INFECTION AND IMMUNITY, June 1980, p. 681-687
0019-9567/80/06-0681/07$02.00/0

Vol. 28, No. 3

In Vivo Evaluation of Pathogenicity of Clinical and Environmental Isolates of *Vibrio cholerae*

SUZANNE P. SIGEL, SUE LANIER, VICKIE S. BASELSKI,† AND CHARLOTTE D. PARKER*

Department of Microbiology, University of Texas, Austin, Texas 78712

Thirty-three minimally passaged clinical and environmental isolates of *Vibrio cholerae* were examined for ability to survive and multiply in the upper bowel of infant mice and to elicit diarrhea. All of 21 smooth O-1 *V. cholerae* isolates from stool were able to multiply and elicit diarrhea. Three rough strains isolated from stool were unable to multiply or to elicit diarrhea. Two smooth O-1 isolates associated with cholera cases (from a sewer and a septic tank) also were able to cause disease. However, four O-1 strains and one non-O-1 strain from sources not associated with cholera cases did not cause mouse disease. A human gall bladder isolate was also avirulent, whereas a Louisiana shrimp isolate showed low mouse virulence. We conclude that smooth human diarrheal isolates of *V. cholerae* of serogroup O-1 are virulent for infant mice. Examination of sequential isolates from single patients showed that some strains isolated later in infection had a reduced ability to induce diarrhea. Comparison of epidemiologically related strains showed that an isolate from crab had a low ability to induce disease in infant mice, whereas the isolates from patients showed the expected ability to multiply and elicit diarrhea in mice.

Vibrio cholerae causes diarrhea in humans by elaborating a protein enterotoxin that elicits excessive fluid and electrolyte secretion into the gut lumen (7). Oral challenge of infant mice with viable *V. cholerae* results in an infection of the upper intestine which closely resembles human cholera. Recent work has shown that a classical *V. cholerae* strain and mutants derived from it show significant differences in their abilities to associate with the infant mouse upper bowel, survive and multiply there, and induce diarrhea (1-5). We studied these three properties in *V. cholerae* strains which had been passaged only a few times in the laboratory. This paper demonstrates that both the ability to survive and multiply in mouse upper bowel and the ability to induce diarrhea are of value in assessing the pathogenicity of clinical and environmental *V. cholerae* isolates.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* strains and their sources and histories are described in Tables 1, 2, and 3. Strain CA 401, a classical Inaba strain isolated at the same time as the classical strains in Table 1 and studied extensively in our laboratory (1, 2, 4), was used in some experiments.

Strain maintenance and preparation of inocula. Strains 1 through 8 were received in original lyophil vials and had been passaged only one or two times since primary isolation. Strains 9 through 16 were received on the original stock slants after isolation from primary plates. Strains 17 through 33 had been passaged a few times after isolation, and were sent to us on slants. On receipt, all strains were streaked on meat extract agar (MEA) (1), and a single colony from each was chosen, subcultured on MEA, quick-frozen in nutrient broth containing 15% glycerol, and maintained frozen at −80°C. Working stocks were obtained by culturing the freezer stock on MEA and holding for ≤14 days.

Working inocula were prepared from an 18-h culture on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.). For studies which did not require labeled cells, vibrios from the BHI agar were grown in BHI broth to early log phase. In studies using radiolabeled vibrios, BHI agar-grown cells were inoculated into minimal asparagine-glucose medium containing $^{35}SO_4$ and labeled as previously described (4). In most experiments, the same radiolabeled inoculum was used for two sets of infant mice: one for determination of mean infective potential (MIP) and retention of radiolabel, and the other for determination of fluid accumulation (FA) ratio. Each experimental group consisted of 6 to 12 infant mice.

Determination of MIP. Infant CFW mice from our colony weighing 3.0 ± 0.5 g (about 4 to 6 days of age) were starved for 6 h before inoculation. Each animal was challenged per os with 0.05 ml of BHI broth containing 4×10^6 to 7×10^6 colony-forming units (CFU) and 150,000 to 250,000 cpm. At 4 h postchallenge, the animals were sacrificed, and their upper bowels were removed and homogenized. Viable counts and radioactivity were determined on individual homogenates. MIP, which reflects multiplication or killing in the upper bowel, was calculated as previously described (2). Briefly, MIP = −log mean (cpm_4/

† Present address: Center for Disease Control, Atlanta, GA 30333.

