Soft-Shell Clam, *Mya arenaria*, a Convenient Laboratory Animal for Screening Pathogens of Bivalve Mollusks

HASKELL S. TUBIASH

Biological Laboratory, National Marine Fisheries Service, Oxford, Maryland 21654

Received for publication 30 April 1971

Attempts to introduce infectious or foreign material into oysters and other bivalve mollusks usually involve force or trauma because of immediate, prolonged adduction of the tightly closing valves. The soft-shell clam, *Mya arenaria*, is unable to seal its valves completely and relaxes readily, exposing soft tissue and a large siphon. This species is free from fouling organisms and is readily available at all seasons in the New England and mid-Atlantic areas. Suspensions of five strains of *Vibrio* sp. that cause bacillary necrosis in larval and juvenile bivalve mollusks were injected into the heart, siphon tissue, and the incumbent and excurrent siphon lumina of soft-shell clams. All vibrio strains caused significant mortality, usually within 2 days. Heaviest losses resulted from heart and excurrent siphon injections. No mortality occurred in control clams injected with seawater, broth, *Serratia* sp., and *Escherichia coli*. The soft-shell clam appears to be a useful animal for testing the pathogenicity of marine microorganisms for bivalve mollusks.

The American or eastern oyster, *Crassostrea virginica*, is the most widely studied and economically valuable bivalve mollusk of the Western Hemisphere (4). During an investigation of the microflora and fauna associated with extensive oyster mortalities in Delaware and Chesapeake Bays (2, 6), numerous bacterial strains were isolated from diseased oysters and enzootic environments (7), and a practical method for evaluating their pathogenicity was sought. Because of the oyster's heavy, tightly closing valves secured by powerful, persistent adductor muscles, attempts to introduce infectious or other foreign material usually involve force and tedious manipulations resulting in trauma to the animal (1, 3, 8–10). Additionally, oyster shells provide habitats for diverse populations of commensal, competitive, and parasitic flora and fauna which may tend to obscure reactions to the agent or substance under test.

The soft-shell clam, *Mya arenaria*, is unable to seal its valves completely, relaxes readily, and when relaxed it extends a large, fleshy siphon. The shells are comparatively smooth, chalky-white, and free from fouling organisms, and they can be readily labeled with ordinary lead pencil or common marking inks. This cosmopolitan inhabitant of sandy coves of the Northern Hemisphere is originally native to the coasts of the North Atlantic. However, as the result of fortuitous introduction into San Francisco Bay around 1880, its geographic range now extends from Alaska to Monterey, Calif. This species is also found along the Asiatic coast from Kamchatka to the southern reaches of the Japanese Islands (5). It is readily available during all seasons in the New England and mid-Atlantic coastal areas. It was, therefore, believed that this clam might serve as a useful laboratory animal for screening potential pathogens of bivalve mollusks.

**MATERIALS AND METHODS**

A group of marine vibrios have been demonstrated to be the etiologic agents causing fatal epizootics of bacillary necrosis among larval and juvenile bivalve mollusks (11, 12). These bacteria were, therefore, utilized as test pathogens, and other species were used as presumably nonpathogenic controls.

**Bacterial strains.** Five type cultures of the etiologic agents of bacillary necrosis, including three strains of *Vibrio anguillarum* (ATCC 19105, 19106, and 19109), one of *V. alginolyticus* (ATCC 19108), and one *Vibrio* sp. (ATCC 19107) were grown on Trypti-

case-glucose-yeast extract (TGY) agar prepared with filtered Chesapeake Bay water and autoclaved at 15 psi for 10 min (11). The salinity of the water was 12%. Cultures were incubated for 24 hr at 26 C, washed from the agar surface, centrifuged, and resuspended in sterile Chesapeake Bay water to turbidimetrically adjusted concentrations of approximately 10⁸ organisms per ml.

To determine whether pathology was caused by ac-
anterior muscular, retractable

After clam, controls consisted for water of Chesapeake water circulating through siphons. Controls consisted of inocula of Vibrio strains (isolated from a Chesapeake Bay oyster), Serratia marcescens (from shucked soft-shell clam meats), and Aeromonas salmonicida. Additional controls consisted of TGY broth and nonsterile Bay water from the laboratory open supply.

