

Production of Enterotoxin by *Vibrio vulnificus* Isolates

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ABSTRACT

Weakly virulent isolates of *Vibrio vulnificus* that were lethal only to simultaneously iron-overloaded and immunosuppressed mice were tested for ability to cause fluid accumulation in the permanently ligated rabbit ileal loop. Unlike the highly virulent isolates, which caused septicemia and death in rabbits, these isolates caused significant fluid accumulation in the rabbit loops. Fluid accumulation was also observed when culture filtrates were tested, indicating the existence of an enterotoxin. Enterotoxin activity did not correlate with the hemolysin or protease activities. Only one of three enterotoxigenic isolates caused diarrhea when administered to temporarily ligated rabbit ileal loops, suggesting involvement of some other pathogenic determinant(s) such as colonization.

Vibrio vulnificus is a halophilic marine bacterium that has been identified as a cause of primary septicemia in individuals with chronic underlying disorders (2,13). Conditions that enhance susceptibility to *V. vulnificus* septicemia include elevated serum iron levels (3,9) and impaired immune function (5). In most instances, the organisms appear to have entered the bloodstream via the gastrointestinal tract, with raw oysters serving as the vehicle (13).

Diarrhea has also been associated with *V. vulnificus* infections (2,6,13). However, Blake et al. (2) reported diarrhea to be an uncommon symptom in patients with *V. vulnificus* septicemia, and Tacket et al. (13) reported that only 40% of patients with septicemia also had diarrhea. These observations suggest either that the diarrhea was caused by another organism present in the food vehicle or that *V. vulnificus* diarrhea is a separate illness with distinct pathogenic determinants. Support for the separate illness hypothesis was provided by Johnston et al. (6), who observed *V. vulnificus*-associated diarrhea in compromised patients that were not septicemic.

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In this study, we report identifying putatively enteropathogenic strains of *V. vulnificus* by their ability to cause fluid accumulation in the permanently ligated rabbit ileal loop (RIL). We also report evidence that (a) the fluid accumulation was due to a soluble enterotoxin that is distinct from both the hemolysin (4,14) and the protease, and that (b) enterotoxin is not the sole determinant of enteropathogenicity.

MATERIALS AND METHODS

Bacterial strains and growth

The *V. vulnificus* strains, their origins, and the individuals who supplied them are listed in Table 1. Cultures were grown in 250-ml flasks containing 50 ml of brain heart infusion broth (BBL, Cockeysville, MD) supplemented with 3% NaCl (BHIB3) or heart infusion broth (Difco Laboratories, Detroit, MI) with no added NaCl (HIB). Starter cultures were inoculated from stocks stored in 40% glycerol at -70°C were incubated at 35°C and 250 rpm. When these cultures reached stationary phase (7-9 h), 0.1 ml of each culture was inoculated into 50 ml of fresh medium, and the flasks were incubated as above. Culture filtrates were prepared by centrifugation of test cultures at 17,000 x g for 15 min at 4°C in a Beckman J21C centrifuge followed by passage through 0.45 µm filters (Gelman acrodisc, Ann Arbor, MI). Before filtration of the supernatant liquids, 5.0 ml of 0.25% bovine serum albumin was passed through the filters to block the protein adsorption sites on the membranes.

Hemolysin and protease assays

Hemolytic activity was measured by a modification of the procedure used by Tison and Kelly (14). Serial two-fold dilutions of culture filtrates were made in 0.85% saline solution plus 0.05 M CaCl₂, and 0.5 ml of a 1% suspension of washed sheep erythrocytes was added to 0.5 ml of each filtrate. After 1 h of incubation at 37°C the suspensions were centrifuged and absorbance at 545 nm was determined. Hemolysin titers were determined as the reciprocal of the last dilution at which there was greater than 50% lysis. Standard curves were prepared from erythrocytes lysed with saponin.

