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In Vivo Clearance of Enteric Bacteria from the Hemolymph of the Hard Clam and the American Oyster

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American oysters, *Crassostrea virginica,* and hard clams, *Mercenaria mercenaria,* were experimentally contaminated with *Escherichia coli, Salmonella typhimurium,* and *Shigella flexneri* either by intracardial injection or via the natural route of ingestion. Bacterial inactivation in the hemolymph was monitored for 72 h after exposure to these enteric pathogens at 20 and 6°C. At 6°C, both mean bacterial uptake by ingestion and subsequent clearance was significantly lower than at 20°C. However, substantial bacterial clearance from the hemolymph occurred for both shellfish at each temperature. At 20°C, viable bacteria were no longer detectable after 24 h in hemolymph of either clams or oysters after exposure to contaminated water containing 4×10^3 bacteria per ml.

Marine mollusks, such as hard clams, *Mercenaria mercenaria,* and American oysters, *Crassostrea virginica,* filter large volumes of seawater during their feeding activities and may concentrate bacteria such as coliforms or *Salmonella* in this water within their bodies (13). Removal mechanisms for the bacteria include the phagocytic activities of cells in the shellfish hemolymph (2). These phagocytic cells are continuously lost to the exterior through epithelial borders (4), a process which provides an efficient clearance mechanism for phagocytized bacteria and other materials (11). The exteriorized phagocytes are carried away in mucus or feces by the water stream set up by natural pumping action in the body of the shellfish (7, 11).

The ability of contaminated marine shellfish to rid themselves of coliform bacteria when placed in clean water for 48 h is well known and has been extensively exploited in artificial depuration systems. The effectiveness of this process in respect of some enteric pathogens in oysters has, however, been questioned. Janssen (8) found that *Salmonella typhimurium* may persist for as long as 7 weeks in the tissues of live oysters. Studies of this kind have not as yet been reported for clams. Also, little is known of the kinetics of the specific processes by which enteric bacteria of public health significance are cleared or destroyed in clams or oysters. It is essential to understand these if more efficient and economic depuration techniques are to be developed that predict reliable estimates of survival time for enteric pathogens. With this in mind, this study was undertaken to examine the inactivation of *S. typhimurium, Shigella flexneri,* and *Escherichia coli* in hemolymph of hard clams and American oysters after exposure by the oral route or by intracardial injection.

MATERIALS AND METHODS

Experimental animals. Shellfish were obtained from commercial sources on the south shore of Long Island, N.Y. Adult *C. virginica* ranged from 10 to 13 cm, and *M. mercenaria* measured 7 to 9 cm. Shellfish were shipped under refrigeration in closed containers and transported for approximately 8 h. No death occurred due to the transportation procedure.

Experimental tanks and containers. Shellfish were maintained at 6°C in a circulating Living Stream unit (Frigid Units, Inc., Toledo, Ohio) for up to 2 weeks. In this system, water was contained in a closed system and circulated through a fiberglass and bone carbon filter every 1.5 min. Water was artificially prepared from Instant Ocean synthetic sea salts (Aquarium Systems, Inc., East Lake, Ohio). Salinity was 20%. Mollusks were fed a variable amount of algal suspension of *Isochrysis galbana* and *Dunaliella tertiolecta* (cell density, 10^3/ml) each week.

After removal from the maintenance tank, shellfish were acclimated for 2 days in experimental containers at the specific temperature of the experiment. These experimental containers were 11.5-quart enamel-coated pots which were placed in a Living Stream unit to maintain the specified temperature. An air supply was provided for each container by means of a commercial aquarium pump. Groups of 12 shellfish were contained in each pot and were separated at sufficient distance for independent functioning. The Living Stream unit was operated at either 6 or 20°C to simulate mean winter and mean summer values.

Bacterial strains. *E. coli* K-12 ATCC 14948 and *S. typhimurium* (247) were obtained from the collection maintained at the Department of Microbiology at the New York State College of Veterinary Medicine.

