Vibrio parahaemolyticus Gastroenteritis in Maryland: Laboratory Aspects

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Received for publication 3 May 1972

Vibrio parahaemolyticus was the etiologic agent in three food-related epidemics of gastroenteritis in Maryland, during August 1971. These outbreaks involved crab food products. Fifteen isolates of V. parahaemolyticus were made which included 11 from patients and 4 from foods. Serotype 04:K11 was the cause of the outbreaks. It was recovered from patients in each outbreak and gave a positive Kanagawa reaction, an indication of enteropathogenicity. Other patient isolates included types 03:K30, 03:K33, and an untypable isolate, all of which were Kanagawa negative. Food isolates included serotypes 03:K30, 02:K28, and two untypable isolates, all of which were Kanagawa negative. The outbreaks reported in this paper constitute the first confirmed foodborne epidemics due to V. parahaemolyticus in the United States. Methods for the isolation and identification of V. parahaemolyticus are presented, including a procedure for the simple conversion of conventional laboratory media into suitable culture media for this halophilic organism.

Vibrio parahaemolyticus is a facultatively halophilic organism which has been repeatedly isolated from seafood products during summer food poisoning outbreaks in Japan. It was first isolated by Fujino et al. (7) in 1951 while investigating a foodborne outbreak in Osaka and was named Pasteurella parahemolytica. Taki-kawa (18) later isolated a similar halophilic organism from a food poisoning outbreak and classified it as Pseudomonas enteritis. Miyamoto et al. (12) proposed establishment of the genus Oceanomonas for this halophile. Finally, Sakazaki et al. (16) in 1963 established the taxonomic position of the organism by means of a detailed study of 1,702 cultures and proposed the name Vibrio parahaemolyticus. At the same time he called attention to two subgroups within the species. Subgroup 1 was considered to be enteropathogenic, but subgroup 2 was not.

Zen-Yoji et al. (28), on the basis of numerical taxonomic studies of these two subgroups, proposed that subgroup 2 be excluded from the species designation. Sakazaki (15) concluded thereafter that a fundamental difference existed between the two subgroups and proposed that subgroup 2 henceforth be given a separate species identification and be known as Vibrio alginolyticus.

Kato et al. (9) from the Kanagawa Prefectural Public Health Laboratory, Yokahama, showed that strains of V. parahaemolyticus which were obtained from stools of enteritis patients were hemolytic on a special high-salt blood agar; on the other hand, those strains which were derived from seafoods or marine waters were not hemolytic on this medium. The terms “Kanagawa” positive and negative have been applied to this particular hemolytic test. This special high-salt blood agar was modified by Wagsutsama (11). Further studies of 3,370 V. parahaemolyticus cultures by Saka-zaki et al. (17) showed that 96.5% of the human patient isolates were Kanagawa positive, whereas 99% of the seafood and marine isolates were Kanagawa negative.

In addition to Japan, the organism has been reported in certain marine forms and environs in Germany (14), the Netherlands (8), Hawaii (26), Korea (5), and in southeast Asia (27). Although the organism has been isolated from seafish and coastal sea waters in Europe, examination of over 3,000 stools of gastroenteritis patients in Germany (1, 13) did not reveal
V. *parahaemolyticus*. It has been recovered from cases of diarrhea in Calcutta (4).

In the United States, *V. parahaemolyticus* has been recovered from shellfish (3, 10, 25), processed crab-meat (6), marine waters and sediments (2, 3), and also from cases of localized tissue infection (20). Although it has been reported as the probable cause of four unconfirmed outbreaks of foodborne illness in the state of Washington (21, 22), its role as an etiological agent of foodborne illness has not been firmly established in this country.

Three outbreaks of food-associated gastroenteritis which occurred in Maryland in August 1971 were studied. Epidemiological investigation (T. Dadisman et al., manuscript in preparation) indicated steamed crabs served at two unrelated picnics as the suspect vehicle in two outbreaks, and a crab salad served at a medical institution in the third. The three incidents involved about 425 persons (57.0%) of an estimated 745 at risk. In this article, we present laboratory data on the isolation and identification of *V. parahaemolyticus* from persons and foods involved in these outbreaks. The epidemiological and clinical details are described elsewhere (T. Dadisman et al., manuscript in preparation; 23).

