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An Enterotoxin-Negative Strain of *Yersinia enterocolitica* Serotype O:3 Is Capable of Producing Diarrhea in Mice

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A strain of *Yersinia enterocolitica* serotype O:3 that consistently produced heat-stable enterotoxin at 22 but not at 37°C and another strain of the same serotype which did not produce enterotoxin at 22°C were both positive for autoagglutination at 35°C, a test that has been related to virulence in yersiniae. Both strains were infective for HeLa cells and produced guinea pig conjunctivitis. Mice infected with either strain through their drinking water developed diarrhea and excreted the organism in high numbers in the feces. A control strain of serotype O:3 positive for enterotoxin and HeLa cell infectivity but negative for autoagglutination was avirulent. Extracts of feces and intestines from mice with diarrhea were negative for enterotoxin. The results indicate that the heat-stable enterotoxin produced in vitro by some strains of *Y. enterocolitica* and measured by the infant mouse assay plays no role in pathogenesis as described by the mouse diarrhea model.

Pai and Mors (9) first reported that *Yersinia enterocolitica* is capable of producing a heat-stable enterotoxin (ST) that gives a positive test with the infant mouse assay, which has been used for detecting the ST produced by *Escherichia coli* (2). Pai and Mors (9) observed that this capability was characteristic of all human isolates of *Y. enterocolitica* serotype O:3, and Pai et al. (11) found that it was present in the majority of human isolates of other serotypes as well. Rao et al. (12) reported that the ST of *Y. enterocolitica* was similar to that produced by *E. coli* in that it stimulated the production of guanylate cyclase. Boyce et al. (1), however, suggested that the ST *Y. enterocolitica* might be larger in molecular size than the ST produced by *E. coli* because of differences in ultrafiltration properties. Whereas the ST produced by *E. coli* has been accepted as important to the pathogenicity of that organism, there has remained some doubt about the role of *Y. enterocolitica* ST, particularly because of the inability of the organism to produce ST in vitro at temperatures above 30°C (9). There also has been no demonstration as yet that *Y. enterocolitica* ST is produced in vivo (10, 15).

Laird and Cavanaugh (6) recently described a mouse diarrhea model for demonstrating virulence in *Y. enterocolitica*. The production of diarrhea in mice was limited to strains which had the ability to autoagglutinate at 35°C in a tissue culture medium. We have found that this phenomenon is demonstrated by a number of serotypes of *Y. enterocolitica*, including O:3, which is the most common serotype isolated from humans in Canada and many other countries (17).

This report describes the production of diarrhea with fecal excretion in mice by a strain of *Y. enterocolitica* serotype O:3 that was autoagglutination positive and invasive but did not produce enterotoxin.

MATERIALS AND METHODS

Test organisms. Three strains of *Y. enterocolitica* serotype O:3 were used for this study. Strains E547 and E664 were obtained from S. Toma, Canadian National Reference Service for *Yersinia*, Toronto, Ontario. These strains had originally been isolated from humans. Both strains produced ST at 22°C that was detectable with the infant mouse assay. Strain E232 was isolated by our laboratory from fresh pork tongue (15). This strain was found repeatedly negative for ST production at 22°C. The three strains were identical biochemically (Wauters biotype 4).

Autoagglutination. Cultures were grown on Trypticase soy-yeast extract (0.6%) agar (TSYE) (pH 7.6) (BBL Microbiology Systems) incubated at 22°C for 48 h. Duplicate plastic tissue culture tubes with 2 ml of Eagle minimal essential medium with Earle salts and 10% fetal bovine serum were inoculated with about 10^6 cells. One tube was incubated at 35°C, and the other was incubated at 22°C. A positive test consisted of agglutinated cells concentrated along the lower wall and bottom, with a nearly clear medium in the upper portion of the tube after overnight incubation at 35°C and a nearly uniform turbid suspension without agglutination at 22°C.

Enterotoxin production. The standard method for enterotoxin production consisted of preparing a culture on TSYE agar as described above and then using it to inoculate 25 ml of TSYE or brain heart

infusion (BHI) broth (pH 7.6) in a 125-ml baffle flask. The flask was incubated at 22°C on a rotary shaker (Psycrotherm model G-26, New Brunswick Scientific) providing 240 rpm for aeration. After 48 h, the cultures were centrifuged (10,000 rpm for 30 min), and the supernatant was passed through a 0.45-μm (pore size) membrane filter. Filtrates were stored at −20°C until tested.

Culture filtrates were concentrated fivefold by ultrafiltration with an Amicon UM10 membrane. Boyce et al. (1) reported that this technique would retain the ST produced by both *Y. enterocolitica* and *E. coli.*

Infant mouse assay. Culture filtrates were prepared for testing by adding 2 drops of 2% Evans blue per ml of broth. This material (0.5 ml) was injected directly into the stomach of a 2- to 4-day-old mouse. The mice were held for 4 h (±15 min) at 22°C before being sacrificed with chloroform. The intestines from three or four mice were combined and weighed, as were the carcasses from the same animals. The result was expressed as the ratio of intestine weight to body weight. A ratio of 0.083 or higher is generally accepted as a positive test for ST (13).