TABLE 1. *In vivo measurements relating to pathogenicity of sequential isolates of V. cholerae*

Strain[a]	FA ratio[b]	Diarrheal response[b]	MIP[c]	Date of isolation	Source and comments[d]
1. CA321	0.085 ± 0.002	pos	0.8	3-3-53	Strains 1–8: CEL
2. CA325	0.081 ± 0.002	pos	0.8	3-7-53	
3. CA381	0.110 ± 0.003	str pos	1.0	3-4-53	Strains 1–5: classical Ogawa
4. CA383	0.085 ± 0.001	pos	0.7	3-6-53	
5. CA412	0.082 ± 0.001	pos	1.0	3-6-53	Strains 6–8: classical rough
6. CA414	0.055 ± 0.001	neg	−0.2	3-8-53	
7. CA415	0.061 ± 0.001	neg	−0.3	3-9-53	
8. CA416	0.057 ± 0.001	neg	−0.3	3-10-53	
9. 7967	0.077 ± 0.002	pos	0.2	10-4-78	Strains 9–16: JGW
10. 7970	0.070 ± 0.002	pos	0.5	10-7-78	
11. 7971	0.066 ± 0.002	±	0.5	9-30-78	El Tor Ogawa
12. 7974	0.069 ± 0.002	±	0.5	10-3-78	
13. 7975	0.073 ± 0.005	pos	0.2	10-4-78	
14. 7979	0.073 ± 0.002	pos	0.2	10-8-78	
15. 7986	0.079 ± 0.003	pos	0.5	9-30-78	
16. 7990	0.081 ± 0.003	pos	0.8	10-4-78	

[a] Strains are listed by original numbers and are assigned consecutive numbers for convenience. Brackets indicate sequential stool isolates from the same patient.

[b] FA ratio is fluid accumulation ratio 16 to 18 h postchallenge; each value represents the mean of 6 to 12 animals ± 1 standard error of the mean. Our interpretation of the data is shown as diarrheal response:

FA ratio	*Diarrheal response*
<0.064	neg = negative
0.065–0.069	± = questionable
0.070–0.099	pos = positive
>0.100	str pos = strong positive

[c] MIP is the mean infective potential 4 h after challenge; each value represents the mean of 6 to 12 animals. A positive number indicates net multiplication; a negative number indicates net killing.

[d] Serotype and biotype were provided by donors. Sources: CEL, C. E. Lankford; Department of Microbiology, University of Texas at Austin (8); JGW, Joy G. Wells, Center for Disease Control, Atlanta, Ga.

$CFU_4)/(cpm_0/CFU_0)$, where cpm_4 is radioactivity at 4 h, CFU_0 is colony-forming units at the time of inoculation, etc. A positive MIP indicates a decrease in the ratio of radioactivity to colony-forming units, thus indicating net multiplication. A negative MIP indicates an increase in this ratio, signifying net killing. Since MIP is a logarithm, small numerical differences are significant.

Calculation of radiolabel retention. The same homogenates used in calculation of MIP were used to determine retention of radiolabel. Percentage of label retained equals $(\overline{cpm}_4/cpm_0) \times 100$, where $\overline{cpm}_4$ is the average counts per minute at 4 h and cpm_0 is counts per minute of inoculum (2).

Determination of FA ratio. Infant mice were inoculated as described above with 4×10^6 to 7×10^6 CFU/animal. At 16 to 18 h postchallenge, the animals were sacrificed and weighed, and the individual guts (including stomach) were removed and weighed. Secretion of fluid into the gut lumen causes an increased FA ratio, since FA ratio = gut weight ÷ (body weight − gut weight). (FA ratios are expressed as the mean ± 1 standard error of the mean.) BHI broth gives an FA ratio of 0.057 ± 0.002 at 16 to 18 h postchallenge (1).

