Occurrence of *Vibrio parahaemolyticus* and Related Hemolytic Vibrios in Marine Environments of Washington State

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Samples of sediment, water, and fauna were tested for the presence of *Vibrio parahaemolyticus* and the related biotype *V. alginolyticus*. Altogether, 379 samples were analyzed quantitatively by using a starch-agar medium. Invertebrate and sediment samples were invariably positive for *V. parahaemolyticus*, whereas water samples were quite variable. Samples of the Pacific oyster (*Crassostrea gigas*), obtained on a regular basis for 26 months from a single environment, showed a close correlation between total numbers of mesophilic vibrios and the overlying water temperature; the seasonal counts of oysters ranged from less than 10 to greater than 100,000 per g. Ecological implications and possible pathogenicity of these vibrios are discussed.

Since Fujino et al. published the first report on food poisoning caused by *Vibrio parahaemolyticus* in 1953 (7), the organism has been repeatedly isolated from food materials and environmental samples taken in Japan (1, 3, 8, 23). The organism may be isolated readily from coastal waters, sediments, and marine fauna with high frequency during summer months but not in winter (1, 13). It is a major cause of gastroenteritis in Japan and outbreaks show the same seasonal pattern (17). In the last 2 years, there have been reports of isolation of *V. parahaemolyticus* from sediments, water, and oyster and crab samples in the United States and from marine fish and water in Germany, Korea, and Hawaii (4, 5, 14, 22, 24). In all isolations from the natural environment, it has been necessary to distinguish *V. parahaemolyticus* from other mildly halophilic vibrios which are abundantly present. There has been no confirmed report of food poisoning caused by *V. parahaemolyticus* in the United States but the organism has been indicted as the cause of localized severe wound infections contracted at marine bathing beaches (21). *V. parahaemolyticus* has also been identified as the probable cause of mortalities in blue crab (*Callinectes sapidus*) in Chesapeake Bay (10), suggesting a diverse host range for the organism.

We have already made a preliminary report (4) of the isolation of *V. parahaemolyticus* from inshore marine waters of Washington State. The present paper reports the results of an extensive study of the distribution of *V. parahaemolyticus* and related haemolytic vibrios in the marine environment of Puget Sound and inshore coastal waters. Evidence is presented showing a seasonal incidence of *V. parahaemolyticus* and *V. alginolyticus* in oysters and the extent of occurrence of these organisms in other mollusks, crustaceae, fin fish, water, and sediment is noted and discussed.

**MATERIALS AND METHODS**

**Sampling.** All samples were obtained from the marine environment and worked up within 8 hr. Sediment, water, and fish samples were obtained either from Puget Sound or from off the coast of Washington by using the M. V. Commando, the College of Fisheries vessel. Sediments and water samples were taken with a geological corer and a sterile Liston-Lighthart water sampler (12), respectively. Sediment and water samples from oyster beds were obtained by using sterile glass bottles.

For the determination of possible seasonal distribution of mesophilic vibrios, including *V. parahaemolyticus*, extensive investigations were carried out at the commercial oyster bed and site of the Northwest Shellfish Sanitation Laboratory at Purdy, Wash., over a period of 26 months. Pacific oysters (*Crassostrea gigas*) and water samples were taken from three areas on the bed, selected because the oysters at these points were submerged under water at all tidal levels. Temperature measurements were made of the overlying water at the time oyster samples were obtained. Samples of 3 to 20 oysters were obtained at least once per month with biweekly samples from July, August, and September.

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Samples of the Olympia oyster (Ostrea lurida), clams (Venerupis japonica and Protothaca staminea), and additional samples of Pacific oysters were obtained on an irregular basis from various environments throughout the Pacific Northwest. Fin fish were obtained by otter trawl.

