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Virulence Characteristics of Clinical and Environmental Isolates of *Vibrio vulnificus*

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Twenty-four randomly selected clinical and environmental *Vibrio vulnificus* isolates were tested for virulence in iron-overloaded mice (250 mg of iron dextran per kg of body weight). The $\log_{10}$ 50% lethal doses of 17 isolates were lower by ≥ 3.5 $\log_{10}$ units in iron-overloaded mice than in control mice. These isolates were classified as virulent. The 50% lethal doses of these virulent isolates were also lower in mice that were immunosuppressed by treatment with cyclophosphamide (150 mg/kg). Four of the seven isolates initially classified as avirulent were virulent in mice that were simultaneously iron overloaded and immunosuppressed. These isolates were classified as moderately virulent. The remaining three isolates were avirulent under all conditions. The incidence of virulent strains among clinical and environmental isolates did not differ. The virulent isolates produced high titers of hemolysin, were resistant to inactivation by serum complement, produced phenolate siderophore, and utilized transferrin-bound iron. The moderately virulent isolates differed from the virulent isolates only in their increased sensitivity to inactivation by serum complement. The avirulent isolates differed from those of the other two classes in their inability to either produce significant amounts of phenolate siderophore or utilize transferrin-bound iron. A modified agar plate diffusion method for transferrin-bound iron utilization was developed to differentiate the two classes of virulent isolates from the avirulent isolates in vitro.

Vibrio vulnificus is a halophilic marine bacterium that causes localized wound infections and primary septicemia. The wound infections are acquired through exposure of a wound to salt water or shellfish, whereas septicemia is usually acquired through oral ingestion of the organisms, with raw oysters as the vehicle (2, 24). The mortality rate of *V. vulnificus* primary septicemia is 61% (24); 89% of patients suffering from *V. vulnificus* primary septicemia reported having an underlying chronic disorder (9). Disorders most frequently associated with *V. vulnificus* septicemia are those that result in iron overload, such as chronic cirrhosis, hemochromatosis, and thalassemia (24). Chronic renal insufficiency and use of immunosuppressive drugs have also been associated with *V. vulnificus* septicemia, suggesting that impaired immune function is also a risk factor (9).

To assess the significance of *V. vulnificus* as a public health problem, it is necessary to have a method that differentiates virulent from avirulent isolates and an estimate of the proportion of *V. vulnificus* strains in the environment that are hazardous to human health. Studies comparing the virulence of clinical and environmental isolates have yielded contradictory results. Oliver et al. (15) reported that 82% of marine isolates were virulent. Tison and Seidler (28) reported that marine strains were genetically indistinguishable from clinical isolates. Tison and Kelly (27) observed no differences between environmental and clinical isolates on the basis of biochemical characterization, production of virulence factors, and pathogenicity for mice. In contrast, Johnson et al. (8) reported that environmental isolates could be distinguished from clinical isolates by their sensitivity to serum bactericidal effects and their lack of pathogenicity to suckling mice.

In this study, we report the use of animal models to classify *V. vulnificus* isolates from various sources as virulent, moderately virulent, or avirulent. Our results showed no significant differences in virulence between clinical and environmental isolates. Our results also suggest that iron overload is a more significant risk factor than impaired immune function and that ability to acquire iron from highly saturated serum transferrin is the most critical of the known virulence factors of *V. vulnificus*.

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MATERIALS AND METHODS

Bacterial cultures. The 24 *V. vulnificus* strains used in this study are listed in Table 1 along with their sources and donors. Eleven of the isolates were clinical, and thirteen were environmental. All strains were stored in 40% glycerol at −70°C.

Fifty percent lethal dose (LD_{50}) determinations. Flasks (250 ml) containing 50 ml of brain heart infusion (BHI) broth (BBL, Cockeysville, Md.) plus 3% NaCl were inoculated from the frozen stocks and incubated at 37°C on a New Brunswick incubator shaker at 250 rpm. When stationary

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TABLE 1. Effects of iron overload on the virulence of clinical and environmental isolates of *V. vulnificus*