Mouse passage experiments. About 5×10^6 CFU of either stock strain CA 401 or Lou 14 (Table 3, strain 33) was used to challenge groups of three to six infant mice. Eighteen hours after challenge, mice were sacrificed; their intestines were removed, pooled, and homogenized in BHI broth. The homogenate was streaked on MEA plates. Single colonies were transferred, grown overnight in BHI broth, and diluted to 4×10^6 to 7×10^6 viable cells/0.05 ml. Individual infant mice were challenged per os with these inocula. FA ratios were determined 16 to 18 h later and recorded.

RESULTS

Kinetics studies of El Tor strains. Earlier work in our laboratory with classical *V. cholerae* strains showed the importance of choosing an early time point (4 h) for examining ability to multiply (2, 3). As compared with classical

TABLE 2. *In vivo measurements relating to pathogenicity of V. cholerae isolates from the western hemisphere*

Strain[a]	FA ratio[b]	Diarrheal response[b]	MIP[c]	Source and comments[d]
Not associated with cholera cases				
17. 1074-78	0.061 ± 0.001	neg	−0.3	IKW; sewage, Sao Paulo, Brazil, 1978, El Tor, Ogawa[e]
18. 2634-78	0.062 ± 0.001	neg	−0.3	IKW; sewage, Rio de Janeiro, Brazil, 1978, El Tor, Ogawa
19. V40	0.058 ± 0.004	neg	−0.9	RRC; water isolate from Chesapeake Bay, 1977, non-O-1 (does not react with O group I antiserum)
20. V69	0.063 ± 0.002	neg	−0.5	RRC; water isolate from Chesapeake Bay, 1977, El Tor, Inaba[f]
21. 1166-77	0.062 ± 0.001	neg	−1.1	JGW; from gall bladder of Alabama resident, El Tor, Inaba (6)
Associated with cholera cases				
22. 479935	0.078 ± 0.002	pos	0.6	JGW; stool from patient, Port Lavaca, Tex., 1973 (10), El Tor, Inaba
23. Lou 4	0.077 ± 0.002	pos	0.4	JGW; stool from patient, Abbeville, La., 1978, El Tor, Inaba
24. Lou 7	0.071 ± 0.001	pos	0.6	JGW; from sewer, Abbeville, La., 1978, El Tor, Inaba
25. Lou 18	0.068 ± 0.002	±	0.4	JGW; from shrimp caught in same water as crab which yielded strain 33, Table 3, 1978, El Tor, Inaba

[a] Strains are listed by original numbers and are assigned consecutive numbers for convenience.

[b, c] See Table 1.

[d] Donors provided information on isolation site, serotype, and biotype. Sources: IKW, I. K. Wachsmuth, Center for Disease Control, Atlanta, Ga.; RRC, R. R. Colwell, Department of Microbiology, University of Maryland, College Park (9); JGW, Joy G. Wells, Center for Disease Control.

[e] Also reacts weakly with Inaba antiserum.

[f] Reacts slowly with some lots of anti-O-1 antisera and does not react with other lots.

TABLE 3. *In vivo measurements relating to pathogenicity of epidemiologically related strains of Vibrio cholerae from Louisiana*

Strain[a]	FA ratio[b]	Diarrheal response[b]	MIP[c]	Source and comments[d]
				JGW
26. Lou 15	0.079 ± 0.002	pos	0.7	From ill patient
27. Lou 22	0.078 ± 0.004	pos	0.6	From ill patient
28. Lou 33	0.076 ± 0.004	pos	0.7	From septic tank of patients Lou 11 and Lou 17
29. Lou 17	0.076 ± 0.003	pos	0.8	From asymptomatic patient
30. Lou 11	0.076 ± 0.003	pos	0.5	From asymptomatic patient
31. Lou 20	0.073 ± 0.003	pos	0.5	From ill patient
32. Lou 13	0.072 ± 0.003	pos	0.6	From ill patient
33. Lou 14	0.066 ± 0.002	±	0.2	From crab eaten by patients All strains El Tor, Inaba

[a] Strains are listed by original numbers and are assigned consecutive numbers for convenience.

[b, c] See Table 1.

