Journal of Food Protection, Vol. 49, No. 8, Pages 643-646 (August 1986)

Recovery of *Aeromonas hydrophila* from Oysters Implicated in an Outbreak of Foodborne Illness

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(Received for publication February 3, 1986)

ABSTRACT

Potentially pathogenic *Aeromonas hydrophila* organism were isolated from oysters frozen at -72°C for 1-1/2 years. The oysters which had been associated with 472 cases of gastroenteritis in Louisiana in November 1982, were examined and found negative for *Salmonella*, pathogenic *Vibrio parahaemolyticus*, and diarrhetic shellfish poison. In 1983, oysters from the same shellfish growing area in Louisiana were implicated in seven cases of gastroenteritis caused by *A. hydrophila*. The oysters collected in 1982 were reexamined and found to contain *A. hydrophila* (MPN 9.3/100 g). Twenty-three of 28 strains identified by the MICRO-IS and API-20E systems were positive for at least one of the tests for virulence which included the suckling mouse test, the adrenal Y-1 mouse cell test, and hemolysin assays. Of five strains tested, all showed activity in the rabbit ileal loop. Although these results do not prove that *A. hydrophila* caused the outbreak in 1982, they suggest that in cases of foodborne illness involving oysters, *A. hydrophila* should be included in the screening tests.

During November and December 1982, approximately 472 cases of gastroenteritis associated with consumption of raw oysters were reported to the Food and Drug Administration (FDA) (*3*) by the Louisiana Department of Health and Human Resources. Affected individuals had nausea, vomiting, diarrhea and stomach cramps which developed from 24 to 48 h after ingestion of oysters. Implicated oysters were harvested from two separate areas of Louisiana (Sister Lake area in Terrebonne Parish and St. Mary's Point area in Jefferson Parish). A background investigation by FDA and Louisiana officials determined that inclement weather conditions coupled with by-passes of sewage from already overloaded wastewater treatment facilities occurred in this area just before the reported illnesses. Although the treatment plants were located 30 miles from the approved shellfishing areas, they were suspected of affecting the water quality of the shellfish-growing area under the prevailing meterological conditions.

Oysters involved in the outbreak were analyzed for *Salmonella* and *Vibrio parahaemolyticus* by FDA laboratories in Cincinnati and Dallas. High levels of weakly hemolytic *V. parahaemolyticus* with low pathogenic potential were present (Stelma, G.N., Jr., C.H. Johnson, A.L. Reyes, R.G. Crawford, P.L. Spaulding, and R.M. Twedt, 1985, Abstr. Annu. Meet. Am. Soc. Microbiol., p. 41, p. 257). No salmonellae were found. Enteric viruses, particularly Norwalk virus were suspected of being causative agents (*12*) as were marine toxins.

We examined these oysters to determine the presence of diarrhetic shellfish poison (DSP) because the symptoms were consistent with those reported for this toxin (*16*); however, no toxins were found. Reserve oysters were stored at -72°C.

Approximately 1 year later, an oyster-borne disease outbreak occurred in St. Petersburg, Florida, involving seven cases (*7*). Oysters and stool specimens from affected individuals were positive for *Aeromonas hydrophila*. The oysters came from the same growing area in Louisiana that had been involved in the November 1982, outbreak. Because of similarities between the two outbreaks, the oysters that had been frozen for 1-1/2 years were examined and found to contain potentially enteropathogenic strains of *A. hydrophila*.

MATERIALS AND METHODS

Media preparation

Trypticase soy broth. Trypticase soy broth with ampicillin (TSBA) (*17*), was prepared according to the manufacturer's (Difco, Detroit, MI) directions. After the TSB was autoclaved at 121°C for 15 min, filter-sterilized ampicillin (Sigma St. Louis, MO) was added to give a final concentration of 30 mg/L.

Tryptone broth. Tryptone broth (TB) (Difco) was prepared by dissolving 8 g of tryptone and 5 g of NaCl per liter and adjusting the pH to 6.9±0.2. The TB was autoclaved for 15 min at 121°C.

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Modified Rimler Shotts broth. Modified Rimler Shotts broth (MRSB) (*9*) contained the following ingredients (g/L): maltose, 3.5; L-cysteine hydrochloride, 0.3; bile salts No. 3, 1.0; yeast extract, 3.0; sodium chloride, 5.0; and bromothymol blue, 0.03. The pH was adjusted to 7.0 and the broth was autoclaved at 121°C for 15 min. After the broth was autoclaved, filter-sterilized novobiocin (Sigma) was added to give a final concentration of 0.005 g/L.

Craig's medium. Craig's medium use for cytotoxicity testing was prepared as indicated (*6*).

Aeromonas hydrophila medium (AHM). The multitest screening medium AHM was prepared as described (*8*).