Protease activity was measured against 0.5% azoalbumin as described by Millet (8). One unit was defined as the amount of protease that caused an increase in absorbance at 440 nm of 0.1 per 1.0 ml per 30 min of incubation at 37°C.

TABLE 1. Activity of live cultures of *V. vulnificus* in the permanently ligated rabbit ileal loop.

Strain designation	Source	Donor	Virulence ^a	Dose ^b	Fluid accumulation ratios ^c		
					Rabbit #1	Rabbit #2	Rabbit #3
MO6-24	Clinical	C. Kaysner, FDA, Seattle, WA	V	7.8x10 ⁹	Dead	Dead	Dead
UNCC 913	Environmental	J. D. Oliver, Univ. of North Carolina, Charlotte, NC	V	1.1x10 ⁹	Dead	Dead	ND ^d
241	Environmental	M. T. Kelly, Univ. of British Columbia, Vancouver, BC	C	7.8x10 ⁸	1.34(+)	1.45(+)	1.05(+)
A9	Environmental	M. T. Kelly	C	1.5x10 ⁹	0.63(+)	0.52(+)	--
141	Environmental	M. T. Kelly	C	1x10 ⁹	1.26(+)	1.47(+)	0.13(-)
E4125-A	Clinical	D. Hollis, CDC, Atlanta, GA	A	1.3x10 ⁹	0.05(-)	0.05(-)	0.16(-)
AO21 ^e	Normal stool	J. J. Farmer III, CDC Atlanta, GA	A	6.0x10 ⁹	0.06(-)	0.08(-)	--

^aV, virulent to iron-overloaded mice; C, avirulent to iron-overloaded mice but virulent to simultaneously iron-overloaded and immunosuppressed mice; A, avirulent under all conditions (A. L. Reyes, J. T. Peeler, C. H. Johnson, P. L. Spaulding and G. N. Stelma, Jr. Abstract, 86th Annual Meeting of the American Society for Microbiology, P2, p. 275).

^bCFU/loop.

^cFluid accumulated in g/cm intestinal loop; (+) indicates a positive result and (-) indicates a negative result.

^dNot determined. Fluid accumulation was observed in all loops, including the negative control loop.

^eNonenterotoxigenic strain of *Aeromonas caviae*.

TABLE 2. Activity of filtrates from 5 h cultures of *V. vulnificus* in the permanently ligated rabbit ileal loop.

Strain designation	Virulence ^a	Growth medium	Hu/ml ^b	Fluid accumulation ratios ^c			
				Rabbit #1	Rabbit #2	Rabbit #3	Rabbit #4
241	C	HIB ^d	2048	0.55(+)	0.18(-)	0.79(+)	0.08(-)
241		BHIB3 ^e	2	0.06(-)	0.73(+)	0.10(-)	0.83(+)
A9	C	HIB	1024	0.95(+)	1.24(+)	0.36(+)	--
A9		BHIB3	2	0.28(+)	1.48(+)	0.08(-)	--
MO6-24	V	HIB	1024	0.95(+)	1.24(+)	0.36(+)	--

^aC, conditionally virulent; V, virulent.

^bHemolytic units/ml expressed as reciprocals of 50% endpoint titers against washed sheep erythrocytes.

^cFluid accumulated in g/cm intestinal loop; (+) indicates a positive result and (-) indicates a negative result.

^dHeart infusion broth.

^eBrain heart infusion broth plus 3% NaCl.

Rabbit ileal loop assay

RIL assays were performed by a modification of the procedure of Twedt and Brown (15,16). Each rabbit contained a positive control loop inoculated with 0.5 µg purified cholera toxin (List Biological Laboratories, Campbell, CA) in 1.0 ml phosphate-buffered saline solution (pH 7.3), a negative control loop inoculated with sterile medium, and no more than two test loops inoculated with culture filtrates or live cultures (10⁹/ml). All inoculations were in 1.0-ml volumes and were done in random order. Individual loops were separated by two inter-loops. A test loop was considered positive if the volume-length ratio was four-fold greater than that of the negative control in the same animal. A strain was considered positive for enterotoxin if loops tested positive at least two of three times in separate animals. These criteria are the minimum required for a significant difference (α=0.05) on the basis of earlier studies (15-17). Data from rabbits in which either control gave an inappropriate response were discarded.