[d] These strains form an epidemiologically related cluster (Paul Blake, personal communication). Five persons shared a boiled crab meal, and three of them developed cholera. Two persons were asymptomatic but culture positive. Culture of the leftover boiled crab yielded strain Lou 14. JGW, Joy G. Wells, Center for Disease Control, Atlanta, Ga.

strains, El Tor strains caused an increase in FA ratio which began 8 to 10 h after challenge and reached a plateau by 14 to 16 h after challenge. This response paralleled the kinetics of fluid accumulation for classical strains, but the increase began 1 to 2 h later. Because of this later response in El Tor strains, MIP was examined at both 4 and 6 h postchallenge for two El Tor strains. Absolute values for MIP were greater at 6 h, but were positive at both time points. Subsequent experiments used a 4-h time point to facilitate comparison with previous studies.

Correlation of pathogenicity for mice with ability to produce disease in humans. Of 24 fresh stool isolates of *V. cholerae*, 21 elicited a positive FA ratio in infant mice (Tables 1-3). Additionally, these 21 strains were able to survive and multiply in the upper bowel. In contrast, the remaining three strains, which were rough strains isolated from a single patient late in infection, were not able to elicit excess fluid accumulation or multiply in the upper bowel. Thus, mouse virulence of smooth *V. cholerae* strains correlated with their ability to infect humans.

Mouse virulence of sequential isolates. For smooth sequential isolates, strains isolated later in infection elicited an FA ratio equivalent to (five sets) or lower than (one set) that of the strain isolated earlier from the same patient (Table 1).

Among the classical isolates, for one set, the later isolate showed a decreased MIP (reflecting a twofold reduction in ability to multiply). In addition, the rough classical strains from one patient showed greatly reduced ability to survive and multiply when compared with the early smooth isolate from the same patient. By contrast, in the El Tor strains, later isolates had either the same MIP or one reflecting a twofold increase in ability to multiply.

Among the smooth classical strains, radiolabel retained was similar for both early and late isolates (about 12% of input). However, the amount of label retained after challenge with the rough isolates was significantly lower (about 2%). Among El Tor sets, retention varied widely (5 to 12% of input).

Mouse virulence of isolates from the western hemisphere. Water isolates from Brazil and Chesapeake Bay and an isolate from gall bladder were unable to induce diarrhea in mice, showing an FA ratio equivalent to the control value (Table 2). These strains also gave a negative MIP, indicating that they were unable to survive and multiply in the upper bowel. An isolate from a cholera patient in Port Lavaca, Tex., and the strains from a patient and a sewer in Abbeville, La., elicited positive FA ratios and positive MIPs (Table 2). The isolate from shrimp had a questionable FA response, although it was able to survive and multiply. For strains that were unable to multiply, the radiolabel retained was <5.0%. Among the strains that were able to multiply, retention of radiolabel varied from 3 to 14%.

Mouse virulence of *V. cholerae* isolates from a single epidemiological cluster. All of the strains from patients in a common-source outbreak in Louisiana elicited positive FA ratios and MIPs (Table 3). The isolate from the crab vehicle was significantly less able to induce diarrhea. The crab isolate also showed an MIP signifying a two- to fourfold-lower ability to multiply in vivo when compared with patient isolates. Radiolabel retention varied widely among the strains (3 to 11% for the patient-associated strains and 5% for the isolate from crab), with no correlation to MIP, FA ratio, or pathogenicity.

Reproducibility of FA ratios in infant mice before and after mouse passage. Figure 1 shows the distribution of FA ratios for individual colonies of *V. cholerae* strains Lou 14 and

