

## Isolation and Characterization of *Vibrio parahaemolyticus* from Cape Cod Soft-Shell Clams (*Mya arenaria*)

PAUL M. EARLE AND F. D. CRISLEY\*

Biology Department, Northeastern University, Boston, Massachusetts 02115

Received for publication 2 August 1974

*Vibrio parahaemolyticus* was isolated from soft-shell clams (*Mya arenaria*) taken from 10 different clamming areas on Cape Cod, Mass., during July and August 1972. Direct plating on thiosulfate-citrate-bile salts-sucrose agar was found to be superior to either direct plating on Vanderzant modified salt starch agar or enrichment with Trypticase soy broth containing 7% salt for isolation from clam samples. Morphological and biochemical characteristics of 33 isolates from 30 samples generally conform to those described for this organism in the literature, except for the production of acid from sucrose, lactose, and sorbitol. Six of the isolates were hemolytic on human blood agar plates, whereas all showed a negative Kanagawa phenomenon. Twenty of the 33 isolates reacted with pooled antisera to the K antigen; 15 of these reacted with 9 different specific K antisera, leaving 5 untypable. Ten of these 15 reacted with 4 different O antisera.

*Vibrio parahaemolyticus* is a major cause of summer gastroenteritis in Japan. Documented cases involving contaminated sea foods have also been reported in the United States (5), including the state of Massachusetts (B. B. McEachern, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, p. 2; 5). The organism has been frequently isolated from marine products and the marine environment; it has also been isolated from infected injuries (13) and from a recently reported case of fatal fulminating septicemia. (22).

Cape Cod (Barnstable County), Mass., is an important source of all types of fish and shellfish consumed locally and commercially. In 1972, Barnstable County was responsible for producing over one-half of the total value of shellfish sold in all the counties of Massachusetts combined (11). Many types of shellfish harvested on the Cape are consumed raw or only partially cooked. Since a significant number of persons may be at risk, particularly during the summer tourist season when the frequency of outbreaks and isolations is known to be the highest (2, 9, 10), a limited survey was carried out in 10 areas along the perimeter of Cape Cod to estimate the distribution of *V. parahaemolyticus* in samples of soft-shell clams from widely separated areas. This species of clam is commonly sought by local clambers and summer residents and is widely consumed. In the process of completing the present survey a similar study (B. B. McEachern, Abstr. Annu.

Meet. Am. Soc. Microbiol. 1973, p. 2), apparently in progress about the same time as our study, came to our attention, confirming the presence of various K serotypes of *V. parahaemolyticus* in Massachusetts soft-shell clams. Our isolates were compared biochemically and serologically to isolates of *V. parahaemolyticus* previously reported in the literature.

### MATERIALS AND METHODS

**Sampling.** Ten clams were selected at random from each of ten clamming areas on both sides of the Cape (Fig. 1). Each clam was immediately put into a sealed plastic bag and transported to the laboratory in a refrigerated container. All specimens were processed within 4 h of collection.

**Isolation procedures.** Samples of clams were washed and shucked by procedures recommended by the American Public Health Association (1), and a 1:10 (g/ml) dilution of each sample in 7% sterile NaCl solution was homogenized in a Waring blender for 2 min.

The mixture was then serially diluted ( $10^{-2}$  to  $10^{-9}$ ) in 7% sterile NaCl, and 0.1 ml of each dilution was plated onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) and onto Vanderzant modified salt starch agar (MT) (19), a modification of a medium proposed by Twedt et al. (16), using the surface spread plate technique. One milliliter of the original dilution was also inoculated into an enrichment medium consisting of Trypticase soy (TS) broth with 7% NaCl.

