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Survival of Virus in Chilled, Frozen, and Processed Oysters

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Samples of whole and shucked Pacific and Olympia oysters, contaminated with 10^4-plaque-forming units (PFU) of poliovirus Lsc-2ab per ml, were held refrigerated at two temperatures, 5 and −17.5 C. To study the survival of virus in the oysters under these conditions, samples were assayed for virus content at weekly intervals for as long as 12 weeks. Results indicated that poliovirus would survive in refrigerated oysters for a period varying from 30 to 90 days, depending upon temperature. The survival rate varied from 10 to 13%. To study the extent of the hazard presented by oysters contaminated with virus, samples of whole and shucked Pacific oysters contaminated with 10^4 PFU of poliovirus Lsc-2ab per ml were heat processed in four ways: by stewing, frying, baking, and steaming. Results indicated that virus in oysters withstood these methods of processing. The survival rate varied from 7 to 10% and appeared dependent upon the processing method used. Heat penetration studies showed that the internal temperature in the oyster was not sufficient to inactivate all of the virus present. These results suggest that not only fresh but also refrigerated and cooked oysters can serve as vectors for the dissemination of virus disease if the shellfish are harvested from a polluted area.

Epidemiological evidence has incriminated bivalve mollusks that have been feeding in sewage-polluted waters as vectors for the enteric virus disease infectious hepatitis (9, 10, 14, 16-18). Research by European and East coast workers has shown that viruses can survive in the sea long enough to be taken up by shellfish (2, 3, 13). Similar findings have been reported recently for West coast species (8). The major site of virus accumulation in these species has proven to be the digestive tract (11, 12, 15). However, the extent of the hazard presented by the survival of virus, through the processes of freezing or cooking, has not been fully examined. Therefore, a study was made of virus survival in chilled and frozen oysters and in oysters processed by stewing, frying, steaming, and baking. This latter study was considered of particular value because the Pacific oyster, the principal oyster grown on the West coast, is normally cooked prior to being eaten.

MATERIALS AND METHODS

Oyster samples. The Pacific oysters (*Crassostrea gigas*) and Olympia oysters (*Ostrea lurida*) used in these experiments were obtained from a Shelton, Wash., oyster grower in lots of 48 to 50 oysters. The shells were cleaned of external debris and superficially disinfected by dipping in a 1% hypochlorite solution; they were then rinsed with tap water and dried. Oysters to be used in experiments were kept in 5-gal (ca. 19 liters) stainless-steel aquaria to which was added 3,500 ml of filtered seawater of 28‰ salinity. Water temperature was maintained at 13 C by cold-air circulation in a constant temperature bath. Aeration and water circulation were provided by means of air hoses placed in the aquaria.

Virus. Attenuated poliovirus Lsc-2ab was used in all experiments. This virus is representative of enteric viruses and has been used commonly in studies dealing with the uptake of virus by shellfish. The strain was obtained from the Department of Preventive Medicine, University of Washington. The seed virus contained 3.5×10^8 virus plaque-forming units (PFU)/ml. Stock virus was propagated on primary African green monkey (MK) kidney cell cultures. The stock virus pool, diluted with Hanks balanced salt solution (BSS) at 30 C and adjusted by plaque formation determinations to contain approximately 10^8 PFU/ml, was kept at −20 C until used.

Tissue culture. Cell suspensions of primary MK kidney tissue (BBL) were used throughout experiments. Monolayer cultures were prepared in 3-oz prescription bottles (ca. 90 ml). Hanks BSS, to which were added 0.2% Casamino Acids and 10% calf serum, was used for cell growth, and Earle's BSS containing 2% calf serum was used for cell maintenance. One hundred units of penicillin G per milliliter, 100 μg of streptomycin sulfate per ml, and 2.5 μg of

Fungizone per ml were incorporated in all media to prevent the growth of bacteria, molds, and yeasts.

