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Production of Vascular Permeability Factor by Enterotoxigenic *Escherichia coli* Isolated from Man

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Enterotoxin preparations derived from *Escherichia coli* strain H-10407 were shown to contain vascular permeability factor (PF) activity as well as diarrheagenic activity. Intradermal injection of *E. coli* enterotoxin (ECT) caused localized induration and permeability of small blood vessels of the skin to intravenously administered Evans blue dye. The PF assay described here demonstrated a linear dose response and was at least as sensitive as the adult rabbit ileal loop assay for detecting ECT. *E. coli* PF activity was heat labile and was neutralized by homologous antiserum. PF production was enhanced by the addition of yeast extract (up to 0.6%) to a Casamino Acids-salts medium. PF activity was detectable as early as 6 h in aerated (shake) cultures in the Casamino Acids-yeast extract-salts medium, pH 8.5, maximal at 18 h and essentially unchanged at 48 h. The skin test (PF) assay for ECT has numerous advantages over current assay methods which involve gastrointestinal challenge of experimental animals.

Filterable enterotoxins elaborated by *Vibrio cholerae* and certain strains of *Escherichia coli* associated with acute diarrhea in man produce diarrheal reactions in the gastrointestinal tract of experimental animals (6, 8, 10, 14). In 1965, Craig (2) showed that intracutaneous injection of culture filtrates of *V. cholerae* caused erythema, induration, and increased permeability of small blood vessels in the skin. The vascular permeability factor (PF) and the diarrheagenic factor appear to be the same substance, since purified preparations of cholera enterotoxin (choleragen) possess both activities. Furthermore, both activities are neutralized by specific anticholeragen serum (1, 3, 13). PF activity of choleragen is the basis of a useful assay for quantitating both choleragen and anticholeragen antibody in serum (3).

One major difference between these enterotoxins has been the reported failure of *E. coli* enterotoxin (ECT) to elicit the vascular permeability (PF) effect characteristic of choleragen (1, 7, 16). In 1971, Moon and Whipp (12) reported heat-labile dermal (PF) activity in an enterotoxin preparation procured from a strain of *E. coli* enteropathogenic for newborn pigs. However, these authors concluded that there was no direct evidence to relate enteropathogenicity with the ability to produce PF.

In this paper we report the presence of PF activity in cell-free culture filtrates of enterotoxigenic *E. coli* isolated from man. We describe here some of the properties of ECT PF and various factors which may explain previous failures to demonstrate this activity. We present evidence here and elsewhere (manuscript submitted for publication) indicating that PF activity is a function of heat-labile ECT.

MATERIALS AND METHODS

Organism and culture conditions. *E. coli* strain H-10407 (serotype 078:H 11) was employed for the production of enterotoxin. This strain was isolated from liquid stool of a patient with severe cholera-like, *Vibrio*-negative diarrhea in Dacca, Bangladesh. Strain H-10407 has been employed in several previous studies (7, 8, 17). Stock cultures were maintained on slants composed of 2.0% peptone (Difco), 0.5% NaCl and 2.0% agar.

The standard medium employed for the production of enterotoxin was composed as follows: 20 g of Casamino Acids (Difco), 6.0 g of yeast extract (Difco), 2.5 g of NaCl, 8.71 g of K_2HPO_4 (0.05 M), and 1.0 ml

of trace salts, added to distilled water in that order, adjusted to pH 8.5 with 0.1 N NaOH, and brought to a final volume of 1 liter. The trace salts mixture consisted of 5.0% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$ dissolved in 0.001 N H_2SO_4. Shake cultures (80 ml per 500-ml Erlenmeyer flask) were loop inoculated from stock slants and agitated in a rotary incubator shaker at 135 to 140 rpm for 18 h at 37 C. Enterotoxin production in the absence of aeration was investigated by incubating cultures (40 ml per 50-ml Erlenmeyer flask) in a 37 C constant temperature water bath without shaking. Growth was monitored by determining the optical density of 1:10 dilutions in saline of culture samples at 640 nm with a Perkin-Elmer (Coleman 124) double-beam spectrophotometer.