Strain designation	Source	Donor	LD_{50}[a] (CFU/20-g mouse)		Virulence
			Untreated mice	Iron-overloaded mice[b]	
121	Clinical	M. T. Kelly	7.6×10^6 (2)	1.0[c] (2)	+
185	Clinical	M. T. Kelly	6.2×10^6 (2)	1.4[c] (2)	+
A3490	Clinical	D. Hollis	9.3×10^6 (2)	11.0[c] (2)	+
C7184	Clinical	D. Hollis	7.1×10^6 (2)	3.1[c] (2)	+
UNCC 890	Environmental	J. D. Oliver	5.5×10^6 (2)	1.0×10^{2}[c] (4)	+
UNCC 913	Environmental	J. D. Oliver	4.3×10^6 (2)	0.8[c] (2)	+
UNCC 1001	Environmental	J. D. Oliver	5.7×10^3 (2)	0.7[c] (2)	+
UNCC 1002	Environmental	J. D. Oliver	6.0×10^5 (2)	2.9[c] (2)	+
J7	Environmental	M. T. Kelly	1.8×10^6 (2)	1.8[c] (2)	+
241	Environmental	M. T. Kelly	6.2×10^7 (2)	3.6×10^{6}[c] (3)	−
A1402	Clinical	D. Hollis	3.0×10^8 (2)	8.4×10^8 (2)	−
140	Environmental	M. T. Kelly	9.3×10^7 (2)	5.3×10^{6}[c] (2)	−
141	Environmental	M. T. Kelly	1.5×10^8 (1)	1.6×10^7 (1)	−
A9	Environmental	M. T. Kelly	4.2×10^7 (1)	2.7×10^6 (1)	−
125	Clinical	M. T. Kelly	3.5×10^7 (1)	6.5[c] (1)	+
298	Clinical	M. T. Kelly	2.8×10^6 (1)	9.5[c] (1)	+
E4125-A	Clinical	D. Hollis	1.4×10^8 (1)	2.4×10^7 (1)	−
A8694	Clinical	D. Hollis	1.1×10^9 (1)	7.0×10^7 (1)	−
LA-MO6	Clinical	C. Kaysner	3.2×10^5 (1)	25.0[c] (1)	+
UNCC 912	Environmental	J. D. Oliver	6.2×10^5 (1)	13.0[c] (1)	+
UNCC 1003	Environmental	J. D. Oliver	6.2×10^4 (1)	89.0[c] (1)	+
UNCC 1014	Environmental	J. D. Oliver	1.1×10^6 (1)	1.1×10^{2}[c] (1)	+
UNCC 1017	Environmental	J. D. Oliver	1.5×10^6 (1)	60.0[c] (1)	+
C7684	Clinical	D. Hollis	1.1×10^6 (1)	7.0[c] (1)	+

[a] Numbers of trials are in parentheses; the variances were 0.27704 for the normal mice (15 df) and 0.40084 for the iron-overloaded mice (17 df).
[b] Injected i.m. with 250 mg of iron dextran per kg of body weight 2 h prior to infection.
[c] Significantly different from the value for untreated controls.

phase was reached (~10 h), 0.1 ml of each starter culture was inoculated into 50 ml of fresh BHI broth plus 3% NaCl, and the cultures were incubated as described above. After 10 h, the cultures were harvested by centrifugation at 3,000 × *g*, washed twice with phosphate-buffered saline (PBS; pH 7.2), and resuspended and diluted in PBS. Viable counts were determined by standard pour plate techniques, using BHI agar plus 3% NaCl. The plates were incubated at 37°C, and colonies were counted after 48 h.

Male and female outbred Swiss white mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). The mice were approximately 30 days old and weighed 18 to 22 g. Groups of five mice were challenged by intraperitoneal (i.p.) injections of 10-fold serial dilutions of washed cells suspended in PBS. At least four concentrations of each strain were used. Control mice were injected with PBS only. Deaths were recorded after 48 h, and the LD_{50} values and 95% confidence limits were estimated by the method of Spearman and Karber as described elsewhere (5). Experiments in which controls died were discarded.

Iron overload was achieved either by i.p. injection of filter-sterilized ferric ammonium citrate (FAC; 4 mg/kg of body weight; Sigma, St. Louis, Mo.) in PBS immediately prior to challenge (30) or by intramuscular (i.m.) injection of iron dextran (250 mg/kg; Steris Laboratory, Inc., Phoenix, Ariz.) 2 h prior to challenge (7). Immunosuppression was achieved by i.p. injection of cyclophosphamide (CY; 150 mg/kg; Sigma) 72 h before challenge (25). In some experiments, iron dextran was also administered to the immunosuppressed mice 2 h prior to challenge. Controls were treated with iron, CY, or both and inoculated with PBS at the time of bacterial challenge.

