

TOXIN DETECTION AFTER STORAGE OR CULTIVATION OF ENTEROTOXIGENIC
WITH COLICINOGENIC *Escherichia coli*: A POSSIBLE MECHANISM
FOR TOXIN NEGATIVE POOLS

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OBJECTIVES : The suckling mouse assay to detect *Escherichia coli* heat-stable toxin and Y-1 adrenal cell assay to detect heat-labile toxin are time consuming and expensive. Recently investigators have proposed pooling schemes to reduce the number of assays required. This study was designed to evaluate the effect of colicinogenic strains on toxin detection to better interpret the effect of pooling isolates to define the epidemiology of enterotoxigenic *E. coli* infections.

BACKGROUND : Both the time and expense required to perform the infant suckling mouse assay in large scale field studies of diarrhea have led investigators to test pools of colonies or pools of supernatants rather than individual colonies (1, 3, 4, 7, 13). Since colicins are bacteriocidal proteins (5), pooling of enterotoxigenic (tox+) with colicinogenic (col+) *E. coli* might have a deleterious effect on the growth and survival of the tox+ strains, and ultimately, upon the detection of toxin. We therefore tested *E. coli* isolated from diarrheal stool for the production of colicin. The effect of pooling col+ strains with tox+ strains on the detection of toxin was then examined in the suckling mouse (2) and Y-1 adrenal cell assays (12).

METHODS :

Bacterial Strains : One hundred non-enterotoxigenic (tox-) and 24 tox+ *E. coli* were isolated from different patients during a study of pediatric diarrhea in Bangkok, Thailand. These strains had been identified as *E. coli*, tested for heat-labile toxin (LT) and heat-stable toxin (ST), and lyophilized immediately by methods previously described (2, 12). *E. coli* K12 Xac (F⁻, lac⁻, pro⁻, met⁻, arg⁻, nal^r) was used as an indicator to detect col+ *E. coli*.

Detection of Colicin Production : Strains to be tested for colicin production were grown overnight in TSBY (trypticase soy broth, BBL, Cockeysville, MD) with 0.6% yeast extract (DIFCO, Detroit, MI) at 37°C, inoculated onto TYC agar plates (1% tryptone, 1% yeast extract, 1% glucose, and 1.2% agar) using a Steers replicator and grown overnight at 37°C. One set of plates was inverted over chloroform for 30 minutes to lyse the bacterial cells and then dried for 60-90 minutes. A duplicate set of plates was not exposed to chloroform. Indicator organism *E. coli* Xac was grown overnight in TSBY at 37°C and diluted to approximately 10⁶ organisms per ml in H-top soft agar (one

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percent tryptone, 0.8% agar, and 0.8% NaCl). Two and one half milliliters of seeded agar was then slowly pipetted onto each plate, allowed to harden, and incubated overnight at 37°C. Plates were examined for clear zones in the agar overlay. The following definitions for the degree of clearing were used: 1+, a zone of partial clearing extending 1-4 milliliters outside of the test colony; 2+, a zone of partial clearing extending >4 mm beyond the test colony; 3+, a zone of complete clearing extending 1-4 mm beyond the test colony; 4+, a zone of complete clearing extending >4 mm beyond the test colony.

Strains that produced clear zones in the indicator organism were retested with trypsin 100-500 µg/ml added to the soft agar. In addition, the strains were grown overnight, the cells lysed by the addition of chloroform, and the cellular debris removed by centrifugation. Ten microliters of 2 fold dilutions of the supernatant were spotted on Xac-seeded soft agar freshly poured onto TYC plates, the plates were incubated at 37°C overnight, and examined for clear zones.

Initially 50 tox- and 24 tox+ *E. coli* were tested against each other to detect production of, and susceptibility to, colicin. *E. coli* Xac, which was susceptible to all the colicins tested, was then used to screen 50 additional tox- *E. coli*. Strains found to produce colicins were then tested against all the tox+ *E. coli*.

Storage and Cultivation : An individual colony of each of various tox+ and col+ *E. coli* was streaked onto MacConkey agar plates. For co-storage studies, a single wooden toothpick was used to pick from one to four tox+, and from one to four col+ colonies (total of five *E. coli* per toothpick), then inoculated onto nutrient agar slant cultures, and stored at 25°C. The strains were chosen such that the col+ *E. coli* was known to be inhibitory to the tox+ *E. coli* it was stored with. Five to ten colonies of the tox+ strains were also picked and stored alone or in combination with col- *E. coli*. After 3 weeks, the slant cultures were inoculated into TSBY, grown, and tested for toxin production (2, 12).

For co-cultivation studies, colonies of tox+ and col+ *E. coli* were picked from MacConkey agar onto wood sticks using the proportions described above, inoculated directly into five milliliters of TSBY, grown, and tested for toxin production. Colonies of tox+ *E. coli* were also tested for toxin production after co-cultivation with col- *E. coli* and after cultivation alone.

RESULTS :

Detection of Colicin Production : Twenty-six of 124 strains of *E. coli* were found to produce clear zones in agar overlays seeded with *E. coli* Xac. All zones were present whether or not the test organisms were lysed by chloroform, although chloroform lysis did increase the amount of clearing by one degree with 20% of the test strains. The size of the zones around the test colonies and the fact that the zones did not increase in size with prolonged incubation indicate that the agent responsible was freely diffusible and was not spreading by cell to cell propagation. Spotting two fold dilutions of supernatants of these test strains failed to produce any evidence of plaque formation, although in many instances, little or no clearing was produced even with the undiluted samples. Twenty-four of the zones were eliminated

by trypsin 100 µg/ml and two were eliminated by trypsin 500 µg/ml. For these reasons, the clear zones were felt to be due to the effect of colicin(s) and not to the effect of lytic phage (6, 9).

