APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1986, p. 1216–1219
0099-2240/86/061216-04$02.00/0

Vol. 51, No. 6

Isolation of Non-O1 *Vibrio cholerae* Serovars from Surface Waters in Western Colorado

JOHN B. RHODES,[1] HARRY L. SMITH, JR.,[2] AND JAMES E. OGG[3]*

Western Slope Animal Diagnostic Laboratory, Colorado State University, Grand Junction, Colorado 80501[1]; Vibrio Reference Laboratory, Department of Microbiology, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107[2]; and Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523[3]

Received 7 February 1986/Accepted 18 March 1986

Non-O1 *Vibrio cholerae* was isolated from rivers, creeks, washes, irrigation canals, and ditches in western Colorado during the summer of 1985. The organism occurred in fresh water (≤5 mmol of Na^+ per liter) as well as in water of higher salinity (≈17 mmol of Na^+ per liter). Sixteen serovars of non-O1 *V. cholerae* were identified among the environmental isolates. All of the isolates were cytotoxic to Y-1 mouse adrenal cells.

Non-O1 *Vibrio cholerae* is biochemically identical to *V. cholerae* O1 but does not agglutinate in O group 1 antiserum. Over 70 serovars (serotypes) are now recognized under the typing scheme developed by Smith (25). Unlike *V. cholerae* O1, which causes epidemics of cholera in humans, non-O1 strains cause isolated or small outbreaks of gastrointestinal disease. Non-O1 strains also can cause systemic infections and have been isolated from the tissues and body fluids of patients with wound, ear, or other extraintestinal lesions (2, 9). Human infections with non-O1 *V. cholerae* are most often associated with seafood consumption, exposure to polluted fresh water, brackish water, or seawater, and foreign travel (5, 9, 18).

Non-O1 *V. cholerae* has been found in natural waters in many countries (19, 20, 21, 27). In the United States it has been recovered from coastal waters and sediments from the Chesapeake Bay (3, 10), the Pacific Ocean (12, 13), and the Gulf of Mexico (18), but its distribution throughout the country has not been determined.

The isolation of non-O1 *V. cholerae* from herbivores with enteritis in western Colorado by Rhodes et al. (23) led to this investigation of the possible sources of this organism in the environment. We report here the occurrence of non-O1 *V. cholerae* in surface waters in Mesa County, Colo.

MATERIALS AND METHODS

Collection of water samples. Single water samples were collected at 24 sites along rivers, creeks, washes, irrigation canals, and ditches in Mesa County, Colo., during August 1985 (Fig. 1). Some of the sites were the same as those used by the United States Department of Interior Bureau of Reclamation for its project monitoring the salinity of waters entering the Colorado River. Surface water samples were collected in sterile, screw-cap glass containers which had been rinsed with water from the site immediately before collection of the sample. The temperature of the water at most of the sites was recorded at the time of sampling. The water samples were held in an insulated container containing ice packs to maintain a temperature of about 4°C until they were processed at the laboratory within 2 to 3 h.

Physical and chemical parameters. The pH of each water sample was measured electrometrically. The electrical conductivity (reported as microsiemens per centimeter) and the amount of sodium cations (Na^+) and chloride anions (Cl^-) of selected water samples were determined by the Soil Testing Laboratory, Colorado State University. The U.S. Bureau of Reclamation, Grand Junction, Colo., provided the summer mean measurements of electrical conductivity and the sodium concentrations in waters at its sampling sites.

Enrichment and isolation. *V. cholerae* was isolated from water by using a two-step enrichment method. Water (10 ml) was added to 10 ml of double-strength alkaline peptone broth (1% peptone [Difco Laboratories; Detroit, Mich.], 1% NaCl, [pH 8.6]), and the tube was incubated overnight at 42°C (A. DePaola, M. L. Motes, R. E. Becker, S. R. Zywino, and R. M. McPhearson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q99, p. 274). A sample (0.2 to 0.5 ml) was removed from the top portion of an alkaline peptone broth culture and transferred to 10 ml of 96S4YL medium (1% peptone [Difco], 4% NaCl, 0.1% yeast extract [Difco] [ph 9.6]) with lincomycin (The Upjohn Co., Kalamazoo, Mich.) added to the cooled, sterile medium to a final concentration of 10 μg/ml. The 96S4YL medium was incubated at 37°C for 24 h. Plates of thiosulfate-citrate-bile salts-sucrose agar and Columbia agar with 10% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) were streaked with growth appearing at the surface of the 96S4YL medium. The plates were incubated at 37°C for 24 h and examined for growth typical of *V. cholerae* (23). Isolated smooth, yellow colonies from the thiosulfate-citrate-bile salts-sucrose agar or colonies showing greenish zones of hemolysis from blood agar were transferred to T1N1 agar (26) slants to establish stock cultures derived from single colonies. The T1N1 agar slant cultures were maintained at room temperature.

