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Analysis of *Vibrio vulnificus* from Market Oysters and Septicemia Cases for Virulence Markers

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Representative encapsulated strains of *Vibrio vulnificus* from market oysters and oyster-associated primary septicemia cases (25 isolates each) were tested in a blinded fashion for potential virulence markers that may distinguish strains from these two sources. These isolates were analyzed for plasmid content, for the presence of a 460-bp amplicon by randomly amplified polymorphic DNA PCR, and for virulence in subcutaneously (s.c.) inoculated, iron-dextran-treated mice. Similar percentages of market oyster and clinical isolates possessed detectable plasmids (24 and 36%, respectively), produced the 460-bp amplicon (45 and 50%, respectively), and were judged to be virulent in the mouse s.c. inoculation–iron-dextran model (88% for each). Therefore, it appears that nearly all *V. vulnificus* strains in oysters are virulent and that genetic tests for plasmids and specific PCR size amplicons cannot distinguish between fully virulent and less virulent strains or between clinical and environmental isolates. The inability of these methods to distinguish food and clinical *V. vulnificus* isolates demonstrates the need for alternative subtyping approaches and virulence assays.

In the United States, *Vibrio vulnificus* is the leading cause of death associated with consumption of seafood (12, 15). Consumption of raw Gulf Coast oysters from April to November is responsible for nearly all of the cases. Although *V. vulnificus* is abundant in oysters during that time of year, cases are rare even in the high-risk group (i.e., those with preexisting liver disease or who are immunocompromised) (7). A major obstacle in developing effective control strategies is the inability to identify in the oysters *V. vulnificus* strains that are capable of causing human illness.

Two major research needs identified at a 1994 *V. vulnificus* workshop sponsored by the Food and Drug Administration (FDA) were to develop methods to distinguish virulent *V. vulnificus* strains from avirulent strains and to determine the infectious dose (22). Since human volunteer studies with *V. vulnificus* are not ethical, a consensus approach was proposed to determine the infectious dose by relating disease frequency with exposure. It was also suggested that a collection of strains from oysters and human septicemia cases associated with oyster consumption should be characterized in various assays in an attempt to determine traits that may be linked to virulence. The Centers for Disease Control and Prevention (CDC), FDA, and various state departments of health collected approximately 75 well-characterized clinical strains from human septicemia cases with known sources of oysters consumed, patient histories, etc. A recent study of the abundance of *V. parahaemolyticus* and *V. vulnificus* in retail oysters by the Interstate Shellfish Sanitation Conference and the FDA generated a large collection of *V. vulnificus* cultures that is seasonally and geographically diverse and well defined (3). Molecular characterization and virulence assays of representative *V. vulnificus* isolates from these two collections might reveal the importance of various traits for human infection and help determine the significance of total *V. vulnificus* numbers in oysters in terms of human illness.

Several putative virulence factors, such as the cytolysin-hemolysin, lipopolysaccharide, capsule, and siderophores, have been identified in *V. vulnificus* (10, 11, 18). The frequencies of occurrence of these factors are similar among clinical and environmental isolates (11); however, few isolates have been tested, and they usually have not been well defined. On the other hand, strains can easily be discriminated with various molecular techniques, such as pulsed-field gel electrophoresis and ribotyping. However, most strains examined have shown different genotypes, and so far the fingerprints generated by these techniques have not been useful in virulence prediction (2, 8, 19, 21).

Recent studies that have revealed the ability to discriminate between environmental and clinical strains present promising new approaches. Plasmids are associated with virulence of many bacterial species. A more sensitive plasmid detection technique for *V. vulnificus*, described by Danish researchers, was used to find plasmids that were not detected in clinical strains by standard plasmid isolation protocols (L. Høi, J. A. Gooch, A. Dalsgaard, and A. DePaola, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. Q-316, p. 594, 1999). A randomly amplified polymorphic DNA (RAPD) PCR procedure was found to produce an extra DNA band (178 to 200 bp) for all 31 clinical *V. vulnificus* isolates tested but for only 2 of 39

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TABLE 1. Source information on *V. vulnificus* strains isolated from market oysters