**Isolation procedures.** Samples of oysters and clams were washed and shucked by procedures recommended by the American Public Health Association (2), and an equal weight by volume (grams/milliliter) mixture of the sample and sterile buffered 3% saline solution at pH 7.5 was homogenized in a Waring Blender for 60 sec. The mixture was serially diluted by decimal intervals in buffer, and samples of 0.1 ml of each dilution (10^{-2} to 10^{-9}) were plated onto salt-starch-agar (0.5% soluble starch; 0.3% peptone (Difco); 0.1% yeast extract (Difco); 5% NaCl; 1.5% agar; and distilled water adjusted to pH 7.5) by using the spread plate technique. Plates were incubated for 24 to 36 hr at 37 C in an anaerobic jar (BBL, gas pack). Starch hydrolysis was indicated by the formation of a precipitate as a halo around positive colonies. Where halos were not distinct, suspect colonies were circled with a marking pencil, and plates were incubated for an additional 6 hr at 37 C aerobically, allowing halos to become definite in positive cultures. Usually plates with more than 100 colonies showed confluent lysis of starch, and colonies had to be isolated on the basis of their morphology.

Colonies which were smooth, transparent, and non-pigmented and showed starch hydrolysis were considered presumptive positive vibrios. Spreading non-pigmented colonies were usually indicative of biotype 2, *V. alginolyticus*.

Sediments and water samples were processed in the same way with the exception that 2.5 units of penicillin per ml was added to the medium for sediment samples to reduce the numbers of gram-positive aerobic and anaerobic *Bacillus*. Sediment samples yielded more diversity of organisms than shellfish or water samples; sediment samples taken in the last part of the study were incubated at 42 C, since this was found to yield a higher proportional recovery of hemolytic vibrios though the total count was reduced.

All fin fish were sampled on board the research vessel by swabbing the gills, skin, and intestinal contents with sterile cotton swabs. The swabs were streaked onto salt-starch-agar which was incubated anaerobically at 37 C. In some cases, the swabs were streaked directly onto the Japanese selective agar media BTB-Teepol, TCBS (16), and AAC (8), or placed immediately after sampling into the Japanese liquid enrichment media, glucose-salt-teepol, TCBS (16), and AE (8).

**Identification of vibrios.** Amylase-positive colonies were streaked for purification onto vibrio maintenance medium [1% Trypticase (BBL), 3% gelatin, 2% NaCl, 1.5% agar, made up with distilled water and adjusted to pH 7.2]. This medium also indicates gelatinase production by the formation of a turbid precipitate around colonies. Gelatinase-positive isolates were Gram stained and tested for their oxidase reaction (9). Gram-negative, oxidase-positive pleomorphic rods were streaked onto blood-agar (BBL blood agar base and 1% NaCl, with 5% human defibrinated whole blood). Organisms that showed beta-hemolysis at 37 C were considered presumptive *V. parahaemolyticus*. All organisms were further tested in accordance with the schemes of Shewan and Hobbs (19) for gram-negative rods and Sakazaki et al. (17) for the differentiation of *V. parahaemolyticus* from the related biotypes *V. alginolyticus* and *V. anguillarum* (Table 1).

**RESULTS**

**Taxonomy.** Sakazaki et al. (17) described three biotypes of *V. parahaemolyticus*. These organisms were found to differ in their ability to grow at various salt concentrations, ferment various sugars, and produce acetylmethylcarbinol (Table 1). Biotype 1, *V. parahaemolyticus*, and biotype 2, *V. alginolyticus*, are considered possible foodborne pathogens, whereas biotype 3, *V. anguillarum*, is not. The hemolytic vibrio isolates obtained from samples of oysters, clams, water, and sediment from Puget Sound were found to fall into three distinct groups. The most commonly occurring vibrio was identical with Saka- zaki’s biotype 1, with the exception that it fermented sucrose. The second group, amounting to about 25% of the isolates, was identical with the biotype 1. The third group of vibrios, identical with biotype 2, *V. alginolyticus*, was found to occur only during the spring and summer months when they would account for greater than 50% of the mesophilic vibrio population isolated at these times. Biotype 2 was easily recognized by its swarming growth on ordinary 1.5% agar medium, production of acetylmethylcarbinol, and ability to grow in media containing 10% NaCl. Biotype 3, *V. anguillarum*, was not encountered, probably because it does not grow on the salt-starch medium employed in this investigation. The fermentation of cellobiose was not considered a good differentiating characteristic since a considerable portion of the Japanese strains of *V. parahaemolyticus* utilized this sugar (4, 21).