Plaque assay technique. The assay method used was essentially that of Hsuing and Melnick (9). The medium was decanted from monolayer bottles, and 0.5 ml of appropriately diluted sample was added to the bottles. All samples and all experiments were run in duplicate. Immediately after inoculation, the bottles were rotated five times to insure even distribution of the inoculum over the cell sheet. All inoculated bottles were incubated at 36 C for 1 hr and 15 min to allow for virus adsorption. After incubation, cell sheets were washed once with 4 ml of warm (30 C) Hanks BSS and then overlaid with 10 ml of growth medium containing 1.5% purified agar (Difco) and 0.0017% neutral red (BBL). This procedure was conducted in semidarkness to prevent photoinactivation of virus and cell sheets by the neutral red. After the agar solidified, the bottles were rotated flat side up and then covered and returned to the 36 C incubator. Bottles were observed for the appearance of plaques on the third to fifth days. Each plaque was marked with a Magic Marker pen, and the total number observed was written on the side of the bottle. The total count on the last day of observation was considered the final count. The sum of the counts of two bottles multiplied by the dilution factor represented the total virus content in each milliliter of inoculum.

Survival of virus in chilled Olympia oysters. To determine the survival rate of virus in Olympia oysters held at storage temperatures, the following study was conducted. Forty-eight 4-year-old Olympia oysters were exposed to attenuated poliovirus I for 48 hr, dipped in a 1% hypochlorite solution to disinfect the shell surfaces, washed in distilled water, and sealed in polymylar pouches, eight per pouch. Samples were placed in a refrigerator set at 5 C and assayed for virus content at 0, 5, 10, 15, 20, 25, and 30 days. To determine the rate of virus inactivation in the various anatomical regions of the oyster, the shellfish were carefully dissected as aseptically as possible. The gills, mantle, and palps were first dissected out, then the digestive area, and the remaining body. Ten per cent (w/v) homogenates of each in nutrient broth were prepared, and the samples were clarified by low-speed centrifugation (1,200 $\times$ g for 20 min at 10 C) in a Sorvall RC 2-B refrigerated centrifuge. Serial decimal dilutions in nutrient broth were prepared, and all samples were assayed.

Survival of virus in frozen oysters. To determine the ability of virus to withstand inactivation in frozen whole oysters, a long-term study was conducted. Fifty shucked Pacific oysters were inoculated directly in the gut region with an inoculum containing 10^4 virus PFU/ml. The inoculum consisted of poliovirus Lsc-2ab in nutrient broth. The oysters were sealed in polymylar pouches, five to a pouch, and quick frozen at 36 C in a blast freezer for 24 hr. Suspensions of poliovirus (3 ml) in nutrient broth were also quick frozen for 24 hr to serve as controls. Samples were removed from the freezer and stored frozen at -17.5 C. Samples were assayed for virus content at 0, 2, 4, 6, 8, 10, and 12 weeks, by using identical assay procedures (*see above*).

Survival of virus in stewed, fried, baked, and steamed oysters. (i) For stewed oyster studies, 20 medium-sized shucked Pacific oysters were inoculated with 0.5 ml of an attenuated poliovirus suspension in nutrient broth containing 10^4 virus PFU/ml. Oyster stew, in milk, was prepared by standard cookbook methods. The inoculated oysters were placed in boiling milk in a 2.5-liter stainless steel kettle. Normal recipe procedure requires the oysters to stew for 5 min; however, cooking time was extended to 8 min. Samples of milk and oysters were removed at 0, 2, 4, and 8 min. A 5-ml amount of milk and five oysters constituted a sample. The milk was aseptically pipetted into 20-ml sterile test tubes, and the oyster samples were sealed in polymylar pouches. All samples were cooled to room temperature (23 C). Samples not assayed immediately were held at -20 C until tested. Controls consisted of inoculated oysters which were not stewed but were sealed in polymylar pouches and held at -20 C until assayed. (ii) For fried oyster studies, 20 medium-sized shucked Pacific oysters were inoculated with a poliovirus suspension in the identical manner described in the preceding experiment. The oysters were prepared for frying by standard cookbook recipes. Oysters were coated in a batter of egg, bread crumbs, and seasoning and fried in Wesson Oil at a temperature of 177 C. Frying time was extended from the 5 to 8 min called for in the recipe to 10 min. Samples of five oysters each were removed for assay at 3-, 6-, and 10-min intervals, sealed, in polymylar pouches, allowed to cool, and tested. The zero-hour and control samples consisted of inoculated but unprocessed oysters. (iii) For the baked oyster study, 20 medium-sized Pacific oysters were inoculated with poliovirus, as described in preceding experiment, and then prepared by standard cookbook methods. The oysters were rolled in a batter of egg and bread crumbs, plus seasoning, and then baked for 20 min in an oven at 121.5 C. Samples were removed for assay at 5, 15, and 20 min. Oysters used as zero-hour and control samples were inoculated and rolled in batter but not baked. (iv) For the steamed oyster study, 25 medium-sized Pacific oysters were placed in three 5-gal (ca. 19 liters) aquaria containing 3,500 ml of filtered seawater (28% salinity) at 13 C. To each aquarium was added sufficient poliovirus Lsc-2ab to yield a count of 10^5 virus PFU/ml of seawater. The oysters were contaminated for 48 hr and then removed from the aquaria. Shell surfaces were disinfected by dipping in a 1% hypochlorite solution and then rinsed in distilled water and dried. Oysters used in experiments were placed in metal pans and held under flowing steam for 30 min in an autoclave. Samples were removed at 0, 5, 15, 20, and 30 min, by using metal tongs. They were placed in polymylar pouches and allowed to cool to 25 C before being assayed. The control samples were contaminated oysters not subjected to steaming.