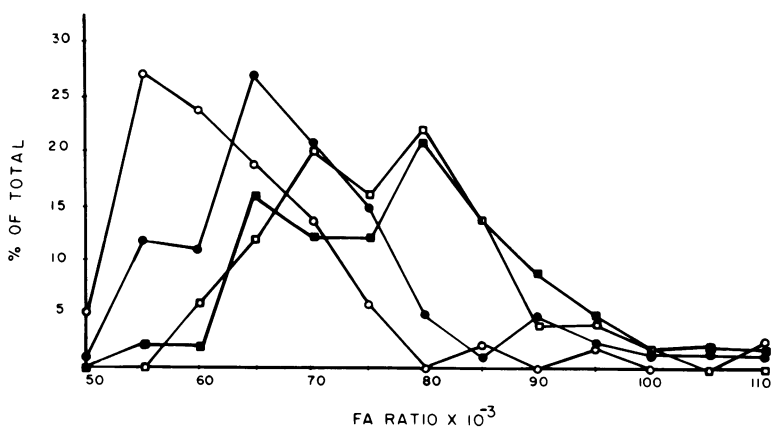

FIG. 1. *Distribution of FA ratios for individual colonies of V. cholerae strains Lou 14 and CA 401. Single colonies of stock Lou 14 (○), mouse-passaged Lou 14 (●), stock CA 401 (□), and mouse-passaged CA 401 (■) were inoculated orally into individual infant mice. FA ratios were determined 16 to 18 h later. Individual values were grouped for graphing so that 0.050 to 0.054 were graphed as 0.050, etc. The numbers of colonies tested were as follows: 96 colonies of stock Lou 14; 94 of mouse-passaged Lou 14; 51 of stock CA 401; and 45 of mouse-passaged CA 401.*

CA 401. For CA 401, the distribution of values approximated a normal curve, with few animals showing extremely high or extremely low values. No differences were detectable in experiments using our preserved stock strain and colonies tested after one mouse passage. However, strain Lou 14 showed a different pattern. In addition to having an obviously lower mean FA ratio, the curve appeared skewed toward low FA values. Additionally, colonies tested after a single mouse passage yielded a significantly shifted curve, with a higher mean FA ratio.

Correlation of ability to multiply with ability to induce diarrhea. Figure 2 shows the correlation between MIP and FA ratio for the 33 strains in the study. Also shown is our control strain, classical strain CA 401. The correlation between ability to induce diarrhea (elevated FA ratio) and ability to multiply in the infant mouse upper bowel (positive MIP) is evident.

Patterns of change in virulence observed for *V. cholerae* isolates tested at two time points. The infant mouse infection model is sensitive enough to quantitate virulence-associated properties of cholera strains. Table 4 summarizes the differences we detected among *V. cholerae* strains with various histories. Among seven sets of sequential cholera case isolates, three sets appeared to be identical by mouse assay when early and late isolates were compared. Four sets showed various patterns of change in FA ratio and MIP. The only predictable variation, perhaps, was that given by the smooth-to-rough alteration (strains 5 through 8). Since differences were detected between most sequential human stool isolates, we believe that *V. cholerae* is more variable in its virulence properties than had been suspected previously.

DISCUSSION

These results conclusively demonstrate that *V. cholerae* isolates from human cholera cases are virulent in infant mice. Correlation between virulence for mice (as determined by FA ratio and MIP) and involvement of strains in human diarrheal disease is excellent. However, mouse virulence does not invariably correlate with human disease, since two mouse-virulent strains were isolated from asymptomatic persons who had eaten contaminated crab. Furthermore, rough *V. cholerae* isolates from a cholera case were avirulent in mice.

We also found that four smooth O-1 strains of *V. cholerae* were avirulent in mice. None of these strains was epidemiologically associated with diarrheal disease. Strain 20 has been reported to make cholera toxin (9) by some workers, whereas others (W. Spira, personal communication) find it non-toxinogenic. Strain 21 appears to be negative for toxin production in vitro (Spira, personal communication), as do strains 17 and 18 (K. Wachsmuth, personal communication). None of these strains can multiply in the upper intestine of mice. This fact, plus the