Table 2 summarizes some of the data showing the similarities between the Japanese strains of *V. parahaemolyticus* and the comparable vibrios isolated from Puget Sound. These data clearly show that the only characteristic difference between these vibrios is their ability to ferment sucrose. However, 7 of the 40 Japanese type strains of *V. parahaemolyticus* were found to utilize this sugar. These data do not include all biochemical characteristics. The characteristics described represent those stressed by the Japanese investigators as being the most important for clinically screening presumptive *V. parahaemolyticus* cultures. In addition to the more familiar
Table 1. Differentiation of *Vibrio* parahaemolyticus (biotype 1) from *V. alginolyticus* (biotype 2) and *V. anguillarum* (biotype 3)*

| Characteristic                        | 1, *V. parahaemolyticus* | 2, *V. algino-lyticus* | 3, *V. anguillarum* |
|---------------------------------------|---------------------------|-------------------------|---------------------|
| Growth in 1% Trypsicase broth         | +                         | -                       | +                   |
| With 0% NaCl                          | +                         | +                       | +                   |
| With 7% NaCl                          | +                         | -                       | -                   |
| With 10% NaCl                         | -                         | +                       | -                   |
| Voges-Proskauer reaction              | -                         | + or -                  |                     |
| Sucrose fermentation                  | +                         | + or -                  |                     |
| Cellobiose fermentation (within 24 hr)| -                         | -                       | +                   |
| Colony morphology on 1.5% agar        | Smooth                    | Swarming                | Smooth              |

* Differentiation scheme based on data of Sakazaki et al. (17).

biochemical tests, many other tests, including serological tests with the O and K antigens, phage typing, guanine plus cytosine content, and deoxyribonucleic acid (DNA)-DNA homologies, have been performed with these organisms. All hemolytic mesophilic vibrios meeting the general classification of *V. parahaemolyticus* without regard to sucrose fermentation are therefore described as *V. parahaemolyticus* in the report and are considered together as far as distribution is concerned. A report dealing with the taxonomic comparison between related marine vibrios is forthcoming.

Distribution. Altogether, 379 samples were analyzed for *V. parahaemolyticus*. The overall results of the analysis are shown in Table 3. In this table, results for the highly variable shallow oyster growing areas and the relatively stable deeper Puget Sound areas are shown separately. The organisms were always found in sediment from both areas, but the incidence in water was much lower in the colder Puget Sound area. Shellfish were found always to harbor hemolytic vibrios, but counts varied at different seasons of the year. The variability in counts of hemolytic vibrios among water samples from shallow intertidal areas and deep Puget Sound water is illustrated in Table 4. Higher counts were more common in intertidal waters than in Puget Sound waters. Moreover, deep water samples from Puget Sound never showed a detectable level of hemolytic vibrios. A somewhat similar pattern for sediment samples is indicated by the data in Table 5. There is indeed evidence of a decreasing incidence of hemolytic vibrios among sediments taken from areas increasingly remote from land influences. However, in view of the mesophilic nature of the...