Of the 26 strains found to produce colicin(s), 24 were tox- and two were tox+. Six of the col+ strains, including the two tox+ *E. coli*, were active only against *E. coli* Xac and had no effect upon any of the clinical *E. coli* isolates. Six col+ strains were active against 22 or more of the tox+ *E. coli*, and one of these 6 strains caused complete clearing (zone 3+ - 4+) in all of the tox+ *E. coli*. Tables 1 and 2 summarize the activity of the col+ *E. coli*.

Storage : Thirteen tox+ *E. coli* were stored in pools with each of several col+ *E. coli* known to inhibit the tox+ strain it was stored with. Ratios of the number of tox+ to the number of col+ colonies which were stored together ranged from 4 : 1 to 1 : 4 (five organisms per pool). Four strains producing LT- only lost the ability to produce LT whether stored alone or stored with col+ *E. coli*. Toxin production by the remaining 9 strains (ST+/LT+ and ST+ only) was inhibited to a variable degree by co-storage with col+ *E. coli*. Fifty-one of 96 pools were negative for ST (Table 3). Nine pools were negative when 4 tox+ colonies were stored with 1 col+ colony; 11 were negative when 3 tox+ were stored with 2 col+ colonies; 14 were negative when 2 tox+ were stored with 3 col+ colonies; and 17 were negative when 1 tox+ was stored with 4 col+ colonies. Of these 96 pools, 72 contained ST+/LT+ strains. Seventeen of these 72 pools were negative for LT. Except for the LT strains already mentioned, no pool in which these tox+ strains of *E. coli* were stored alone (5-10 control slants per strain), or with col- *E. coli*, was negative for toxin (Table 3).

Cultivation : When col+ and tox+ *E. coli* were grown together, without prior co-storage, 12 of 96 pools were negative for ST. Six were negative for ST when four col+ colonies were mixed with one tox+ colonies; one was negative at a ratio of three col+ to two tox+ colonies; four were negative at a ratio of three col+ to two tox+ *E. coli*; and one was negative for ST at a ratio of 2 col+ to 3 tox+ *E. coli*. Of the 72 combinations expected to produce LT, only one was negative. No combination was negative for toxin when only one col+ *E. coli* was grown with col- *E. coli*.

This study is complete.

Table 1. Activity of 21 Col+ *E. coli* Against Tox+ *E. coli*

Colicin producing					
strain	1+ ^a	2+	3+	4+	Total
C19	2 ^b	4	1	15	22
C32	1	0	6	15	22
C44	0	0	2	20	22
C52	5	9	0	2	16
C64	0	0	1	23	24
C68	2	3	11	3	19
C69	4	2	0	0	6
C72	4	0	0	0	4
C84	2	2	0	0	4
C85	1	2	0	0	3
C94	0	0	2	22	24
C98	1	1	0	0	2
C99	4	3	1	0	8
C111	4	5	6	1	16
C115	1	5	3	1	10
C118	3	0	0	0	3
C121	7	3	2	1	13
C122	3	6	9	4	22
C123	1	4	6	4	15
C126	0	9	2	1	12
C128	2	0	0	2	4

Total 21^c

^a Degree of lysis produced; see text for definition

^b Number of strains of tox+ *E. coli* lysed

^c Five strains, not listed here, were active against *E. coli* Xac but not against any of the clinical tox+ *E. coli*

Table 2, Susceptibility of Tox+ to Col+ *E. coli*

Enterotoxigenic <i>E. coli</i>	# of Col+ strains active against each ETEC
ET3	18 ^a
ET4	18
ET6	15
ET7	12
ET8	13
ET10	7
ET11	15
ET12	12
ET14	11
ET15	14
ET23	10
ET25	12
ET28	10
ET29	8
ET70	2
ET71	18
ET72	12
ET73	7
ET74	9
ET75	20
ET76	10
ET77	6
ET78	2
ET79	12

^a 26 Col+ strains tested

Table 3. Detection of ST Production after Co-storage of Tox+ *E. coli* with Col+ *E. coli*

Tox+ <i>E. coli</i> x Col+ <i>E. coli</i>			5:0 ^a	4:1	3:2	2:3	1:4
ET 6	x C44		+ ^b	-	-	-	-
"	x C123		+	+	+	+	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 7	x C44		+	+	-	-	-
"	x C111		+	+	-	+	-
"	x C122		+	-	+	-	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 8	x C44		+	-	-	-	-
"	x C122		+	+	+	-	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 9	x C44		+	+	-	-	-
"	x C64		+	+	+	+	+
"	x C122		+	+	-	-	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 12	x C19		+	+	+	+	+
"	x C64		+	+	+	+	+
"	x C122		+	+	+	+	+
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 13	x C44		+	-	-	-	-
"	x C111		+	-	-	-	-
"	x C122		+	-	-	-	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 23	x C111		+	-	+	-	-
"	x C122		+	+	+	+	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 26	x C44		+	+	+	+	+
"	x C94		+	+	+	+	+
"	x C111		+	+	+	+	+
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+
ET 75	x C44		+	-	-	-	-
"	x C111		+	+	+	-	-
"	x C115		+	-	-	-	-
"	x tox-, col- <i>E. coli</i>		+	+	+	+	+

^a Ratio of tox+ *E. coli* to col+ *E. coli* stored together for three weeks on nutrient agar slants

^b Results of infant suckling mouse assay (done in triplicate)

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