Uptake of Bacteriophage and Their Subsequent Survival in Edible West Coast Crabs After Processing

R. DiGIROLAMO, L. WICZYNSKI, M. DALEY, F. MIRANDA, AND C. VIEHWEGER

Department of Biology, College of Notre Dame, Belmont, California 94002

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Edible West Coast crabs (Cancer magister and Cancer antennarius), when in seawater contaminated with coliphage T4, were found to accumulate high titers of this virus. To study the extent of the hazard presented by crabs contaminated with virus, samples of edible crabs were contaminated with coliphage T4 and then processed by boiling. Results indicated that virus in crabs withstood this method of processing. The survival rate varied from 2.5 to 20%, depending upon the processing time. Heat penetration studies showed that, although internal temperature in the crabs was sufficient to inactivate virus, the processing times normally used to cook crabs were not. These results suggest that processed crabs could serve as vectors for the dissemination of virus diseases if the crabs are harvested from a polluted area.

It has been well proven that bivalved molluscs (oysters, mussels, and clams) when harvested from sewage polluted waters, even if later cooked, could serve as vectors of viral disease, e.g., infectious hepatitis (2, 5, 6, 8, 9, 10). Recently, it has also been shown that not only these shellfish, but also certain species of crabs, can become contaminated with virus, either from feeding off of contaminated shellfish or as a direct result of residing in sewage-polluted waters (3). However, the actual sites of virus accumulation within these animals is unknown, as is the extent of the hazard presented by the possible survival of virus in edible crabs after processing. Therefore, a series of studies was made to determine the major sites of viral residence within crabs and the amount of virus surviving in edible crabs after processing by boiling. This latter study was considered of particular value because the Dungeness crab and the "Red Rock" crab, the two principal edible crabs found along the Northern California coast, are normally cooked in this fashion prior to being eaten.

MATERIALS AND METHODS

Crab samples. The Dungeness crabs (Cancer magister) and "Red Rock" crabs (C. antennarius) used in these studies were collected off of Pillar Point, California, by using commercial crab traps. Only crabs having a carapace width of 15 to 18 cm were used in these studies. The carapaces and surfaces of the crabs were cleaned of any debris and sanitized by briefly dipping them in a 1% hypochlorite solution. They were then rinsed with filtered seawater. Crabs to be used in experiments were kept in 38-liter stainless-steel aquaria to which were added 3,500 ml of filtered seawater of 30% salinity. Water temperature was maintained at 10 C by cold-water circulation in a constant-temperature bath. Aeration and water circulation were provided by means of air hoses placed in the aquaria.

Virus. The virus model used in all experiments was coliphage T4. Phage and host cells, Escherichia coli B, were obtained from Presque Isle Microbiological, Presque Isle, Pa. Stock virus cultures were propagated in 250-ml nutrient broth (Difco) cultures of E. coli. The cells were removed by centrifugation and filtration. The supernatant fluid, containing 10^9 phage particles per ml, was stored over chloroform at 5 C.

Uptake studies. To determine the amount of virus acquired by edible crabs during a 48-hr period and the major anatomical sites of this uptake, the following study was conducted. Sufficient coliphage T4 was added to the seawater to yield a total virus count of approximately 2.6 × 10^4 plaque-forming units (PFU)/ml. Samples of seawater and crabs were collected at 0, 12, 24, and 48 hr intervals. Three crabs were used per sample. Ten ml of seawater was routinely used as a representative water sample. Crabs were removed from the aquaria, dipped in a 1% hypochlorite solution to sanitize the shell surfaces, rinsed in distilled water, sealed in polymy lar bags, and frozen at -20 C for 1 hr or until used. The
purpose of the freezing was to kill the crabs. Samples were prepared for assay by allowing the crabs to first thaw to room temperature (22°C). Carefully, and as aseptically as possible, incisions were made in the carapace of the animals and the blood was drained into sterile petri dishes. The carapace was removed, and first the digestive system and then the crab muscle tissue were dissected out. Then homogenates of crab digestive glands and muscle were prepared by blending samples with an equal weight of nutrient broth for 2 min in a Waring Blender at 6,500 rev/min. Homogenation was not deemed necessary for blood samples.

**Assay technique.** Ten-ml samples of the homogenates, blood, and seawater were placed in sterile 20-ml screw-capped tubes to which were added 2 ml of chloroform. The tubes were capped and shaken and then centrifuged at 25,000 × g at 10°C for 30 min. A 1-ml sample of supernatant fluid and serial decimal dilutions prepared in nutrient broth were uniformly spread over a base layer of nutrient agar plus 0.0025% CaCl₂. After 15 min this was overlaid with 1.5% nutrient agar adjusted to pH 7.6 with trihydroxyethylaminomethane buffer and containing approximately 10⁶ host cells per ml. The host cells, *E. coli* B, were propagated in 250-ml flasks of nutrient broth at 37°C. Plates were incubated for 12 hr at 37°C.