Table 2. Biochemical characteristics of Japanese serotypes of *Vibrio* parahaemolyticus and U.S. strains of related hemolytic vibrios, excluding *V. alginolyticus*, from oyster beds

| Reactiona | Japanese serotypesb, *V. parahaemolyticus* (40)c | Oyster bed isolates |
|-----------|--------------------------------------------------|---------------------|
|           | *Oysters* (410) | *Clams* (98) | *Water* (86) |
| Growth in 1% Trypsicase broth | + | - | - |
| With 0% NaCl | 0d | 0 | 0 |
| With 3% NaCl | 40 | 410 | 98 |
| With 7% NaCl | 40 | 410 | 98 |
| With 10% NaCl | 0 | 0 | 0 |
| Acetyl methyl-carbinol | 0 | 0 | 0 |
| Methyl red | 40 | 410 | 98 |
| Nitrate reduction to nitrite | 40 | 410 | 98 |
| Hemolysis of human blood | 40 | 410 | 98 |
| Growth at 40 C | 40 | 410 | 98 |
| Sucrose (acid only) | 7 | 293 | 68 |
| Gelatin liquefaction | 40 | 410 | 98 |
| H2S from triple sugar iron agar | 40 | 410 | 98 |
| Starch hydrolysis | 40 | 410 | 98 |
| Chitin hydrolysis | 33 | 410 | 98 |
| Pleomorphic formation of spirals and spheroplasts | 40 | 410 | 98 |
| Sensitivity to 0-129 (19) | 40 | 410 | 98 |
| Sensitivity to 2.5 units of penicillin | 0 | 0 | 0 |

* All media, unless designated, were made up with seawater or 3% NaCl. All reactions were run at 37 C, except chitin hydrolysis which was run at 22 C.
* Cultures received from R. Sakazaki, Japan, and R. R. Colwell, Georgetown University, Washington, D.C.
* Numbers in parentheses indicate the number of cultures tested.
* Values in table indicate total positive.

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TABLE 3. Incidence of Vibrio parahaemolyticus in marine samples

| Sample          | Sampling area |
|-----------------|---------------|
|                 | Oyster areas  | Puget Sound |
|                 | Total no.     | Per cent positive | Total no. | Per cent positive |
| Water           | 67            | 78               | 39        | 18               |
| Sediment        | 7             | 100              | 16        | 100              |
| Fish            | 0             |                  | 174       |                  |
| Oysters         | 61            | 100              | 0         |                  |
| Clams           | 9             | 100              | 0         |                  |
| Crab intestine  |               |                  | 6*        | 100              |

* All samples were from Bellingham Bay.

TABLE 4. Total counts of mesophilic hemolytic vibrios from Puget Sound and intertidal water samples

| Location         | No. of samples | Range of vibrio counts/ml of water sample |
|------------------|----------------|------------------------------------------|
|                  |                | 10 | 11-100 | 101-1,000 | 101-5,000 | 5,000 |
| Purdy oyster bed | Lagoon         | 24 | 10    | 5        | 9        | 0      | 0      |
| Creek            | 18             | 7  | 5      | 7        | 1        | 0      |
| Oyster bed A     | 13             | 7  | 3      | 3        | 0        | 0      |
| Oyster bed B     | 13             | 7  | 3      | 2        | 1        | 0      |
| Oyster waste     | 4              | 2  | 0      | 1        | 0        | 1      |
| Puget Sound      | Surface water  | 15 | 12     | 1        | 2        | 0      | 0      |
| Bottom water     | 24             | 24 | 0      | 0        | 0        | 0      |

vibrios, this may reflect more an effect of temperature rather than of land contamination.

The distribution of counts among oyster and clam samples is shown in Table 6. In the majority of cases, the number of hemolytic vibrios was in excess of 1,000 per g of shellfish meat. This should be compared with the much lower level of incidence of V. alginolyticus which is shown in Table 7. Of course, as has already been noted, high counts of this organism occurred only in the summer.

The seasonal incidence of V. parahaemolyticus in Purdy oysters is illustrated in Fig. 1. Mean water temperatures are included in the graph for comparison. There is a very clear correlation between season, water temperature, and the level of incidence of V. parahaemolyticus in the oysters. This correlation with water temperature is shown clearly in Fig. 2.