Culture identification no.	Harvest state	Harvest date (mo/day/yr)	MPN/g[a]
99–624 DP-C10	Texas	1/5/99	17
99–779 DP-D2	Louisiana	4/16/99	490
99–736 DP-C7	Florida	4/5/99	1,100
99–645 DP-C4	Texas	5/1/99	4,900
98–624 DP-C9	Louisiana	8/18/98	130,000
99–581 DP-C7	Louisiana	12/8/98	1,300
99–796 DP-E7	Florida	4/6/99	<240
99–584 DP-B12	Texas	3/31/99	1,300
98–640 DP-E9	Louisiana	8/23/98	170,000
99–743 DP-B6	Texas	5/8/99	9,500
98–783 DP-A1	Louisiana	5/1/99	790
99–780 DP-E1	Louisiana	4/14/99	7.8
99–625 DP-D8	Texas	1/5/99	4.9
99–738 DP-B5	Florida	4/19/99	700
99–537 DP-G7	Maryland	11/9/98	13
99–540 DP-B6	Texas	11/21/98	4,600
99–742 DP-A9	Mississippi	5/11/99	330
99–578 DP-B1	Louisiana	11/5/98	13,000
99–623 DP-F5	Florida	12/2/98	>1,600
99–520 DP-B8	Rhode Island	12/29/98	0.2
99–505 DP-C8	Texas	11/9/98	3,400
99–609 DP-A4	Oregon	2/1/99	<0.18
98–641 DP-G8	Louisiana	8/23/98	23,000
99–622 DP-E4	Texas	12/9/98	>1,600
99–509 DP-A6	Texas	1/8/99	220

[a] Most probable numbers (MPN) of *V. vulnificus* were determined as described by Cook et al. (3).

environmental isolates (21). However, no particular gene was associated with this DNA band. University of Florida researchers recently reported a 1,000-fold difference in the doses of virulent and selected naturally attenuated *V. vulnificus* required to cause disease in subcutaneously (s.c.) inoculated, iron-dextran-treated mice (16). In each of these studies, either few isolates were tested or the source of the isolates and their association with oysters were not reported.

The objective of this study was to test the hypothesis that proposed molecular markers of virulence (RAPD-PCR amplicons or plasmids) or virulence (s.c.-inoculated, iron-dextran-treated mouse model) are more prevalent in clinical isolates of *V. vulnificus* than in isolates from market oysters.

MATERIALS AND METHODS

***V. vulnificus* isolates.** Culture designations, sources, and other information for 50 representative *V. vulnificus* isolates are shown in Table 1 (oyster isolates) and Table 2 (clinical isolates). Half of the isolates were from human primary septicemia cases linked to raw-oyster consumption, and half were from a nationwide market survey of shell oysters (3). All of the isolates were judged to be encapsulated on the basis of their opaque colony morphology after overnight incubation at 35°C on tryptic soy agar (Difco, Sparks, Md.). Most of the clinical isolates had been previously tested for lipopolysaccharide type (23). The isolates were coded to conceal their source from the investigators. The investigators were Anders Dalsgaard of the Royal Veterinary and Agricultural University of Denmark (for plasmid analysis), James Oliver of the University of North Carolina—Charlotte (for RAPD-PCR typing), and Paul A. Gulig of the University of Florida (for virulence testing with s.c.-inoculated mice).

Plasmid extraction and detection. Isolates were grown in Luria broth (Difco) supplemented with 1% (wt/vol) NaCl at 37°C for 18 to 24 h. Control strains included *V. vulnificus* strain CDC 9344-95 (containing an 11.5-kb plasmid); *V. cholerae* O1 strain 1075/25 (150-kb plasmid); and the molecular size marker *E. coli* strains V517 (54-, 7.4-, 5.6-, 5.1-, 4.4-, 3.0-, 2.7-, and 2.1-kb plasmids) and 39R861 (147-, 63-, 36-, and 7-kb plasmids). Plasmid extraction was by the method of Birnboim and Doly (1) as modified by Høi et al. (Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999). Briefly, plasmids were extracted using SET buffer (50 mM Tris [pH 8], 50 mM EDTA, 0.58 M saccharose), lysozyme, and RNase enzyme. Following alkaline lysis at 56°C, potassium acetate buffer (3 M CH_3COOK, 1 mM EDTA, 2 M CH_3COOH) was added to precipitate protein, and plasmids were extracted using phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol).

The plasmid DNA was separated in a 0.8% (wt./vol) agarose gel (SeaKem GTG; Medinova, Hellerup, Denmark) in TAE buffer (89 mM Tris, 89 mM acetic

TABLE 2. Source information on *V. vulnificus* strains from primary septicemia cases