**Processing studies.** To determine the survival rate of bacteriophage in crabs processed by boiling, the following study was conducted. Fifteen "Red Rock" crabs (Cancer productus) with a 15- to 18-cm carapace width were exposed to coliphage T4 (4.0 × 10⁴ PFU/ml of seawater) for 48 hr, sanitized, sealed in polymyler pouches (three per pouch), and frozen at −20°C as described above. After thawing to room temperature (22°C), the crabs were processed according to standard cooking procedures in 5 liters of boiling water (100°C) containing 0.5 g of NaCl per liter. Samples were removed at 0, 5, 10, 15, and 20 min by using metal tongs. They were placed in polymyler pouches and allowed to cool to 25°C before being assayed. To determine the rate of inactivation of phage in the various anatomical regions of the crabs, the shellfish were dissected as aseptically as possible. The carapace was removed and the coagulated blood was scooped out, and, following this, the digestive area and then the crab muscle tissue were dissected out. For these studies the digestive system and muscle were pooled and reported as one (crab tissue). Homogenates were prepared in nutrient broth. Serial decimal dilutions in nutrient broth were prepared from the homogenates and all samples were assayed. Control samples for these experiments were contaminated crabs not subjected to boiling.

**Temperature determinations.** Temperature determinations were made in the processing studies to correlate the rate of phage inactivation with the rise in temperature inside processed crabs. All processing studies were repeated in duplicate with noncontaminated crabs. Five crabs were used in each experiment. A thermocouple wire was inserted into the digestive area of each crab and the temperature change recorded on a recording potentiometer. The shellfish were processed as described above. Thus, temperature increase inside the crabs could be determined for the duration of boiling. To insert thermocouples, small holes (4 mm) were drilled into the central part of the carapace and the thermocouple wire inserted. The thermocouple wires were taped down with waterproof tape to prevent them from slipping out of the shells.

**RESULTS**

**Uptake of bacteriophage by edible crabs.** The results of uptake studies are presented in Fig. 1. After 24 hr, total uptake of coliphage T4 was found to be 1.5 × 10⁴ particles or, on a per-unit-weight basis, approximately 58% of the virus present in 1 ml of seawater. Of the total virus present in the crabs, 8.0 × 10⁴ or 31% was recovered from the muscle, 5.5 × 10³ or 21% from the digestive system, and 1.5 × 10³ or 6% from the blood. Total phage uptake at the end of 48 hr was found to be 1.9 × 10⁴ or approximately 73%. Phage titer in the muscle was found to have decreased slightly to 7.0 × 10³ or approximately 29%. However, the greatest single increase observed was in the number of phage recovered from the blood, 4.5 × 10³ or 17% of the total phage recovered. This represents an almost 200% increase in the number of phage recovered from the blood. This increase in phage titer in the blood was a

![Fig. 1. Uptake of coliphage T4 by edible West Coast crabs in contaminated seawater for 48 hr (salinity, 30%; temperature, 10°C). Symbols: x, phage/ml of seawater; □, phage/g of crab muscle; ○, phage/ml of crab blood; ▲, phage/g of crab digestive system.](image-url)
persistent phenomenon observed in all uptake experiments.

**Processed crab studies.** The inactivation of bacteriophage in boiled crabs was relatively rapid. However, after 10 min of processing, 10% of the phage still survived, and 2.5% still survived after 20 min (Table 1).

**Heat penetration studies.** The results of heat penetration determination for the above study are presented in Fig. 2 and Table 1. Temperature rise was rapid inside the crabs. Routinely, exposure to a temperature of 70°C for 30 min is required for fully heat-inactivate virus (1). In these studies, 5 min of heating was required for the crabs to reach this temperature, and the processing times used were under 30 min. Under experimental conditions the internal temperature of the crabs never obtained that of input (processing temperature). This was as expected and is due, in part, to the kinetics of heat penetration and, in part, to the processing times used.

**DISCUSSION**

**Uptake of bacteriophage by edible crabs.** We have previously reported that West Coast shore crabs accumulate a considerable level of at least one enterovirus, type 1 poliovirus (3). Thus, we observed that *Pachygrapsus* sp. and *Hemigrapsus* sp., if allowed to feed on contaminated shellfish, would accumulate within their tissues 74 to 94% of the virus contained in the shellfish tissue. We also observed that these same animals, if placed in contaminated water, would acquire in 48 hr 63% of the virus present. The results of our new studies confirm this latter observation and show that edible crabs also accumulate virus from seawater. The difference in uptake observed between the two studies probably reflects the difference in size of the experimental animals. Significantly, under experimental conditions, 26 to 29% of the virus was recovered from crab muscle, which is the portion of the animal normally eaten.