Similar information is shown for V. alginolyti-

TABLE 5. Comparative mean and range of counts (in logs) of hemolytic mesophilic vibrios obtained from oceanic, Puget Sound, and oyster bed sediments

| Area sampled                  | No. of samples | Mean count/g of sediment | Range /g of sediment |
|-------------------------------|----------------|--------------------------|----------------------|
| Off coast of Washington       | 3              | ~1                       | <4 → 2               |
| Puget Sound (off shore)       | 8              | 2.7                      | 2.2 → 3.5            |
| Puget Sound (inshore)         | 9              | 3.2                      | <2 → 4.1             |
| Oyster beds                   | 10             | 4.3                      | 2.5 → 7.1            |

cus in Fig. 3 and 4. This organism occurred at much lower average numbers during the colder months of the year and responded more sharply to temperature increase. Thus, V. parahaemolyticus counts showed an increase of about 2 log units per 10 °C increase in temperature, whereas V. alginolyticus showed nearly a 3-log-unit increase.

The occurrence of hemolytic vibrios in fish samples is shown in Table 8. Only Pacific herring, catshark, and sable fish showed no vibrios. Among the other 12 species sampled, 88% of the gill samples, 61% of the skin samples, and 32% of the gut samples showed vibrios. Since the skin and gill flora tend to be moderately stable in composition (11) whereas the gut flora varies with the food ingested, these results suggest that the Vibrio is a common member of the fish microflora.

DISCUSSION

It is clear from the results that V. parahaemolyticus can be isolated with relative ease from a number of environments in the inshore marine waters of the Pacific Northwest of the United States. The organism seems to be most abundant in inshore sediments and molluscan shellfish. This is particularly significant in view of the known capability of V. parahaemolyticus to produce gastroenteritis in humans. Japanese workers have reported a seasonal incidence in cases of gastroenteritis caused by V. parahaemolyticus, with a peak during the warmer months of the year (17). From an ecological point of view, this correlates well with our observation of seasonal variations in the numbers of V. parahaemolyticus in oysters and the good correlation of
TABLE 6. Total counts of hemolytic mesophilic vibrios isolated from oysters and clams

| Sample        | Location | No. of samples | No. of samples and counts of vibrios |
|---------------|----------|----------------|-------------------------------------|
|               |          |                | 0-100 | 101-1,000 | 1001-10,000 | 10,001-100,000 | >10⁵ |
| Pacific oyster| Purdy    | 40             | 8     | 9         | 11         | 11           | 1    |
|               | Seabeck  | 10             | 1     | 0         | 1          | 4            | 5    |
|               | Shelton  | 2              | 0     | 1         | 0          | 1            | 0    |
|               | British Columbia | 2      | 0     | 1         | 1          | 0            | 0    |
|               | Willapa Bay | 1        | 0     | 1         | 0          | 0            | 0    |
|               | Oyster Bay | 1        | 0     | 0         | 0          | 1            | 0    |
|               | Astoria  | 1              | 1     | 0         | 0          | 0            | 0    |
| Olympia oyster| Shelton  | 4              | 1     | 0         | 0          | 3            | 0    |
| Clams         | Purdy    | 6              | 2     | 1         | 4          | 0            | 0    |
|               | Willapa Bay | 2       | 0     | 0         | 1          | 1            | 0    |
|               | Seabeck  | 1              | 0     | 0         | 0          | 0            | 1    |