The "removable intestinal tie-adult rabbit diarrhea" (RI-TARD) assay was performed as described by Spira et al. (11), except that rompin (0.05 ml/kg) was used as the tranquilizer and kataset (0.35 ml/kg) was the anesthetic. *V. vulnificus* was recovered from diarrheal stools by streaking stool samples on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. The identities of the stool isolates were verified by the API-20E system (Analytab).

RESULTS

Potentially enteropathogenic strains of *V. vulnificus* were identified by testing live cultures for ability to cause fluid accumulation in the permanently ligated RIL. The results (Table 1) showed that the enteropathogenic potential of the virulent strains could not be determined because of their lethality to rabbits. The conditionally virulent strains (A. L. Reyes, J. T. Peeler, C. H. Johnson, P. L. Spaulding, and G. N. Stelma, Jr., Abstract, 86th Annual Meeting of the American Society for Microbiology, P2, p. 275), which were not lethal to the rabbits, were more useful for this study. The three conditionally virulent strains that were tested caused significant (α=0.05) fluid accumulation and were considered to be potentially enteropathogenic. The one avirulent strain tested did not cause fluid accumulation, although a high dose (1.3 x 10⁹ CFU/ml) was used. The data from the nonenterotoxigenic *A. caviae* strain were included to show that the positive reactions observed with the *V. vulnificus* cultures were not due to excessive doses at which any organism might elicit a response.

Filtrates from strains that had caused fluid accumulation in the RIL were tested for the presence of an enterotoxin. The

TABLE 3. Time course of enterotoxin hemolysin and protease production by heart infusion broth cultures of *V. vulnificus* strains A9 and 241.

Strain designation	Culture ^a age	Fluid accumulation ratios ^b			Hu/ml ^c	Protease ^d (units/ml)
		Rabbit #1	Rabbit #2	Rabbit #3		
A9	3 h	0.05(-)	0.03(-)	0.07(-)	8	--
241	3 h	0.07(-)	0.06(-)	0.05(-)	32	--
A9	5 h	0.95(+)	1.24(+)	0.36(+)	1024	1.7
241	5 h	0.55(+)	0.18(-)	0.79(+)	2048	1.4
A9	10 h	0.82(+)	1.10(+)	--	16	10.8
241	10 h	0.04(-)	0.05(-)	--	64	5.3
A9	14 h	0.15(-)	0.07(-)	0.05(-)	--	14.7
241	14 h	0.06(-)	0.07(-)	0.05(-)	--	16.0

^aThe end of exponential growth occurred at approximately 5 h.

^bFluid accumulated in g/cm intestinal loop; (+) indicates a positive result and (-) indicates a negative result.

^cHemolytic units/ml expressed as the reciprocals of 50% endpoint titers against washed sheep erythrocytes.

^dOne unit of protease activity was defined as the amount of protease that caused an increase in A_{440} of 0.1/ml/30 min.

TABLE 4. Biological activity of *V. vulnificus* isolates in the RITARD^a.