Test clams. Soft-shell clams (average shell length, 7 cm) from the Miles River, a Chesapeake Bay tributary, were obtained commercially. Before use, the animals were acclimatized by being held for at least 24 hr in shallow laboratory trays (wet tables) of flowing Chesapeake Bay water at 20 C. Acclimatized clams extended their siphon tubes, “pumped” or circulated water through the siphons vigorously, and retracted quickly when disturbed.

Routes and method of injection. Most bivalve molusks are filter-feeders, obtaining food and oxygen by circulating water through incurrent and excurrent siphons. The routes of inoculation most closely approximating natural exposure are via the incurrent and excurrent siphon orifices [although adult soft-shell clams, as well as eastern oysters, hard clams (Mercenaria mercenaria), and blue mussels (Mytilus edulis), are refractory to 24 hr of exposure in large concentrations of these vibrios (11)]. Other inoculation sites chosen were the siphon tissue and the primitive heart lying directly below the shell hinges (Fig. 1).

Acclimatized clams were removed from the water, dried, and labeled with a marking pen. The points of 18-gauge needles were ground off and smoothed to form 35 mm long cannulas. Clams were challenged by injection with 1 ml of bacterial suspensions into the incurrent and excurrent siphons (Fig. 2). With a little practice, the cannula could be readily inserted about 25 mm into the siphon lumina even though the animals retracted completely. Handling the clams and insertion of the cannulas induced voiding of excess water, and very little of the inocula was expelled. Separate groups of animals were injected in the heart and siphon tissue (Fig. 3 and 4) with 0.2-ml inocula by means of a 25-gauge needle (2 X 10^8 cells). Intracardiac injection alone was used for challenge with filtrates and heat-inactivated cells. Fifty clams were injected with each organism by each route, except in the case of the inactivation studies and the controls, for which 10 rather than 50 animals were used for each test point. In total, 1,300 clams were utilized: 1,000 for exposure to the

tive infection or possible toxemia, three 24-hr cultures of each Vibrio strain were prepared in TGY broth. One suspension was untreated, one was heated at 80 C for 10 min to inactivate viable cells, and the third was filter-sterilized through a 0.45-μm pore-size membrane filter. Controls consisted of 24-hr suspensions of Escherichia coli (isolated from a Chesapeake Bay oyster), Serratia marcescens (from shucked soft-shell clam meats), and Aeromonas salmonicida. Additional controls consisted of TGY broth and nonsterile Bay water from the laboratory open supply.

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Table 1. Response to *Vibrio* challenge

| Pathogen          | Per cent mortalitya |
|-------------------|---------------------|
|                   | Heart | Siphon tissue | Incurrent siphon | Excurrent siphon |
| *V. anguillarum* 19105 | 60    | 28            | 10               | 42               |
| *V. anguillarum* 19106 | 48    | 32            | 26               | 20               |
| *Vibrio* sp. 19107 | 82    | 50            | 22               | 28               |
| *V. alginolyticus* 19108 | 58    | 42            | 12               | 74               |
| *V. anguillarum* 19109 | 72    | 38            | 2                | 20               |

aN = 50 clams with each organism by each route.

Table 2. Effect of pathogens by all routes

| Pathogena       | Mean mortality (%) |
|-----------------|--------------------|
| *V. anguillarum* 19105 | 35    |
| *V. anguillarum* 19106 | 32    |
| *Vibrio* sp. 19107 | 45    |
| *V. alginolyticus* 19108 | 37    |
| *V. anguillarum* 19109 | 33    |

aN = 200 clams with each organism.

five vibrios, 150 for the inactivation study, and 150 for the controls.

Inoculated animals were held out of water at room temperature for 4 hr, and then were returned to trays of flowing sea-water maintained at 20 to 22 °C and were observed for 5 days.

**RESULTS**

Morbidity was usually apparent within 24 hr. The earliest clinical or gross pathological signs were flaccidity and loss of siphonal tone, with reduced ability to retract. In infected animals, the siphon gradually acquired a tough, leathery texture. Once this texture was achieved, pumping activity and reflexes slowed, and death inevitably followed. Most losses occurred during the first 72 hr postinjection, and mortality was usually completed within 4 days. The animals characteristically died with siphons partly extended and shells gaping.

