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A Research Note

Evidence That *Clostridium perfringens* Produces Only One Enterotoxin

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ABSTRACT

Thirteen *Clostridium perfringens* isolates classified as nonenterotoxigenic by radioimmunoassay (RIA) were tested for biological activity in rabbit ileal loops to determine whether these organisms produced enterotoxins serologically unrelated to the classical *C. perfringens* enterotoxin. None of these strains was active in the ileal loop assays. The large number of RIA-negative isolates obtained from food-poisoning outbreaks is more likely due to the failure to isolate causative strains rather than to the existence of novel enterotoxins.

Isolates of *Clostridium perfringens* implicated in food-poisoning outbreaks have frequently failed to produce enterotoxin detectable by serological assays (*1,8*). One of several possible explanations for this is the existence of unknown *C. perfringens* enterotoxins that do not cross-react serologically with the classical enterotoxin. Since many other bacterial species that cause food-poisoning symptoms produce more than one type of enterotoxin, this explanation is plausible.

Representative isolates from food-poisoning outbreaks that had been classified as nonenterotoxigenic by radioimmunoassay (RIA; *8*) were tested for biological activity using the rabbit ileal loop assay. This model was chosen because earlier studies had shown that the rabbit ileal loop assay was highly effective in distinguishing strains capable of producing the food-poisoning syndrome in monkeys (*3*) and in human volunteers (*9*). The stages of growth or sporulation required for the production of possible new enterotoxins were not known, therefore, these preliminary tests were done using live cultures rather than culture supernatant fluids.

MATERIALS AND METHODS

All of the *C. perfringens* strains used in this study were isolated from foods implicated in food-poisoning outbreaks. *C. perfringens* FD2, FD6, FD7, FD20, FD26, S45 and S80 were obtained from S. Harmon, Division of Microbiology, Food and Drug Administration, Washington, DC; isolates PS16, PS17 and PS18 were obtained from G. Lombard, Centers for Disease Control, Atlanta, GA; and isolates CW122, CW324 and T65 were obtained from E. Sommers, Food Research Institute, University of Wisconsin, Madison, WI. The control strains NCTC 8239 (enterotoxigenic) and FDl (nonenterotoxigenic) were obtained from V. Scott, Food Research Institute, Madison, WI.

Working stock spore suspensions of the *C. perfringens* strains were produced in modified cooked meat medium (*5*) or in modified Duncan-Strong (D-S) medium (*8*) and stored at -20°C. Spores were activated by inoculating 1.0 ml from thawed spore stocks into 10 ml of fluid thioglycollate medium, heat shocking at 75°C for 20 min, and incubating at 37°C. After 16 h, 1.0 ml was transferred from each fluid thioglycollate culture into 10 ml of freshly boiled skim milk medium. These cultures were incubated for 5 h at 37°C. These 5-h cultures, which contained from 10^6 to 10^7 CFU/ml, were used to inoculate the rabbit ileal loops. The ileal loop assays were performed by the procedure of Twedt and Brown (*11,12*). Each rabbit contained a positive control loop inoculated with *C. perfringens* NCTC 8239 and a negative control loop inoculated with sterile skim milk medium. Some of the rabbits contained an additional negative control loop inoculated with *C. perfringens* FDl, a well-characterized, nonenterotoxigenic strain (*3*). A test culture was considered positive if the volume-to-length ratio was three-fold greater than that of the negative control in the same animal. A strain was considered positive if loops tested positive at least two of three times in separate animals (*13*).

Two of the RIA-negative strains (FD2 and FD6) were tested because their culture supernatant fluids had shown some effect on Vero cell monolayers (*8*). Strain T65 was tested because it had been reported as enterotoxigenic in earlier studies (*2,4*). The other RIA-negative strains used in this study were chosen at random.

RESULTS AND DISCUSSION

Results of the rabbit ileal loop experiments (Table 1) showed no evidence for the production of novel enterotoxins or for the presence of other pathogenic mechanisms in the 13 strains that were tested. These results suggest that the existence of enterotoxins serologically unrelated

TABLE 1. *Activity of selected strains of* C. perfringens *in the rabbit ileal loop assay.*

C. perfringens strain	Source	RIA activity[a]	Rabbit ileal loop activity[b]
8239	Positive control	+	11/11
FD1	Negative control	–	0/4
FD2	Chicken broth	–	0/3
FD6	Smoked salmon	–	0/3
FD7	Chili	–	0/3
FD20	Roast beef	–	0/2
FD26	Barbecued beef	–	0/2
S45	Dried beef	–	0/2
S80	Chicken	–	0/2
PS16	Shrimp	–	0/2
PS17	Chicken	–	0/2
PS18	Turkey	–	0/2
T65	Turkey	–	0/2
CW122	Dormitory meal	–	0/2
CW324	Chicken gravy	–	0/3

[a]Stelma et al. (*8*).

[b]Number of loops with a volume to length ratio three or more times greater than that of the sterile medium control after 18 h of incubation.

to the classical *C. perfringens* enterotoxin is not the cause for the appearance of so many RIA-negative strains among isolates from food-poisoning outbreaks. The most likely explanation for this phenomenon is the presence of a mixture of enterotoxigenic and nonenterotoxigenic populations of *C. perfringens* in the foods, with too few isolates being tested for enterotoxigenicity.

Considering the large number of spores of *C. perfringens* present in the environment, it would be surprising to find contaminated foods that contained pure cultures of *C. perfringens*. Thus, it is essential to test a sufficient number of isolates from a food or a stool sample to be sure that any enterotoxigenic strain present is found. Our laboratory has recently developed an enzyme-linked, immunosorbent assay (ELISA) that detects enterotoxin production by individual colonies of *C. perfringens* (*7*). Use of this assay should facilitate the testing of large numbers of isolates.

Another possible explanation is that some outbreaks from which nonenterotoxigenic *C. perfringens* have been isolated were caused by other species. The tendency may be to diagnose an outbreak merely by the presence of the typical *C. perfringens* syndrome and the detection of *C. perfringens* organisms in the suspect food, ignoring the presence of other organisms. At least one other *Clostridium, C. sphenoides,* has recently been implicated in an outbreak of diarrheal disease (*10*). Other genera should also be considered. The clinical syndrome of a *Bacillus cereus* diarrheal outbreak parallels that of a *C. perfringens* outbreak, and *B. cereus* is sometimes found in the same types of foods frequently implicated in *C. perfringens* outbreaks (*6*).

A third possible explanation is that some of these strains lost their enterotoxigenicity after repeated subculturing. Live cultures of *C. perfringens* T65 produced positive rabbit ileal loop responses in studies that were published in 1968 and 1969 (*2,4*). This strain was negative for enterotoxin when tested recently by RIA (*8*) and produced no ileal loop activity in this study. However, this explanation cannot account for the absence of enterotoxigenicity of all of these strains, the majority of which had been stored as lyophilized spore stocks between their initial isolation and the present studies.

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