Serum iron. Serum iron levels were determined colorimetrically by using an assay kit (American Monitor Corp., Indianapolis, Ind.).

Hemolysin assays. Cultures were grown under conditions described above except that heart infusion (HI) broth without added NaCl was substituted for BHI broth plus 3% NaCl to optimize hemolysin production (26). At various time intervals (5 to 9 h), 10-ml samples were removed and centrifuged at 17,000 × *g*. Twofold serial dilutions of culture supernatants were made in 0.02 M Tris buffer (pH 7.5), and equal volumes of a 1% suspension of washed sheep erythrocytes were added. After 30 min of incubation at 37°C, the suspensions were centrifuged and the A_{545} was determined on a Bausch & Lomb Spectronic 2000 spectrophotometer. Standard curves were prepared from erythrocyte suspensions lysed with saponin. Hemolytic titers were determined as the reciprocal of the last dilution at which there was >50% lysis. The hemolytic titers reported are the maximum titers observed in replicate cultures.

Serum inactivation. Sera were collected aseptically from healthy donors and mixed in pools, combining sera from at least six individuals. The pooled sera were stored in 0.5-ml aliquots at −70°C until used. Cultures were grown by the method used in the LD_{50} determinations except that final cultures were harvested at 5 h. The cultures were centrifuged at 10,000 × *g* for 10 min at room temperature. The cells were washed once with gelatin-Veronal-buffered saline plus Mg^{2+} and Ca^{2+} (6) and resuspended in the same buffer at approximately 5×10^7 CFU/ml; 0.5 ml of each suspension was mixed with 0.5 ml of pooled normal human serum (NHS). Samples (0.1 ml) were removed immediately after mixing and after incubation at 37°C for 30, 60, 90, and 120 min. Serial dilutions were made in alkaline peptone water, and viable counts were obtained by standard pour plate methods. Replicate experiments (two to four) were performed. Results were recorded as mean decreases in viable CFU after incubation in 50% pooled NHS.

Preparation of deferrated media. A 10× concentrated

solution of Syncase medium (3) was prepared with no iron added. Contaminating iron was removed by coprecipitation with magnesium carbonate (12), and then the medium was treated for 30 min with iron-free conalbumin (10 mg/ml in 5 mM $NaHCO_3$) (Sigma). The final concentration of conalbumin was 1.0 mg/ml of medium (17). The conalbumin-iron complexes were removed by ultrafiltration through a PM-10 filter (Amicon Corp., Lexington, Mass.). The final iron concentration of each lot was verified to be less than 0.5 μM by the colorimetric assay of Stookey (23). For preparation of solid deferrated Syncase, purified agar (BBL) was added at a concentration of 15 g/liter. All glassware used in the preparation of these media and in subsequent experiments used was acid washed with 6 N HCl and rinsed 10 times with double-distilled and deionized water (20).

Phenolate siderophore assay. Starter cultures were grown in BHI broth as previously described. After overnight growth (16 h), the cultures were centrifuged at 10,000 × *g*, washed twice with 0.01 M Tris plus 0.85% NaCl (pH 7.3), and resuspended in the Tris buffer. Flasks (250 ml) containing 50 ml of deferrated Syncase broth were inoculated with sufficient washed cells to give a concentration of 10^5 CFU/ml and were incubated on a New Brunswick Psychrotherm incubator shaker at 26°C and 250 rpm. After 24 h, the cultures were centrifuged at 10,000 × *g*, and the supernatants were lyophilized and concentrated 10-fold. The concentrated supernatants were adjusted to pH 1.5 with HCl, and the phenolate siderophore was extracted with ethyl acetate (19). The Arnow phenolic acid assay (1) was used to detect the phenolate siderophore.