Mouse diarrhea model. The growth from a TSYE agar plate was washed off, and this suspension was used to inoculate 100 ml of 0.1% peptone to give a density of about 10^9 cells per ml. The inoculated peptone solution was provided to two mice who had been deprived of drinking water for 24 h. The peptone solution was replaced after 24 h with fresh drinking water. After 2 days, the mice were placed in a jar on a wire screen held over a piece of filter paper for about 2 h. The feces were examined for evidence of diarrhea by using as criteria the consistency, shape, fluid and mucous content, and color, compared with normal fecal material. The feces were collected in a tared tube containing 5 ml of phosphate-buffered saline, and the density of *Y. enterocolitica* was determined by colony counts on CIN agar (14). Recovered organisms were identified by typical colony appearance on CIN agar and agglutination with specific antiserum.

Extraction of feces and intestines. The feces from two mice with diarrhea were collected and mixed well with a small quantity of phosphate-buffered saline and then held overnight at 4°C. The material was centrifuged, and the supernatant was tested for ST activity by the infant mouse assay. The intestines of the same mice were removed after sacrificing and suspended in a small quantity of phosphate-buffered saline. The material was homogenized in a Stomacher and held overnight at 4°C. The next day it was processed in a manner similar to that of the feces extract.

Guinea pig conjunctivitis. A 10-μl amount of the bacterial suspension containing about 10^9 cells was introduced into the right eyes of two guinea pigs. The animals were examined daily for symptoms of conjunctivitis. A positive result was recorded when there was evidence of swelling of the conjunctivae, eyeball depression, and accumulation of fluid or mucous with recovery of the organism that was introduced at 2 days or thereafter.

HeLa cell penetration. The ability to penetrate HeLa cells was determined by a roller tube technique, which provides a quantitative index of relative infectivity (3). Nonpenetrating strains of *Y. enterocolitica* show an index of about 3.0 and lower, whereas penetrating strains give an index of 3.7 to 5.0 by this procedure (unpublished data).

RESULTS

Strain E547 consistently produced ST at 22°C in both TSYE and BHI broths. However, repeated attempts to produce ST at 37°C by this strain were always negative. Some attempts to induce ST production at 37°C by manipulations in media and inocula included: (i) addition of mitomycin C at late log phase of growth; (ii) addition of bile salts at late log phase of growth; (iii) growth of the organism at 22°C and transfer at various times during incubation to the same medium incubated at 37°C; (iv) variation in the size of the inoculum grown at 22°C and added to medium at 37°C; (v) supplementation of media with bile salts, calcium chloride, or horse serum.

Strain E232 was repeatedly found negative for ST by standard in vitro methods used for toxin production. Tests completed at different times on this culture for autoagglutination and mouse diarrhea were always positive. A final experiment was completed in which all tests were done simultaneously by using a single cell suspension. The results of this experiment are presented in Tables 1 and 2.

Strains E232 and E547 were tested for ST production in three separate flasks of TSYE and BHI broths. The results shown in Table 1 indicate that infant mouse assays were always negative for E232 and positive for E547. A test on the composited broths for E547 after heating at 100°C for 15 min verified that the enterotoxin produced by this organism was heat stable.

The composited broth filtrates were subjected to ultrafiltration, using an Amicon UM10 membrane in a stirred cell with a 5-to-1 concentration of volume. The results in the infant mouse assay were exactly the same with the retentates; that is, E232 was negative, and E547 was positive for ST. The filtrates were also negative, except for the BHI broth from culture E547, which showed a reduced activity from that observed with the unconcentrated material, but, nevertheless, high enough to be considered a positive test for ST.

Extracts of the feces and intestines from mice with symptoms of diarrhea were negative for ST whether originating from animals infected by strain E547, an ST-positive strain, or E232, an ST-negative strain.

Strains E232 and E547 were autoagglutination positive, whereas E674 was autoagglutination negative. All strains were able to penetrate HeLa cells, giving infectivity indexes ranging from 3.92 to 4.14. Strains E232 and E547 produced diar-

rhea in mice after administration through drinking water. Diarrhea was accompanied by fecal excretion of *Y. enterocolitica* in high numbers by the infected mice. Both strains also produced guinea pig conjunctivitis. Symptoms in the guinea pigs were mild and transitory as Schiemann and Devenish have observed previously with other strains of serotype O:3 (16). The control strain of the same serotype (E674), which was ST positive and infective for HeLa cells but negative for autoagglutination, was avirulent in both mice and guinea pigs.

DISCUSSION

The mouse diarrhea model of Laird and Cavanaugh (6) provides an excellent system for evaluating virulence in *Y. enterocolitica*, particularly since it so closely duplicates the infectious process in humans. Not only is the presence of diarrhea easily verifiable, but fecal counts provide further evidence that infection and multiplication have occurred. Examination of both virulent and avirulent strains by this system have found a consistent correlation with autoagglutination (17; unpublished data).