Enterotoxin preparations. A 60-fold concentration of enterotoxin from the spent culture fluid was achieved by the following procedure. Cells were removed from 18-h cultures by centrifugation at 12,000 $\times$ *g* for 45 min followed by membrane (0.45 μm, Millipore Corp.) filtration of the supernatant fluid. Enterotoxin was precipitated by slowly adding solid $(NH_4)_2SO_4$ (AMS) to 90% saturation at 4 C with stirring, followed by 20 min without stirring. After centrifugation at 12,000 $\times$ *g* for 45 min at 4 C, the pellet was dissolved in 0.02 M tris(hydroxymethyl) aminomethane (Tris)-chloride buffer, pH 8.0 (10 ml per liter original volume), and dialyzed for 18 h against 650 volumes of the same buffer at 4 C. Essentially all of the enterotoxin activity (both heat stable and heat labile) was recovered in the dialysand. A typical X60 ECT concentrate contained approximately 1.5 mg of protein per ml as determined by the method of Lowry (11).

The heat-labile ECT was further purified by precipitation with protamine sulfate (PRS), extraction of the precipitate with NaCl, and reprecipitation with AMS as follows. PRS (0.222 volumes of a 1.0% solution in water) was added dropwise to the X60 ECT concentrate at room temperature with rapid stirring, followed by 20 min without stirring to enhance precipitate formation. The precipitate was collected by centrifugation at 16,000 $\times$ *g* for 20 min at 4 C, and the supernatant was discarded. The pellet was extracted by homogenization in 0.02 M Tris-chloride buffer, pH 8.0, containing 0.10 M NaCl (1.5 ml per ml of X60 ECT concentrate). After an additional 20 min at room temperature, the insoluble precipitate was collected by centrifugation as above. The resultant pellet was extracted by homogenization in 0.02 M Tris-chloride buffer, pH 8.0, containing 0.15 M NaCl, as above, followed by centrifugation under the same conditions. The final pellet was discarded and the two extracts were pooled. ECT was then precipitated by the addition of solid AMS to 90% saturation at room temperature, followed by 30 min at 4 C. The resultant precipitate was collected by centrifugation for 30 min at 16,000 $\times$ *g* at 4 C and resuspended to $\frac{1}{100}$th of the original culture supernatant fluid. A typical X600 PRS-ECT preparation contained approximately 1.3 mg of protein per ml as determined by the method of Lowry (11), including an undetermined amount of PRS. Stored ECT preparations were maintained at −45 C.

Source of rabbits. Rabbits employed for the ileal loop assay were procured from Mission Laboratory Supply, Inc., Rosemead, Calif. Rabbits employed for the PF assay were procured mainly from the same source; an additional study included animals from Curd's Caviary and Animal Supply, La Puente, Calif., and ABC Animal Supply. Pomona, Calif.

Rabbit intestinal ligated loop assay. The adult rabbit intestinal loop assay (4) was performed essentially by the modifications of Pierce (8, 14) employing albino rabbits weighing 1.5 to 2.0 kg. Each ligated segment received a 1.0-ml intraluminal injection of test material diluted in 0.01 M phosphate-buffered saline (PBS) or PBS (negative control) or cell-free *E. coli* H-10407 culture fluid (positive control). Details of this assay method were reported recently (8). A 6-h gut exposure time was employed here. Enterotoxin activity detectable after boiling for 30 min is referred to as stable toxin (ST); activity destroyed by such treatment is referred to as labile toxin (LT).

Assay of vascular PF activity. Unless otherwise specified, New Zealand albino rabbits weighing 1.5 to 2.0 kg were employed. Hair was shaved from the back of the animal with small animal clippers, and the remainder was removed by application of a commercial depilatory cream. The cream was then gently removed by rinsing several times with warm water. Test and control materials were injected intradermally, 0.1 ml per injection site, approximately 2.5 cm apart in a random pattern. All specimens were coded and assayed in a "blind" manner. The standard diluant, which also served as the negative control, consisted of sterile PBS, pH 7.2, plus 0.02% bovine serum albumin (BSA). The animals were then caged individually to avoid scratching. Eighteen hours later the animals were administered intravenously a solution (2.0% in 0.15 M NaCl) of Evans blue dye (Matheson Coleman and Bell) at a dose of 40 mg/kg of body weight. Two hours were allowed for permeation of the dye, after which the induration and blueing of the lesions were recorded. The diameters of the zones were measured to the nearest millimeter in two different directions. The score was derived by squaring the average of the two values.