S. flexneri (E129) type 2a was obtained from the Diagnostic Laboratory at the New York State College of Veterinary Medicine. Mutants of each of these bacterial strains resistant to >40 μg of chloramphenicol per ml were selected and used for the experiments. Use of these mutants greatly facilitated plate counting because the indigenous flora of the clam and oyster was chloramphenicol sensitive. Brain heart infusion agar with 40 μg of chloramphenicol per ml was used for isolation and for plate counts.

Each bacterial species was grown in brain heart infusion both for 18 h, washed in sterile 0.85% saline, and brought to a concentration of 10^9 colony-forming units (CFU) per ml for each experiment.

Experiment 1. Intracardial route of exposure. Experiments on groups of 10 to 15 clams or oysters were conducted in separate containers for filtered synthetic sea salts. A hole was bored with a 0.25-inch (ca. 0.64-cm) drill in the valve of the shellfish. The thin layer of nacre was peeled away by forceps by the procedure of Feng (5). An incision was made through the mantle to expose the pericardial cavity. Approximately 0.02 ml of a bacterial suspension containing 10^3 CFU was injected. Control shellfish were injected with sterile synthetic seawater. Shellfish displaying significant leakage were discarded. No mortality was noted during the procedure. Bore holes in the valves were sealed with cotton and melted paraffin.

Each shellfish was bled at 4-h intervals until no viable test bacteria were detected in the hemolymph. At each sampling time, the shellfish were removed from the containers and washed in large quantities of filtered synthetic seawater and exposed to ultraviolet light (Ultraviolet Products, Inc., San Gabriel, Calif.) with intensity 480 μW/cm² at 254 μm at 15 cm for 5 minutes to remove surface bacteria. Hemolymph was withdrawn with a tuberculin syringe attached to a 27-gauge needle, and 0.1-ml portions were spread plated on brain heart infusion agar plates in triplicate. After spreading, plates were incubated at 37°C for 24 h. After bleeding, shellfish were returned to fresh water in the holding containers.

Clearance of all three bacterial species was studied at 20°C. At 6°C, only *E. coli* and *S. typhimurium* were studied. At 72 h, when significant numbers of bacteria were no longer cultured from the hemolymph, individual shellfish were shucked and homogenized in a Sorvall Omnimixer (Ivan Sorvall, Inc., Newton, Conn.) in an equal quantity of filtered synthetic seawater. A 0.05-ml amount of the homogenates was plated in triplicate by the spread plate method. After incubation at 37°C for 24 h, bacteria were counted.

Experiment 2. Oral route of exposure. Groups of 10 to 15 shellfish in containers were exposed for 15 min to approximately 4×10^3 bacteria per ml in 4 liters of filtered synthetic seawater. The shellfish valves were then washed in water, treated with UV irradiation, and placed in clean containers of 4 liters of filtered synthetic seawater. Sampling was performed as in experiment 1.

RESULTS

Figure 1 shows the bacterial clearance curves for *C. virginica* and *M. mercenaria* over a 72-h period at 20°C after intracardial inoculation. Mean values of CFU from triplicate plates were calculated as percentages of the CFU found at zero time. At 72 h, homogenates were prepared from individual shellfish, and the percent CFU recovered was calculated from the mean number of bacteria per gram of tissue. These values which represent residual CFU were plotted as points on the graph. After 24 h at 20°C, bacterial counts in both the clam and the oyster hemolymph went below detectable levels. At 72 h, the mean CFU of either *S. typhimurium* or *S. flexneri* in tissue homogenates was less than 10/g.

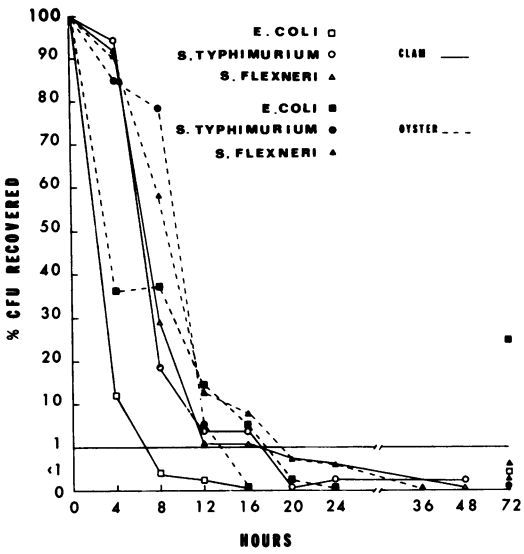

FIG. 1. *In vivo bacterial clearance of selected enteric pathogens from the hemolymph of C. virginica and M. mercenaria after intracardial inoculation at 20°C. CFU values were obtained per milliliter of hemolymph. At 72 h, CFU values were obtained per gram of homogenate.*