Culture identification no.	LPS[a]	Harvest state of oysters consumed	Harvest date (mo/day/yr)	Death
NSV 5829 (CDC 9149-95)	unk	Florida/Louisiana	5/23/95	No
NSV 5736 (CDC 9349-95)	1/5	Alabama	7/16/95	Yes
DAL 6-5000 (CDC 9345-95)	unk	Louisiana	9/30/95	Yes
ATL 9579	unk	Texas	8/23/94	No
ATL 71503 (CDC 9075-96)	2	Florida	10/24/96	Yes
ATL-9824	1/5	Texas	11/6/94	No
DAL 79040 (CDC 9070-96)	4	Texas	10/3/96	No
NSV 5830 (CDC 9348-95)	3	Florida	5/23/95	No
ATL 9823 (CDC 9352-94)	1/5	Louisiana	10/23/94	Yes
ATL 71504 (CDC 9076-96)	1/5	Louisiana	10/29/96	No
DAL 7-9087 (CDC 9005-97)	unk	Louisiana	5/14/97	No
ATL-9572	unk	Florida	6/30/94	No
ORL 8324 (CDC 9340-95)	1/5	Florida/Louisiana	7/26/95 or 8/7/95	Yes
ATL-9580	1/5	Texas/Louisiana	9/2/94	Yes
DAL 7-9002 (CDC 9060-96)	3	Texas	8/28/96	Yes
FLA 9509 (CDC 9003-97)	1/5	Louisiana	4/29/97	Yes
LOS 7343 (CDC 9062-96)	1/5	Louisiana	5/18/96	Yes
FLA 8869 (CDC 9053-96)	1/5	Texas	8/16/96	No
LOS 6966 (CDC 9342-95)	3	Texas/Louisiana	8/2/95 or 7/24/95	No
ATL 6-1306 (CDC 9031-96)	unk	Florida	4/30/96	No
ORL 1506 (CDC 9030-95)	3	Florida	5/95–8/95	No
LOS 7318 (CDC 9038-96)	unk	Texas	4/27/96	Yes
DAL 7-9000 (CDC 9067-96)	1/5	Texas	9/23/96	Yes
ATL 71491 (CDC 9074-96)	1/5	Texas/Louisiana	10/9/96	Yes
ORL 8074 (CDC 9032-95)	1/5	Texas	5/13/95	No

[a] Lipopolysaccharide (LPS) determined previously by Zuppardo et al. (23). unk, unknown.

acid, 2.5 mM EDTA; pH 8) at 10°C with a current of 135 mA for 4 h. The gels were stained with ethidium bromide (2 μg/ml; Sigma, St. Louis, Mo.) for 15 min, destained in distilled water, and photographed over a 354-nm UV transilluminator. Further, two-dimensional (2-D) gel electrophoresis was conducted according to the method of Hintermann et al. (6), which discriminates between covalently closed circular, open circular, and linear forms of plasmid DNA. Two consecutive steps of agarose gel electrophoresis with a single DNA sample were used. UV irradiation was performed between the steps to introduce single-strand nicks in ethidium bromide-stained DNA, converting covalently closed circular into open circular forms. Thus, differently configured forms of the same plasmid could be identified. Plasmid analyses were repeated at least twice for each strain.

RAPD analysis. Cells were grown in 1 to 3 ml of heart infusion broth (Difco) overnight at 22°C with aeration. A 200-μl volume of the overnight culture was centrifuged at 14,000 rpm (Marathon Micro A centrifuge; Fisher Scientific, Pittsburgh, Pa.) for 5 min, the supernatant was discarded, and the pellet was suspended in 200 μl of sterile water. At a cell-free station, a master mix containing 2.5 μl of 10× reaction buffer (Promega), 3.5 μl of 25 mM $MgCl_2$ (Promega), 1 to 2 μl of 5 mM deoxynucleoside triphosphate solution (Promega), 1 to 2 μl of a 5 mM primer solution (5′ GGATCTGAAC 3′; Biosynthesis), 0.5 μl of *Taq* polymerase (Promega), and 8.5 to 9.5 μl of distilled water was made, and 20.0-μl volumes of this mix were placed into sterile 0.5-ml microcentrifuge tubes (USA Scientific, Inc., Ocala, Fla.). All solutions were held on ice. A total of 5.0 μl of each bacterial culture was added to the master mix to give a final reaction volume of 25 μl. Samples were vortexed and overlaid with 20 μl of sterile mineral oil (Sigma) to prevent evaporation. Thermal cycling was performed in a Techne (Princeton, N.J.) model PHC-3 thermal cycler. The cycling profile was as follows: 1 cycle of 94°C for 5 min; 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min; and a final extension cycle of 72°C for 5 min. Fifteen to twenty microliters of PCR product was loaded on a 3% (wt/vol) agarose gel (Fisher Scientific) containing 0.5 μg of ethidium bromide/ml and electrophoresed in 0.5× TBE buffer (prepared from a 10× stock consisting of 0.89 M Tris, 0.89 M boric acid, and 25 mM EDTA) for as long as necessary to yield well-separated bands. The gels were photographed with a Polaroid Quick Shooter model QSP camera (International Biotechnologies, New Haven, Conn.). A 100-bp ladder (BioWhittaker Molecular Applications, Rockland, Maine) was used as a molecular size marker. Each time RAPD-PCR was performed, a clinical strain of *V. vulnificus* was included as a positive control while heart infusion broth served as a negative control.