Preparation of antitoxin. Three adult albino rabbits received seven sequential doses of approximately 65 μg of PRS-ECT at 4-day intervals. For the first inguinal (intramuscular) injection, the enterotoxin was emulsified in incomplete Freund adjuvant. The remaining doses of enterotoxin, diluted in physiological saline, were injected intramuscularly at multiple sites. Fourteen days after the last injection, the rabbits were bled and sera were collected and pooled, distributed in 2.0-ml samples, and stored at −45 C. Control serum was a pool derived from the same rabbits prior to immunization.

Neutralization of PF activity by antitoxin. Rabbit serum containing anti-(H-10407)-enterotoxin was obtained as described above. In the experiment reported here, samples of X600 PRS-ECT diluted 1:61 in PBS plus 0.02% BSA were combined with equal volumes of immune serum (twofold dilutions starting at 1:16). Mixtures were incubated at 37 C for 30 min prior to injection for PF determination. Control mix-

tures consisted of PBS, ECT plus control serum (1:32 final dilution), and ECT (1:122) without serum.

RESULTS

PF and diarrheagenic activity of crude ECT. Cell-free culture supernatant fluid of *E. coli* strain H-10407 was employed to investigate the response to ECT in both the PF and the rabbit ligated intestinal loop assay systems (Fig. 1). Both assay systems produced a near-linear dose response curve, although the PF assay was more sensitive. For example, a 1:81 dilution of culture supernatant fluid had no detectable loop activity, whereas there was a 25-mm² (5-mm diameter) mean blueing reaction.

PF versus diarrheagenic activity of E. coli LT. Preliminary work concerning the purification of ECT led to the preparation of a PRS-ECT concentrate, as described in Materials and Methods. This material was shown to be more pure on the basis of immunological analysis, and lacked detectable ST activity in the ileal loop assay system. PRS-ECT was employed to investigate the effect of large doses of LT in the PF assay system, including a study of the induration component of the PF lesion. Figure 2 shows that PRS-ECT produced a linear response as a function of dose in both the PF and ileal loop assay systems. PF (blueing) and ileal loop activity were both half-maximal at a dose

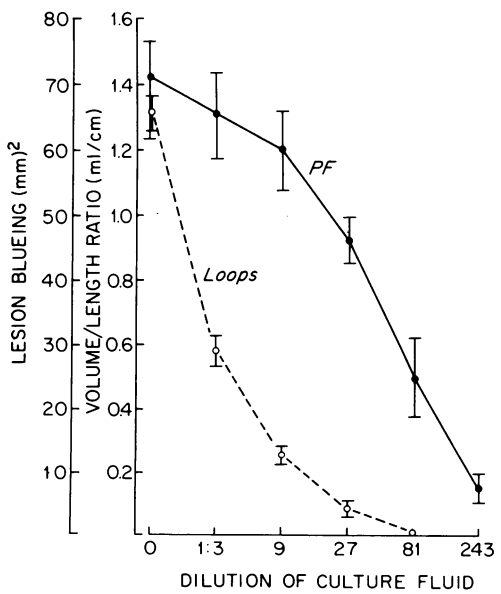

FIG. 1. *PF and rabbit intestinal loop activities in cell-free culture filtrates of E. coli strain H-10407. PF: mean of 22 values ± 1 SEM; fluid accumulation: mean of 31 values ± 1 SEM.*

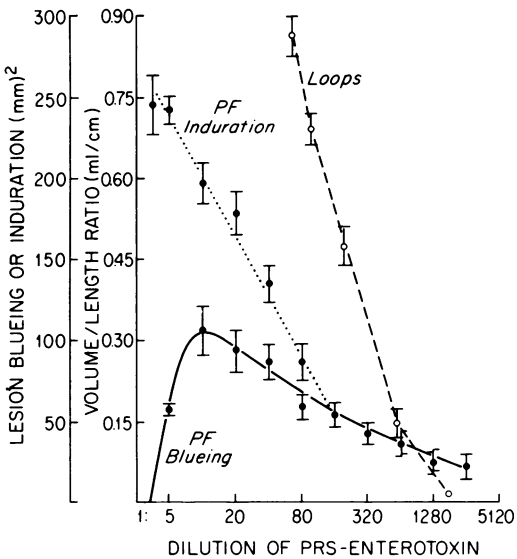

FIG. 2. *PF and rabbit intestinal loop activities in 600-fold concentrated E. coli H-10407 enterotoxin preparation. PF: mean of 15 values ± 1 SEM; fluid accumulation: mean of 36 values ± 1 SEM.*

