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Vibrio anguillarum and Larval Mortality in a California Coastal Shellfish Hatchery

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Vibrio anguillarum was isolated as a pathogen in the commercial culture of oyster spat at Pigeon Point, Calif. A water-soluble, heat-stable exotoxin extracted from cultures of the vibrio inhibited larval swimming and contributed to larval mortality. Although the vibrio was insensitive to penicillin in standard plate testing, this antibiotic proved useful in preventing mass larval mortalities in the hatchery.

The Pigeon Point Shellfish Hatchery, on the exposed California coastline, 60 miles (ca. 96.5 km) south of San Francisco, has had a mixed history of success in the commercial production of juvenile oysters (spat). A major problem, encountered numerous times in the past under several ownerships, has been mass mortality of oyster larvae in the culture systems before their setting and metamorphosing to form spat. *Vibrio anguillarum*, a well-known aquatic pathogen of both vertebrates and invertebrates (4), has now been identified as a major cause of larval mortality at the hatchery, and remedial measures are being implemented.

The hatchery operation is basically a scaled-up version of juvenile oyster culture methods developed in the laboratory by Loosanoff and Davis (5). In routine hatchery operation, oceanic seawater is pumped into settling tanks to remove large particulates and then filtered to remove particles down to 5 μm in size. This water is then heated in an all-glass heat exchanger to 22 to 25°C before its transfer into culture tanks. Spawning of *Crassostrea gigas* (Japanese oyster) brood stock is carried out by the method of Loosanoff and Davis (5) with proprietary modifications. Zygotes from spawning are allowed to develop to straight-hinge veliger larvae over a 48-h period. These larvae are screened off and replaced in clean water approximately every 3 days for periods of up to 21 days, during which they are constantly fed microscopic algae cultured in another area of the hatchery. Upon reaching the 325-μm size, the larvae are transferred to (proprietary) setting systems, where they metamorphose and set. The spat are then maintained on submerged screens and fed cultured phytoplankton until reaching marketable size (>3 mm in diameter). *Ostrea edulis* larvae are also cultured by proprietary techniques. Incipient development of mass mortality is usually preceded by failure of the veliger larvae to maintain themselves in the water column of the rearing tanks, with larvae progressively accumulating in spots on the bottoms of the tanks.

Among the possible causes for larval mortality, bacterial attack was discounted in early hatchery research, since successful spat production was routinely carried out in spite of the presence of high total counts of bacteria (Table 1).

In March 1975, when larval mortality occurred in the hatchery during research on larval culture techniques, it was fortuitously demonstrated that treatment of moribund larval cultures with 50 mg of penicillin G (Technical, Pfizer Co.) per liter saved the larvae and allowed them to metamorphose and set. Experimental rearing experiments were set up at a 1/10 production scale, and comparative bacteriological monitoring of both healthy and moribund larval cultures was carried out with TCBS agar (thiosulfate, bile salts, sucrose; BBL). Bacterial colonies routinely associated with larval morbidity were subcultured and used in larval pathogenicity experiments. Pure cultures of suspect bacterial species were grown in hatchery seawater supplemented with 5% peptone (Difco) and used to challenge healthy *O. edulis* larvae. Petri dishes containing 30 ml of seawater with 1 to 2 larvae per ml were inoculated with either 1 ml or one loop (ca. 0.01 ml) of a dense, seawater culture of suspect bacterial species. Uninoculated control and inoculated test systems were run for 72 h. Of the few bacterial colony types arising on TCBS agar, a yellow-pigmented isolate, forming small colonies, was found to routinely cause complete mor-

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TABLE 1. *Bacterial total count data for several sampling sites in the Pigeon Point Shellfish Hatchery*[a]

Sample	Plate count (CFU[b]/ml)
April–May 1973	
Intake water	30
Primary settling tank	25–65
Starter algae,[c] initial	10
Starter algae,[c] log phase	12,000–134,000
Algae feed stock,[d] log phase	220,000–740,000
New fill water, larvae	70–220
Larvae culture water, subsurface (3)[e]	400–13,700
Larvae culture water, surface foam (3)[e]	680,000
June–July 1974	
Starter algae[c] (5)[e]	10,000–80,000
Starter algae[c] (12)[e]	220,000
New fill water, larvae tank	2,500
Same water, aged for 48 h	28,000
Larvae water (11)[e]	1,700,000
October 1974	
Intake water	1,700
Algae feed stock,[d] post-inoculation	78,000
Algae feed stock[d] (7)[e]	620,000
Larvae culture water, initial	1,500–1,700
Larvae culture water (2)[e]	1,000,000

[a] No mortality of larvae evident.
[b] CFU, Colony-forming units.
[c] *Isochrysis galbana* or *Monochrysis lutheri*.
[d] *Cyclotella nana*.
[e] Age (days).

tality in preliminary dish tests, with 100% survival of untreated control larvae. Bacterial swarming was routinely observed on moribund larvae, by using light microscopy at ×200.

The suspect pathogen was maintained on TCBS medium supplemented with 3% NaCl, as were all media used in the screening process. One of us (J.B.) carried out diagnostic screening tests at the California State Department of Public Health, Microbial Diseases Laboratory, Berkeley. Standard procedures were used throughout the screening tests (1, 2), supplemented by specific tests for pathogenic marine vibrios (4, 7).