Temperature determinations. Temperature determinations were made in all processing studies to correlate the rate of virus inactivation with the rise in temperature inside processed shellfish. All processing procedures were repeated in duplicate with noncontaminated oysters. Four oysters were used in each experiment. A thermocouple wire was inserted into the

gut area of each shellfish, and the temperature change was recorded on a Brown Recording Potentiometer. The shellfish were processed as described above. Thus, temperature increase inside the shellfish could be determined for the duration of each processing procedure. To insert thermocouples into oysters used in steaming experiments, small holes (4 mm) were drilled into the bills of the shells and thermocouple wires were inserted. The thermocouple wires were taped down with plastic tape to prevent them from slipping out of the shells.

RESULTS

Survival of virus in chilled olympia oysters. Poliovirus in Olympia oysters proved to be very stable under storage conditions (Table 1). After 5 days of storage at 5 C, virus numbers in the oysters were reduced by 10%, with 75% of the total virus still viable in the digestive area. After 15 days of storage, the total virus count was reduced by 60%, with 36% of the total virus still surviving in the digestive area.

Because of decomposition, it was impossible to dissect the oysters after more than 15 days of storage. A second study was made over a 30-day period in which virus survival in the whole oyster was measured. Results are summarized in Fig. 1. After 10 days of storage, there was less than a log reduction in the total virus present and 46% of the virus were still viable. After 30 days of storage, the oysters were badly decomposed. At this time, the total virus count was reduced by 2 logs, but 13% of the virus still remained viable even under these conditions.

Survival of virus in frozen oysters. Poliovirus was found to be very stable in whole Pacific oysters held in the frozen state. Loss of virus titer was gradual during the entire period of the study (Fig. 2). After 4 weeks of storage, the total virus count was reduced by little more than 0.5 log. By 12 weeks of storage, the original virus titer in the oysters was reduced by one log. Approximately 91% of the virus was viable after 2 weeks of storage, 40% was still viable at 6 weeks, and 10% still survived after 12 weeks of storage (Table 2).

Stewed oyster studies. The inactivation of poliovirus in stewed oysters was relatively rapid. The stewing process caused the oysters to split open after 2 min of cooking time, thereby releasing some of the virus into the hot milk. This fact probably accounted for some of the virus inactivation. However, even after 8 min of stewing, 10% of the virus still survived in the oyster, and 7% was recovered from the milk (Table 3).

Fried oyster studies. The initial inactivation of poliovirus in the oyster was slow, with 61% of the

TABLE 1. *Recovery of poliovirus from the organs and body of O. lurida stored for 15 days at 5 C*

Sample	Time (days)	Virus PFU/g	Per cent of virus survival
Gill, mantle, palps	0	3.8×10^2	2.4
Body		1.74×10^3	9.4
Digestive area		1.36×10^4	88.2
Gill, mantle, palps	5	2.6×10^2	1.6
Body		1.36×10^3	8.8
Digestive area		1.18×10^4	75.4
Gill, mantle, palps	10	1.46×10^2	1.0
Body		6.8×10^2	4.4
Digestive area		6.4×10^3	41.4
Gill, mantle, palps	15	5.0×10^2	0.32
Body		6.90×10^2	4.4
Digestive area		5.70×10^3	36.3