The cultures were incubated at 42 C for 18 h. At the end of 18 h, 0.1 ml of each serial dilution ( $10^{-2}$  to  $10^{-9}$ ) of the enrichment culture was inoculated on

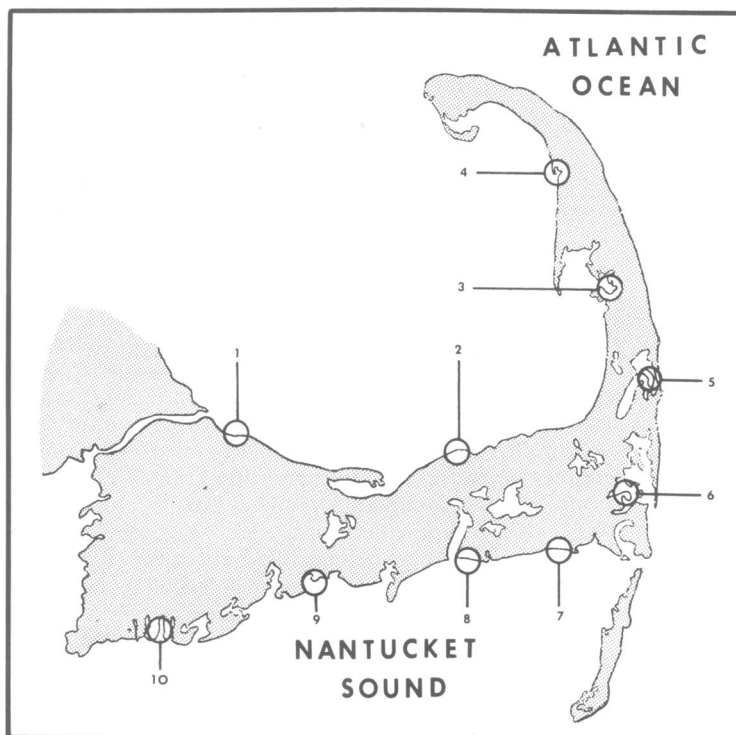


FIG. 1. Cape Cod, Mass. Clamming areas from which soft-shell clam samples were tested for *V. parahaemolyticus*. (Numbers correspond to sample areas in Table 2.)

to TCBS and MT agar and incubated in the same manner.

Sucrose-negative colonies with dark green centers on TCBS were selected for further testing. On MT agar, smooth, nonspreading, nonpigmented colonies showing a large zone of starch hydrolysis at the end of 18 h were considered suspect.

Suspected isolates which were nonspreading on 1.5% agar, gram negative, motile, pleomorphic rods, oxidase positive, and negative for acetylmethylcarbinol production were considered presumptive for *V. parahaemolyticus*. These isolates were grown on *Vibrio* maintenance medium (17) and overlaid with paraffin oil for further study.

**Identification.** Table 1 summarizes the morphological and biochemical tests used for identification of the suspected isolates. Gelatinase production was determined by production of a turbid precipitation around colonies on *Vibrio* maintenance medium. Human blood cells were used in preparation of both the blood agar and Wagatsuma agar. Wagatsuma agar was prepared without sterilization by the methods of Sakazaki et al. (14). In view of the variation in carbohydrate utilization frequently reported, initial carbohydrate fermentation of sucrose and glucose was determined by three methods repeated sequentially. First employed (method 1) was Hugh-Liefson medium in which 1% carbohydrate was autoclave sterilized in the complete basal medium. The second (method 2) was similar to the

first, except for the addition of a sterile Vaspar overlay. A third experiment, which we call the anaerobic method, involved the addition of concentrated membrane-filtered (Millipore) carbohydrate to the sterilized hot Hugh-Liefson basal medium to a final concentration of 1%. This was rapidly cooled to insure stratification, inoculated, and then overlaid with 2% agar (Difco) containing 0.2% sodium thioglycolate.