Strain designation	Challenge dose	No. of animals challenged	No. responding with	
			Watery diarrhea	Uniform stool
A9	2.3×10^5	4	0	0
A9	3.2×10^7	3	0	1
A9	$0.7-1.3 \times 10^8$	5	3	2
241	3.2×10^8	3	1	1
141	1.7×10^8	3	0	0

^aRemovable intestinal tie-adult rabbit diarrhea.

initial assays were performed by using filtrates of cultures harvested at the end of exponential growth (5 h). Cultures were grown both in a medium that supported the production of high titers of hemolysin (HIB) and in a medium that was inhibitory to hemolysin production (BHIB3) (14). Filtrates from strain 241 were marginally positive (Table 2), with significant accumulation in only two of four loops and fluid accumulation ratios <1.0. This was true whether the hemolysin titers were high (2048 hu/ml) or low (2 hu/ml). Filtrates from strain A9 were positive in three of three rabbits when the hemolysin titer was 2048 hu/ml and in two of three rabbits when the hemolysin titer was < 2 hu/ml (Table 2). Filtrates from M06-24, a highly virulent strain, were tested to determine whether the enterotoxin could also be synthesized by virulent strains. These filtrates were positive in three of three rabbits, indicating that M06-24 is an enterotoxin producer (Table 2).

The time course of enterotoxin production was estimated by assaying the RIL activities of filtrates from cultures of various ages. Hemolysin and protease activities were also measured. The results (Table 3) showed that biologically detectable enterotoxin was produced in the early postexponential stage of growth. Enterotoxin was detected in the 5-h filtrates of strain 241 and in both 5-h and 10-h filtrates of strain A9. Neither strain produced biologically detectable enterotoxin during exponential growth (3 h) or in cultures >14 h in age. Enterotoxin activity did not correlate with hemolysin activity or protease activity. The hemolysin titers of the nonenterotoxic 3-h cultures were higher (8 hu and 32 hu) (Table 3) than those of the enterotoxic 5-h BHIB3 cultures

(<2 hu) (Table 2). The filtrate from the 10-h culture of strain A9, which had a hemolysin titer of only 16 hu/ml, was active in the RIL; the filtrate from the same age culture of strain 241 was inactive in the RIL, although it had a higher hemolysin titer (64 hu/ml). The filtrates from the 14-h cultures of both strains had the highest titers of protease, but both were inactive in the RIL. Attempts to concentrate the enterotoxin in the filtrates by ultrafiltration or lyophilization have resulted in loss of biological activity.

The results of the RITARD experiments are summarized in Table 4. At a dose of ca. 10^5 CFU per rabbit, strain A9 did not cause diarrhea. At a dose of ca. 10^7 CFU per rabbit, strain A9 caused a mild diarrhea in one of three rabbits. This diarrhea consisted of unformed stools and lasted for ca. 36 h. At a dose of ca. 10^8 CFU per rabbit, strain A9 caused diarrhea in five of five rabbits. In two of the rabbits the diarrhea consisted of unformed stool and only lasted 1-2 d. The other three rabbits had a more severe watery diarrhea lasting 3-4 d. *V. vulnificus* was isolated from both types of diarrheal stool, including the watery stool of a rabbit that did not develop diarrhea until the third day after surgery. Strain 241 was weakly diarrheagenic at a dose of ca. 10^8 CFU per rabbit. Although two of three rabbits inoculated with ca. 10^8 CFU developed diarrhea, the duration of the illness of each rabbit was only 1 d. Strain 141 did not cause diarrhea in any of three rabbits at a dose of 10^8 .

DISCUSSION

When highly virulent strains of *V. vulnificus* were inoculated into permanently ligated rabbit ileal loops, nearly all of the animals died during the course of the experiment (Table 1). This lethality of *V. vulnificus* toward rabbits, also observed by Poole and Oliver (9), was probably due to the high level of iron normally present in the serum of rabbits (7). In subsequent experiments, we used only conditionally virulent strains that were not lethal when iron-overload was the only risk factor (A. L. Reyes, J. T. Peeler, C. H. Johnson, P. L. Spaulding, and G. N. Stelma, Jr., Abstract, 86th Annual Meeting of the American Society for Microbiology, P2, p.275). All three conditionally virulent isolates tested caused

fluid accumulation in the RIL, suggesting that they might be capable of causing human diarrhea (Table 1).