Identification. The preliminary screening tests for the isolates were the oxidase reaction (Kovacs oxidase), the String reaction (24), and sensitivity to 2,2-diamine-6,7-diisopropylpteridine phosphate (0/129 phosphate) with 10-μg disks. Organisms showing positive reactions in these tests were identified by methods outlined in the *Manual of Clinical Microbiology* (16), supplemented when necessary by reference to *Bergey's Manual* (14), and by use of the API 20E system (Analytab Products, Plainview, N.Y.). Isolates identified as *V. cholerae* were tested for ability to ferment sucrose, mannose, and arabinose, and were grouped according to the scheme of Heiberg (7).

Serotyping. Serotyping of isolates was performed by H. L. Smith, Jr. (Vibrio Reference Laboratory, Jefferson Medical

* Corresponding author.

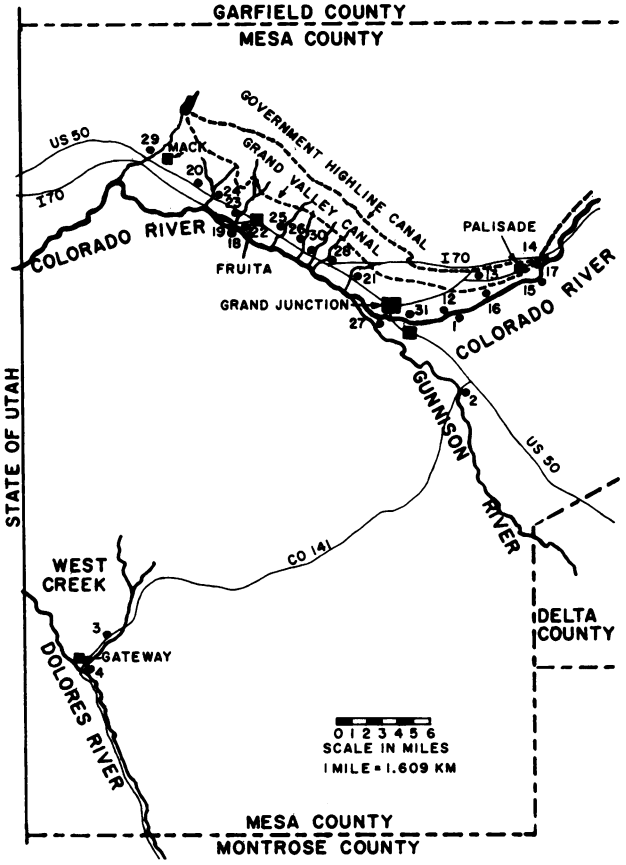

FIG. 1. Water sampling sites in Mesa County, Colo.

College, Philadelphia, Pa.). Using live organisms, cultures were typed according to the slide agglutination test procedure (26) and separated into serovars by the scheme developed by Smith (25).

Cytotoxicity. Y-1 mouse adrenal cells (6) were grown in 24-well plastic dishes containing Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 0.45% glucose, and penicillin-streptomycin at 37°C in a 4% carbon dioxide atmosphere. After sufficient growth had occurred, the medium was replenished, and the test materials were added. Morphologic changes in the cells were observed with an inverted microscope after overnight incubation of the mixtures.

All *V. cholerae* cultures were grown overnight in 1% Trypticase (BBL)–1% NaCl broth at 37°C. After centrifugation, the supernatant fluids were tested for their action on the Y-1 cells. When testing heat-treated preparations, the supernatants were placed in a boiling water bath for 5 min, cooled, and added to the wells containing the Y-1 tissue cultures.

Controls included supernatants from cultures of *V. cholerae* 569B (the classic producer of cholera toxin) and cholera toxin, lot 001, prepared by the Craig method (4).