Utilization of transferrin-bound iron. A modification of the agar plate diffusion method of Kochan et al. (12) was used. Purified human transferrin (98% iron free; Sigma) was added to deferrated Syncase agar to a final concentration of 0.2 mg/ml immediately before the plates were poured. A well 10 mm in diameter was cut in the center of each plate, and 0.1 ml of FAC solution containing 0.5 μg of Fe^{3+} was added to each well. The plates were held at room temperature for 24 h to allow diffusion of the iron. Cultures grown 6 h in BHI broth plus 3% NaCl were centrifuged at 10,000 × *g* and washed twice with deferrated Syncase. The washed cells were diluted in deferrated Syncase to a final concentration of ~10^5 CFU/ml, and 0.1 ml was spread on each plate. A zone of growth around the well after 12 to 16 h of incubation at 35°C was indicative of a positive test. *V. vulnificus* A1402 was the negative control, and strain C7184 was the positive control (20).

RESULTS

Effect of iron overload. In preliminary experiments, mice were given i.p. injections of FAC (80 mg/kg) or i.m. injections of iron dextran (250 mg/kg), and their serum iron levels were measured at various times. The serum iron levels of the mice treated with FAC returned to normal 3 h after treatment, whereas those of the mice treated with iron dextran remained high for >24 h (data not shown). Because the iron dextran treatment created a condition more similar to the chronic iron overloads that predispose humans to *V. vulnificus* infections, the iron dextran-treated mice were used in classifying the virulence of the *V. vulnificus* isolates.

Several concentrations of each of five clinical and seven environmental isolates were injected into normal and iron-overloaded mice, and the LD_{50}s were estimated. The LD_{50}s of these first 12 isolates (Table 1) are the geometric means of replicate determinations. The mean LD_{50}s ranged from 5.7 × 10^3 to 3.0 × 10^8 CFU in the normal mice and from 0.7 to 8.4 × 10^8 CFU in the iron-overloaded mice. The LD_{50}s of 11 of these 12 isolates were significantly lower in the iron-overloaded mice ($\alpha = 0.05$). However, only the nine isolates with LD_{50}s of ≤10^2 CFU in the iron-overloaded mice were classified as virulent. The other two isolates (241 and 140) had LD_{50}s of >10^6 CFU in the iron-overloaded mice, which was approximately the same as the LD_{50}s of typical *V. vulnificus* isolates in normal mice. The LD_{50}s of the virulent isolates were all lower by ≥3.5 $\log_{10}$ units in the iron-overloaded mice than in the normal mice, whereas the LD_{50}s of the avirulent isolates were only ≤1.2 $\log_{10}$ units lower. The LD_{50}s of the virulent isolates were at least 4 $\log_{10}$ units lower in iron-overloaded mice than were those of the avirulent isolates.

TABLE 2. Lethality of *V. vulnificus* UNCC 890 in mice after treatment with iron dextran or FAC

UNCC 890 CFU/mouse	Lethality[a]	
	Fe-dextran[b]	FAC[c]
5.5 × 10^5	5	4
5.5 × 10^4	5	3
5.5 × 10^3	3	0
5.5 × 10^2	3	0
5.5 × 10^1	1	0

[a] Number of deaths in 48 h among five mice injected.
[b] Mice injected i.m. with iron dextran (250 mg/kg) 2 h prior to infection.
[c] Mice injected i.p. with FAC (80 mg of Fe/kg) immediately prior to infection.

The LD_{50}s of the 12 additional isolates (6 clinical and 6 environmental) were then compared in normal and iron-overloaded mice. Because of the large differences in mean LD_{50}s observed with the first 12 isolates, replicate experiments were not performed. The significance at the $\alpha = 0.05$ level was determined by calculation of the confidence limits for the LD_{50}. The LD_{50}s of eight of these isolates were significantly lower in the iron-overloaded mice than in the controls. The LD_{50}s of these virulent isolates ranged from 7.0 to 1.1 × 10^2 CFU per mouse, whereas the LD_{50}s of the avirulent isolates were all ≥2.7 × 10^6 CFU, a difference of >4 $\log_{10}$ units (Table 1).

Altogether, 17 of the 24 isolates (8 of 11 clinical isolates and 9 of 13 environmental isolates) were virulent in the iron-overloaded mouse model. All 17 of these isolates had LD_{50}s of ≤1.1 × 10^2 CFU in the iron dextran-treated mice. The remaining seven isolates, all of which had LD_{50}s of ≥2.7 × 10^6 CFU in the iron dextran-treated mice, were classified as avirulent.