Figure 2 summarizes the results of the experiment conducted at 6°C. It can be seen that clearance was slower than at 20°C. *E. coli* and *S. typhimurium* were still detectable in oyster hemolymph at 72 h. The clam appeared to be more efficient at 6°C because viable bacteria were no longer detectable in hemolymph at 72 h. Counts of *E. coli* and *S. typhimurium* in hemolymph of clams and oysters after oral exposure at 6 and 20°C for 15 min are shown in Table 1. Uptake by both shellfish species was much greater at 20°C and oysters appeared to take up more *S. typhimurium* than did clams.

On Figure 3 are shown bacterial clearance curves for shellfish exposed by the oral route for 72 h at 20°C. Bacteria were not detectable in hemolymph after 24 h at 20°C.

DISCUSSION

Because shellfish are poikilotherms and affected by temperature fluctuations in the unstable estuarine region where they commonly live, experiments in this study were conducted at both 20 and 6°C to simulate mean summer and winter values in Long Island waters, respectively. At 20°C, *C. virginica* and *M. mercenaria* were able to clear themselves of an appreciable number of pathogens within a 24-h period when exposed either by intracardial injection or by oral exposure. At 6°C, the efficiency of the inactivation process was reduced. This is in accord with previous findings of Liu et al. (9) who observed that oysters displayed little depuration of either liquor or meat at 7 to 8°C. In our experiment at 6°C, a few (≤1%) viable *E. coli* and *S. typhimurium* were still present in the hemolymph of clams and oysters 72 h after intracardial inoculation. At the lower temperature, mean bacterial uptake by ingestion was 10 times less possibly because of lowered pumping action which would also have resulted in decreased circulation of the hemolymph. In this connection, Foley and Cheng (6) have established that the influence of temperature on particle clearance is proportional to its effect on molluscan heart rate.

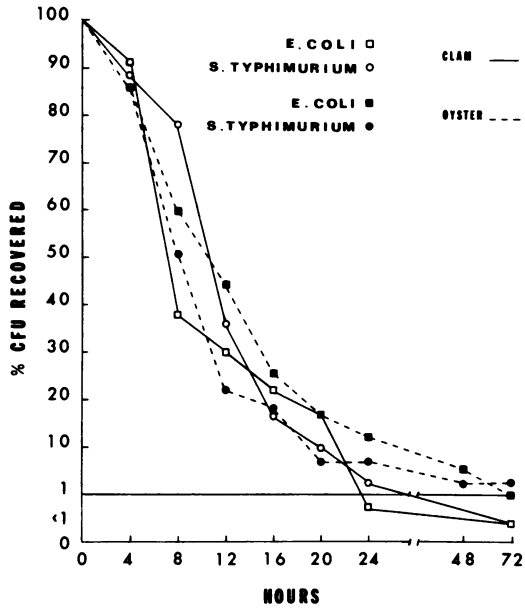

FIG. 2. *In vivo bacterial clearance of selected enteric pathogens from the hemolymph of C. virginica and M. mercenaria after intracardial inoculation at 6°C. CFU values were obtained per milliliter of hemolymph. At 72 h, CFU values were obtained per gram of homogenate.*