**Efficiency of recovery.** A brief estimate of the efficiency of recovery that might be expected from clam homogenate was made by parallel plating of dilutions of a TS broth inoculum of *V. parahaemolyticus* directly onto TS agar, MT, and TCBS media, and dilutions of sterile clam homogenate were seeded with the same inoculum and plated as follows. A 1:10 dilution of sterile clam homogenate in 7% NaCl solution (150 ml) was blended for 2 min in a Waring blender, seeded with 1 ml of an 18-h TS broth culture containing  $10^8$  cells of *V. parahaemolyticus* (strain 6-IBOH4-421, Puget Sound, Liston), and again blended for 1 min to disperse the inoculum. This homogenate (0.1 ml) and serial tenfold ( $10^{-2}$  to  $10^{-6}$ ) dilutions of the homogenate (0.1 ml) were inoculated on to TS agar, MT, and TCBS plates by the spread plate technique to determine the number of cells recovered. Seeded plates were inoculated at 42 C for 18 h and counted on a Quebec colony counter. MT and TCBS agars recovered 81 and 84% of the cells, respectively, as compared to the number recovered from homogenates plated on TS agar,

indicating that the efficiency of these media in the presence of clam homogenate substance was approximately equal.

**Serology.** Isolates were identified serologically by means of slide agglutination with O and K antisera (Nichiman Co., New York). The presence of K antigens was determined by the method of Fishbein et al. (6) using both polyvalent and monovalent K antisera. Typing for O antigens was done by the procedure of Montague et al. (12).

## RESULTS AND DISCUSSION

**Isolation and identification of *V. parahaemolyticus*.** Initially we decided to employ both MT and TCBS agars in parallel as primary isolation media. This was to increase the probability of detecting strains of *V. parahaemolyticus* that were either amylase negative, sucrose negative, or sucrose positive, amylase positive, and because preliminary tests with sterile homogenate indicated that the plating efficiency of these two media was approximately equal. However, in direct platings of raw clam samples, "positive" colonies on MT plates were found to be more difficult to recognize (even when using Lugol iodine) than those on the TCBS plates and resulted in a much greater number of false "positive" colonies than on TCBS. Consequently, direct plating on MT media produced only three isolates of *V. parahaemolyticus*, whereas direct plating on TCBS was responsible for 29, and TS broth-7% NaCl enrichment followed by plating on TCBS produced one isolate. Vanderzant et al. (20) also found that fewer "false positives" were picked from TCBS than from MT plates made from enrichment cultures. Their report contrasts the earlier studies of Vanderzant and Nickelson (19) which suggested that of various types of enrichment broths, enrichment in TS broth with 7% NaCl and subsequent plating on MT medium produced the best results. As used by us with 1-ml quantities of clam homogenate, this TS broth-7% NaCl-MT enrichment procedure yielded no isolates presumptively to be considered *V. parahaemolyticus*. This was mainly due to heavy overgrowth and spreading on isolation plates by clam flora. The TS broth-7% NaCl-TCBS enrichment procedure accounted for only one isolation due to the overgrowth and spreading of sucrose utilizing types.

Based upon the approach of Fishbein and Wentz (7) to the testing scheme suggested by R. Sakazaki (symposium presented by the Food and Drug Administration, Washington, D.C., 30 July 1971), and those in the Food and Drug Administration bacteriological analytical manual (18), Table 1 lists the biochemical reac-

TABLE 1. Morphological and biochemical characteristics of isolates considered to be *V. parahaemolyticus*, taken from soft-shell clam samples

Biochemical or morphological test	No. of isolates out of 33	
	Positive	Negative
Growth in 1% Trypticase broth:		
With 0% NaCl	0	33
With 3% NaCl	33	0
With 7% NaCl	33	0
With 10% NaCl	0	33
Malonate utilization	0	33
Citrate utilization	33	0
Nitrate reduction	33	0
Hemolysis on blood agar (human)	6	27
Kanagawa phenomenon	0	33
Growth at 42 C	33	0
Triple sugar iron agar (alkaline/acid)	32	1 <sup>a</sup>
Microscopic morphology (pleomorphic)	33	0
Gram reaction	0	33
Motility	33	0
Spreading on 1.5% agar	0	33
Methyl red test	33	0
Production of:		
β-Galactosidase	0	33
Lysine decarboxylase	33	0
Ornithine decarboxylase	25	8
Arginine dihydrolase	0	33
Tryptophane deaminase	5	28
Catalase	33	0
Urease	0	33
Indole	33	0
Amylase	22	11
Oxidase	33	0
Gelatinase	33	0
Acetylmethylcarbinol	0	33
H <sub>2</sub> S from triple sugar iron agar	0	33
Fermentation of:		
Lactose	29	4
Glucose	33	0
Sucrose	32	1
Cellobiose (within 24 h)	33	0
Arabinose	33	0
Mannitol	33	0
Maltose	33	0
Sorbitol	33	0
Rhamnose	0	33
Inositol	0	33
Adonitol	0	33
Xylose	0	33
Salicin	0	33
Trehalose	33	0