RESULTS

V. cholerae non-O1 was isolated from surface waters at 21 of the 24 sites sampled in Mesa County, Colo., during August 1985 (Fig. 1). This high rate of isolation (88%) indicates that the two-step enrichment procedure is a reliable method for isolating the organism from water. In most cases, *V. cholerae* was the only organism detected in the 96S4YL broth.

Non-O1 *V. cholerae* appears to be ubiquitous in water in the Grand Valley area of Mesa County, with water from only two sites (sites 15 and 19) testing negative for the organism at the time of collection. Failure to detect *V. cholerae* at these two sites along the Colorado River may have been due to the single sample and small volume (10 ml) of water tested, since the organism was isolated from two other samples of Colorado River water collected the same day at sites 1 and 14. Site 14 was located at a tunnel discharging Colorado River water into the High Line Canal (Fig. 1). *V. cholerae* also was not isolated from water collected at site 3 on West Creek, a rapidly flowing, cold mountain stream; however, the organism was found at site 4 along the Dolores River about 5 km from site 3.

All isolates were positive in the following tests: oxidase, lysine and ornithine decarboxylase, indole production, citrate utilization, nitrate reduction, hemolysis (sheep blood), gelatinase, String test reaction, acid production from glucose and sucrose, and growth at 42°C and in 0, 1, 3, and 5% NaCl. Negative reactions were obtained in tests for urease, acid production from inositol and arabinose, gas production from glucose, and growth in 6 and 7% NaCl. Variation among isolates was observed for the Voges-Proskauer test at 37°C (78% positive), acid production from mannose (53% positive), and sensitivity to 50 IU of polymyxin B (22% positive). All isolates were inhibited by 10 μg of 0/129 phosphate.

The data on serotyping and grouping of the *V. cholerae* isolates are presented in Table 1. Sixteen serovars were detected in waters of Mesa County during the survey. Serovar 27 had been isolated previously from a lamb and a herd of bison suffering from enteritis in this part of Colorado (23).

Isolates 965 (serovars 115 and 146) and 984 (serovars 27 and 49) each reacted with two specific antisera, an indication that two distinct epitopes were being expressed by a cell. A similar condition is present in *V. cholerae* O1 serovar Hikojima, which reacts with both Ogawa and Inaba type-specific antisera.

The non-O1 *V. cholerae* strains isolated during this survey belonged to either Heiberg group I or II. There does not appear to be any correlation between Heiberg fermentation patterns and serovars; for example, isolates of serovar 31 were either Heiberg group I or II (Table 1).

V. cholerae isolates tested for toxin production exhibited cytoxicity; none showed cytotonic activity. When the Craig toxin or the supernatant from a *V. cholerae* 569B culture was used, the Y-1 mouse adrenal cells became rounded, indicating cytotonicity. These cells were refractile and well delineated by a surrounding membrane. However, when the supernatants from the non-O1 *V. cholerae* cultures were tested, the Y-1 cells in the wells became granular, lost their outer membranes, and aggregated. This effect (cytoxicity) generally could be observed within 3 h. In the few instances where titrations were conducted, the effect could be detected even when the supernatant was diluted 1:80. The toxin was destroyed by heating at 100°C for 5 min.

V. cholerae was isolated from waters having a wide range of sodium concentrations (2.3 to 16.9 mmol of Na^+ per liter) and electrical conductivities (865 to 2,596 μS/cm) (Table 1). Since there was good correlation of the concentrations of sodium cations (Na^+) and chloride anions (Cl^-), only the Na^+ concentrations are recorded in the table. The electrical conductivity indicates soluble salt content; sodium ion concentration measures salinity. In this survey, *V. cholerae* was isolated from fresh waters of low salinity (about ≤5.0 mmol of Na^+ per liter) to those of higher salinity (11.0 to 16.9 mmol

TABLE 1. Non-O1 *Vibrio cholerae* isolated from surface waters in Mesa County, Colo.

