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# Oral Infectivity of *Vibrio vulnificus* in Suckling Mice

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ABSTRACT

Lethal doses of 11 clinical and environmental isolates of *Vibrio vulnificus* were determined in suckling mice after oral challenge. With one exception, isolates that were virulent to iron-overloaded adult mice after intraperitoneal inoculation were highly lethal to the infant mice (>50% lethality at $10^5$ CFU/mouse). The virulent isolate that failed to kill infant mice at $10^5$ CFU had lost its invasiveness. Conditionally virulent isolates that were virulent only to simultaneously iron-overloaded and immunosuppressed adult mice required $>10^9$ CFU to kill the infant mice. Avirulent isolates failed to kill at $>10^9$ CFU/mouse. There were no significant differences in the lethalities of clinical and environmental isolates. These findings demonstrated a close correlation between virulence in the iron-overloaded adult mouse and infectivity by the oral route.

*Vibrio vulnificus* is a halophilic marine vibrio that is capable of causing primary septicemia in patients with chronic underlying illness *(1,4,8)*. An epidemiological report by Tacket et al. *(8)* indicated that 87% of patients with *V. vulnificus* septicemia recalled eating raw oysters. This suggests that most victims acquired the infection by the oral route.

Because iron-overload has been identified as a risk factor for *V. vulnificus* septicemia, by both epidemiological *(8)* and experimental *(12)* data, we used an iron-overloaded adult mouse model to determine the pathogenicity of 24 clinical and environmental *V. vulnificus* 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). Our data showed that 70% of clinical and environmental strains were highly lethal to iron-overloaded mice after intraperitoneal (I.P.) injection ($LD_{50} \leq 250$ CFU/mouse). These strains were classified as "virulent." More than one-half of the strains that were avirulent in iron-overloaded mice were virulent in mice subjected simultaneously to iron-overload and immunosuppression, a condition that exists in humans with cirrhosis of the liver. These strains were classified as conditionally virulent. Only 3 of 24 strains were avirulent under all conditions. These results correlate with the observation by Tison and Kelly *(10)* that environmental strains of *V. vulnificus* were biochemically identical to clinical strains and pathogenic to mice, and suggest that nearly all environmental isolates are hazardous to some segment of the population.

In contrast, Johnson et al. *(3)* reported that environmental isolates of *V. vulnificus* were not virulent when given to suckling mice by the oral route. Their observations raised the possibility that many of our environmental isolates that were virulent after IP-injection into iron-overloaded mice might not be infective when given orally.

We investigated the oral infectivity of 11 representative clinical and environmental isolates that had been previously classified by our IP models. This was done to identify those isolates that would represent a hazard if ingested with food and to estimate the proportion of environmental isolates that represent a potential foodborne health hazard.

## MATERIALS AND METHODS

### *Organisms*

Ten representative strains were selected from clinical and environmental sources. These strains included representatives of each of our three virulence classes and included strains that were efficient and inefficient at binding to human buccal cells (Table 1). Stock cultures were maintained at -70°C in cryotubes containing brain heart infusion broth plus 2.5% NaCl and 40% glycerol.

### *Mice*

Infant mice were of the Swiss Webster strain and were bred in our facility. Suckling mice (6 to 8-d-old) weighing 4-6 g were placed in groups of five and separated from their mothers 2 h before oral challenge.

### *Infection studies*

A modification of the suckling mouse test developed by Johnson et al. *(3)* was used. *V. vulnificus* strains were grown at 35°C overnight in flasks (250 ml) containing 50 ml of pep-

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TABLE 1. *Lethality of* V. vulnificus *isolates to infant mice after oral inoculation.*

| Strain designation | Virulence[a] | Buccal cell[b] binding | Source | Donor | CFU | No. dead/ no. inoculated |
|---|---|---|---|---|---|---|
| MO6-24 | V | High | Clinical | C. Kaysner | $1.0\times10^5$ | 3/5 |
| | | | | | $3.2\times10^5$ | 5/5 |
| J7 | V | High | Environmental | M. T. Kelly | $3.5\times10^5$ | 4/5 |
| | | | | | $1.8\times10^5$ | 4/5 |
| UNCC 913 | V | High | Environmental | J. D. Oliver | $1.0\times10^5$ | 3/5 |
| | | | | | $3.9\times10^5$ | 3/5 |
| 185 | V | Low | Clinical | M. T. Kelly | $8.5\times10^4$ | 4/5 |
| | | | | | $5.0\times10^5$ | 3/5 |
| UNCC 1001 | V | Low | Environmental | J. D. Oliver | $4.3\times10^5$ | 3/5 |
| | | | | | $4.2\times10^5$ | 4/5 |
| UNCC 1003 | V | Low | Environmental | J. D. Oliver | $5.0\times10^5$ | 4/5 |
| | | | | | $2.5\times10^5$ | 4/5 |
| C7184 | V | High | Clinical | D. Hollis | $2.9\times10^9$ | 0/5 |
| | | | | | $1.4\times10^9$ | 2/5 |
| A9 | C | High | Environmental | M. T. Kelly | $1.5\times10^9$ | 0/6 |
| | | | | | $4.0\times10^9$ | 2/5 |
| 241 | C | Low | Environmental | J. D. Oliver | $2.1\times10^9$ | 0/5 |
| | | | | | $5.1\times10^9$ | 1/5 |
| A1402 | A | High | Clinical | D. Hollis | $6.7\times10^9$ | 0/5 |
| | | | | | $2.1\times10^9$ | 0/5 |
| E4125-A | A | High | Clinical | D. Hollis | $1.1\times10^9$ | 0/5 |
| | | | | | $5.2\times10^8$ | 0/5 |