A virulent isolate with a relatively high LD_{50} (UNCC 890) was used to compare the effect of using iron dextran to achieve an iron overload with that of using FAC. This was done by estimating the LD_{50}s in a group of mice given i.m. injections of 250 mg of iron dextran per kg and a parallel group of mice given i.p. injections of 80 mg of FAC per kg. The results (Table 2) suggested that the iron dextran method was more sensitive and more likely to identify borderline virulent isolates. The LD_{50}s were 6.9 × 10^2 CFU in the mice treated with iron dextran and 6.9 × 10^4 CFU in the mice treated with FAC, a 2-$\log_{10}$-unit difference. None of the FAC-treated mice receiving doses of ≤5.5 × 10^3 CFU died, whereas one of the iron dextran-treated mice died after receiving a dose of only 55 CFU.

Effect of immunosuppression. Seven isolates that were virulent in iron-overloaded mice were tested for virulence in

TABLE 3. Effects of immunosuppression and of simultaneous immunosuppression and iron overload on the virulence of *V. vulnificus* isolates

Strain designation	Virulence[a]	LD_{50}[b] (CFU/20-g mouse)		
		Normal mice	Immunosuppressed mice[c]	Immunosuppressed and iron-overloaded mice[d]
UNCC 913	V	4.3×10^6 (2)	3.8×10^4 (2)[e]	ND
A3490	V	9.3×10^6 (2)	8.6×10^5 (3)	ND
LA-MO6	V	3.2×10^5 (2)	42 (2)[e]	ND
J7	V	1.8×10^6 (2)	6.2×10^3 (2)[e]	ND
UNCC 912	V	6.2×10^5 (1)	1.7×10^2 (1)[e]	ND
121	V	7.6×10^6 (2)	2.3×10^2 (2)[e]	ND
UNCC 1003	V	6.2×10^4 (2)	8.7×10^2 (2)[e]	ND
A9	M	4.2×10^7 (2)	1.1×10^6 (4)	4.0 (2)[e]
241	M	6.2×10^7 (2)	9.6×10^7 (3)	29.0 (4)[e]
140	M	9.3×10^7 (2)	1.0×10^8 (2)	9.0×10^3 (3)[e]
141	M	1.5×10^9 (1)	1.0×10^8 (2)	1.3×10^3 (2)[e]
A1402	A	3.0×10^8 (2)	4.7×10^7 (1)	9.6×10^6 (3)
E4125	A	1.4×10^8 (1)	2.5×10^8 (1)	1.9×10^7 (3)
A8694	A	1.1×10^9 (1)	8.2×10^7 (1)	6.3×10^5 (3)[e]

[a] V, virulent; M, moderately virulent; A, avirulent.
[b] Numbers of trials are in parentheses. ND, not done.
[c] Injected i.p. with 150 mg of CY per kg of body weight 72 h prior to infection.
[d] Injected i.p. with 150 mg of CY per kg of body weight 72 h prior to infection and i.m. with 250 mg of iron dextran 2 h prior to infection.
[e] Significantly different from the value for controls ($\alpha = 0.05$).

mice subjected to immunosuppression by CY treatment (Table 3). The LD_{50}s of six of these isolates were significantly lower in the immunosuppressed mice than in the controls ($\alpha = 0.05$). However, with one exception (strain LA-MO6), the LD_{50}s were ≥ 2 $\log_{10}$ units higher in the immunosuppressed mice than in the iron-overloaded mice. The LD_{50}s of the seven isolates avirulent in iron-overloaded mice were not significantly lower in the immunosuppressed mice (Tables 1 and 3).

The seven avirulent isolates were also tested in mice that were simultaneously iron overloaded and immunosuppressed, since both conditions are present in human cirrhosis. The results (Table 3) showed that these isolates were of two different types. The LD_{50}s of four isolates were at least 4 $\log_{10}$ units lower in the iron-overloaded and immunosuppressed mice than in the normal mice. These isolates, which all had LD_{50}s of $<10^4$ CFU in the iron-overloaded and immunosuppressed mice, were classified as moderately virulent. The LD_{50}s of the other three isolates were also significantly lower ($\alpha = 0.05$) in doubly compromised mice than in control mice. However, these LD_{50}s were still very high ($\geq 6.3 \times 10^5$ CFU), considering the degree to which the mice were compromised. These isolates were classified as avirulent. Because one of these avirulent isolates (E4125) was virulent in the hands of other researchers (14a), a second copy was obtained and tested in iron-overloaded mice. The second copy (E4125-V) was virulent (LD_{50} = 3.7 CFU).

