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Virulence of Three Clinical Isolates of *Vibrio cholerae* Non-O-1 Serogroup in Experimental Enteric Infections in Rabbits

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Three *Vibrio cholerae*, non-O-1 isolates, pathogenic in both adult rabbit ligated ileal loop and infant rabbit assays, produced no heat-labile or heat-stable toxins. Their pathogenicity did not correlate with the presence of plasmids.

Cholera has not been epidemic in the United States since 1911 (15); however, in 1978, eleven people in Louisiana became infected with *Vibrio cholerae*, O-1 (causative agent of Asiatic cholera), after eating insufficiently cooked and/or recontaminated cooked crabs (3). Although *V. cholerae* O-1 infections had occurred sporadically in the United States between 1911 and 1978, these infections were either in isolated individuals or were laboratory acquired (15, 17, 23). The incidence of diarrhea caused by *V. cholerae*, serogroup non-O-1 (often referred to as NAG, nonagglutinable, or NCV, noncholera vibrios), in the United States has increased since 1972 (12). The most recent outbreak occurred in November 1979 (5) and was associated with the consumption of raw oysters harvested from Florida waters. Bacterial cultures obtained from three of the affected individuals were designated strains 2193c, 2194c, and 2227c; these isolates are the subject of this report.

The three strains were characterized biochemically, using the following dehydrated media: Kligler iron agar (Difco), lysine iron agar (Difco), Simmon's citrate agar (Difco), motility-indole-ornithine medium (Difco), 1% tryptone medium (Difco), and nutrient gelatin medium (BBL Microbiology Systems). Andrade carbohydrate broth was made by the formula provided in the *Manual of Clinical Microbiology* (22), using 0.2 g instead of 0.5 g of acid fuchsin. Carbohydrates were filter sterilized and added aseptically to the basal medium. The biochemical profiles of the three strains are shown in Table 1. The strains were serotyped according to the method and scheme of Smith (18). Two strains, 2193c and 2194c, were untypable with Smith's serotyping scheme (18); strain 2194c was Smith serotype 17.

The tests for heat-labile toxin, heat-stable toxin, and cholera toxin consisted of the Y1 mouse adrenal cell assay for heat-labile toxin (8, 16), the solid-phase radioimmunoassay for cholera toxin (11), and the suckling mouse assay for heat-stable toxin (6, 10). Bacterial cultures were grown with shaking for 18 to 24 h at 35°C in T_1N_1 broth (18) before animal or toxin testing. Known toxigenic cultures of *V. cholerae* O-1 (strains 569B and MP15434) and of *V. cholerae* non-O-1 (strains 61892, G12R, and 62058) as well as uninoculated T_1N_1 broth were included as controls for animal and toxin testing. Known toxigenic strains of *V. cholerae* O-1 and non-O-1 were obtained from William Spira, Johns Hopkins University School of Medicine, Baltimore, Md. None of the isolates gave positive results in these tests. Adult rabbit ileal loops and infant (8 to 12 days old) rabbit intraintestinal inocula-

TABLE 1. *Biochemical profiles of three isolates of V. cholerae non-O-1*[a]

Test	Reaction[b]
Kligler iron agar	K/A,[c] H_2S-
Lysine iron agar	K/K, H_2S-
Simmon's citrate	+
Motility-indole-ornithine	M+, I+, O-
1% Tryptone broth (indole)	+
Nutrient gelatin (23°C)	+
Esculin hydrolysis	-
Fermentation of:	
Salicin	-
Inositol	-
Mannitol	+
Arabinose	-
Mannose	-
Melibiose	-
Lactose	-
Dextrose	+
Sucrose	+

[a] Strains 2193c, 2194c, and 2227c yielded identical biochemical profiles.

[b] Positive reaction = +; negative reaction = -.