Site code	Location	Temp (°C)	Electrical conductivity (μS/cm)	Amt of sodium (mmol of Na^+/liter)	pH	Isolate code no.	*V. cholerae* serovar	Heiberg group[a]
1	Colorado River	21	880	3.9	8.2	965	115, 346	I
2	Gunnison River	20	901	2.3	8.2	966	172	II
3	West Creek	ND[b]	ND	ND	8.2			
4	Dolores River	22	2,520	16.9	8.1	967	16	I
12(GV70)[c]	Lewis Wash	21	1,111	4.7	8.2	969	361	II
13	Price Ditch	22	ND	ND	8.4	970	999[d]	I
14	High Line Canal	21	865	4.0	8.3	971	27	I
15	Colorado River	ND	ND	ND	8.2			
16 (GV30)	Clifton Drain	21	2,596	11.0	7.9	972	29	II
17	High Line Canal Ditch	ND	ND	ND	8.2	973	31	I
18	Little Salt Wash	22	ND	ND	8.0	974	31	I
						974A	31	II
19	Colorado River	20	ND	ND	8.3			
20	Kiefer Extension	21	ND	ND	8.3	975	148	I
21(GV170)	Leach Creek	20	1,571	5.7	8.2	976	107	I
22(GV250)	Little Salt Wash	23	1,267	5.9	8.1	997	23	II
						979A	106	II
23(GV260)	Big Salt Wash	21	1,720	6.0	8.1	978	23	I
24(GV271)	Reed Wash	21	1,665	5.5	8.0	979	361	II
25(GV230)	Adobe Creek	20	1,504	5.7	8.0	980	999	II
26(GV220)	Hunter Wash	21	1,663	5.9	8.0	981	31	I
						981A	31	II
27(GV140)	Orchard Mesa Drain	20	1,645	5.9	8.1	982	352	I
28(GV200)	Persigo Wash	21	1,483	5.9	8.1	983	13	I
						983A	999	II
29(S40)	Mack Wash	23	1,599	5.3	8.1	984	27, 48	I
30(GV210)	Pritchard Wash	21	1,745	6.5	8.0	985	23	I
31(GV120)	Indian Wash	20	1,336	5.6	8.2	986	11	II

[a] Heiberg group I, sucrose$^+$, mannose$^+$, arabinose$^-$; Heiberg group II, sucrose$^+$, mannose$^-$, arabinose$^-$.
[b] ND, Not done.
[c] GV or S indicates site sampled by the U.S. Bureau of Reclamation. The Bureau in Grand Junction, Colo., supplied the corresponding summer mean data for electrical conductivity and sodium content.
[d] Strain not typable by any antiserum in Vibrio Reference Laboratory collection.

of Na^+ per liter); however, none of the water could be considered brackish (≥25 mmol of Na^+ per liter). Others have reported the isolation of non-O1 *V. cholerae* from freshwater rivers and streams (<5 mmol Na^+ per liter (15, 20). Like the sodium content, the total soluble salt content of the waters as measured by electrical conductivity varied from medium (800 to about 1,500 μS/cm) to high (1,500 to 2,596 μS/cm). The soluble salt content and the pH (about 8.0) of these surface waters of Mesa County are favorable for the survival of *V. cholerae*.

DISCUSSION

Waters from three rivers in Mesa County (the Colorado, the Gunnison, and the Dolores) contained *V. cholerae* at the time of our sampling in August 1985. Water from the Colorado River is used for irrigation in the Grand Valley, where many of the sites in our survey are located. A variety of *V. cholerae* serovars were isolated from water samples taken from this irrigation system (ditches, washes, streams, and creeks). The isolation of *V. cholerae* from such diverse water sources indicates that the organism is prevalent throughout Mesa County, and may also be present in other counties in western Colorado.

During the time of our survey, we tested water from a ranch in the neighboring county of Montrose and detected *V. cholerae* non-O1 serovar 113 (data not shown). This ranch contained a herd of bison that had experienced an outbreak of gastroenteritis in 1984. At that time *V. cholerae* serovar 27 was isolated from two dead bison (23). Mesa and Montrose Counties are located in western Colorado on the western slope of the Rocky Mountains. Our survey sites are located at 1,400 meters of elevation and are approximately 900 km from the nearest seacoast on the Pacific Ocean. Although *V. cholerae* non-O1 has been found in coastal waters of the United States (3, 5, 8, 12), we are not aware of reports of the isolation of this organism from surface waters this far inland.

This study was initiated after non-O1 *V. cholerae* was detected in specimens taken from herbivores with enteric disease in this region of Colorado (23). The cases of enteritis in a horse, lamb, and bison occurred during the summer season; it was our hypothesis that the animals acquired *V. cholerae* from an environmental source, perhaps the pools of brackish water which appear in this region during late summer. However, the results of our study indicate that *V. cholerae* is prevalent in all types of water in Mesa County, from flowing fresh water (about ≤5 mmol of Na^+ per liter) to waters of higher salinity (11 to 17 mmol Na^+ per liter).