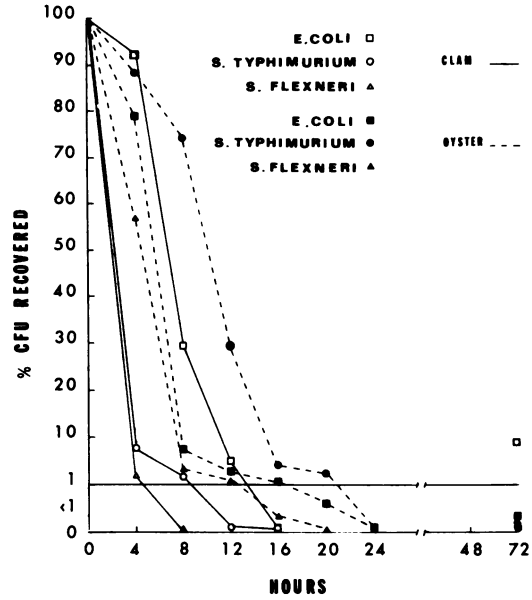

FIG. 3. *In vivo bacterial clearance of selected enteric pathogens from the hemolymph of C. virginica and M. mercenaria following exposure through natural ingestion at 20°C. CFU values were obtained per ml of hemolymph. At 72 h, CFU values were obtained per gram of homogenate.*

Oysters and clams inoculated via the intracardial route generally exhibited slightly greater

TABLE 1. *Counts of E. coli and S. typhimurium in hemolymph of the hard clam (M. mercenaria) and the American oyster (C. virginica) after 15 min of oral exposure to 4 × 10^3 bacteria per ml*

Bacterial species	Mean CFU/ml of hemolymph (range)			
	Clam		Oyster	
	20°C	6°C	20°C	6°C
E. coli	234 (28–500)	10 (9–11)	145 (105–203)	21 (17–25)
S. typhimurium	194 (5–500)	20 (9–28)	567 (500–600)	63 (43–83)
S. flexneri	127 (77–175)		560 (500–600)	

persistence of bacteria than when exposed in the surrounding water (Fig. 1 and 3). The reason for this is not clear, but it may have been the result of the trauma of the inoculation process. After intracardial injection, bacteria are known to be transported from blood vessels to adjacent tissues and then removed by migrating phagocytes to the water stream (14). Presumably, some bacteria that are ingested by shellfish and enter the hemolymph are removed by a similar process. Unpublished data obtained in our laboratory suggest, however, that another influence is contributing to bacterial disappearance in the shellfish. We have observed that bacterial counts in the water surrounding the inoculated shellfish are lower than would be expected if appreciable numbers of bacteria in migrating phagocytes were being dumped into the water stream. Possibly some of these bacteria enter the digestive tract and are destroyed by enzyme action (15). Some may also be destroyed by lysozomal enzymes released from hemolymph cells (3). Previous work by Janssen (8) in the oyster suggested that *S. flexneri* and *S. typhimurium* are retained for markedly different times. *S. flexneri* was removed within 24 h, whereas small numbers of *S. typhimurium* persisted up to 7 weeks. In our study, the latter organism was also noted to persist in greater numbers in the oyster after exposure by the oral route than in the clam. It should, however, be pointed out that the initial uptake of *S. typhimurium* by oysters was more than twice that of clams. Greater persistence of *S. typhimurium* in oysters than in clams in polluted waters has already been observed (1).

Although uptake of bacteria was greatly reduced, clearance from hemolymph still occurred to a significant degree at 6°C for both species of shellfish. Thus, shellfish would have the ability to depurate themselves at winter temperatures even after pumping action had ceased. This is consistent with the well-established observation that shellfish harvested from polluted waters in winter exhibit very low bacterial counts (10).

With the exception of *E. coli* in the oyster and clam, only small numbers of bacteria were found in homogenates at 72 h. Because shellfish sanitation standards have traditionally been based on coliform and fecal coliform counts, our findings suggest that standards based on these bacteria give a maximum statement of risk and at times may not correlate with the presence of other pathogenic enteric bacteria. Other investigators have similarly questioned the correlation of these organisms with enteric pathogens such as *Salmonella* (1, 12, 13).

It should be stressed that our shellfish were initially exposed to numbers of bacteria much larger than would be encountered in most naturally occurring polluted estuarine waters. The ability of these animals to clear themselves of heavy contamination by enteric bacteria supports the potential of artificial depuration systems as a means of utilizing shellfish from contaminated waters.

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