[c] Slant/butt; K = alkaline, A = acid.

tions were performed to ascertain the biological activity of the isolates and their culture filtrates (4, 8, 9, 21). Infant rabbits were inoculated intraintestinally with 0.1 ml of a suspension containing 10^5 colony-forming units. The results of the rabbit ligated ileal loop assays are presented in Table 2. In the infant rabbit pathogenicity assays, all 15 animals inoculated (5 per strain) showed a positive reaction. Fluid accumulated in the intestines, and the animals either died or became sick with diarrhea after intraintestinal inoculation with 10^5 bacterial cells.

Scanning electron microscopy was performed on ileum sections taken from the infant rabbits.

TABLE 2. *Rabbit ligated ileal loop results with three isolates of Vibrio cholerae non-O-1*

Isolate	Culture filtrate[a]	Live culture[b]
2193c	0/3[c]	5/9[c]
2194c	0/3	8/9
2227c	0/3	2/9

[a] A 1-ml amount of culture filtrate was inoculated into each ligated ileal loop.

[b] Bacterial cells (10^8) were injected into each ligated ileal loop.

[c] Number of positive loops per number of loops inoculated.

Sections were removed 2 cm above the cecum and were fixed immediately with 2.5% glutaraldehyde in 0.04 M phsophate-buffered saline. Intestinal sections were cut longitudinally, pinned to styrofoam blocks, and fixed overnight at 4°C. The specimens were dehydrated in a graduated ethanol series and dried at the critical point in carbon dioxide. They were then mounted on scanning electron microscope pegs, sputter-coated with gold, and viewed at an accelerating voltage of 10 kV in an ISI Super IIIA scanning electron microscope (Fig. 1 and 2).

Previous studies (13) of the pathogenicity of *V. cholerae* non-O-1 clinical isolates for infant rabbits did not report electron microscopic examinations of the intestinal lumen and included no information as to whether the isolates studied produced cholera toxin. Other studies (14) have reported that infection with cholera toxin-producing *V. cholerae* gave the intestinal villi of the infant rabbit a rough appearance. Our research showed that infection with the non-O-1 strains of *V. cholerae* studied in this investigation caused total destruction of the infant rabbit intestinal villi, followed by infiltration with polymorphonucleocytes (Fig. 2).

The methods of Birnboim and Doly (2) and Portnoy, White, and Falkow (D. A. Portnoy, F.

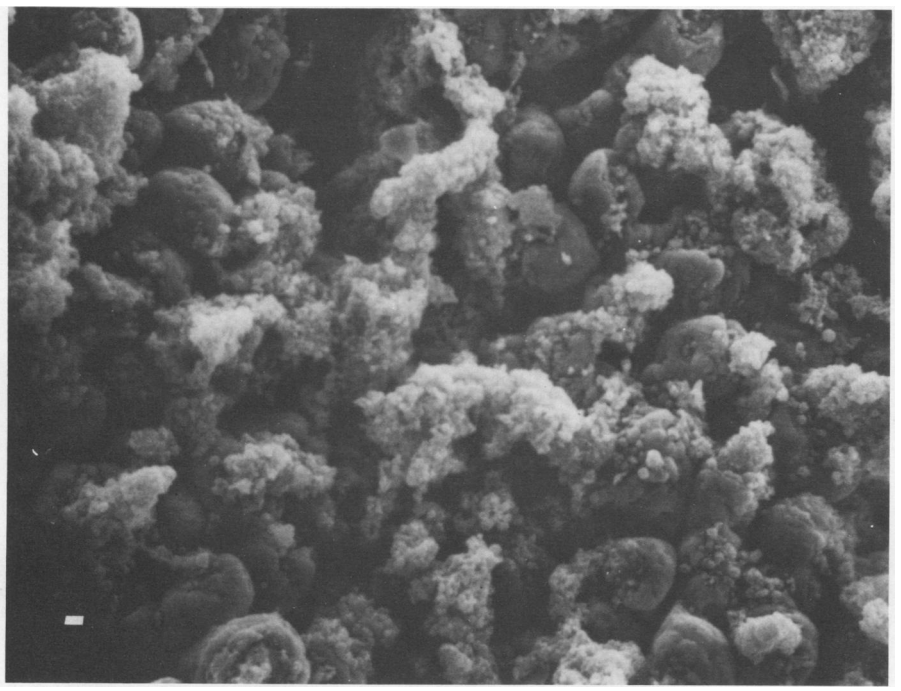

FIG. 1. *Scanning electron micrograph of infant rabbit intestine (ileum) 18 h after infection with 10^5 colony-forming units of V. cholerae non-O-1 strain 2227c. Bar represents 1 μm.*