The incidence of *V. cholerae* in water has been related to the ionic and sodium content (10, 15) and to changes in temperature (3, 15, 27). Water temperature appeared to be a critical factor in the isolation of *V. cholerae* from the Chesapeake Bay. Colwell and co-workers (3) detected the organism in late fall and early spring when the water temperature was 15 to 20°C, but not during the winter when water temperature was 0 to 10°C. A distinct seasonal incidence of *V. cholerae* was also apparent in the waters of Kent, England, where the highest frequency of isolation and counts occurred during the summer months (15, 27). The temperature of the waters in our study area ranged between 18 and 23°C at the time of sampling in August. Further

ecological studies will be required to determine whether this parameter influences the incidence of *V. cholerae* in waters in Mesa County. We have sampled routinely one body of brackish water (≈30 mmol Na^+ per liter; electrical conductivity, 5,140 μS/cm) located in a drainage ditch in Mesa County (data not shown). During the period of July through October 1985, *V. cholerae* non-O1 was isolated over a temperature range of 13 to 23°C.

Our environmental isolates failed to agglutinate in O group 1 antisera and would not be considered to have the potential of producing epidemics of cholera in humans. However, some non-O1 strains are pathogens, producing either cholera enterotoxin or a very similar choleralike toxin (11), and other virulence factors like cytotoxins and cytolysins (17, 22). All of our isolates exhibited cytotoxic activity; the toxin destroyed Y-1 mouse adrenal cells in a manner which differed from the cytotonic action of cholera enterotoxin. The cytotoxic effect was still evident at a dilution of 1:80.

Some of the serovars from the Mesa County waters are of the same types reported in other countries. For example, serovars 11, 23, 31, 107, 113, 115, and 346 were isolated originally from human cases of diarrhea in Bangladesh; serovar 148 was obtained from a patient with diarrhea in the Philippines in 1964 (25). We assume that our isolates of non-O1 *V. cholerae* are potentially pathogenic for humans and animals and may present a public health hazard similar to other waterborne disease agents, including *Campylobacter* spp. and *Giardia lamblia*, which are found in untreated surface waters throughout Colorado and many other states.

The sources of *V. cholerae* in waters in western Colorado remains unknown. The results of surveys conducted in Kent, England, (15) suggest that farm animals are an unlikely source since no *V. cholerae* was isolated from feces of sheep that drank water containing high numbers of the organism. *V. cholerae* also was not isolated from the feces of cattle, sheep, pigs, or poultry, or even from animals with an enteric disorder. However, in the United States *V. cholerae* non-O1 was isolated from intestinal specimens from cases of gastroenteritis in a horse, lamb, and bison (23). The water may be contaminated from the feces of animals harboring the organism; on the other hand, the animals may be transient hosts that become infected by consuming water already contaminated from another source, such as feces from migrating water fowl (1, 15) or aquatic animals harboring *V. cholerae*. Others have proposed that non-O1 *V. cholerae* belongs to the autochthonous flora of streams and rivers, and brackish, estuarine, and coastal waters (3, 10, 15, 20). The results of our study tend to support their theory; the organism may indeed be indigenous to the aquatic environment in parts of western Colorado.

ACKNOWLEDGMENT

We thank Betty J. Ogg for preparing Fig. 1 and for typing the manuscript.