Ileal loop assays performed on filtrates of cultures grown in HIB (Table 2) showed that a soluble enterotoxin was produced by *V. vulnificus* cultures at the end of exponential growth when hemolysin titers were high. Because the β -hemolysin (cytotoxin) produced by *A. hydrophila* has enterotoxic activity (1,12), it seemed possible that the *V. vulnificus* hemolysin could also possess enterotoxic activity. The enterotoxic activities of *V. vulnificus* isolates were again assayed by using filtrates from cultures grown in BHIB3, a condition that inhibits hemolysin production (14). The results showed that enterotoxic activity did not correlate with hemolytic activity (Table 2). Experiments performed with cultures of various ages substantiated the lack of correlation between enterotoxic activities and hemolysin titers (Table 3). These experiments also showed that fluid accumulation was not caused by protease. Fluid accumulation was observed in 5-h culture filtrates that had low protease activities but not in 14-h culture filtrates that had very high protease activities (Table 3). The absence of fluid accumulation in the filtrates that were high in protease activity is probably due to degradation of the enterotoxin by the protease.

The observation that three of three conditionally virulent isolates were positive in the RIL assay (Table 1) suggests that some *V. vulnificus* strains cause diarrhea in the absence of septicemia. It is probable that the three cases described by Johnston et al. (6) were caused by that type of strain. All three compromised patients suffered from gastroenteritis but none developed septicemia. Although the cases of diarrhea without concurrent septicemia described by Johnston et al. (6) occurred in compromised individuals, there is no reason to assume that *V. vulnificus* diarrhea could not occur in normal individuals.

The ability of filtrate from a highly virulent strain (M06-24) to cause fluid accumulation in the RIL (Table 2) showed that enterotoxin production was not restricted to the conditionally virulent strains. This observation was also consistent with epidemiological data. Tacket et al. (13) reported that 40% of patients with septicemia also suffered from diarrhea.

Although the single avirulent strain tested did not cause fluid accumulation (Table 1), it is conceivable that some avirulent strains (incapable of causing septicemia) could nevertheless produce enterotoxin. No conclusions can be made until more strains have been tested.

The results of the RITARD assays suggest that *V. vulnificus* is a less potent causative agent of diarrhea than *V. cholerae*. *V. cholerae* caused a fatal diarrhea in seven of eight rabbits at a dose of 4×10^5 CFU per rabbit (11), whereas *V. vulnificus* A9 (the most potent of our strains) caused diarrhea in none of four rabbits at doses of $2-3 \times 10^5$ CFU per rabbit (Table 4). The volumes of diarrheal stool produced by rabbits infected with high doses (ca. 10^8 CFU) of *V. vulnificus* were also less copious than the volumes reported for rabbits infected with *V. cholerae* (11).

The RITARD results (Table 4) also suggest that enterotoxin production and fluid accumulation in the permanently ligated RIL do not necessarily correlate with causation of

diarrhea by *V. vulnificus*. This is not surprising; the permanently ligated RIL measures fluid accumulation in a closed system in which bacteria that cannot colonize or invade the intestine may grow and produce an enterotoxin. The RITARD, on the other hand, is an open system in which diarrhea is the endpoint. In the RITARD enterotoxin producers that do not colonize or invade the intestinal mucosa are washed through the intestine without producing an effect (11). Only one (strain A9) of three enterotoxigenic isolates was strongly diarrheagenic at ca. 10^8 CFU per rabbit. The other two enterotoxigenic isolates were either weakly diarrheagenic (strain 241) or not diarrheagenic (strain 141) at that dose (Table 4). In an earlier study (10), strain A9 was observed to be an avid binder to human buccal cells, whereas strains 241 and 141 were weak binders. These observations suggest that buccal cell binding may be a measure of adherence to and colonization of the intestine. No firm conclusions can be drawn from data derived from only three isolates. Isolation and characterization of additional enterotoxigenic isolates of the conditionally virulent class will be necessary to substantiate the correlation between buccal cell binding and diarrhea.

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