MacConkey's agar (MAC), and Plate Count agar (PCA). Both agars were prepared according to the manufacturer's (Difco) directions.

Rimler Shotts agar. Rimler-Shotts agar (RSA) (*13*) contained the following ingredients in g/L: L-lysine-hydrochloride, 5.0; L-ornithine-hydrochloride, 6.5; maltose, 3.5; sodium thiosulfate, 6.8; L-cysteine hydrochloride, 0.5; bromothymol blue, 0.03; ferric ammonium citrate, 0.8; sodium deoxycholate, 1.0; novobiocin, 0.005; yeast extract, 3.0; sodium chloride, 5.0; and agar, 13.5. The pH was adjusted to 7.0, the mixture was boiled for 1 min and cooled to 45°C before plates were poured.

Wagatsuma agar. Modified Wagatsuma agar contained in g/L: yeast extract, 3; bacto-peptone, 10; sodium chloride, 5; dipotassium phosphate, 5; mannitol, 10; crystal violet, 0.001; and bacto agar, 15. The agar also contained 1 L distilled water and 50 ml fresh human red blood cells.

Cholera toxin. Cholera toxin (CT) was obtained from Schwarz/Mann (Orangeburg, NY).

Enrichment and isolation

Oysters were received frozen from the Cincinnati FDA laboratory in 3 sterile 8-oz. plastic containers. One container was examined immediately for marine toxins. The remaining two were stored at -72°C for 1-1/2 years before examination for bacteria. Oysters were thawed at 25°C for 2 h. Those for microbiological analysis were homogenized with enrichment broths TB, TSBA, and MRSB to give the following oyster concentration for each enrichment broth: 0.1 g/10 ml; 1.0 g/10 ml; 10 g/50 ml; and 20 g/180 ml. Enrichment broths were incubated at 35°C for 24 h, streaked onto MAC and RSA which were incubated at 35°C for 24 h.

Typical colonies were picked for multitest screening (*8*). Strains that produced an alkaline surface and an acid butt and were motile in AHM were considered presumptive positive and tested further for cytochrome oxidase. Identification of *A. hydrophila* strains was based on the following reactions (at 35°C unless otherwise specified): cytochrome oxidase (+); motility (+); growth on MAC (+); indole (±); methyl red, 35°C (+); methyl red, 26°C (±); lysine decarboxylase (±); L-arginine dihydrolase (+); ornithine decarboxylase (−); H_2S triple sugar iron (−); I-inositol (−); and D-mannitol (+). Biochemical tests were based on the MICRO-IS identification program IDDNEW (IBM 370 computer National Institutes of Health) (*11*). Twenty-eight strains identified as *A. hydrophila* by the MICRO-IS were confirmed with the API 20E system (Analytical Products, Plainview, New York).

Quantitative microbiological analyses

Aerobic plate count (APC). Duplicate pour plates for dilutions of 10^0 to 10^{-6} were prepared using PCA (*1*). Inoculated plates were incubated at 35°C for 48 h at which time colonies were large enough to be counted.

Total coliforms and Escherichia coli. The 3-tube most probable number (MPN) method (*6*) was used to enumerate coliforms and *E. coli*.

Aeromonas hydrophila. The 3-tube MPN method using TSBA was used to enumerate *A. hydrophila*.

Preparation of cell-free extracts

A. hydrophila strains were inoculated into 10 ml of TSB and incubated at 35°C for 24 h in an environmental incubator-shaker set at 300 rpm. Broth cultures were centrifuged at 23,000 × *g* for 20 min at 4°C. Cells were discarded and the supernatant fluid was filter-sterilized through a 0.45-μm filter. Filtrates were stored at 4°C for futher toxigencity studies.

Suckling-mouse test for enterotoxin

Test supernatant liquids (100 μl) containing about 0.02 ml of 0.1% ethyl violet/ml were administered perorally into the stomachs of 3-d-old infant Swiss albino mice, using a size 24 stainless steel animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY) connected to a 1-ml syringe. After 3 h of incubation at 26°C, mice were killed using chloroform. Large and small intestines were removed and weighed (IW), remaining body weight (BW) was measured, and the ratio of IW to BW was calculated. Ratios over 0.08 were scored as positive (*2*). This assay was used to identify the presence of nondialyzable, heat-labile enterotoxin of *Aeromonas* spp. A positive control isolated from an environmental sample was done with each group of tests.

Hemolysin assay

Test supernatant liquids (500 μl) of twofold dilutions in phosphate-buffered saline (PSB) solution were added to equal volumes of 1% washed (3×) rabbit erythrocytes (0.85% saline solution) in microtiter trays. Hemolysis was recorded after incubation for 1 h at 37°C and again after 24 h of incubation at 4°C. Hemolysis of ≥50% of the erythrocytes was considered positive. Positive controls from environmental isolates were done with each assay.