The isolated species was a gram-negative, asporogenous, non-acid-fast, motile, small rod, which did not form pigmented colonies on marine agar. Motility was due to a single polar flagellum. Structures interpreted as spheroplasts were seen in some hatchery cultures that had been refrigerated for 48 h at 4°C. The organism grew well at 25 and 35°C but failed to grow at 42°C. The organism did not grow without salt in the medium and would not grow at 8% NaCl, although growth was positive in 6% NaCl. The organism was able to reduce nitrate but not nitrite. It showed beta-hemolytic activity on sheep blood agar. Additional test results conformed with literature descriptions of *Vibrio anguillarum*, including insensitivity to penicillin. This diagnosis was confirmed by the U.S. Public Health Service Communicable Disease Center, Atlanta, Ga.

In tests for bacterial exotoxins, pure cultures of the pathogenic isolate were spread-plated to produce a dense culture on marine agar. Cells were harvested from the plates with a flat-ended spatula after 24, 48, 96, and 120 h of growth at 20°C. Approximately 1 ml of cells from each culture was suspended in 10 ml of sterile seawater, mixed with a vibrator for 5 min, centrifuged from the seawater, and aseptically filtered off on a membrane filter (0.45-μm pores, Millipore Corp.) to give a clear supernatant. This fluid was divided into two equal portions, one of which was heat-treated in a boiling-water bath for 10 min.

The immediate response of the larvae to the untreated and heat-treated cell wash water was determined by immersing about 200 larvae in 0.5 ml of seawater plus 0.5 ml of wash water in depression slides and observing the larvae after a 30-min exposure. Results were tallied by counting 100 larvae at random with a light microscope at ×100 and recording (i) normal swimmers, (ii) weak swimmers, (iii) closed valves, but showing internal movement, and (iv) closed valves, with no signs of life.

The treated and untreated wash water from all cultures was pooled and serially diluted 1:2, 1:4, etc., to 1:512, and approximately 100 larvae were placed in 10 ml of each dilution in test tubes. Tubes were maintained at 16°C over a period of 5 days and were observed daily for larval mortality.

Both fresh and heat-treated seawater extracts of cells of the suspect pathogen showed deleterious effects on larvae of *O. edulis* after 30 min (Table 2). There was no apparent difference between fresh and heat-treated extracts when examining grouped mean values comparing swimmers with nonswimmers (Table 2). Culture age had no effect when summing organisms that showed movement (live) for comparison with those that showed no movement (dead), particularly with the untreated extracts.

Five-day test tube observations showed mortality of larvae in both fresh and heat-treated extracts up to and including the 1:16 dilution.

Guillard (3) originally isolated pathogens from moribund larvae and demonstrated that substances present in culture fluid separated from the pathogens inhibited larval swimming. Diagnosis of the pathogen as *V. anguillarum* was not unexpected, since vibriosis of cultured larvae by this and closely related organisms has been documented by Tubiash et al. (6, 7). Our preliminary observations on the pathology and etiology

TABLE 2. *Behavior of O. edulis veliger larvae after 30 min of incubation in fresh and heat-treated seawater used to wash cells of V. anguillarum that had been grown on marine agar*

Age of culture (h)	No. of larvae showing behavior[a]							
	Seawater extract				Heat-treated seawater extract			
	1	2	3	4	1	2	3	4
24	13	54	16	17	11	53	15	22
48	0	56	20	24	1	91	4	4
96	3	65	13	19	9	46	8	37
120	1	83	1	15	0	56	5	39
Grouped mean values	68.75[b]		31.25[c]		66.75[b]		33.5[c]	

[a] 1, Normal swimming; 2, weak swimming; 3, closed valves, internal movement; 4, closed valves, no movement. Controls were all no. 1.
[b] Swimmers.
[c] Nonswimmers.

of this disease at Pigeon Point compare favorably to those made by Tubiash et al. (6). *V. anguillarum* is routinely found in hatchery waters, and it is likely that vibriosis in larvae develops due to external stresses on the larvae that make them susceptible to infection. Such factors could include variations in the quality of phytoplanktonic feed algae, variations in ocean water quality, and contamination of hatchery systems with excess organic wastes, which could promote selective growth of pathogenic bacteria. In the latter case, it may be hypothesized that larval disease does not occur unless a critical threshold population of the pathogen is allowed to build up in the hatchery system.

Production of toxins by the bacteria that inhibit larval swimming (Table 2) may be the cause of "spotting" phenomena seen in the hatchery, where large numbers of larvae aggregate on the bottoms of culture tanks. This would be of benefit to the bacteria, since the bottom detritus in the tanks, consisting of larval feces and sedimented feed algae, may stimulate bacterial growth and promote larval infection. Although *V. anguillarum* was not susceptible to penicillin in standard testing, the use of this antibiotic has shown great promise in the control of larval mortality in the hatchery. The reasons for this are unclear, although this observation may indicate that there is more than one pathogen active in the hatchery system which has not as yet been identified.

Improvements in basic sanitation have aided in lowering hatchery system bacterial counts, prevented detrital buildups in culture systems, and prevented carryovers of bacteria from one spawning to the next.

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