Subcutaneously inoculated, iron-dextran-treated mouse model. Virulence was measured using the s.c.-inoculated, iron-dextran-treated mouse model as previously described (16). Mice were injected intraperitoneally with iron-dextran (Sigma) at 250 μg/g of body weight 2 h before *V. vulnificus* inoculation. Groups of five mice were initially injected s.c. (lower back) with 10^3 CFU of bacteria suspended in phosphate-buffered saline containing 0.01% (wt/vol) gelatin. Four parameters were used to determine virulence: CFU per gram of skin lesion, CFU per gram of liver tissue, lesion size and quality score, and body temperature (rectal temperature recorded with a Traceable digital temperature probe [Fisher Scientific]). The most critical criterion for determining virulence was CFU per gram of skin lesion. A strain was labeled as virulent if three or more of five injected mice had 10^7 CFU/g of lesion or if the average for all of the lesions was 10^6 CFU/g. The CFU per gram of liver tissue, a decrease in body temperature below 37°C (a sign of severe illness), and the lesion score (0 = no lesion, 1 = discoloration without hemorrhage, 2 = hemorrhagic lesion <2 cm^2, and 3 = hemorrhagic lesion >2 cm^2) were also noted. If a strain did not meet the virulence criteria at the initial inoculation dose, the dose was increased to 10^5 CFU/mouse. If the higher dose caused symptoms of virulence, the strain was labeled as attenuated. However, if at a dose of 10^5 CFU the strain still did not meet the criteria for virulence, it was labeled as avirulent.

Differences in values for virulence measures between oyster and clinical strains were compared using the Student *t* test or Mann-Whitney U test. Because the 4-point lesion score does not yield a normal distribution, it was not subjected to statistical analysis.

RESULTS

Table 3 lists the virulence markers for each of the *V. vulnificus* isolates from market oysters and cases of oyster-associated *V. vulnificus* primary septicemia. Plasmids were detected in 30% of the isolates; the proportions for clinical and oyster isolates were similar (36 and 24%, respectively). The plasmid profiles of the clinical strains CDC 9149-95, CDC 9030-95, CDC 9031-96, and CDC 9075-96 contain a band of approximately 40 kb that is a relaxed form of the 29-kb plasmid; two of these strains also contained a second plasmid (Fig. 1).

A 460-bp band was found in approximately half of the iso-

TABLE 3. Virulence markers in *V. vulnificus* isolates from market oysters and oyster-associated *V. vulnificus* primary septicemia cases

Culture identification no.	Plasmid(s) (kb)	460-bp band[a]	Mouse s.c.
Oyster			
99-624 DP-C10	—[b]	−	Virulent
99-779 DP-D2	4.5	−	Virulent
99-736 DP-C7	—	+	Virulent
99-645 DP-C4	—	+	Virulent
98-624 DP-C9	—	−	Attenuated
99-581 DP-C7	—	+	Virulent
99-796 DP-E7	23	ND	Virulent
99-584 DP-B12	—	−	Avirulent
98-640 DP-E9	—	−	Virulent
99-743 DP-B6	—	+	Virulent
98-783 DP-A1	—	+	Virulent
99-780 DP-E1	—	+	Virulent
99-625 DP-D8	—	+	Virulent
99-738 DP-B5	112	+	Virulent
99-537 DP-G7	—	−	Virulent
99-540 DP-B6	—	−	Virulent
99-742 DP-A9	25	ND	Virulent
99-578 DP-B1	—	ND	Virulent
99-623 DP-F5	—	−	Attenuated
99-520 DP-B8	—	−	Virulent
99-505 DP-C8	—	−	Virulent
99-609 DP-A4	55	ND	Virulent
98-641 DP-G8	—	ND	Virulent
99-622 DP-E4	44	+	Virulent
99-509 DP-A6	—	−	Virulent
Total (%)	24	45	88
Clinical			
NSV 5829 (CDC 9149-95)	29	+	Virulent
NSV 5736 (CDC 9349-95)	12	−	Virulent
DAL 6-5000 (CDC 9345-95)	—	−	Attenuated
ATL 9579	—	+	Virulent
ATL 71503 (CDC 9075-96)	29, 6.9	+	Virulent
ATL-9824	—	−	Virulent
DAL 79040 (CDC 9070-96)	—	−	Virulent
NSV 5830 (CDC 9348-95)	—	+	Virulent
ATL 9823 (CDC 9352-94)	—	+	Virulent
ATL 71504 (CDC 9076-96)	—	−	Virulent
DAL 7–9087 (CDC 9005-97)	—	+	Virulent
ATL-9572	12	+	Avirulent
ORL 8324 (CDC 9340-95)	10.5	−	Virulent
ATL-9580	—	+	Virulent
DAL 7-9002 (CDC 9060-96)	—	−	Virulent
FLA 9509 (CDC 9003-97)	—	−	Virulent
LOS 7343 (CDC 9062-96)	—	ND	Virulent
FLA 8869 (CDC 9053-96)	—	−	Attenuated
LOS 6966 (CDC 9342-95)	—	+	Virulent
ATL 6-1306 (CDC 9031-96)	50, 29	+	Virulent
ORL 1506 (CDC 9030-95)	29	+	Virulent
LOS 7318 (CDC 9038-96)	—	−	Virulent
DAL 7-9000 (CDC 9067-96)	41	ND	Virulent
ATL 71491 (CDC 9074-96)	—	−	Virulent
ORL 8074 (CDC 9032–95)	2	ND	Virulent
Total (%)	36	50	88