**MATERIALS AND METHODS**

**Food samples.** Approximately 1 dozen steamed crabs was obtained from each outbreak from picnic attendees who had taken some home for later consumption. All samples obtained from these sources had been refrigerated after being taken home. The maximum period of such refrigeration prior to bacteriological examination was 10 days. A sample of leftover crab salad involved in the institutional outbreak was obtained, which had been refrigerated 2 days prior to examination.

**Stool specimens.** Stools were initially examined in each outbreak to determine *Salmonella* or *Shigella* involvement. Subsequent stool specimens, and rectal swabs in some cases, were collected and reexamined for these agents as well as *V. parahaemolyticus* and enteropathogenic *Escherichia coli*. Of 63 stools and 11 rectal swabs examined, 44 were examined for *Salmonella* and *Shigella*, and 40 were examined specifically for the vibrio. Enteropathogenic *E. coli* examinations were limited to those stools found to contain the vibrio.

Food and fecal materials were collaboratively analyzed by the Laboratories and Research Administration, Maryland State Department of Health and Mental Hygiene, Baltimore, Md., and the Division of Microbiology, Food and Drug Administration, Washington, D.C. Materials were initially received and cultured by the Maryland State Laboratory. Suspect *V. parahaemolyticus* isolates, as well as food and stool specimens, were sent to the Food and Drug Laboratory for confirmation both of identity and isolation.

**Media.** Media were prepared either from original formulas or from dehydrated products obtained from Difco Laboratories, Detroit, Mich., or from BBL, Division of BioQuest, Cockeysville, Md. Where necessary, the media were modified as described in *Bacteriological Analytical Methods* (24) to contain an increased concentration of sodium chloride.

After isolation of the organism on thiosulfate citrate bile salts sucrose (TCBS) media, experiments were carried out to determine the feasibility of characterizing this halophilic vibrio by using conventionally prepared biochemical test media supplemented by the dropwise addition of a concentrated salt solution. Two drops of a sterile aqueous 30% NaCl solution were added to 5 ml of the conventional broth medium. In the case of conventional slanted media, the surface was moistened with a few drops of the salt solution prior to inoculation with the organism.

**Control culture.** A culture of *V. parahaemolyticus* 33C10 was furnished to the Maryland State Laboratory by the Division of Microbiology, Food and Drug Administration, Washington, D.C., which was used as a control culture. This enteropathogenic, Kanagawa-positive isolate was supplied to the latter laboratory by Riichi Sakazaki of the Japanese Institute of Health, Tokyo, Japan.

**Bacteriological procedures.** Initially, stools were collected and delivered to the laboratory in buffered glycerol saline containers for *Salmonella* and *Shigella* examination. Subsequent stool specimens for vibrio examination in the steamed crab outbreaks were collected in clean, untreated containers and delivered the same day to the laboratory for examination. In the crab salad outbreak, additional stools were collected, and a few grams was placed in a 1% Trypticase-5% NaCl solution contained in a 50-ml bottle and was mailed to the laboratory. In addition, 11 rectal swabs were collected later in this outbreak, placed in tubes which contained 1 ml of 1% peptone water-3% NaCl, pH 7.4, and delivered immediately to the laboratory.

Examination for the vibrio was made by directly streaking the stool specimens on TCBS agar plates and also by inoculation into a tube of 1% peptone water-3% NaCl, pH 7.4, for enrichment. Incubation was at 35 C for 18 hr. After incubation, the enriched broth cultures were streaked on TCBS agar plates which were incubated at 35 C for 18 hr. Individual blue-green colonies were inoculated on triple sugar iron slants which contained 3% NaCl. After 18 hr of incubation at 35 C, isolates which produced an alkaline slant and an acid butt with no gas or H₂S, and proved to be gram-negative rods, were tested further biochemically by the methods of Fisher et al. (6) and *Bacteriological Analytical Methods* (24). The Kanagawa test (11) was performed with freshly drawn human blood.

Samples of picked, steamed crab meat and crab salad were prepared in 3% NaCl diluent in dilutions up to 1:100,000. These dilutions were streaked directly on TCBS agar, enriched overnight (1-ml sam-
parahaemolyticus in 3% NaCl Trypsicase soy broth at 35 C, or both, before streaking on TCBS agar for isolation of the vibrio.

