APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1986, p. 1004–1006
0099-2240/86/051004-03$02.00/0

Vol. 51, No. 5

Virulence of *Vibrio vulnificus* Strains from Marine Environments

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Received 2 December 1985/Accepted 26 February 1986

***Vibrio vulnificus* strains isolated from geographically diverse marine sources were compared with clinical isolates for phenotype and in vitro and in vivo production of virulence factors. There were no differences between environmental and clinical strains on the basis of biochemical characteristics or antimicrobial susceptibility patterns. Cytolysin and cytotoxin titers produced by environmental strains were generally comparable to those of clinical strains. Of 29 environmental isolates tested, 25 were pathogenic for mice. These data show that environmental *V. vulnificus* strains are phenotypically indistinguishable from clinical isolates and that approximately 90% of the environmental strains tested produced in vitro virulence factors and in vivo pathogenicity for mice comparable to those produced by clinical *V. vulnificus* isolates.**

The marine bacterium *Vibrio vulnificus* was first described by Reichelt et al. (14) as *Beneckea vulnifica*. Subsequent taxonomic revisions have placed this organism in the genus *Vibrio* as *V. vulnificus* (2, 8). Early case reports of clinical infections referred to this organism as the lactose-positive, LAC+, or L+ vibrio (9, 11). Two major clinical presentations are associated with *V. vulnificus* infections: wound infections, which frequently require extensive debridement, and primary septicemia, which has a mortality rate of near 50%. Wound infections are generally acquired from exposure to seawater or marine animals, while primary *V. vulnificus* septicemias are most commonly associated with the consumption of raw oysters (3).

Several factors have been described which may be associated with the virulence of *V. vulnificus* strains. These include the resistance of *V. vulnificus* strains to the bactericidal activity of human serum (6), the role of increased iron availability (22), and the production of cytolytic and cytotoxic extracellular factors (12). The role of these virulence factors in the in vivo pathogenesis of *V. vulnificus* infections largely remains to be determined.

Vibrio vulnificus has been isolated from numerous marine environments (10, 13, 16); the highest densities are generally found in areas of high temperature (>20°C) and low to moderate salinity (5 to 20 ‰).

The majority of environmental isolates of *V. cholerae* and *V. parahaemolyticus* differ from strains of these species isolated from individuals with clinical infections. Environmental isolates of *V. cholerae* are predominantly nontoxigenic, non-O1 serovars, whereas the majority of clinical strains are toxigenic, O1 *V. cholerae* (4). Similarly, most *V. parahaemolyticus* strains isolated from marine sources fail to produce the Kanagawa hemolysin, while the majority of strains isolated from cases of gasteroenteritis produce the Kanagawa hemolysin (15). It was our goal to determine whether differences between environmental and clinical strains of *V. vulnificus* could be identified. Phenotypic characteristics, the ability to produce certain virulence factors, and animal pathogenicity were examined.

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MATERIALS AND METHODS

V. vulnificus strains from geographically diverse marine and clinical sources were characterized for potential biotyping by the API 20E system (Analytab Products, Plainview, N.Y.) and standard biochemical tests as described previously (18, 19). Antimicrobial susceptibility testing was done by the standardized disk diffusion method (1). The cytolytic activity of heart infusion broth (Difco Laboratories, Detroit, Mich.) cultures against sheep erythrocytes was assayed as described previously (17). Cytotoxic activity was determined by using McCoy cells and a modification of the procedure for the detection of *Clostridium difficile* cytotoxin (7). McCoy cell monolayers in a 96-well microtiter tray were inoculated with 1:10, 1:20, 1:100, and 1:200 dilutions of heart infusion broth culture supernatants. Cytotoxicity was determined by microscopic examination of the cell monolayer after 18 to 24 h of incubation at 35°C. The highest dilution producing cytopathic effects, identified as rounding of the McCoy cells, was designated the cytotoxin titer. Animal pathogenicity was determined by using Institute of Cancer Research (ICR) strain male mice, weighing 30 g each. *V. vulnificus* strains were grown for 4 h at room temperature in tryptic soy broth without glucose (Difco). These conditions were chosen since previous studies showed that they are the conditions under which cytolysin production is minimal (17). Cell densities were adjusted by diluting to a turbidity comparable to a McFarland 0.5 nephelometer standard. Three mice were injected intraperitoneally with about 10^8 cells (0.5 ml) for each *V. vulnificus* strain tested. This inoculum concentration was also used in previous virulence studies in mice (5). Our preliminary studies showed that this inoculum concentration produced no nonspecific toxicity and that there was no difference between washed and unwashed cells when test strains were grown under the conditions described above. Mice were examined about 1 h after inoculation to ensure that no deaths resulted from inoculum toxicity or trauma as a result of inoculation. Bacterial cell densities in the inocula were checked by plate counts. Sterile broth controls were included in each set of experiments. Mice were examined after 24 and 48 h, and the number of survivors was recorded.