[a] −, band absent; +, band present; ND, not determined.
[b] —, no plasmid present.

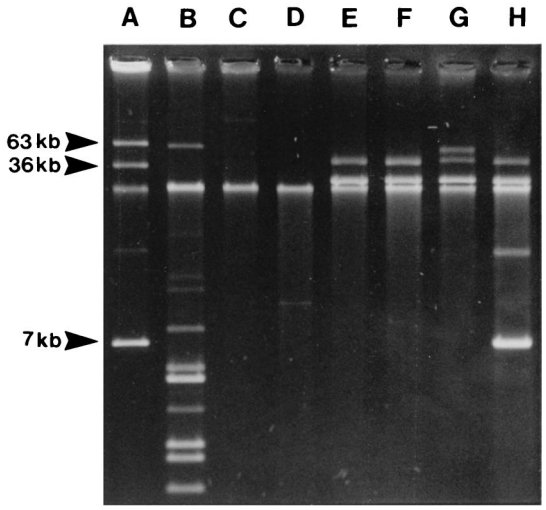

FIG. 1. Plasmid profiles of selected *V. vulnificus* strains. Lanes: A, *E. coli* 39R861; B, *E. coli* V517; C, *V. cholerae* 1075/25; D, *V. vulnificus* CDC 9344-95; E, *V. vulnificus* CDC 9149-95; F, *V. vulnificus* CDC 9030-95; G, *V. vulnificus* CDC 9031-96; H, *V. vulnificus* CDC 9075-96. Strains in lanes E to H contain a band of approximately 40 kb that is a relaxed form of the 29-kb plasmid, and lane H also contains a relaxed form of the 6.9-kb plasmid.

lates by RAPD-PCR, with similar proportions in clinical and oyster isolates (50 and 45%, respectively). DNA from 8 (16%) of the 50 strains could not be amplified by this method. Measurement of CFU per gram of skin lesion in the s.c.-inoculated, iron-dextran-treated mouse model indicated that 88% of the isolates were virulent, 8% were attenuated, and only 4% were avirulent; these characteristics were evenly divided among clinical and oyster isolates. The mean log CFU per gram of skin lesion for the clinical isolates was not significantly different from that for oyster isolates (7.24 ± 0.72 and 7.27 ± 0.98, respectively; $P = 0.87$, Student t test) (Fig. 2), and the mean lesion scores were 2.5 and 2.3, respectively. However, the log CFU per gram of liver tissue for the clinical strains was nearly significantly higher than that for the oyster isolates (4.04 ± 0.70 and 3.30 ± 0.69, respectively; $P = 0.06$, Student t test). Upon examining the ordered data, we observed that most of the oyster isolates were at the low end of the spectrum, and a Mann-Whitney U test confirmed that despite the insignificant difference in mean CFU per gram of liver tissue, the rank order of clinical isolates was significantly different from that of oyster isolates ($P < 0.005$). Finally, the mean body temperature at sacrifice of mice infected with clinical isolates was significantly different from that of mice infected with oyster isolates (33.4 ± 2.6 and 34.9 ± 1.2; $P = 0.01$, Student t test). Lower body temperature is correlated with severe systemic disease. Furthermore, body temperatures of 33°C or less (a correlate for death) were observed with 11 clinical isolates but with only 2 oyster isolates ($P = 0.004$, chi-squared test).