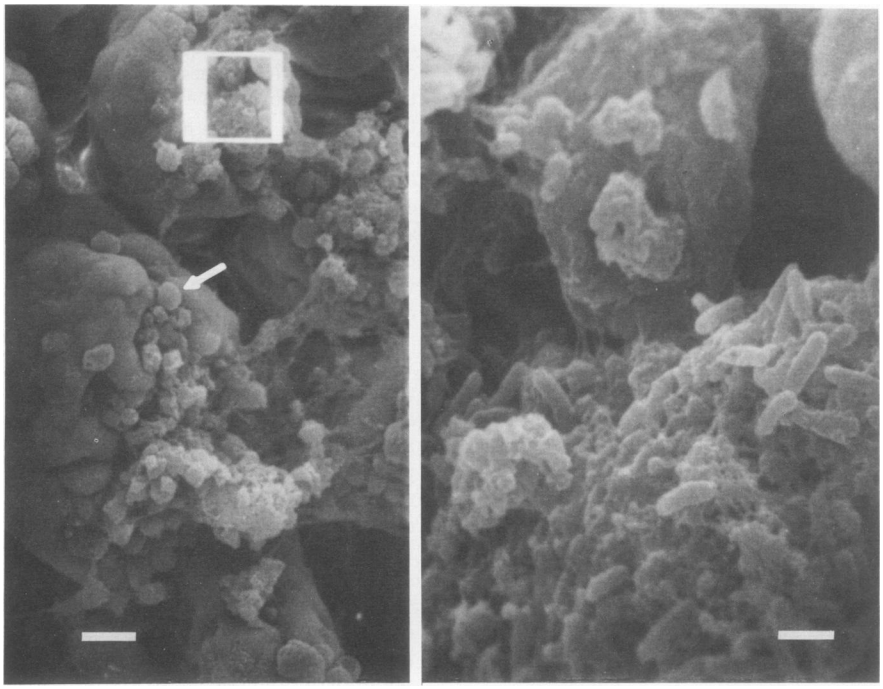

FIG. 2. *Scanning electron micrograph of infant rabbit intestine (ileum) 18 h after infection with 10^5 colony-forming units of V. cholerae non-O-1 strain 2227c. Note infiltration of polymorphonucleocytes (arrow). Bar represents 1 μm on left and 10 μm on right.*

F. White, and S. Falkow, submitted for publication) were used to screen the *V. cholerae* isolates for the presence of plasmids; however, no plasmids were observed when these methods were used. When lysates were prepared by the method of So et al. (19), a small extrachromosomal element (3 to 5 megadaltons) was observed in strain 2193c.

V. cholerae non-O-1 strains can produce a toxin that is similar to but not identical with cholera toxin (1, 24, 25). Some *V. cholerae* non-O-1 strains also produce heat-stable toxin, and some human isolates do not produce either a cholera toxin-like or heat-stable toxin (20). Whole-cell cultures of these isolates are positive in either the infant rabbit or the rabbit ileal loop assay (20). Except for the illnesses reported in Bangladesh (20), *V. cholerae* non-O-1 isolates from human diarrhea that do not produce cholera toxin-like or heat-stable toxin have not been reported to cause human illness.

The isolates discussed here appear to possess a virulence mechanism similar to that recently described by Spira and Daniel (20), i.e., pathogenicity in the infant rabbit or adult rabbit ileal loop assay or both by whole-cell cultures without production of either a heat-labile or a heat-stable toxin by conventional assays.

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