RESULTS AND DISCUSSION

Biochemical characteristics. The phenotypic characteristics of 31 environmental *V. vulnificus* isolates are shown in

TABLE 1. Phenotypic characteristics of environmental strains of *V. vulnificus*

Characteristic	% Positive (N = 31)[a]	% Positive (predicted from literature)[b]
Oxidase	100	100
Gelatinase	100	97
Nitrate	100	100
Voges-Proskauer	0	0
Indole	94	100
ONPG[c]	100	100
Arginine dihydrolase	0	0
Lysine decarboxylase	100	100
Ornithine decarboxylase	81	97
Gas from glucose	0	0
Acid from		
Glucose	100	100
Sucrose	0	0
Arabinose	0	0
Mannitol	61	62
Lactose	100	98
Salicin	100	ND[d]
Amygdalin	100	ND
Cellobiose	100	ND
Growth in 1% peptone with		
0% NaCl	0	0
3% NaCl	100	100
6% NaCl	100	95
10% NaCl	0	0

[a] Includes two strains of *V. vulnificus* biogroup 2.
[b] See reference 21.
[c] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[d] ND, Data not presented.

Table 1. Also shown are the predicted results based largely on cumulative data for clinical strains generated by a reference laboratory (21). The results for the environmental strains are nearly identical to the reference data, particularly since two of the strains included in the environmental group are members of biogroup 2 of *V. vulnificus*, which is indole and ornithine decarboxylase negative (19). Similar results, comparing a more limited number of marine and clinical *V. vulnificus* strains, have been reported (20).

Antimicrobial susceptibilities. A summary of antimicrobial susceptibilities for 30 marine *V. vulnificus* isolates is shown in Table 2. These data are comparable to those previously

TABLE 2. Antimicrobial susceptibility of 30 environmental strains of *V. vulnificus*

Antimicrobial agent	Mean zone size (mm) ± SD	Susceptibility[a]
Amikacin	17.4 ± 1.1	S
Ampicillin	22.0 ± 1.2	S
Carbenicillin	25.4 ± 1.1	S
Cephalothin	20.5 ± 1.1	S
Chloramphenicol	31.3 ± 1.1	S
Colistin	7.4 ± 0.9	R
Erythromycin	20.2 ± 1.7	S
Gentamicin	18.6 ± 1.5	S
Kanamycin	17.1 ± 1.3	I–S
Novobiocin	22.3 ± 3.1	S
Penicillin	19.8 ± 1.8	I
Trimethoprim-sulfamethoxazole	28.3 ± 2.2	S
Tetracycline	24.6 ± 2.1	S
0/129 (10 μg)	20.8 ± 1.7	S

[a] S = susceptible, I = indeterminant, R = resistant.

TABLE 3. Comparison of cytolysin titers produced by clinical and environmental isolates of *V. vulnificus*

Source	% of strains producing cytolysin titer of:				
	≤1:20	1:40	1:80	1:160	≥1:320
Clinical (n = 20)	0	10	35	40	15
Environmental (n = 29)	10	24	24	24	18

reported for clinical *V. vulnificus* strains (9). The only notable difference is that the 30 marine strains reported here were somewhat less susceptible to erythromycin (mean zone diameter, 20.2 mm) than were the 33 clinical strains (mean zone diameter, 26.4 mm) reported by Hollis et al. (9).

In vitro and in vivo virulence assays. Summaries of cytolysin and cytotoxin titers produced by clinical and environmental isolates of *V. vulnificus* are shown in Tables 3 and 4. All clinical isolates tested in these in vitro assays produced significant titers of both cytolysin (≥1:40) and cytotoxin (≥1:20). Of 29 marine strains tested, 3 (10%) produced cytolysin (hemolysin) titers below those produced by clinical isolates of *V. vulnificus* (1:40). Of 27 marine strains, 1 produced a cytotoxin titer of ≤1:20, which was less than the lowest cytolysin titer (1:20) produced by the 13 clinical strains tested.