of approximately 2.0 μg of protein per ml. The zones of induration and blueing were essentially identical in size when the PF lesions were less than 8 by 8 mm in diameter. However, with larger doses of PRS-ECT the zones of induration were increasingly larger than the zones of blueing. The maximum area of blueing was approximately 121 mm², whereas the maximum area of induration was approximately 256 mm². This may be due to the increasing pressure of extracellular fluid at maximum induration which prevented the movement of dye into the surrounding tissue.

Table 1 summarizes the results of several experiments in which the heat lability of PF was tested. The PF activity of crude culture fluid was completely heat labile, although such preparations also contained significant ST activity as determined by the ileal loop assay. The PRS-ECT concentrate contained no detectable ST activity but did contain heat-labile loop activity and heat-labile PF activity. This indicates that ST did not contribute to the PF activity, as determined by the method reported here.

Effect of yeast extract concentration on ECT PF production. Several experiments were performed to determine optimal conditions for production of PF activity. The concentrations of various constituents of the culture medium such as Casamino Acids, phosphate, and yeast extract were investigated. Yeast extract concen-

tration was the only factor which was found to effect PF production independently of cell yield. The basal medium without yeast extract yielded no detectable PF activity in the unconcentrated culture fluid. There was an increasing production of PF activity with concentrations of yeast extract from 0.025% to 0.6% (Fig. 3); further addition of yeast extract did not increase the results. Loop activity was also augmented at the higher concentrations of yeast extract. Medium containing 1.0% Casamino Acids and 0.2% yeast extract yielded more PF than that containing 2.0% Casamino Acids and 0.02% yeast extract, other factors remaining constant.

TABLE 1. *Heat lability of PF activity of ECT*

ECT preparation[a]	Ileal loop		PF	
	Dilution	Activity[b]	Dilution	Activity[c]
X1 ECT		1.32 ± 0.05		70.73 ± 7.84
X1 ECT (boiled)		0.45 ± 0.05		0.0
PRS-ECT	1:100	0.69 ± 0.04	1:20	92.75 ± 12.71
PRS-ECT (boiled)	1:100	0.0	1:20	0.0

[a] X1 ECT denotes undiluted cell-free culture fluid; PRS-ECT denotes X600 concentrated ECT preparation. Boiling was for 30 min.

[b] Ileal loop activity is expressed as ml/cm of fluid accumulated after a 6-h gut exposure; mean of 30 values ± 1 SEM.

[c] PF activity is expressed as (diameter of blueing, in mm)^2; mean of 15 values ± 1 SEM.

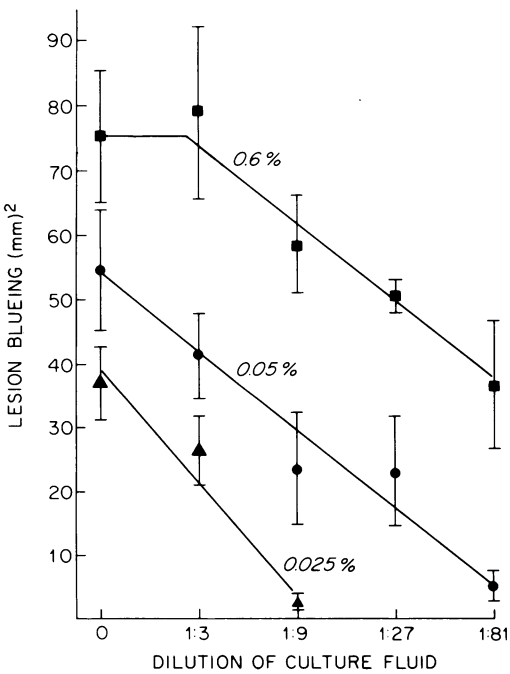

FIG. 3. *Effect of yeast extract concentration on yield of PF activity by E. coli strain H-10407. Data: mean of six values ± 1 SEM.*

Time course of PF production. PF production as a function of incubation time was investigated by employing the Casamino Acids-0.6% yeast extract medium and the aeration conditions previously described. Samples were removed at sequential times for determination of optical density, pH, and PF activity. The appearance of PF in the culture fluid paralleled the growth curve (Fig. 4). PF activity was detectable as early as 6 h after inoculation and attained maximum levels by 18 h. The plateau observed here was not a function of the assay system. The pH, initially a value of 8.5, was somewhat lower (pH 8.2) at 6 h but returned to initial levels by 24 h.