<sup>a</sup> Acid/acid.

tions of the 33 isolates selected out of 86 considered to be presumptive for *V. parahaemolyticus*. They were biochemically sufficiently similar to other United States isolates previously reported (21) to warrant further study, except that 32 were able to produce acid from sucrose, 29 from lactose, and all 33 from sorbitol within 24 h. In addition, 11 isolates did not produce amylase, and one produced acid butt and alkaline slant on triple sugar iron agar.

Only six of the isolates produced beta lysis on human blood agar with 7% NaCl, and none produced hemolysis on Wagatsuma agar (Kanagawa phenomenon).

The literature contains many reports of strains that produce acid from sucrose (3, 8, 17, 20, 21). Also, strains producing acid from lactose have been reported by Twedt et al. (17), and all of the isolates of Bartley and Slanetz (4) from the coastal waters of New Hampshire produce acid from sorbitol. We, however, found a higher proportion of isolates which produced acid from sucrose, lactose, and sorbitol than these workers. Repetition of sucrose and glucose utilization tests, sequentially under three different conditions of exposure to oxygen, revealed that of the 86 isolates considered positive presumptive, the reaction of two isolates was recorded sucrose negative by methods 1 and 2 and positive by the anaerobic method. Three isolates initially sucrose positive by methods 1 and 2 were negative by anaerobic culture. Of the final 33 confirmed isolates, one recorded sucrose positive by methods 1 and 2 was sucrose negative by the anaerobic method, and one isolate showed the opposite change in reaction. We therefore consider the sucrose reaction to be a stable characteristic of our isolates.

In general, similar stability was noted for the other carbohydrates tested. Therefore, we feel that the higher frequency of sucrose-, lactose-, and sorbitol-utilizing types as compared to previous reports may well reflect an environmentally or otherwise induced divergence peculiar to the Cape Cod isolates.

Of the 33 isolates, 11 (33%) were amylase negative on MT medium. Again this represents a higher proportion than previously reported, except for the report of Thomson and Trenholm (15), who found 20 amylase-negative out of 27 isolates (77%). These results may open the question of the general procedural utility of the amylase reaction for making isolations on MT and other starch-containing media, which has been found to be so useful by Vanderzant and Nickelson (19) and others.

**Serotyping.** When typed for O and K antigens, 20 of the 33 isolates reacted with pooled

antisera, and 14 of these reacted with 9 different specific K antisera.

In her isolates from Massachusetts, McEachern (Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, p. 2) reported the presence of 17 distinct K antigens, all of which were different from those found in our cultures. This represents a total of 26 different K serotypes from Massachusetts or 50% of the recognized Japanese types. Only 10 of the 15 typable with specific K antisera reacted with O antisera (Table 2). Four of these isolates had combinations of O and K antigens which have been reported in previous isolations in the United States. These were: O4:K8, O4:K42, O6:K18, and O1:K25 (2). The latter two serotypes have been previously reported (2) from processed lobster meat incriminated in an episode of gastroenteritis in Massachusetts.

Two isolates, O4:K3 and O3:K48, contained combinations of O and K antigens which have never been reported in the United States, and the combination of O4:K3 has not been, to the best of our knowledge, previously reported anywhere.