These findings are significant because crabs caught in so-called clean water have always been assumed to be free of contamination. However, unlike bivalved molluscs, crabs are not sedentary but do migrate either in search of food or with tidal changes. Hence, it is possible for crabs to enter sewage-polluted water even for a brief period of time and become contaminated either from their food source or the water, or both. Thus, even if caught in pollution-free water, the crabs still could be potential vectors of virus diseases if they had resided for any length of time in polluted water. The reasons for the accumulation and persistence of virus in crabs are not known. It has been shown that accumulation of virus by shellfish is due to ionic bonding between the virus and shellfish mucous (R. DiGirolamo, Ph.D. thesis, Univ. of Washington, Seattle, 1969). Possibly some form of ionic bonding, plus the tendency of virus to aggregate, may be the means by which the virus enters and persists in crabs. However, this would not explain the increase in titer observed in crab blood. Hence, further research is needed to clarify these matters.

**Survival of bacteriophage in boiled crabs.** Viruses are known to be inactivated by heat, which causes coagulation and breakdown of the virus protein coat. However, the medium in which viruses are held has been shown to influence virus sensitivity to thermal inactivation (1, 4, 7, 11, 12).

Results of our processing studies show that virus in crabs survived the inactivating effects of heat. The survival rate varied from 2.5 to 20%, depending upon processing time. Heat penetration studies indicate that, although the internal temperature of the crabs is sufficient to inactivate virus, the duration of processing is not. Significantly, crabs normally are not boiled commercially or in the home for more than 10 to 15 min. Under experimental conditions the amount of virus surviving at these

| Processing time (min) | Internal temp (C) | Virus PFU/ml of crab blood | Virus PFU/g of crab muscle | Survival (%) |
|-----------------------|------------------|---------------------------|---------------------------|--------------|
|                       |                  |                           |                           | Crab blood   |
|                       |                  |                           |                           | Crab muscle  |
|                       |                  |                           |                           | Total        |
| 0                     | 20               | $2.0 \times 10^2$         | $1.1 \times 10^4$         | 64.5         |
| 5                     | 70               | $2.3 \times 10^2$         | $1.0 \times 10^4$         | 11.5         |
| 10                    | 75               | $1.6 \times 10^2$         | $8.0 \times 10^2$         | 8.0          |
| 15                    | 79               | $1.0 \times 10^2$         | $5.4 \times 10^2$         | 5.0          |
| 20                    | 84               | $1.0 \times 10^2$         | $2.1 \times 10^2$         | 0.5          |

*PFU = plaque-forming units.*
LITERATURE CITED
1. Andrewes, C., and H. G. Pereira. 1967. Viruses of vertebrates, p. 50. The Williams & Wilkins Co., Baltimore.
2. DiGirolamo, R., J. Liston, and J. Matches. 1970. Survival of virus in chilled, frozen, and processed oysters. Appl. Microbiol. 20:58-63.
3. DiGirolamo, R., L. Wiczynski, M. Daley, and F. Miranda. 1971. Preliminary observations on the uptake of poliovirus by West Coast shore crabs. Appl. Microbiol. 23:170-171.
4. Dimmock, I. F. 1967. Differences between the thermal inactivation of picorna viruses at "high" and "low" temperatures. Virology 31:338-342.
5. Dougherty, W. J., and R. Altman. 1962. Viral hepatitis in New Jersey. Amer. J. Med. 32:704-716.
6. Mason, J. O., and W. R. McClean. 1962. Infectious hepatitis traced to consumption of raw oysters. Amer. J. Hyg. 75:90-112.
7. Milo, G. E. Jr. 1971. Thermal inactivation of poliovirus in the presence of selective organic molecules (cholesterol, lecithin, collagen, and β carotene). Appl. Microbiol. 21:198-202.
8. Rindge, S. J., R. F. Johnson, J. W. Moseley, J. B. Atwater, M. A. Rosetti, and J. C. Hart. 1964. An epidemic of clam associated hepatitis. J. Amer. Med. Ass. 208:649-655.
9. Roos, B. 1956. Hepatitis epidemic conveyed by oysters. Sv. Lekkartin. 53:984-1003.
10. Rully, S. J., R. F. Johnson, J. W. Moseley, J. B. Atwater, M. A. Rossetti, and J. C. Hart. 1964. An epidemic of clam associated hepatitis. J. Amer. Med. Ass. 208:649-655.
11. Wallis, C., and J. L. Melnick. 1965. Thermostabilization and thermosensitization of herpesvirus. J. Bacteriol. 90:1632-1637.
12. Wallis, C., C. Young, and J. L. Melnick. 1962. Effects of cations on thermal inactivation of vaccinia, herpes simplex, and adenovirus. J. Immunol. 84:41-47.