Examination of stool specimens and food samples for enteropathogenic E. coli was made according to the procedures described in Bacteriological Analytical Methods (24).

Serology. Serological identification was accomplished by the slide agglutination test employing V. parahaemolyticus antisem obtained from the Nichimien Company, New York, N.Y., by the methods described by Fishbein et al. (6).

RESULTS AND DISCUSSION

Of 40 stools and rectal swabs examined for V. parahaemolyticus, this organism was recovered from 11 different individuals; 10 isolations were from stools and one was from a rectal swab. Of these individuals, four were involved in the first outbreak, one in the second, and six in the third. The organism was also isolated from four different crab samples. Two isolations were made from steamed crabs from the first outbreak, one from steamed crabs involved in the second episode, and one from the crab salad involved in the third epidemic. Biochemical and serological data used to identify the isolates are presented in Table 1.

Identical results were obtained with the control and all of the isolates by using either regularly prepared high-salt V. parahaemolyticus media or conventionally prepared media supplemented by the dropwise addition of a 30% NaCl solution.

Best recovery results were obtained from the specimens enriched in 1% peptone water-3% NaCl broth prior to TCBS inoculation. A few specimens were found to contain only a few colonies when directly streaked, but yielded the organism in abundance after overnight enrichment in the broth prior to TCBS inoculation.

Twelve of the isolates were serotypable, but three were not. Serotype 04:K11 was the only Kanagawa-positive isolate made from the fecal specimens. It was recovered from patients in each epidemic and, therefore, was considered as the cause of all three separate and unrelated outbreaks. Other stool isolates included types 03:K30, 03:K33, and an untypable isolate, all of which were Kanagawa negative. Food isolates included serotypes 03:K30, 02:K28, and two untypable isolates, all of which were Kanagawa negative. Salmonella, Shigella, and enteropathogenic E. coli were not found in any of the stool or food samples examined for these agents.

It is noteworthy that serotype 04:K11, which was recovered from patients in all three epidemics, was not recovered from the incriminated foods. This is not unusual inasmuch as this epidemic behavior pattern parallels the usual Japanese experience with this organism. Teramoto et al. (19) described three restaurant outbreaks occurring in a period of 2 years in Kobe which involved 130 diners of whom 59 became ill. In these epidemics, six serotypes, only one of which was Kanagawa positive, were recovered from the patients. Significantly the latter isolate was serotype 04:K11. Analysis of the incriminated foods disclosed the recovery of five serotypes, not one of which matched those found in the patients. This was similar to our experience in the Maryland outbreaks.

Zen-Yoji et al. (29) and Sakazaki (R. Sakazaki, Symposia presented by the Division of Microbiology, Food and Drug Administration, Washington, D.C., 30 July 1971) reported the recovery of multiple serotypes among patients in a single outbreak, such as we observed, and multiple pathogenic types from individual patients in Japan and India, respectively.

Epidemiological investigation of these outbreaks (T. Dadisman et al., manuscript in preparation) revealed their cause to be the same as in most foodborne outbreaks, i.e., improper food handling techniques. The steamed crabs involved in the first and second outbreaks (after preparation) were shipped, stored with live crabs, or both, which led to cross-contamination prior to consumption. The crab salad involved in the third outbreak was prepared from contaminated canned crab meat. The latter consisted of the meat of steamed crabs which was picked and packed into cans which were then lidded with snap-on-type lids with no further heat treatment applied to the cans. Such cans are supposedly refrigerated from packing until opening for consumption. It was concluded that the crab meat involved in this outbreak was probably contaminated with the vibrio during the picking and packing process.

The foregoing data provided laboratory support to clinical and epidemiological evidence, thereby establishing the incidents reported herein and elsewhere (T. Dadisman et al., manuscript in preparation; 23) to be the first confirmed foodborne outbreaks due to V. parahaemolyticus in the United States.

