (2) examined a sweep of bacterial growth obtained from the confluent portion of the agar plate. Sweep specimens from three patients with confirmed diarrhoea due to ETEC showed negative for ETECs. However, when individual colonies were examined, nearly half were found to be ETECs. Gorbach *et al.* (3) observed that unless ETECs are present in high concentrations in stools, they would normally be obscured, and escape detection.

The last issue we would like to raise is that of the sensitivity of methods to detect toxin-producing *E. coli*. Small numbers of ETECs have been isolated from asymptomatic individuals in several studies. Merson *et al.* (4) isolated ETECs from 7% of healthy people, and Gorbach *et al.* (3) from 15%. In a previous study, (5) we detected ETECs in 20% of asymptomatic contacts of cases (ETEC). It is, therefore, possible that, when the number of colonies tested is increased (sensitivity), low numbers of ETECs may be detected from a significant number of healthy individuals (5). This, however, may be misleading as the mere presence of an ETEC in low numbers does not necessarily explain the aetiology of disease.

We feel that for the diagnosis of diarrhoea due to ETEC, the examination of 3 colonies is to be preferred to that of pooled colonies. This will yield approximately 85% ETEC isolations, even in populations where as few as 50% of the *E. coli* are toxin producers. If greater sensitivity is desired, the examination of additional single colony isolates seems to be better than the examination of a pool. However, higher sensitivity may also lead to confusion as the examination of a large number of colonies may result in the identification of ETEC which are not associated with clinical illness.

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