[a]V = virulent; C = conditionally virulent; A = avirulent as determined in iron overloaded adult mice.
[b]Reference 5.

tone broth plus 3% NaCl, pH 7.0, on a New Brunswick psychrotherm incubator shaker at 250 rpm. One-tenth ml of each starter culture was transferred into 50 ml of fresh peptone broth and the cultures were incubated for 6 h as above. Cultures were spun down at 3,000 × *g* for 10 min at 5°C. The cells were washed twice in a 5% $NaHCO_3$ buffer plus 0.85% NaCl (pH 8.0). Mice were inoculated through a feeding needle with 0.1 ml of the appropriate dilutions of washed cells suspended in the bicarbonate buffer containing 0.2% Evans blue dye. Mice were discarded if inoculum went into the lungs rather than the stomach. Mortality was recorded for 42 h. Control mice that only were given bicarbonate buffer plus 0.2% Evans blue dye did not die within 42 h.

Viable counts were determined by the standard pour plate method using brain-heart infusion agar plus 2.5% NaCl. Cell suspensions were diluted in alkaline peptone water before plating. Plates were incubated at 35°C for 48 h. When $LD_{50}$ estimates were made, the method of Spearman and Karber *(2)* was used.

## RESULTS

Preliminary experiments showed that high doses ($>10^9$ CFU) of virulent *V. vulnificus* strains did not kill iron-overloaded 20-g adult mice when the organisms were administered orally. Infective doses were observed when 10-g weanling mice were substituted for 20-g mice. However, lethal doses were still too high to effectively demonstrate oral infectivity. Therefore, the suckling mouse test was used.

Using the suckling mouse model for oral infectivity we determined that the $LD_{50}$ of *V. vulnificus* MO6-24, a highly virulent clinical isolate, was $<10^5$ CFU/mouse. The $LD_{50}$ of *V. vulnificus* UNCC 913, a virulent environmental strain was also $<10^5$ CFU/mouse. Strain A1402, our avirulent negative control, did not cause deaths at $>10^9$ CFU/mouse. These greater than 10,000-fold differences in the lethalities of the virulent and avirulent isolates enabled us to establish a screening procedure by which groups of mice were inoculated with $10^9$ and with $10^5$ CFU of each test isolate. This minimized the number of mice required to test each strain. A total of 11 clinical and environmental strains were tested.

The results (Table 1) showed that, with one exception (C7184), isolates that had been virulent to iron-overloaded adult mice killed >50% of the infant mice at approximately $10^5$ CFU/mouse. Strain C7184 only caused occasional deaths at $>10^9$ CFU/mouse. There were no differences in the lethalities of clinical and environmental isolates and no correlation between oral infectivity and ability to bind to buccal cells. Conditionally virulent strains A9 and 241 did not kill at doses $<10^9$ CFU and only caused occasional deaths at doses $>10^9$ CFU.

To determine if the failure of strain C7184 to cause deaths at $10^5$ CFU by the oral route was due to a loss of invasiveness, we compared its lethality by subcutaneous (S.C.) injection with its lethality by I.P. injection. Strains that are invasive kill as effectively by S.C. injections as by I.P. injections *(5)*. The $LD_{50}$ of C7184 in iron-overloaded mice was only 3.3 when the organisms were administered by I.P. injection. However, when S.C. injections were administered the $LD_{50}$ was $5.2\times10^6$. In contrast, the $LD_{50}$ of a typical invasive strain (UNCC 1002) was ≤4.0 CFU/mouse whether I.P. or S.C. inocu-