**Occurrence of *V. parahaemolyticus*.** Table 2 shows that out of 10 areas on Cape Cod, Mass., *V. parahaemolyticus* was demonstrated in soft-shell clams from 8. Because only 10 samples were analyzed from each area, and also because the 2 negative sampling areas were separated by 3 positive areas geographically along the same coast (Fig. 1), no particular ecological significance can be attached to the absence of the organism in the clams from these areas on the basis of the present study. It is interesting to note that the area of highest percentage of clam samples yielding positive isolations (Table 2) and one of the areas with the second highest percentage were both closed to shellfishing due to pollution.

Of greater significance is the number of samples containing *V. parahaemolyticus*. We found this to be 30%; McEachern (Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, p. 2), who reported 46%, employed an enrichment technique often considered to be more sensitive in detection but less suitable for quantification of individual samples than direct plating from which most of our positive cultures were isolated. We feel that these two results, which are quite compatible when differing techniques are considered, indicate a relatively high rate of occurrence of this organism. Consequently, a high potential exists for cross-contamination of uncontaminated clams or other seafood being collected at the same time. The potential hazard is apt to be multiplied under careless handling and summer temperature conditions, with the result that consumers may be com-

TABLE 2. Comparative means and range counts of *V. parahaemolyticus* obtained from soft-shell clam samples collected from clamming areas on Cape Cod, Mass.

Sample areas	Location	No. of samples containing <i>V. parahaemolyticus</i>	Serotypes isolated	Comparative mean and range of total counts of <i>V. parahaemolyticus</i> per gram of clam	
				Mean count	Range
1	Town Neck Beach, Sandwich <sup>a</sup>	5/10 <sup>b</sup>	04:K8, 04:K3 O- <sup>c</sup> :K20	280	100-400
2	Brewster Flats, Brewster	1/10	O-:K1	100	
3	The Cove, Wellfleet	4/10	01:K25	175	100-400
4	Pammet Harbor, Truro	1/10		200	
5	Salt Pond Bay, Eastham	3/10		133	100-200
6	Ryder Cove, North Chatham	0/10			
7	Wychmere Harbor, Harwichport	2/10		100	
8	West Dennis Beach, West Dennis <sup>a</sup>	9/10	03:K48, O-K48 04:K42, O-K57	1,000	300-1,700
9	East Bay Inlet, Centerville	5/10	O-:K20, 06:K18	166	100-300
10	Great Pond, Falmouth	0/10			

<sup>a</sup> Closed to shellfishing due to pollution.

<sup>b</sup> Number positive/number of samples.

<sup>c</sup> Untypable

monly ingesting *V. parahaemolyticus* in larger numbers than is presently realized.

Although all of our isolates were negative for the Kanagawa test, which has thus far been shown to be positive for most isolates from patients (14), the relationship of this test to enteropathogenicity is not yet clear (10), nor is the biological mechanism by which Kanagawa-positive strains originate and predominate in isolations from food-poisoning victims, or the role of probability of selection to Kanagawa positive at various population levels of ingested *V. parahaemolyticus*, if such a selection mechanism is operative.

#### ACKNOWLEDGMENT

We wish to thank J. Liston, University of Washington, for the Puget Sound isolate used as a reference strain in this study.