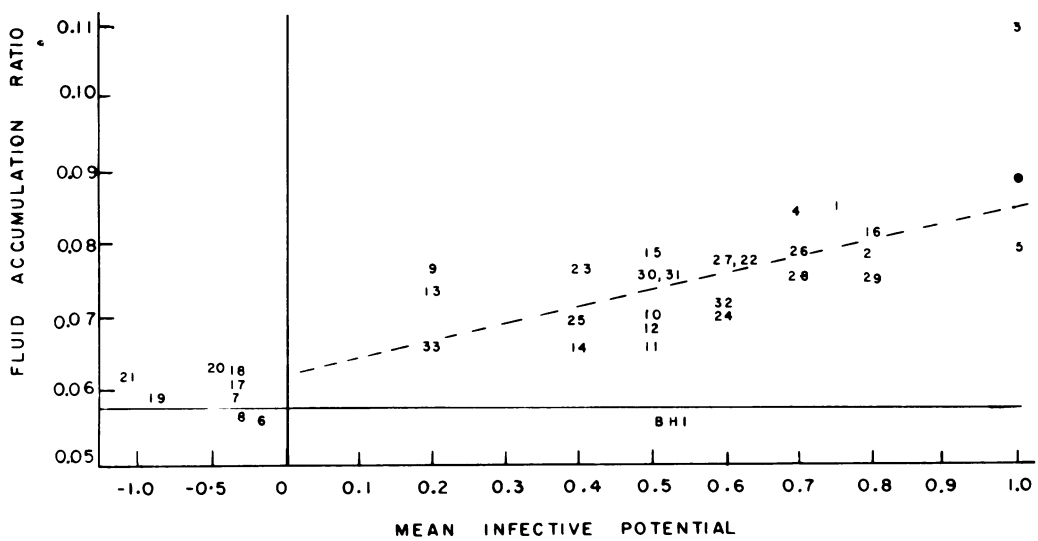

FIG. 2. *Correlation of diarrhea-inducing ability and infective potential of strains of V. cholerae. Each value represents 6 to 12 animals. FA ratios, reflecting induction of diarrhea, were determined 16 to 18 h postchallenge with 4 × 10⁶ to 7 × 10⁶ CFU/mouse. MIP values, reflecting ability to survive and multiply, were determined 4 h after challenge with the same dose. The symbol (●) represents CA 401, a classical strain, serotype Inaba, isolated in Calcutta, India, in 1953 from a patient who died the following day (8). Numbers refer to strains as listed in Table 1. Note change in scale of MIP to the left of zero.*

TABLE 4. *Summary of changes in mouse virulence observed among V. cholerae isolates subjected to human or mouse passage*

Isolate category[a]	Experimental design[b]	Results of second assay compared with first assay			
		FA ratio		MIP	
Sequential human diarrhea	assay ↑ Human gut ---------- assay ↑	pos or ±	(no change)	pos	(no change)
		pos	(no change)	pos	(increase)
		pos	(decrease)	pos	(decrease)
		neg	(decrease)	neg	(decrease)
Individual human diarrhea Strain CA 401	assay ↑ Human gut ----------Mouse gut ---------- assay ↑	pos	(no change)	Not done	
Environment Strain 33; Lou 14	assay ↑ Crab ----------Mouse gut ---------- assay ↑	pos	(increase)	Not done	

[a] Sequential human diarrheal isolates are from Table 1 and consist of seven sets of 16 strains. Strain CA 401 is described in reference 1. Strain 33 (Lou 14) is described in Table 3.

[b] Horizontal bars represent time. Arrows represent point in time at which the strain was isolated and preserved for assay in infant mice. See footnote *b*, for Table 1, for definition of positive (pos) ± (questionable), and negative (neg) FA responses.

observation that diarrhea-inducing strains show net multiplication, suggests that multiple parameters including toxigenicity and ability to multiply in vivo contribute to the virulence of a particular strain. Retention of radiolabel, on the other hand, is ill understood and does not appear to correlate with ability to multiply in the mouse gut or with ability to induce diarrhea.

One would expect net multiplication to be required for induction of a diarrheal response when small oral challenges are used, as in these experiments. Because toxin production in the gut has not been shown to affect vibrio survival, one would expect that non-toxinogenic as well as toxinogenic strains of *V. cholerae* would multiply in the infant mouse bowel. However, among the strains we tested, we found that poorly or non-toxinogenic cholera strains fail to multiply in the upper bowel. This observation, coupled with the fact that toxin-deficient mutants of a virulent strain show net killing in the infant mouse upper bowel (3), suggests that cholera toxin may play a role in initiation of infection.

In testing sequential isolates from patients, we found that several of the isolates from later in infection showed increased ability to multiply without a concomitant increase in ability to induce diarrhea. In one set (strains 3 and 4) the diarrheal response was significantly lower in the later isolate than in the homologous earlier isolate. This reduction in fluid accumulation accompanied by an increase in ability to multiply remains unexplained.