Hemolytic activity was also determined by the observation of alpha or beta hemolysis on modified Wagatsuma agar (spot agar plate method). Bacterial cultures were inoculated into 10 ml of TSB and incubated at 35°C for 24 h. Broth cultures were spotted onto Wagatsuma agar and incubated at 35°C for 24 h.

Cytotoxicity tests

Aermonas spp. (incubated at 35°C in 16 × 125-mm test tubes containing Craig's medium) were inoculated in a volume of 0.2 ml into a 125-ml Erlenmyer flask containing 25 ml of Craig's medium and incubated with shaking at 200 rpm at 35°C. Cultures were centrifuged at 1200 × *g* at 35°C, cells were discarded and the supernatant liquid was filter-sterilized through a 0.45- μm filter. For heat-treated filtrates, 2 ml of each supernatant liquid was heated at 80°C for 30 min. Standard cell culture techniques were used to prepare the Y-1 cell culture (*10*).

Cytotoxin activity was observed by using a light microscope (40×), after 24 h of incubation and determined by the presence of completely rounded, granulated and shriveled Y-1 cells. Negative and CT controls (1 ng CT/ml in PBS) were done with each assay.

Rabbit ileal loop assays

Starter cultures were grown in 250-ml flasks containing 50

ml of trypticase soy broth + 0.6% yeast extract at 37°C and shaken at 250 rpm. When the cultures reached stationary growth phase (8-10 h), 0.05 ml was transferred to 50 ml of fresh medium, and the flasks were incubated as described above. After 10 h, the cells were removed by centrifugation at 10,000 × *g* for 20 min and the culture supernatant liquid was passed through a 45- μm filter (Gelman Acrodisc, Gelman Science, Ann Arbor, MI). Reactivity of the culture supernatant liquids was determined by a modification of the rabbit ileal loop technique (*14,15*). Supernatant liquids (1-ml volumes) were injected in random order into individual loops separated by two inter-loops. Positive control loops contained 0.5 μg of purified CT in sterile PBS. Negative controls contained either sterile PBS or sterile TSB + 0.6% yeast extract. A test culture was considered positive if the volume-to-length ratio was at least fourfold greater than that of the negative control loop in the same animal. A strain was considered positive if loops tested positive at least two of three times in separate animals. Results from animals in which the response of either control was inappropriate were discarded.

Tests for diarrhetic shellfish poison (DSP)

Approximately 10 g of thawed oysters were homogenized for 40 s and extracted three times with 5 volumes of acetone followed by filtering through Whatman No. 3 filter paper and evaporation under nitrogen using a rotary evaporator.

The residue was dissolved in 5 ml of PBS containing 0.4% Tween 60 (PBS-T). Two groups (5 mice/group) of Swiss Webster mice (Tyler Laboratories, Bellevue, WA) weighing approximately 20 g each were injected intraperitoneally with 0.5 ml of undiluted and diluted (1:5 in PBS-T) reconstituted oyster residue and observed for 3 d.

RESULTS AND DISCUSSION

No DSP was found in the oysters; however, low levels (9.3 MPN/100 g) of *A. hydrophila* were detected in oysters stored at -72°C for 1-1/2 years. APC and total coliform counts were also low at 1.1×10^3/g and 1.1×10^2/100 g, respectively. No *E. coli* cells were detected. Colonies of different visual types were randomly picked from APC plates to determine the microbial identities. API identification strips were used to make determinations. Approximately 50% of the microbial population was identified as fluorescent *Pseudomonas* groups (spp. *putida, aeruginosa* and *putrefaciens*). Others were *Klebsiella pneumoniae* (6%), *Enterobacter cloacae* (12%) and *Acinetobacter calco* var. *antiatus* (6%). The remaining 25% were not identified.

A. hydrophila was isolated from all three enrichment broths tested, with highest recoveries in TSBA (Table 1). *A. hydrophila* was found in all of the different homogenates with TSBA. Although the plating media did not differ significantly for isolation of *A. hydrophila,* MAC allowed better recovery, but gave more false positives than did RSA.

Twenty-eight *A. hydrophila* strains identified by the MICRO IS system (*11*) were tested and confirmed with the API-20E system. Of the 28 strains identified as *A. hydrophila,* 23 were subjected to pathogenicity testing.