TABLE 7. Total counts of Vibrio alginolyticus isolated from oysters and clams

| Sample        | Location | No. of samples | No. of samples and counts of Vibrio alginolyticus |
|---------------|----------|----------------|-----------------------------------------------|
|               |          |                | 0-100 | 101-1,000 | 1001-10,000 | 10,001-100,000 | >10⁵ |
| Pacific oyster| Purdy    | 40             | 19    | 14        | 5           | 2             | 0    |
|               | Seabeck  | 10             | 7     | 1         | 2           | 0             | 0    |
|               | Shelton  | 2              | 2     | 0         | 0           | 0             | 0    |
|               | British Columbia | 2      | 2     | 0         | 0           | 0             | 0    |
|               | Willapa Bay | 1        | 1     | 0         | 0           | 0             | 0    |
|               | Oyster Bay | 1        | 0     | 1         | 0           | 0             | 0    |
|               | Astoria  | 1              | 1     | 0         | 0           | 0             | 0    |
| Olympia oyster| Shelton  | 4              | 3     | 0         | 0           | 0             | 1    |
| Clams         | Purdy    | 6              | 2     | 0         | 4           | 0             | 0    |
|               | Willapa Bay | 2       | 2     | 0         | 0           | 0             | 0    |
|               | Seabeck  | 1              | 0     | 0         | 0           | 1             | 0    |

count levels with temperature of the overlying water. We have been unable to obtain growth of the organism at temperatures below 8 C in the laboratory, and it is tempting to propose that the actual occurrence of the organism is related to its ability to grow. However, the situation is obviously somewhat more complex than this, since the seasonal variation of counts in water and sediment is quite random. This suggests that some characteristic of growth and survival of V. parahaemolyticus in the oyster may be involved. The case of V. alginolyticus is much simpler, since the organism is quite rare in winter although abundant in summer. Moreover, the steepness of the line indicating the relationship of the V. alginolyticus count to water temperature and the intersection of this line with the temperature axis close to 8 C (the minimum growth temperature for this organism) suggest that temperature is the determinative factor in this case.

In general, V. parahaemolyticus appears to be associated with habitats with high organic nutrient content. This correlates well with the overall biochemical versatility of the genus Vibrio which is capable of utilizing a wide variety of organics (6). Limited data also indicate that, in marine environments which receive animal wastes, the total counts of V. parahaemolyticus and related vibrios are generally higher than in water low in organic material. This possible correlation, however, should be further investigated before any general statement can be confidently made.

The absence of any confirmed outbreak of
gastroenteritis caused by *V. parahaemolyticus* in the United States raises the question as to whether the vibrios isolated from the Pacific Northwest are capable of producing human gastroenteritis. In the present investigation, we have stressed the hemolysis reaction on human blood as being the most important biochemical criterion for pathogenicity. Recently, Sakazaki et al. (18) demonstrated that cultures of *V. parahaemolyticus* isolated from patients afflicted with *Vibrio* gastroenteritis could be differentiated from biochemically identical vibrios isolated from environmental samples by their ability to hemolyze a special blood-agar. This reaction was named the Kanagawa phenomenon. We are currently investigating this phenomenon and its idiosyncrasies with our isolates. Preliminary results with 30 strains of vibrios isolated from Puget Sound clam and fish samples and 10 strains from Japan indicate that the hemolytic activity on Kanagawa blood-agar is a breakdown of heme pigment and probably involves a hemodigestive enzyme rather than a true hemolysin. Of the strains tested, there was no correlation between Kanagawa-positive strains and the ability to ferment sucrose.
There is presumptive evidence of the involvement of *V. parahaemolyticus* in a series of food poisoning incidents caused by the consumption of shellfish gathered from a beach in Washington State (15). The shellfish samples implicated in these outbreaks showed a high level of incidence of *V. parahaemolyticus* with counts ranging from 100,000 to 10 million per g of shellfish meat, whereas other enteropathogenic bacteria and virus were not detected. Unfortunately, samples of feces from patients were available for testing only after cold storage for 1 month. Since most strains of *V. parahaemolyticus* appear to be sensitive to refrigerator temperatures, the failure to identify the organism in these samples is uninformative. The incident suggests, however, that it would be desirable that procedures for detection of *V. parahaemolyticus* be included in future investigations of gastroenteritis among persons consuming seafood.

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