Taken together, these results indicated that neither plasmid analysis nor RAPD-PCR analysis could distinguish between oyster and clinical isolates of *V. vulnificus*. Furthermore, nearly

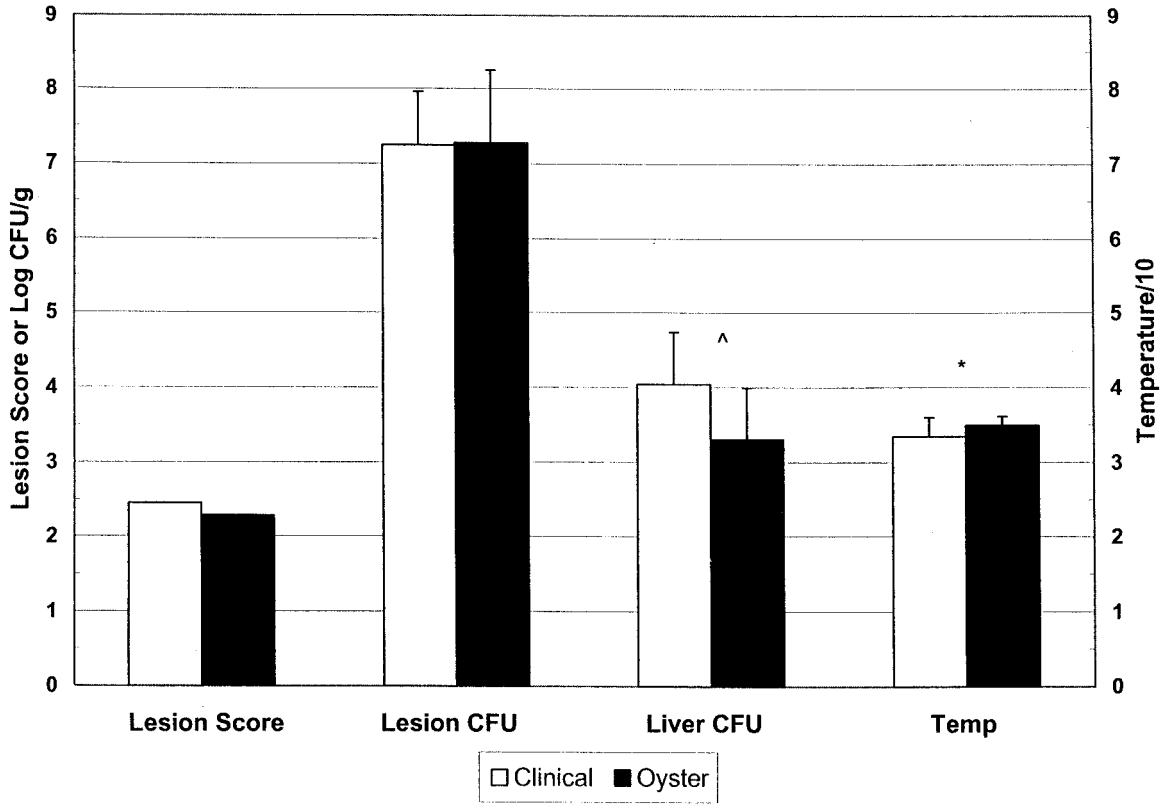

FIG. 2. Summary of quantitative data from mouse infection studies. Iron-dextran-treated mice were inoculated s.c. with 10^3 CFU of *V. vulnificus*. Data are means ± standard deviations of values for log CFU per gram of skin lesion (Lesion CFU), log CFU per gram of liver tissue (Liver CFU), and rectal temperature (Temp). For all groups, $n = 25$. *, $P = 0.01$ (Student t test); ^, $P < 0.005$ (Mann-Whitney U test).

all *V. vulnificus* isolates, regardless of their origin, were virulent according to the s.c. inoculation–iron-dextran method.

DISCUSSION

We undertook this study to determine whether RAPD-PCR genotyping and plasmid analysis could be used to differentiate oyster and clinical isolates of *V. vulnificus*. Furthermore, we wanted to determine whether oyster and clinical isolates had different virulence potentials as measured in an animal model of disease. Several bacteria, such as *Salmonella*, *Shigella*, and *Yersinia* species, contain plasmids encoding virulence attributes (13, 14). We therefore examined a collection of *V. vulnificus* strains for the presence of plasmids. Plasmids were found in 30% of the *V. vulnificus* strains in this collection; their proportions in clinical and oyster isolates were similar. This level is considerably higher than the 12% plasmid carriage for *V. vulnificus* previously reported by Davidson and Oliver (5) but lower than that determined in a Danish study of both clinical and environmental *V. vulnificus* strains, in which 11 of 18 strains (60%) contained single plasmids of typical small molecular sizes (4). In our study, a 29-kb plasmid was found in four clinical isolates but in none of the oyster isolates. Apparently, plasmids of different sizes, as seen in traditional one-dimensional electrophoresis, were often shown to be identical in 2-D gel electrophoresis. This result suggests that because they may yield different configurations of the same plasmid (i.e., open circular versus covalently closed circular), existing methods for plasmid extraction from *V. vulnificus* can still be improved. Southern hybridization using the 29-kb plasmid as a probe may detect additional strains containing few copies of this plasmid. Low copy number could limit detection by the conventional ethidium bromide-based methods that were used to detect the 29-kb plasmid in the present study. However, Southern hybridization using different plasmids from another strain failed to detect additional plasmid-bearing strains among these 50 isolates (data not shown).