Characteristics of each virulence class. To relate specific characteristics of *V. vulnificus* to the virulence classes, nine representative virulent isolates, all four conditionally virulent isolates, and all three avirulent isolates were characterized with respect to several putative virulence factors: (i) production of phenolate siderophore, (ii) utilization of transferrin-bound iron, (iii) production of hemolysin, and (iv) resistance to the bactericidal effects of NHS. The colonial morphologies of these isolates were also observed on the HI agar streak plates.

Concentrated culture supernatant extracts of all nine virulent isolates and all four moderately virulent isolates were strongly positive for phenolate siderophore. Extracts of avirulent isolate A1402 were negative, and extracts of the other two avirulent isolates contained trace amounts of phenolate siderophore (Table 4). All isolates that were strongly positive for phenolate siderophore were also positive for utilization of iron from highly saturated human transferrin. When 10^4 CFU of these organisms was spread on deferrated agar containing human transferrin, confluent growth occurred in the zones around the wells to which iron was added. These zones extended 1.1 to 1.5 cm from the edges of the wells after incubation at 37°C for 16 to 18 h. This

TABLE 4. Characteristics of virulent, moderately virulent, and avirulent Isolates of *V. vulnificus*

Strain designation	Virulence[a]	Phenolate siderophore[b]	Transferrin-bound Fe^{3+} utilization[c]	Hemolysin[d]	Serum inactivation[e]
121	V	+	+	1,024	0.14
A3490	V	+	+	1,024	1.10
LA-MO6	V	+	+	2,048	0.70
C7684	V	+	+	512	0.00
J7	V	+	+	1,024	2.20
UNCC 913	V	+	+	2,048	0.85
UNCC 1001	V	+	+	2,048	0.24
UNCC 1003	V	+	+	4,096	0.0
UNCC 890	V	+	+	2,048	1.4
A9	M	+	+	2,048	3.5
241	M	+	+	2,048	4.8
140	M	+	+	4,096	3.6
141	M	+	+	1,024	2.0
A1402	A	−	−	512	4.5
A8694	A	±	−	256	5.1
E4125	A	±	−	16	4.7

[a] V, virulent in iron-overloaded mice; M, virulent in simultaneously iron-overloaded and immunosuppressed mice (moderately virulent); A, avirulent.
[b] Determined by colorimetric assay (1).
[c] Determined by a modification of the agar plate diffusion assay (12).
[d] Maximum hemolytic titers observed in replicate experiments recorded as the reciprocal of the last dilution at which lysis was ≥50%.
[e] Mean decreases in $\log_{10}$ CFU after 1 h of incubation in pooled 50% NHS. Replicate experiments were performed.

pattern of growth indicated ability to utilize transferrin-bound iron and was observed with both virulent and moderately virulent isolates. No growth was observed around the wells of plates inoculated with the negative control (avirulent strain A1402). Only a few pinpoint colonies were observed within 1.0 cm of the wells of plates inoculated with the other two avirulent strains (E4125-A and A8694), indicating that the ability of these strains to utilize transferrin-bound iron was severely impaired.

Hemolysin titers were measured over a 5-h period so that the maximum titer produced by each isolate could be determined. The titers (Table 4) were the highest observed in replicate experiments. The maximum titers produced by virulent isolates ranged from 512 to 4,096 hemolytic units (HU)/ml; those produced by the moderately virulent isolates were not significantly different (1,024 to 4,096 HU/ml). The maximum titers produced by the three avirulent isolates were somewhat lower (16 to 512 HU/ml); however, the 512 HU/ml produced by strain A1402 was within the range of titers produced by the virulent isolates (Table 4).

The susceptibilities of the isolates to serum bactericidal effects were determined by measuring decreases in viable CFU after 1 h of incubation in 50% NHS (Table 4). Six of the nine virulent isolates were highly resistant to serum bactericidal effects. The decreases in viable CFU of these isolates were <1 $\log_{10}$ CFU in 1 h. The other three virulent isolates were somewhat less resistant, with decreases in viable CFU ranging from 1.1 to 2.2 $\log_{10}$ CFU. The four moderately virulent isolates were more sensitive, with decreases in viable CFU ranging from 2.0 to 4.8 $\log_{10}$ CFU. The decreases observed with three of these isolates were ≥ 3.5 $\log_{10}$ CFU. The three avirulent isolates were the most sensitive to NHS, with decreases in viable CFU ranging from 4.5 to 5.1 $\log_{10}$ CFU. Treatment of the NHS at 56°C for 30 min abolished the bactericidal effects, verifying the role of complement in the observed losses in viability.