TABLE 2. *Iron-enhanced*[a] *lethality of* V. vulnificus *isolates by subcutaneous and intraperitoneal inoculations into adult mice.*

| Organism | Route of inoculation | CFU/mouse | No. dead/ No. inoculated | $LD_{50}$[b] |
|---|---|---|---|---|
| C7184 | I.P. | $5.0\times10^3$ | 5/5 | |
| | | $5.0\times10^2$ | 5/5 | |
| | | $5.0\times10^1$ | 5/5 | |
| | | $5.0\times10^0$ | 4/5 | 3.3 |
| | | 0.0 | 0/5 | |
| C7184 | S.C. | $2.6\times10^9$ | 5/5 | |
| | | $2.6\times10^8$ | 5/5 | |
| | | $2.6\times10^7$ | 4/5 | $5.2\times10^6$ |
| | | $2.6\times10^6$ | 2/5 | |
| UNCC 1002 | I.P. | $4.9\times10^3$ | 5/5 | |
| | | $4.9\times10^2$ | 5/5 | |
| | | $4.9\times10^1$ | 3/5 | |
| | | $4.9\times10^0$ | 5/5 | 3.9 |
| | | 0.0 | 0/5 | |
| UNCC 1002 | S.C. | $6.5\times10^3$ | 5/5 | |
| | | $6.5\times10^2$ | 5/5 | |
| | | $6.5\times10^1$ | 5/5 | 2.1 |
| | | $6.5\times10^0$ | 5/5 | |
| | | 0.0 | 0/5 | |

[a]250 mg/kg iron dextran administered I.P. 2 h before challenge.
[b]CFU/20 g mouse.

lations were used (Table 2). All of the strains that were virulent in the suckling mice (Table 1) were also virulent in iron-overloaded adult mice after S.C. injection (data not shown). When strains A9 and 241 were administered to simultaneously iron-overloaded and immunosuppressed mice, both strains were lethal at $10^3$ CFU/mouse by either I.P. or S.C. inoculation, indicating that they were also invasive.

## DISCUSSION

The inability of oral doses of our *V. vulnificus* isolates to kill adult mice reaffirms a similar observation by Poole and Oliver *(5)* that doses lethal to adult mice by the I.P. and S.C. routes were not lethal when given orally. The relatively high dose required to kill adult mice by the oral route appeared to be related to failure of the *Vibrio* spp. to survive the conditions of the stomach. Pathogenic *V. cholerae* was also non-infective when given to adult rodents by the oral route *(11)*. This problem was circumvented by using very young animals for oral feedings, a method that was effective in earlier studies with *V. cholerae (11)* and *V. vulnificus (3)*.

In contrast to the observations of Johnson et al. *(3)*, our oral inoculation studies showed that most environmental isolates were virulent. With the exception of strain C7184, those strains virulent by I.P. injections into iron-overloaded mice were also virulent to suckling mice by the oral route.

The differences in the virulence of the environmental isolates studied by Johnson et al. *(3)* and our environmental isolates could have been due to differences in isolation procedures. Simpson et al. *(7)* reported that virulent strains of *V. vulnificus* produced mixtures of opaque and translucent colonies on heart infusion agar, whereas avirulent strains produced only translucent colonies that were never observed to revert either to virulence or to production of opaque colonies. It is possible that the procedure used by Johnson et al. *(3)* to isolate their environmental strains led to the inadvertent selection of avirulent colonies.

Adherence to human buccal cells has correlated with enteropathogenicity in some bacterial species *(6,9)*. However, there was no correlation between adherence to buccal cells and the oral infectivity of our *V. vulnificus* isolates (Table 1). Therefore, buccal cell adherence cannot be used to distinguish *V. vulnificus* isolates that will bind to and invade the intestinal mucosa.

Strain C7184 was infective by I.P. and S.C. routes in earlier studies *(5)*. However, in this study $10^5$ CFU of C7184 failed to cause deaths by the oral route. The loss of oral infectivity of C7184 correlated with a loss of virulence after S.C. inoculation into iron-overloaded mice (Table 2). This is an indication of a loss of invasive capacity.

These investigations suggest that a screening procedure in which iron-overloaded mice are injected S.C. rather than I.P. would disclose invasiveness, as well as ability to kill, and would yield results that correlate with infectivity by the oral route. All of our strains that were virulent to iron-overloaded mice after S.C. injections were also virulent to the suckling mice after oral inoculation. Furthermore, all of our strains that were avirulent to iron-overloaded mice after S.C. inoculations were avirulent to the suckling mice by the oral route.

The potential virulence of the isolates (A9 and 241)

that were conditionally virulent in the adult mice (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) is still questionable. They were not infective by the oral route (Table 1), but they were invasive. It is possible that these strains are unable to survive in the stomach and would not cause septicemia in any host. However, it is also possible that infant mice are not sufficiently immunocompromised to allow these strains to cause systemic infections. In that case, these strains might still be a hazard to severely compromised human hosts via the oral route.

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