The original study by Warner and Oliver (21) determined that a ca. 178-bp amplicon was present in 100% of the clinical isolates tested. Subsequent studies (Y. Yano and J. D. Oliver, data) unpublished found that only 71% of clinical isolates possessed this band. At the same time, however, a band of ca. 460 bp was identified in 86% of these same strains. Thus, in the studies reported here, we focused on the 460-bp band as an indicator of virulence. Approximately half of both the oyster and the clinical cultures produced this band, as determined by RAPD-PCR. Therefore, the presence of this band did not correlate with the source of the strain, and since nearly all of the isolates were virulent when injected s.c. into iron-dextran-treated mice, the band did not correlate with virulence using that model. However, note that all strains employed in the present study were exclusively from primary septicemia cases associated with oyster consumption, whereas the clinical strains examined in the unpublished study were from a variety of sources.

A final goal of the present study was to determine whether the virulence of clinical isolates of *V. vulnificus* differs from that of oyster isolates, as measured in an animal model. When CFU per gram of skin lesion was measured after s.c. injection of *V. vulnificus* into iron-dextran-treated mice, 88% of both clinical and oyster isolates were classified as virulent. Most previous animal studies indicated that the majority of *V. vulnificus* strains are virulent regardless of their source (9, 17, 20); however, there is some evidence that not all strains present in oysters have the ability to cause human disease (8a). In contrast to our use of quantitative microbiology of infected mice, most of the previous studies used 50% lethal dose assays with death as an endpoint. We attempted to devise some combination of virulence criteria (e.g., decrease in body temperature or shorter time to euthanasia due to severity of illness) as a surrogate for death as an endpoint; however, no such marker was found to separate the two groups of *V. vulnificus* strains. Consistent with the lack of difference in virulence assigned by minimal levels of bacteria in skin lesions, local infection by clinical isolates, measured by determining the mean CFU per gram of skin lesion, was not significantly different from that by *V. vulnificus* strains from oysters. In contrast, when two criteria for systemic disease, CFU per gram of liver tissue and decreased body temperature (Fig. 2), were employed, clinical strains were capable of causing significantly more severe systemic disease than oyster strains. This result is not entirely unexpected because the clinical isolates were preselected by their ability to cause systemic disease whereas the oyster isolates did not undergo such a selection. Therefore, there may be two populations of virulent *V. vulnificus* strains in oysters, both of which are capable of causing skin disease but only one having the potential to cause sepsis.

In conclusion, no clear distinctions between *V. vulnificus* strains isolated from market oysters and those isolated from patients with oyster-associated *V. vulnificus* primary septicemia were observed with the three approaches used in this study. This collection of encapsulated *V. vulnificus* isolates should be useful for evaluating additional screening methods for their ability to distinguish between fully virulent and less virulent strains. For reasons of safety, essentially all encapsulated strains should be considered capable of causing human disease until effective screening methods have been identified. Since the incidence of serious *V. vulnificus* disease is relatively low, even among people in the populations deemed most at risk who consume raw oysters, attention should also be directed toward *V. vulnificus* levels in oysters and additional, unidentified predisposing conditions of people, with the goal of being able to better predict lethal outcomes of human-oyster-*V. vulnificus* encounters.

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REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
2. **Buchrieser, C., V. V. Gangar, R. L. Murphree, M. L. Tamplin, and C. W. Kaspar.** 1995. Multiple *Vibrio vulnificus* strains in oysters as demonstrated by clamped homogeneous electric field gel electrophoresis. Appl. Environ. Microbiol. **61:**1163–1168.
3. **Cook, D. W., P. O'Leary, J. C. Hunsucker, E. M. Sloan, J. C. Bowers, R. J. Blodgett, and A. DePaola.** 2002. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. retail shell oysters: a national survey, June 1998 to July 1999. J. Food Prot. **65:**79–87.