All virulent and moderately virulent isolates and two of the avirulent isolates (E4125-A and A8649) produced opaque colonies on HI agar; however, occasional translucent colonies were observed. The colonies of the moderately virulent isolates and of E4125-A and A8694 appeared somewhat less opaque than those of the virulent isolates; however, this could be discerned only if the two types were observed side by side. Only strain A1402 produced all translucent colonies.

DISCUSSION

The suitability of an iron-overloaded mouse model for identifying virulent *V. vulnificus* isolates was suggested by both the epidemiological association of *V. vulnificus* septicemia with elevated serum iron levels (2) and the observation by Wright et al. (30) that injection of mice with iron lowered the LD_{50} of strain C7184 from 10^6 to 1.1 CFU. Our comparison of the LD_{50}s of 24 *V. vulnificus* isolates in normal versus iron-overloaded mice (Table 1) showed that all *V. vulnificus* isolates were lethal to normal mice at very high doses, a phenomenon also observed with other opportunistic pathogens (7, 22), and confirmed that virulent isolates can be distinguished more easily and accurately in iron-overloaded mice. Although virulent isolates were usually more lethal to normal mice than were avirulent isolates, the differences were small ($\log_{10}$ LD_{50} of <2), and two of the virulent isolates (A3490 and 125) were indistinguishable from the avirulent isolates by LD_{50}s in normal mice. The LD_{50}s of the virulent isolates were all ≤ 100 CFU in iron-overloaded mice, whereas the LD_{50}s of the avirulent isolates were all $\geq 2.7 \times 10^6$ CFU, a difference of 4.43 $\log_{10}$ LD_{50}. These data suggest that isolates hazardous to individuals with iron overload as the primary risk factor can be identified in iron-overloaded mice with little possibility of either false-positive or false-negative results.

The comparison of the lethality of strain UNCC 890 to mice given i.m. injections of 250 mg of iron dextran per kg and mice given i.p. injections of 4 mg of FAC per kg (Table 2) suggested that iron dextran treatment was the more reliable method for achieving iron overload and for identifying weakly virulent isolates, probably because the iron dextran persisted in the bloodstream for a longer time. Some of the less virulent strains of *V. vulnificus* may not become established in the bloodstream during brief periods of iron overload. However, the more typical virulent isolates behave similarly in both models. The LD_{50} of strain C7184 in the mice injected with iron dextran (3.1 CFU per mouse) did not differ significantly from the LD_{50} obtained by Wright et al. (30) in mice injected with FAC (1.1 CFU per mouse).

The immunosuppressed mouse model was tested because a case control study of *V. vulnificus* infections indicated that chronic renal insufficiency and use of immunosuppressive drugs were also risk factors for *V. vulnificus* septicemia (9). The results showed that although six of seven virulent isolates were more lethal to immunosuppressed mice than to control mice, the LD_{50}s of five of these isolates were still at least 1 to 3 $\log_{10}$ units higher in immunosuppressed mice than in the iron-overloaded mice (Tables 1 and 3). These data suggest that iron overload is the more significant risk factor and that iron-overloaded mice are more effective than immunosuppressed mice for identifying virulent isolates.

Since victims of cirrhosis suffer from both iron overload and immunosuppression, we tested the seven presumably avirulent isolates in mice that were simultaneously iron overloaded and immunosuppressed. The LD_{50}s of four of these isolates were significantly lower ($\alpha = 0.05$) in the iron-overloaded and immunosuppressed mice than in normal mice (Table 3), suggesting that they are moderately virulent and could be hazardous to individuals with both underlying conditions.