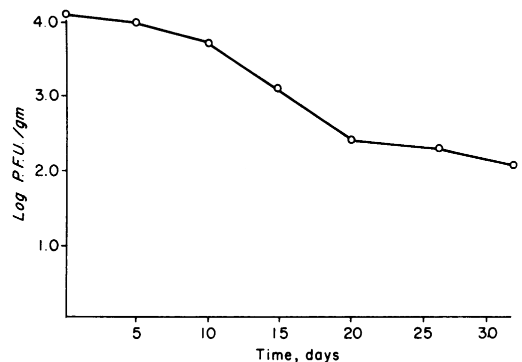

FIG. 1. *Survival of poliovirus in Olympia oysters refrigerated at 5 C for 30 days.*

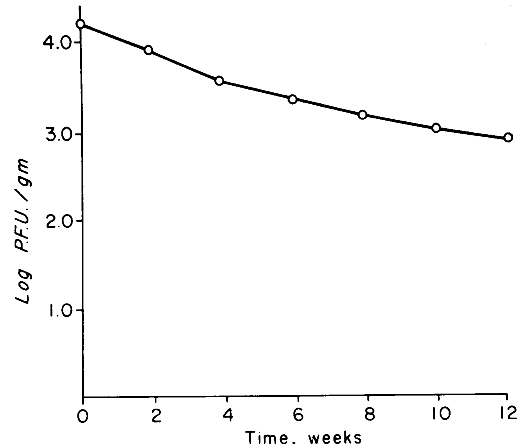

FIG. 2. *Recovery of poliovirus from Pacific oysters frozen at −36 C and held in the frozen state at −17.5 C.*

virus still viable after 3 min of processing. The virus were rapidly inactivated as processing continued (Table 4). When the experiment was terminated, after 10 min of frying, the virus count had been reduced by approximately 2.5 logs. This represents a 13% survival of poliovirus.

TABLE 2. *Recovery of poliovirus from Pacific oysters frozen at −36 C and stored for 30 days at −17.5 C*

Time (weeks)	Virus PFU/g of oyster	Per cent of virus survival
0	1.03×10^4	100
2	9.7×10^3	94.1
4	9.4×10^3	91.1
6	4.2×10^3	40.7
8	2.9×10^3	28.1
10	2.0×10^3	19.4
12	1.0×10^3	9.7

TABLE 3. *Recovery of poliovirus from oysters processed by stewing*

Sample	Processing time (min)	Virus PFU/g or Ml	Per cent of survival	Internal temp (C)
Oyster	0	1.0×10^4	100	18.5
	2	2.7×10^2	27	33.5
	4	1.4×10^2	14	57.0
	8	1.0×10^2	10	75.0
Milk	0	0	0	
	2	1.4×10^2	14	
	4	1.2×10^1	12	
	8	7.0×10^1	7	

TABLE 4. *Recovery of poliovirus from fried oysters processed for 8 min*

Processing time (min)	Virus PFU/g	Per cent of survival	Internal temp (C)
0	1.2×10^4	100	23.0
3	7.6×10^3	61	40.0
6	4.4×10^2	36	72.5
8	1.7×10^1	13	100.0

TABLE 5. *Recovery of poliovirus from baked oysters processed for 20 min*

Processing time (min)	Virus PFU/g	Per cent of survival	Internal temp (C)
0	1.9×10^4	100	25
5	4.6×10^3	24.2	41
15	3.9×10^2	20.5	68
20	2.7×10^1	12.7	90

Baked oyster studies. The rate of virus inactivation was initally rapid but declined as processing proceeded (Table 5). Virus titer was reduced by approximately 2.5 logs after 20 min of baking, at which time the experiment was terminated. Virus titer was rapidly reduced during the first 5 min of baking, with only 24% of the virus still surviving at this time. However, at the conclusion of the study, 13% of the virus still survived in the baked oysters.