4. **Dalsgaard, A., N. Frimodt-Møller, B. Bruun, L. Høi, and J. L. Larsen.** 1996. Clinical manifestations and epidemiology of *Vibrio vulnificus* infections in Denmark. Eur. J. Clin. Microbiol. Infect. Dis. **15:**227–231.
5. **Davidson, L. S., and J. D. Oliver.** 1986. Plasmid carriage in *Vibrio vulnificus* and other lactose-fermenting marine vibrios. Appl. Environ. Microbiol. **52:** 211–213.
6. **Hintermann, G., H.-M. Fischer, R. Crameri, and R. Hütter.** 1981. Simple procedure for distinguishing CCC, OC and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid **5:**371–373.
7. **Hlady, G.** 1994. Risk assessment and current data: Florida risk assessment and factors associated with risk, p. 27–33. *In* W. Watkins and S. McCarthy (ed.), Proceedings of the 1994 *Vibrio vulnificus* Workshop. U.S. Food and Drug Administration, Washington, D.C.
8. **Høi, L., A. Dalsgaard, J. L. Larsen, J. M. Warner, and J. D. Oliver.** 1997. Comparison of ribotyping and randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for characterization of *Vibrio vulnificus*. Appl. Environ. Microbiol. **63:**1674–1678.

8a. **Jackson, J. K., R. L. Murphree, and M. L. Tamplin.** 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. J. clin. Microbiol. **35:**2098–2101.

9. **Kaysner, C. A., C. Abeyta, Jr., M. M. Wekell, A. DePaola, R. F. Stott, and J. M. Leitch.** 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. Appl. Environ. Microbiol. **53:**1349–1351.
10. **Linkhous, D. A., and J. D. Oliver.** 1999. Pathogenesis of *Vibrio vulnificus*. FEMS Microbiol. Lett. **174:**207–214.
11. **Oliver, J. D.** 1989. *Vibrio vulnificus*, p. 569–600. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York, N.Y.
12. **Oliver, J. D., and J. B. Kaper.** 2001. *Vibrio* species, p. 263–300. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montvile (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
13. **Revell, P. A., and V. L. Miller.** 2001. *Yersinia* virulence: more than a plasmid. FEMS Microbiol. Lett. **205:**159–164.
14. **Rotger, R., and J. Casadesus.** 1999. The virulence plasmids of *Salmonella*. Int. Microbiol. **2:**177–184.
15. **Shapiro, R. L., S. Altekruse, S. Hutwagner, R. Bishop, R. Hammond, S. Wilson, B. Ray, S. Thompson, R. V. Tauxe, P. M. Griffin, and the** *Vibrio* Working Group. 1998. The role of Gulf Coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988–1996. J. Infect. Dis. **178:**752–759.
16. **Starks, A. M., T. R. Schoeb, M. L. Tamplin, S. Parveen, T. J. Doyle, P. E. Bomeisl, G. M. Escudero, and P. A. Gulig.** 2000. Pathogenesis of infection by clinical and environmental strains of *Vibrio vulnificus* in iron-dextran-treated mice. Infect. Immun. **68:**5785–5793.
17. **Stelma, G. N., Jr., A. L. Reyes, J. T. Peeler, C. H. Johnson, and P. L. Spaulding.** 1992. Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. Appl. Environ. Microbiol. **58:**2776–2782.
18. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect. **2:**177–188.
19. **Tamplin, M. L., J. K. Jackson, C. Buchrieser, R. L. Murphree, K. M. Portier, V. Gangar, L. G. Miller, and C. W. Kaspar.** 1996. Pulsed-field gel electrophoresis and ribotype profiles of clinical and environmental *Vibrio vulnificus* isolates. Appl. Environ. Microbiol. **62:**3572–3580.
20. **Tison, D. L., and M. T. Kelly.** 1986. Virulence of *Vibrio vulnificus* strains from marine environments. Appl. Environ. Microbiol. **51:**1004–1006.
21. **Warner, J. M., and J. D. Oliver.** 1999. Randomly amplified polymorphic DNA analysis of clinical and environmental isolates if *Vibrio vulnificus* and other *Vibrio* species. Appl. Environ. Microbiol. **65:**1141–1144.
22. **Watkins, W., and S. McCarthy (ed.).** 1994. Proceedings of the 1994 *Vibrio vulnificus* Workshop. U.S. Food and Drug Administration, Washington, D.C.
23. **Zuppardo, A. B., A. DePaola, J. C. Bowers, K. L. Schully, J. A. Gooch, and R. J. Siebeling.** 2001. Heterogeneity of environmental, retail, and clinical isolates of *Vibrio vulnificus* as determined by lipopolysaccharide-specific monoclonal antibodies. J. Food Prot. **64:**1172–1177.