A comparison was made of shaking versus standing cultures after 18 h of incubation; 1:3 dilutions of the culture fluids were tested. The shaking culture produced greater PF activity (mean 74.0 ± 18.6) and higher optical density (8.90 at 640 nm) than the still culture (PF mean 40.6 ± 3.7; OD 1.25 at 640 nm).

Effect of commercial source and rabbit size on PF assay. Rabbits weighing 1.5 to 2.0 kg were obtained from three independent sources, and PF assays were performed by using the same ECT preparation. Figure 5 shows that essentially the same results were found with animals of the same size obtained from these

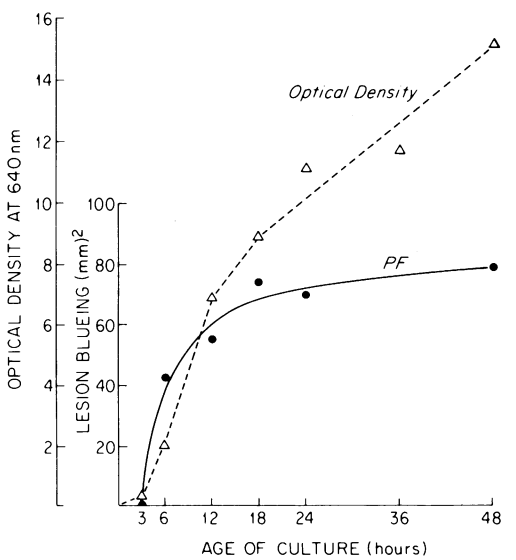

FIG. 4. *Time course of PF production of E. coli strain H-10407 in a 2.0% Casamino Acids-0.6% yeast extract-salts medium in shake culture. PF: mean of six values, determined at 1:3 dilution.*

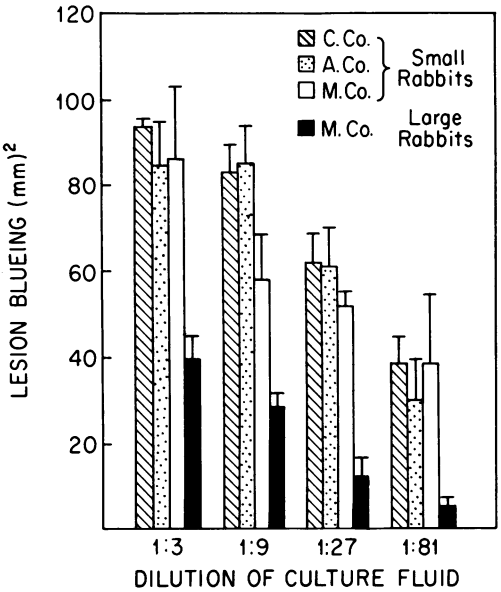

FIG. 5. *Effect of commercial source and size of rabbits on PF reaction. Vertical bars represent mean of six values ± 1 SEM.*

three different commercial sources. In another experiment, a comparison was made of the PF (blueing) response in rabbits of small size (1.5 to 2.0 kg) and of large size (4.0 to 4.5 kg) obtained from our standard commercial source (Mission Laboratory Supply, Inc.). Larger rabbits consistently exhibited a reduced PF response to the same preparations of ECT (Fig. 5). The same effect was observed with the induration component of the PF lesion.

Neutralization of ECT PF activity by homologous antiserum. Neutralization of PF by antibody was investigated by employing immune serum prepared against PRS-ECT. A standard challenge dose of 1:122 dilution of PRS-ECT was used. Complete neutralization was seen with a 1:512 dilution of serum, and 50% of the PF activity was neutralized with a 1:2048 dilution (Fig. 6).