Most reports on the ST produced by *Y. enterocolitica* have at least implied that this toxin is important in pathogenesis. Kapperud (5) commented on the close correlation between HeLa cell invasiveness and ST production. Pai et al. (11) reported a higher incidence of toxigenicity in human isolates than in environmental isolates of *Y. enterocolitica*. On the other hand, other evidence has been presented suggesting that the ST of *Y. enterocolitica* plays no role in pathogenesis. Pai and Mors (9) observed that ST was not generated in vitro at temperatures above 30°C. My studies found that various manipulations in media and inocula could not induce ST formation at 37°C. Pai et al. (10) later reported that the enterotoxin could not be detected in feces from rabbits that were infected with *Y. enterocolitica* and had diarrheal symptoms. I could not detect ST in fecal or intestinal extracts from mice with diarrhea. Although ST production is common in human isolates of *Y. enterocolitica*, it is also not unusual in environmental strains, which do not biochemically or serologically resemble most human strains.

First reports on the frequent occurrence of toxigenicity in strains of *Y. enterocolitica* serotype O:3, which is commonly associated with human illness in many countries, suggested that ST was an important factor in pathogenesis. However, there are now conflicting reports on the incidence of enterotoxigenicity among strains of serotype O:3. Pai et al. (11) and Schiemann (15) found that practically all strains of serotype O:3 are toxigenic, whereas Okamoto et al. (7) found only 6 out of 21 positive, and Olsson et al. (8) reported 20 out of 24 positive for ST.

Strain E232 of serotype O:3 used in this study was repeatedly negative in tests for ST production. A 5-to-1 concentration of culture broth filtrate also showed no ST activity by the infant mouse assay. This strain, which was autoagglutination positive, was equally virulent in mice, compared with strain E547, an autoagglutination-positive and ST-positive organism of the same serotype. Since completing these studies, another strain of a different serotype (O:5,27)

TABLE 1. *ST production by two strains of Y. enterocolitica serotype O:3 cultured in two different media*

Test material	Intestine wt/body wt ratio[a] for following strain:		
	E232	E547	Uninoculated control
TSYE broth[b]			0.070
Culture 1	0.076	0.113	
CUlture 2	0.061	0.105	
Culture 3	0.071	0.117	
Composite[c]	0.059	0.110	
Composite[d]	ND[e]	0.132	
BHI broth[b]			0.070
Culture 1	0.069	0.113	
Culture 2	0.068	0.109	
Culture 3	0.066	0.107	
Composite[c]	0.074	0.095	
Composite[d]	ND	0.139	
Ultrafiltrate retentate[f]			
TSYE broth	0.069	0.119	0.060
TSYE broth diluted 1:5	ND	0.123	
BHI broth	0.073	0.130	0.077
BHI broth diluted 1:5	ND	0.119	
Ultrafiltrate filtrate			
TSYE broth	0.062	0.074	0.066
BHI broth	0.074	0.088	0.065
Extract of mouse feces collected during diarrhea	0.070	0.060	
Extract of mouse intestines removed during diarrhea	0.066	0.064	

[a] A ratio of 0.083 or higher is generally considered positive (13).
[b] Incubated at 22°C for 48 h on a rotary shaker at 240 rpm.
[c] Cultures 1, 2, and 3, unheated.
[d] Cultures 1, 2, and 3, heated at 100°C for 15 min.
[e] ND, Not done.
[f] Five-to-one concentration of volume.

TABLE 2. *Virulence characteristics of three strains of Y. enterocolitica serotype O:3*

Strain	Presence (+) or absence (−) of following characteristic:						Mouse fecal count 2 days after oral infection (no. of cells)	
	ST production	Autoagglutination	HeLa cell infectivity	Guinea pig conjunctivitis	Mouse diarrhea		Animal 1	Animal 2
					Animal 1	Animal 2		
E323	−	+	+	+	+	+	1.4×10^7	2.1×10^7
E547	+	+	+	+	+	+	2.0×10^7	1.2×10^8
E674	+	−	+	−	−	−	$<1 \times 10^3$	$<1 \times 10^3$

has been discovered which demonstrated the same virulence characteristics as E232 but also failed to produce ST in TSYE broth at 22°C (unpublished data). This strain of serotype O: 5,27 and the two strains of serotype O:3 used in this study were capable of penetrating HeLa cells and producing guinea pig conjunctivitis. Gemski et al. (4) described a similar observation with *Shigella dysenteriae*, where a toxin-positive strain and a toxin-negative mutant were equally invasive and pathogenic in animal models.

Strain E674, used as a negative control for animal tests, was positive for ST but negative for autoagglutination and avirulent in mice and guinea pigs. This is consistent with the report of Laird and Cavanaugh (6) on the relationship of autoagglutination to virulence in yersiniae. Strain E674 was equally infective for HeLa cells, compared with virulent strains. I have found with isogenic pairs from the same strain of *Y. enterocolitica* that the loss of autoagglutination ability and virulence does not alter the organism's ability to infect HeLa cells (unpublished data).

The findings of this study indicate that the ST produced in vitro by some strains of *Y. enterocolitica* and measured by the infant mouse assay is not necessary for pathogenesis as described by the mouse diarrhea model.

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