All *Vibrio* strains caused significant mortality by at least two routes. Mortality varied widely according to the route of inoculation and with the individual pathogens, ranging from 82% via the heart with *Vibrio* sp. 19107 to 2% via the incurrent siphon injection with *V. anguillarum* 19109 (Table 1). In all instances, cardiac injection was most sensitive, with mean mortality of 64% for all of the vibrios. Injection into the siphon tissue and the excurrent siphon yielded approximately equal mean mortalities of 38 and 37%, whereas, surprisingly, injection into the incurrent siphon averaged only 14% mortality. These results are summarized for all routes and all pathogens in Tables 2 and 3.

An appreciable number of clams were refractory to infection by any route or pathogen, as in no case was 100% mortality achieved. Cardiac injections with control fluids (TGY broth and sea-water) and bacteria other than the vibrios (*E. coli*, *S. marcescens*, and *A. salmonicida*) were uniformly negative, and caused no mortality. Similarly, membrane-filtered and heat-inactivated 24-hr broth cultures caused no mortality by cardiac challenge, although viable portions of the identical cultures caused high mortalities with all of the vibrios (Table 4). Pathology is, therefore, directly related to the activity of viable microorganisms rather than to the presence of soluble toxins or other metabolites.

**DISCUSSION**

The finding of heavier mortality by the excurrent than the incurrent route seemed surprising, or at least anomalous, but an examination of the animal’s anatomy (Fig. 1) offers grounds for a logical explanation. The incurrent siphon leads to a relatively open area where a reverse rejective mechanism exists—alogous, perhaps, to that in the human esophagus. Injection into the excurrent siphon deposits material into a relatively closed space, the epibranchial cavity, which seems to be unprotected by a reflex rejective mechanism. The excurrent siphon route is, therefore, an interesting

Table 3. Effect of routes with all pathogens

| Routea       | Mean mortality (%) |
|--------------|--------------------|
| Heart        | 64                 |
| Siphon tissue| 38                 |
| Incurrent    | 14                 |
| Excurrent    | 37                 |

aN = 250 clams by each route.

Table 4. Inactivation or removal of bacteria

| Pathogen          | Per cent mortalitya |
|-------------------|---------------------|
|                   | Live | Heated or cell-free |
| *V. anguillarum* 19105 | 80   | 0                |
| *V. anguillarum* 19106 | 50   | 0                |
| *Vibrio* sp. 19107 | 80   | 0                |
| *V. alginolyticus* 19108 | 90   | 0                |
| *V. anguillarum* 19109 | 70   | 0                |

aN = 10 clams with each organism in each form.
and probably effective manner for introducing or “force-feeding” experimental substances into *Mya*. However, for economy of inoculum, technical simplicity, and optimal response to bacterial pathogens, routine challenge via the cardiac or siphon tissue routes, or even concurrently by both, is recommended.

An open, flowing seawater system is essential for holding clams where mortalities are anticipated, since dead animals decompose quickly, producing massive fouling in closed systems, with ancillary pathology unrelated to the primary challenge. Recirculated systems or closed aquaria should only be used for small populations under constant observation.

The fact that not one of the control animals succumbed testifies to the innate resistance of adult mollusks to bacterial infection and trauma. By contrast, it was found in a current study that blue crabs (*Callinectes sapidus*) experienced high mortalities after parenteral challenge with smaller doses of *S. marcescens* and *A. liquefaciens*. It had been noted previously that soft-shell clams as well as eastern oysters, hard clams (*Mercenaria mercenaria*), and blue mussels (*Mytilus edulis*) were refractory to 24 hr of immersion in heavy concentrations of all five vibrios (11).

Although the idiosyncrasies of individual tolerances in *Mya* do not permit the expression of tidy quantitative ID<sub>90</sub> or LD<sub>90</sub> values, the method described does offer a practical qualitative procedure for estimating microbial pathogenicity in a phyllum which seems to be extraordinarily refractory to bacterial infection.

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