ACKNOWLEDGMENT

We are indebted to Riichi Sakazaki of the Japanese National Institute of Health, Tokyo, Japan, for the biochemical, serological and Kanagawa confirmation of all isolates obtained in these outbreaks.
Table 1. Characteristics of *V. parahaemolyticus* isolates from foodborne outbreaks in Maryland, August 1971

| Reactions                              | Outbreak 1  | Outbreak 2  | Outbreak 3  | Rectal Swab | Crab Salad |
|----------------------------------------|-------------|-------------|-------------|-------------|------------|
|                                        | Stools      | Crabs       | Stool       | Crabs       | Stools     |
|                                        | 33°C        | 33°C        | 33°C        | 33°C        | 33°C       |
|                                        | 8657*       | 8658        | 8659        | 8700        | 2882       |
|                                        | 3659        | 3565        | 3565        | 3574        | 3574       |
|                                        | 3574        | 33AC        | 10782       | 10782       | 10782      |
|                                        | 11497       | 11590       | 11590       | 12105       | 12105      |
|                                        | 12105       | 11983       | 11983       | 12204       | 12204      |
|                                        | 12204       | 4263        | 4263        | 4263        |            |

**V. parahaemolyticus** 33°C control culture

| Triple sugar iron | + | + | + | + | + | + | + | + | + | + |
| (Alkaline/Acid)   | - | - | - | - | - | - | - | - | - | - |
| (H₂S, Gas)        | - | - | - | - | - | - | - | - | - | - |
| Growth in 1% Trypticase | - | - | - | - | - | - | - | - | - | - |
| with 0 or 10% NaCl | + | + | + | + | + | + | + | + | + | + |
| with 3, 6, or 8% NaCl | + | + | + | + | + | + | + | + | + | + |
| Purple broth sugars | A<sup>+</sup> | A | A | A | A | A | A | A | A | A |
| Glucose           | A<sup>c</sup> | A | A | A | A | A | A | A | A | A |
| Malrose           | A | A | A | A | A | A | A | A | A | A |
| Mannitol          | A | A | A | A | A | A | A | A | A | A |
| Trehalose         | A | A | A | A | A | A | A | A | A | A |
| Arabinosine       | A | A | A | A | A | A | A | A | A | A |
| Sucrose           | - | - | - | - | - | - | - | - | - | - |
| Lactose           | - | - | - | - | - | - | - | - | - | - |
| Inositol          | - | - | - | - | - | - | - | - | - | - |
| Salicin           | - | - | - | - | - | - | - | - | - | - |
| Xylose            | - | - | - | - | - | - | - | - | - | - |
| Adonitol          | - | - | - | - | - | - | - | - | - | - |
| Rhamnose          | - | - | - | - | - | - | - | - | - | - |
| Hugh Leifson glucose | F<sup>a</sup> | F | F | F | F | F | F | F | F | F |
| Lysine decarboxylase | + | + | + | + | + | + | + | + | + | + |
| Ornithine decarboxylase | + | + | + | + | + | + | + | + | + | + |
| Arginine dehydrodrolase | - | - | - | - | - | - | - | - | - | - |
| Phenylalanine deaminase | - | - | - | - | - | - | - | - | - | - |
| Gelatin liquefication | + | + | + | + | + | + | + | + | + | + |
| Indole production  | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction  | + | + | + | + | + | + | + | + | + | + |
| Methyl red         | + | + | + | + | + | + | + | + | + | + |
| Voges-Proskauer    | - | - | - | - | - | - | - | - | - | - |
| Motility           | + | + | + | + | + | + | + | + | + | + |
| Urease             | - | - | - | - | - | - | - | - | - | - |
| Catalase           | + | + | + | + | + | + | + | + | + | + |
| Citrate (Simmons)  | + | + | + | + | + | + | + | + | + | + |
| Oxidase            | + | + | + | + | + | + | + | + | + | + |
| Hemolysis on       | - | - | - | - | - | - | - | - | - | - |
| Sheep blood agar   | - | - | - | - | - | - | - | - | - | - |
| Human blood agar   | - | - | - | - | - | - | - | - | - | - |
| Growth at 43°C     | + | + | + | + | + | + | + | + | + | + |
| Kanagawa test      | U | 04:03 | 04:04 | 04:05 | 04:06 | 04:07 | 04:08 | 04:09 | 04:10 | 04:11 |
| Serology           | K11 | K11 | K11 | K11 | K11 | K30 | K11 | K28 | K33 | K11 |

<sup>a</sup> Strain of *V. parahaemolyticus*.

<sup>b</sup> Negative for H₂S after 18 hr of incubation, trace noted after a few days.

<sup>c</sup> Acid, no gas.

<sup>d</sup> Fermentation.

<sup>e</sup> Untypable.

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