Strain 33 (Lou 14), the crab isolate from the Louisiana cluster, has questionable ability to induce diarrhea, but it does multiply in the gut. The strains isolated from patients who ate contaminated crab show significantly greater ability to multiply and to induce diarrhea. This experiment of nature suggests that a single passage through the human intestine enhances the disease-producing ability to Lou 14. Mouse passage of strain Lou 14 confirms that it shows a change in virulence after only 16 to 18 h in the gut. The basis for this change is unknown, but seems likely to involve toxin synthesis or delivery, since the mouse-passaged isolates are more diarrheagenic.

Strain CA 401, on the other hand, shows no change in diarrheagenic ability after animal passage. Since it was isolated from a patient during a severe cholera epidemic, it may be assumed that this strain was at peak virulence. It was isolated on the first day of illness, but unfortunately, no isolates from later in the patient's disease are available for test. We suggest, based on admittedly slight evidence, that optimally virulent *V. cholerae* strains are excreted early in the course of the disease. Stool isolates later in disease may show reduced virulence.

The variability in virulence we saw between the Bahrain El Tor isolates is not so surprising as the variability between the classical isolates. The Bahrain outbreak represented reintroduction of cholera disease after an absence, and one might expect that *V. cholerae* virulence would change. We expected that if changes occurred, we would see enhancement of virulence during the individual infection, or through the course of the epidemic. However, we saw decreased virulence of the later isolates from patients without smooth-to-rough transition.

From the data presented, it appears that residence in the mammalian gut may lead to a variety of changes in virulence properties of *V. cholerae*. Virulence enhancement can occur

(Lou 14, for example), but the most common change we saw was a decrease in virulence among strains isolated later in infection. The ability to produce cholera toxin is necessary for virulence, but all O-1 cholera strains can probably make cholera toxin. The ability to multiply in the upper intestine appears to be a primary determinant of virulence for *V. cholerae* strains and a better predictor for ability to cause human disease.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI12819 from the National Institutes of Health to C.P. S.P.S. was supported by the Texas Department of Health during part of this work.

We thank R. R. Colwell, C. E. Lankford, I. K. Wachsmuth, and Joy G. Wells for their gifts of cholera strains used in this research.

LITERATURE CITED

1. **Baselski, V., R. Briggs, and C. D. Parker.** 1977. Intestinal fluid accumulation induced by oral challenge with *Vibrio cholerae* or cholera toxin in infant mice. Infect. Immun. **15**:704–712.
2. **Baselski, V. S., R. A. Medina, and C. D. Parker.** 1978. Survival and multiplication of *Vibrio cholerae* in the upper bowel of infant mice. Infect. Immun. **22**:435–440.
3. **Baselski, V. S., R. A. Medina, and C. D. Parker.** 1979. In vivo and in vitro characterization of virulence-deficient mutants of *Vibrio cholerae*. Infect. Immun. **24**: 111–116.
4. **Baselski, V. S., and C. D. Parker.** 1978. Intestinal distribution of *Vibrio cholerae* in orally infected infant mice: kinetics of recovery of radiolabel and viable cells. Infect. Immun. **21**:518–525.
5. **Baselski, V. S., S. Upchurch, and C. D. Parker.** 1978. Isolation and phenotypic characterization of virulence-deficient mutants of *Vibrio cholerae*. Infect. Immun. **22**:181–188.
6. **Center for Disease Control.** 1977. *Vibrio cholerae*—Alabama. Morbid. Mortal. Weekly Rep. **26**:159–160.
7. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. **2**: 553–623.
8. **Husain, S. S., and W. Burrows.** 1956. Studies on immunity to Asiatic cholera. J. Infect. Dis. **99**:90–102.
9. **Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph.** 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl. Environ. Microbiol. **37**:91–103.
10. **Weissman, J. B., W. E. De Witt, J. Thompson, C. N. Muchnick, B. L. Portnoy, J. C. Feeley, and E. J. Gangarosa.** 1975. A case of cholera in Texas, 1973. Am. J. Epidemiol. **100**:487–498.