No significant differences were found either in the LD_{50}s of the clinical and environmental isolates or in the proportions of clinical and environmental isolates that were virulent ($\alpha = 0.05$). The three avirulent isolates observed were all originally from clinical sources. Strain A1402 was from an atypical site (corneal ulcer) and was reported to be avirulent in earlier studies (20, 30). The other two strains (A8694 and E4125-A) were blood isolates and were originally virulent. The copies used in this study could have been attenuated after numerous laboratory transfers or could have resulted from the inadvertent transfer of avirulent colonies from thiosulfate-citrate-bile salts-sucrose agar when they were purified in our laboratory. Simpson et al. (21) observed that virulent isolates produced a mixture of virulent (opaque) and avirulent (translucent) colonies on HI agar and that these two types could not be distinguished on thiosulfate-citrate-bile salts agar. Our results show that moderately virulent and some avirulent isolates produce opaque colonies that cannot easily be distinguished even on HI agar. A second copy of E4125 (E4125-V) obtained from J. D. Oliver was virulent in the iron-overloaded mice (LD_{50} = 3.7 CFU).

Our observation of no differences in virulence between clinical and environmental isolates agree closely with the observations of Tison and Kelly (27), who reported that 25 of their 29 isolates were virulent, Oliver et al. (15), who reported 82% virulence, and Kaysner et al. (10), who re-

ported comparable lethalities among clinical and environmental isolates. Our observations, however, do not agree with those of Johnson et al. (8), who reported that their environmental isolates were avirulent in suckling mice following oral challenge. These differences were not due to the different methods of challenge used in the two studies. Four of our virulent environmental isolates were tested in the peroral suckling mouse model, and all four were virulent (18). The differences were more likely due to the inadvertent selection for avirulent colonies by Johnson et al. (8) in their isolation procedures or loss of virulence during storage or after subculturing.

The analyses of isolates from each class for several putative virulence factors provided a plausible explanation for the differences in virulence observed in the animal models (Table 4). Isolates that were virulent in the mice subjected only to iron overload possessed all of these activities. The moderately virulent isolates differed from the virulent isolates only in their greater susceptibility to the serum bactericidal effects. Results of earlier studies with *V. vulnificus* suggested that susceptibility to serum bactericidal effects correlated with susceptibility to phagocytosis (8). Increased susceptibility of the moderately virulent isolates to phagocytosis would explain why mice treated only with iron were resistant to infection by these strains and why additional treatment with CY caused the mice to become susceptible. Phagocytosis by polymorphonuclear cells is the first line of defense against *V. vulnificus* infection (29); CY causes a depletion of polymorphonuclear cells and consequently of phagocytosis (25). It appears that both iron overload and impaired phagocytosis are necessary for infection by these isolates. Differences or deficiencies in capsular material may be the cause of the increased susceptibility of the moderately virulent isolates to phagocytosis. Tsuru et al. (29) observed that the capsular material of *V. vulnificus* strains susceptible to phagocytosis was either incomplete or absent.

The three avirulent isolates were not significantly different from the moderately virulent isolates in susceptibility to serum inactivation. Their lack of virulence, even to the iron-overloaded and immunosuppressed animals, was most likely due to their inability to acquire transferrin-bound iron (Table 4). This correlation is not unexpected; iron is an essential nutrient required by all bacteria, and virtually all of the iron present in serum is bound by transferrin and consequently unavailable to most microorganisms (4, 11). The inability of these isolates to utilize transferrin-bound iron was probably due to their inability to produce significant amounts of phenolate siderophore. Simpson and Oliver (20) also observed that avirulent strain A1402 did not produce phenolate siderophore, although it did produce hydroxymate siderophore. Their results and ours suggest that the phenolate siderophore enables the virulent isolates to acquire iron from highly saturated transferrin. This association was also noted by Morris et al. (14).

The relatively low titers of hemolysin produced by the avirulent isolates were probably not important. Both Oliver et al. (16) and Morris et al. (14) observed a lack of correlation between virulence and hemolysin production; also, Massad et al. (13) reported that mutants deficient in the production of hemolysin were still virulent for mice.

Our results suggest that the best in vitro method for identifying virulent and moderately virulent environmental isolates is to test for ability to utilize transferrin-bound iron. A modification of the agar plate diffusion method of Kochan et al. (12) appears to be better than the phenolate siderophore assay because it is easier to perform and will simultaneously test for ability to produce phenolate siderophore and ability to transport the siderophore-iron complex across the cell membrane. Although this test will not distinguish the highly virulent isolates from the moderately virulent isolates, it should be possible to develop a rapid and simple method to distinguish these two types by identifying differences in their surface properties.

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