Sixteen strains (70%) were positive for at least one of the tests for virulence which included the suckling mouse test, the mouse Y-1 adrenal cell test and the hemolysin assays (Table 2). Five of these 16 strains were tested for activity in the rabbit ileal loop and all were positive (Table 2). All 16 strains were Voges-Proskauer (VP)-positive and arabinose-negative. Seven strains that were negative in tests for virulence gave positive reactions in both VP and arabinose. Of the 16 potentially virulent strains, 13 (81%) were enterotoxigenic in suckling mice; however, all 16 strains, including the nonenterotoxigenic ones were positive for hemolysis and cytotoxin produc-

TABLE 1. *Recovery of* Aeromonas hydrophila *from oysters using three enrichment broths: tryptone broth (TB), modified Rimler Shotts broth (MRSB) and tryptic soy broth with 30 mg ampicillin (TSBA) and two agars MacConkey agar (MAC) and Rimler Shotts agar (RSA).*

Test homogenates (g oyster/ml broth)	TB		MRSB		TSBA	
	MAC	RSA	MAC	RSA	MAC	RSA
0.1 g/10 ml	−	−	−	+	+	+
1.0 g/10 ml	+	+	−	−	+	+
10 g/50 ml	−	−	−	−	+	−
20 g/180 ml	+	−	+	+	+	+

TABLE 2. *Results of virulence testing and biochemical characteristics of* A. hydrophila *isolated from oysters associated with gastroenteritis outbreaks in Louisiana, November 1982.*[a]

No. of strain	Biochemical Reactions			Test of virulence		
	Voges-Proskauer	Arabinose fermentation	Hemolysin	Suckling mice	Cytotoxin Y-1	Rabbit ileal loop
13	+	−	+	+	+	+[b]
7	+	+	−	−	−	NA[c]
3	+	−	+	−	+	NA

[a]Oysters were frozen at -72°C for 1-1/2 years.

[b]5 of 13 isolates tested in the rabbit ileal loop with positive results/number tested: 4/6; 5/5; 3/3; 3/3/; 3/3.

[c]Not analyzed.

tion. There was a 100% correlation between hemolysin-positive strains and the response to Y-1 cells. All of the isolates reacting in the Y-1 cell assay demonstrated cytotoxic activity. The cells were completely disrupted and appeared granulated and shriveled. This activity was altered when culture filtrates were held at 56°C for 30 min, giving a cytotonic response, i.e., a complete rounding of cells, as seen with CT.

Similar correlations were found by Burke et al. (*2*) with 686 strains of *Aeromonas* spp. from human and environmental (water) sources. More than 80% of VP-positive strains were enterotoxigenic in suckling mice as were 90% of VP-positive, arabinose-negative strains. An association between VP-positive, arabinose-positive strains and failure to produce enterotoxin was found only with strains from water isolates (*2*).

Correlations have also been reported between the production of lysine decarboxylase and toxin production (*2,4,9*). Our study, however, did not show that relationship. Of 16 cytotoxic strains, seven (44%) were lysine decarboxylase-negative.

CONCLUSION

The ability of *A. hydrophila* to survive low temperatures of -72°C in oysters for 18 months indicates that these organisms can withstand stressful conditions. In our study, strains of *A. hydrophila* possessing cytotoxin, enterotoxin and hemolysins, were detected in oysters involved in 472 cases of gastroenteritis. Although these results do not prove that *A. hydrophila* was the etiological agent for the oyster-borne outbreaks in Louisiana in 1982, they support the advisability of screening for *A. hydrophila* when oysters are implicated in such cases.

In the past *A. hydrophila* was recognized as an opportunistic pathogen of low virulence; it is now accepted as a primary pathogen (*5*). Although the potential of *A. hydrophila* to induce outbreaks of such magnitude is unknown, gastroenteritis caused by *A. hydrophila* is being recognized more frequently; however, cases involving shellfish have not been well-documented. For example, the oyster-borne outbreaks in St. Petersburg, Florida on January 1, 1984, caused by *A. hydrophila,* had an attack rate of 100% (7 of 7). Patient stool specimens and remaining oysters were positive for *A. hydrophila.* Onset time was from 22 to 34 h (median 28 h). Affected individuals reported nausea, vomiting, cramps and diarrhea. Oysters in question were harvested from the Terrebonne locale on December 21 (27 sacks) and December 22 (202 sacks). Water samples collected near the area from November 7 to 12, 1983 had fecal coliform MPNs/100 ml of 79, 49, 33 and 23 (approved growing area bacteriological criteria in a median fecal coliform level of 14/100 ml). Another sampling station near the area during 11/8-12/83 had fecal coliform MPNs of 140, 14, 170 and 22/100 ml. In addition, an investigation revealed that fish had been placed on top of the oysters (on ice) before the oysters were consumed, indicating possible cross contamination of the product. Since the fish were not analyzed, it is not known whether the aeromonads originated in the oysters or in the fish (or both). No other outbreak was reported.

Aeromonads are considered indigenous inhabitants of aquatic environments. Consumption of shellfish containing high levels of these bacteria that are positive for virulence factors may present a health hazard. In cases of foodborne bacterial illness in which oysters are implicated, *A. hydrophila* should be included in the general screening for causative microorganisms.

ACKNOWLEDGMENTS

The authors thank C. H. Johnson, P. L. Spaulding and R. G. Crawford for technical assistance, and Linda Vernon for typing the manuscript.

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