Results of the in vitro and in vivo virulence assays and the source of individual marine strains are shown in Table 5. All clinical *V. vulnificus* strains tested killed at least two mice in each set of three inoculated. Of 29 marine *V. vulnificus* strains, 21 killed at least two of three mice in each set, while 4 marine strains killed one of three mice. Four environmental *V. vulnificus* strains failed to kill mice. All *V. vulnificus* strains which killed fewer than two mice per set were retested and confirmed as low-virulence strains. Three of the four environmental *V. vulnificus* isolates which were avirulent (failed to kill mice) also produced low cytolysin (hemolysin) titers in vitro (<1:40). The in vitro assay for hemolytic activity of culture supernatants appears to be a reliable, simple, and inexpensive method for screening *V. vulnificus* strains for further virulence studies.

In conclusion, there were no notable differences in phenotypic characteristics between clinical and environmental isolates of *V. vulnificus* when either biochemical characteristics or antimicrobial susceptibilities were compared. No distinct biogroups can be identified on the basis of the characteristics presented in Tables 1 and 2. Production of virulence factors both in vitro (cytolysin and cytotoxin) and in vivo (mouse pathogenicity) was similar among clinical and marine *V. vulnificus* strains. Of the environmental *V. vulnificus* isolates tested, 90 and 96% produced cytolysin and cytotoxin titers, respectively, which were comparable to results for clinical isolates. Of the marine strains, 86% were pathogenic for mice. Several marine *V. vulnificus* strains (4 of 29) which could be regarded as avirulent were identified. These strains produced low titers of cytolysin and cytotoxin

TABLE 4. Comparison of cytotoxin titers produced by clinical and environment isolates of *V. vulnificus*

Source	% of strains producing cytotoxin titer of:			
	1:10	1:20	1:100	≥1:200
Clinical (n = 13)	0	8	15	77
Environmental (n = 27)	4	15	63	18

TABLE 5. Source, cytolysin and cytotoxin titers, and relative virulence of environmental *V. vulnificus* strains

Strain	Source, site, and yr of isolation	Cytolysin titer[a]	Cytotoxin titer[b]	Relative virulence[c]
33-O	Fish, Oregon, 1978	160	100	1
34-O	Crab, Oregon, 1978	80	100	2
50-O	Eel, Japan, 1976	320	≥200	3
52-O	Eel, Japan, 1976	80	100	3
125-O	Crab, Louisiana, 1980	320	≥200	1
151-O	Crab, Louisiana, 1980	8	10	3
195-O	Clam, Oregon, 1981	80	100	3
458-O	Water, Oregon, 1982	40	100	0
491-O	Water, N. Carolina, 1981	40	100	3
512-O	Water, N. Carolina, 1981	80	100	3
1-T	Water, Texas, 1979	160	100	0
29-T	Water, Texas, 1979	160	100	1
33-T	Water, Texas, 1980	16	20	0
76-T	Water, Texas, 1980	640	≥200	1
143-T	Oyster, Texas, 1980	20	100	2
150-T	Oyster, Texas, 1980	40	20	2
170-T	Oyster, Texas, 1980	320	100	3
188-T	Oyster, Texas, 1980	80	100	3
236-T	Water, Texas, 1980	40	ND[d]	3
275-T	Oyster, Texas, 1981	40	ND	0
287-T	Water, Texas, 1981	160	100	3
305-T	Water, Texas, 1981	40	20	2
E49-T	Water, Texas, 1983	40	20	2
EBOT-T	Water, Texas, 1983	320	≥200	3
F52-T	Water, Texas, 1983	160	200	2
G64-T	Water, Texas, 1983	160	100	3
G65-T	Water, Texas, 1983	160	100	3
3AG5-T	Water, Texas, 1983	80	100	3
3AI3-T	Water, Texas, 1983	80	100	2

[a] Inverse of cytolysin titers of heart infusion broth supernatant against sheep erythrocytes.

[b] Inverse of cytotoxin titers of heart infusion broth supernatant to McCoy cell monolayers.

[c] Relative virulence in mice: 0 through 3 represents the number of mice killed in each set of three mice inoculated with the test strain.

[d] ND, Not determined.

in vitro or failed to kill mice when about 10^8 bacteria were injected intraperitoneally, or both. However, approximately 90% of the *V. vulnificus* strains isolated from geographically diverse marine sources were comparable in virulence to clinical strains tested. These data support the conclusion that exposure to *V. vulnificus* either by the consumption of raw shellfish, particularly oysters, or contact with brackish or marine waters represents a potential public health hazard. This hazard is apparently most significant during summer months in areas of low to moderate salinity (5 to 20‰) with water temperatures greater than 20°C, when numbers of *V. vulnificus* strains would be expected to be maximal (10, 16).

ACKNOWLEDGMENT

Support of this work was provided by a University of Texas Medical Branch, James W. McLaughlin Fellowship Fund award to D.L.T.

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