#### LITERATURE CITED

- American Public Health Association. 1970. Recommended procedures for the examination of sea water and shellfish, 4th ed. American Public Health Association, New York.
- Barker, W. H. 1974. Public health: *Vibrio parahaemolyticus* outbreaks in the United States. *Lancet* 1:551-554.
- Baross, J., and J. Liston. 1970. Occurrence of *Vibrio parahaemolyticus* and related hemolytic vibrios in marine environments of Washington State. *Appl. Microbiol.* 20:179-186.
- Bartley, C. H., and L. W. Slanetz. 1971. Occurrence of *Vibrio parahaemolyticus* in estuarine waters and oysters of New Hampshire. *Appl. Microbiol.* 21:965-966.
- Center for Disease Control. 1973. Surveillance summary. *Vibrio parahaemolyticus* gastroenteritis—United States, 1969-1972. *Morbidity and Mortality Weekly Report* 22:231-232.
- Fishbein, M., I. J. Mehlman, and J. Pitcher. 1970. Isolation of *Vibrio parahaemolyticus* from the processed meat of Chesapeake Bay blue crabs. *Appl. Microbiol.* 20:176-178.
- Fishbein, M., and B. Wentz. (1973). *Vibrio parahaemolyticus* methodology for isolation from seafoods and epidemic specimens. *J. Milk Food Technol.* 36:118-123.
- Kampelmacher, E. H., L. M. Van Noorle, D. A. Jensen, A. Mossel, and F. J. Groen. 1972. A survey of the occurrence of *Vibrio parahaemolyticus* and *V. alginolyticus* on mussels and oysters and in estuarine waters in the Netherlands. *J. Appl. Bacteriol.* 35:431-438.
- Kaneko, T., and R. R. Colwell. 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. *J. Bacteriol.* 113:24-32.
- Liston, J., and J. Baross. 1973. Distribution of *Vibrio parahaemolyticus* in the natural environment. *J. Milk Food Technol.* 36:113-116.
- Massachusetts Department of Natural Resources, Division of Marine Fisheries. 1973. Annual report, p. 14-15. Massachusetts State Department of Administration and Finance, Boston, Mass.
- Montague, T. S., R. A. LeClair, and H. Zen-Yoji. 1971. Typing of O antigens of *Vibrio parahaemolyticus* by a slide agglutination test. *Appl. Microbiol.* 21:949-950.
- Roland, F. B. 1970. Leg gangrene and endotoxin in shock due to *Vibrio parahaemolyticus*; an infection acquired in New England coastal waters. *N. Engl. J. Med.* 283:319.
- Sakazaki, R., K. Tamura, T. Kato, Y. Obara, S. Yamai,

- and K. Hobo. 1968. Studies of the enteropathogenic facultatively halophilic bacteria, *Vibrio parahaemolyticus*. III. Enteropathogenicity. *Jpn. J. Med. Sci. Biol.* **21**:325-331.
15. Thomson, W. K., and D. A. Trenholm. 1971. The isolation of *Vibrio parahaemolyticus* and related halophilic bacteria from Canadian Atlantic shellfish. *Can. J. Microbiol.* **17**:545-549.
16. Twedt, R. M., B. E. Ivins, R. M. E. Novelli, R. G. Thompson, and H. E. Hall. 1970. Selective and differential medium for *Vibrio parahaemolyticus*. *Bacteriol. Proc.* p. 6.
17. Twedt, R. M., P. L. Spaulding, and H. E. Hall. 1969. Morphological, cultural, biochemical, and serological comparison of Japanese strains of *Vibrio parahaemolyticus* with related cultures isolated in the United States. *J. Bacteriol.* **98**:511-518.
18. U.S. Department of Health, Education, and Welfare. 1972. Food and Drug Administration bacteriological analytical manual 14.20.07. U.S. Department of Health, Education, and Welfare, Washington, D.C.
19. Vanderzant, C., and R. Nickelson. 1972. Procedure for isolation and enumeration of *Vibrio parahaemolyticus*. *Appl. Microbiol.* **23**:26-33.
20. Vanderzant, C., C. A. Thompson, and S. M. Ray. 1973. Microbial flora and level of *Vibrio parahaemolyticus* of oysters (*Crassostrea virginica*), water and sediment from Galveston Bay. *J. Milk Food Technol.* **36**:447-452.
21. Zen-Yoji, H., R. A. LeClair, K. Ohta, and T. S. Montague. 1973. Comparison of *Vibrio parahaemolyticus* cultures isolated in the United States from those isolated in Japan. *J. Infect. Dis.* **127**:237-241.
22. Zide, N., J. Davis, and N. J. Ehrenkranz. 1974. Fulminating *Vibrio parahaemolyticus* septicemia. *Arch. Intern. Med.* **133**:479-481.