LITERATURE CITED

1. **Bisgaard, M., and K. K. Kristensen.** 1975. Isolation, characterization and public health aspects of *V. cholerae* NAG isolated from a Danish duck farm. Avian Pathol. **4**:271–276.
2. **Blake, P. A., R. E. Weaver, and D. G. Hollis.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34**:341–367.
3. **Colwell, R. R., J. Kaper, and S. W. Joseph.** 1977. *Vibrio cholerae*, *Vibrio parahaemolyticus*, and other vibrios: occurrence and distribution in Chesapeake Bay. Science **198**:394–396.
4. **Craig, J.** 1966. Preparation of the vascular permeability factor of *Vibrio cholerae*. J. Bacteriol. **92**:793–795.
5. **DePaola, A.** 1981. *Vibro cholerae* in marine foods and environmental waters: a literature review. J. Food Sci. **46**:66–70.
6. **Donta, S. T., and M. King.** 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. Nature (London) New Biol. **243**:246–247.
7. **Heiberg, B.** 1934. Des réactions de fermentation chez les vibrions. C.R. Soc. Biol. (Paris) **115**:984–986.
8. **Hood, M. A., and G. E. Ness.** 1982. Survival of *Vibrio cholerae* and *Escherichia coli* in estuarine waters and sediments. Appl. Environ. Microbiol. **43**:578–584.
9. **Hughes, J. M., D. G. Hollis, E. J. Gangarosa, and R. E. Weaver.** 1978. Non-cholera vibrio infections in the United States. Ann. Intern. Med. **88**:602–606.
10. **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.
11. **Kaper, J. E., S. L. Moseley, and S. Falkow.** 1981. Molecular characterization of environmental and nontoxigenic strains of *Vibrio cholerae*. Infect. Immun. **32**:661–667.
12. **Kenyon, J. E., D. C. Gilles, D. R. Piexoto, and B. Austin.** 1983. *Vibrio cholerae* (non-O1) isolated from California coastal waters. Appl. Environ. Microbiol. **46**:1232–1233.
13. **Kenyon, J. E., D. R. Piexoto, B. Austin, and D. C. Gilles.** 1984. Seasonal variation in numbers of *Vibrio cholerae* (non-O1) isolated from California coastal waters. Appl. Environ. Microbiol. **47**:1243–1245.
14. **Kreig, N. R., and J. G. Holt (ed.).** 1984. Bergey's Manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
15. **Lee, J. V., D. J. Bashford, T. J. Donovan, A. L. Furniss, and P. A. West.** 1982. The incidence of *Vibrio cholerae* in water, animals and birds in Kent, England. J. Appl. Bacteriol. **52**: 281–291.
16. **Lennette, E. H., A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.).** 1985. Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
17. **McCardell, B. A., J. M. Madden, and D. B. Shah.** 1985. Isolation and characterization of a cytolysin produced by *Vibrio cholerae* serogroup non-O1. Can. J. Microbiol. **31**:711–720.
18. **Morris, J. G., R. Wilson, B. R. Davis, I. K. Wachsmuth, C. F. Riddle, H. G. Wathen, R. A. Pollard, and P. A. Blake.** 1981. Non-O group 1 *Vibrio cholerae* gastroenteritis in the United States: clinical, epidemiologic, and laboratory characteristics of sporatic cases. Ann. Intern. Med. **94**:656–658.
19. **Muller, G.** 1977. Non-agglutinable cholera vibrios (NAG) in sewage, riverwater, and seawater. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. I Orig. B **165**:487–497.
20. **Muller, H. E.** 1978. Occurrence and ecology of NAG vibrios in surface waters. Zentralbl. Bakteriol. Mikrobiol. Abt. I. Orig. B **167**:272–284.
21. **Nacescu, N., and C. Cuifecu.** 1978. Serotypes of NAG vibrios isolated from clinical and environmental sources. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. I. Orig. **240**;334–338.
22. **O'Brien, A. D., M. E. Chen, R. K. Holmes, J. Kaper, and M. M. Levine.** 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga)-like cytotoxin. Lancet **i**:77–78.
23. **Rhodes, J. B., D. Schweitzer, and J. E. Ogg.** 1985. Isolation of non-O1 *Vibrio cholerae* associated with enteric disease of herbivores in western Colorado. J. Clin. Microbiol. **22**:572–575.
24. **Smith, H. L., Jr.** 1970. A presumptive test for vibrios: the "String" test. Bull. W.H.O. **42**:817–818.
25. **Smith, H. L., Jr.** 1979. Serotyping of non-cholera vibrios. J. Clin. Microbiol. **10**:85–90.
26. **Smith, H. L., Jr., and K. Goodner.** 1965. On the classification of vibrios, p. 4–8. *In* O. A. Bushnell and C. S. Brookhyser (ed.), Proceedings of the Cholera Research Symposium, Honolulu, Hawaii.
27. **West, P. A., and J. V. Lee.** 1982. Ecology of *Vibrio* species, including *Vibrio cholerae*, in natural waters of Kent, England. J. Appl. Bacteriol. **52**:435–448.