DISCUSSION

The original recognition of the enterotoxin of *V. cholerae* (choleragen) was based on the ability of cell-free culture fluids to produce fluid accumulation in the rabbit ileal loop model (5). It was subsequently shown that choleragen caused increased capillary permeability, erythema, and induration when injected into the skin (2). On the basis of the data reported here and elsewhere (manuscript submitted for publication), it now appears that ECT also possesses PF activity as well as diarrheagenic activity. Thus, we conclude that the production of PF activity is an important characteristic of enterotoxigenic *E. coli* isolated from man.

There are several factors which influence detection of the PF activity of ECT. Failure to recognize these factors may have led to the inability of many investigators to demonstrate PF activity in culture fluids of enterotoxigenic (tox⁺) *E. coli*. One critical factor is that the PF assay, as described here, only responds to the LT, although tox⁺ *E. coli* isolated from humans also produce an ST (8). Thus, enterotoxin preparations obtained by culture methods which enhance the yield of LT possess easily detectable PF activity, whereas those obtained under conditions unfavorable for LT production may be PF negative. For example, inclusion of carbohydrate in a weakly buffered culture medium causes a decrease in pH which in turn may decrease the yield of LT (8) and thus the PF response. We employed a noncarbohydrate medium preadjusted to pH 8.5 for ECT production.

It has been a common practice to employ Casamino Acids-based media for the production of ECT. In this investigation we have found a requirement for yeast extract such that PF activity is undetectable in crude filtrates prepared with media containing less than approximately 0.02%. At least two of the standard media employed for choleragen production, TRY (15) and Syncase (9), do not meet this minimal requirement. Aeration of the culture is another factor, since shake cultures produce more PF activity than standing cultures. With strict observation of these various factors, we have demonstrated PF activity in crude culture

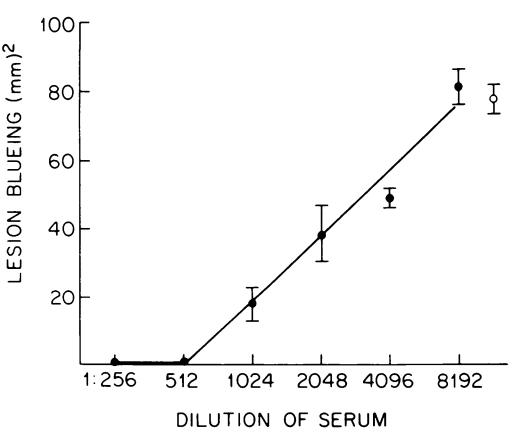

FIG. 6. *Neutralization of E. coli strain H-10407 PF activity by homologous antiserum. Symbols: ●, toxin plus antiserum; ○, toxin without antiserum. Vertical bars represent mean of six values ± 1 SEM.*

filtrates as early as 6 h after inoculation with *E. coli* strain H-10407.

The animal employed for the assay may also influence the ability to detect PF activity in ECT preparations. For example, we have observed that it is a common, and economical, practice to employ large rabbits for measuring PF activity of choleragen. In the present investigation, it was demonstrated that large animals are less responsive to ECT PF than the smaller, younger animals. On the other hand, no significant differences were observed when the PF response was studied in small rabbits obtained from three different commercial sources.

The PF activity of *E. coli* produces a linear dose-response curve with clear-cut end points when ECT is prepared and assayed according to the conditions described here. PF lesions produced by ECT are generally similar to those of choleragen (erythema, induration, blueing), but there are some obvious differences. The relatively diminished response of larger rabbits to ECT PF is one example. Also, induration of an ECT PF lesion is not as raised as a PF lesion of equal diameter elicited by choleragen and the blueing is generally less intense. Another characteristic of the ECT PF lesion is diminished blueing at relatively large doses; we did not observe such diminished blueing with doses of choleragen producing lesions of equal diameters. However, diminished blueing of PF lesions at high dosage of ECT does not present a technical problem since the lesion is well indurated and usually has a central, although small, blue zone.

The PF reaction should provide investigators with a simple test for detecting ECT. The test is easy to perform and fairly economical, since multiple assays can be done in the same animal along with positive and negative controls. This assay can be used to great advantage in the continuing efforts to purify the LT of *E. coli*. In addition, it appears to be applicable to field studies of the incidence of tox^+ *E. coli* in diarrheal disease in man. The ability to neutralize ECT PF activity by anti-ECT antiserum, as shown here, should prove to be a useful epidemiological tool in determining antitoxin levels in patient populations.

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