Steamed oyster studies. The inactivation of poliovirus in steamed oysters appeared to progress in four distinct stages, two of rapid decline and two of longer, more gradual decline. The total virus count was decreased by 1 log after 15 min of steaming and by 2 logs at the end of the experiment. At this time, although the oysters had been steamed for 30 min, approximately 7% of the virus was still viable (Fig. 3).

Heat penetration studies. The results of heat penetration determinations for the above studies are presented in Tables 2 through 6. Temperature rise was rapid inside oysters processed by stewing and frying, but less so in baked and steamed oysters. Routinely, exposure to a temperature of

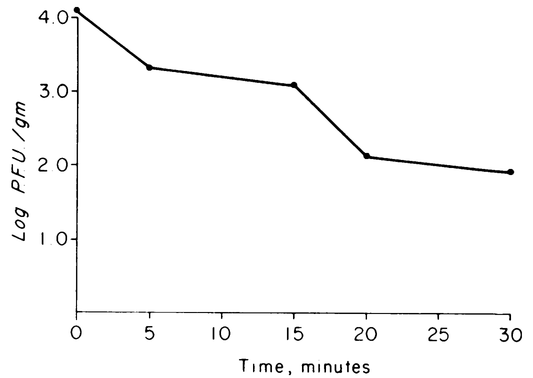

FIG. 3. *Kinetic of poliovirus inactivation in steamed oysters.*

TABLE 6. *Temperature increase in the digestive area of Pacific oysters processed by steaming*

Time (min)	Temp (C)
0	15.0
5	38.4
10	50.2
15	60.0
20	67.2
25	88.0
30	93.7
35	100.0

70 C for 30 min is required to fully heat-inactivate poliovirus (1). In these studies, 6.5 min of heating was required for stewed oysters to reach this temperature, 5.5 min for fried oysters, 16 min for baked oysters, and 20 min for steamed oysters. Under experimental conditions, only in the case of steaming did the actual internal temperature of the processed oysters attain that of input (processing temperature). This was as expected and is due in part to the processing times used and in part to the kinetics of heat penetration.

DISCUSSION

Survival of virus in chilled Olympia oysters. It has been reported that enteroviruses would persist for a considerable length of time in inoculated, chilled, commercial food products. Thus, Lynt (14) observed that type 1 poliovirus and types B1 and B6 coxsackievirus would survive for at least 1 month in representative samples of commercially prepared foods stored at 10 C. The results of our studies are in agreement with the observation of Lynt and show that virus can survive for a considerable length of time in chilled oysters. After 15 days of storage at 5 C, the total poliovirus remaining in the Olympia oysters was reduced by only 60%. Significantly, three-fourths of the remaining virus was recovered from the digestive tract. After 30 days of storage, although the oysters were badly decomposed, 13% of the poliovirus was still viable, indicating that the breakdown products of oyster decomposition have little effect on the virus.

These findings are significant since the Olympia oyster, a small cocktail oyster, is normally not held either in grocery or household refrigeration for more than 5 days. Obviously, refrigeration for this length of time will not be sufficient to inactivate even low numbers of virus.

The reasons for the prolonged survival of virus in shellfish are not known. The tendency of virus to aggregate and their incorporation by ionic bonding into shellfish mucous may be means by which virus are able to survive in chilled oysters. However, further research is needed to clarify this matter.

Survival of virus in frozen oysters. Lynt (14) reported that poliovirus type 1 and coxackievirus types B1 and B6 would survive for 5 months in inoculated frozen foods held at −20 C, findings subsequently confirmed by Heidelbaugh and Giron (6). Results of the present studies with Pacific oysters show that virus also survive for considerable lengths of time in frozen oysters. In our studies, the titer of poliovirus in frozen Pacific oysters was reduced by less than 10% after 4 weeks of storage at −17.5 C, and by only one log after 12 weeks of storage at this temperature.

The de facto survival of virus in frozen oysters is of public health significance. Since the advent of modern freezing techniques, there is considerable interstate shipment of frozen shellfish. If the shellfish are harvested from an area contaminated with virus, or if the animals become secondarily contaminated during handling, freezing will not inactivate these pathogens. Hence, these shellfish can serve as vectors for the dissemination of virus diseases.

Survival of virus in stewed, fried, baked, and steamed oysters. 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 (4, 19, 20).

Results of processing studies show that virus in oysters survived the inactivating effects of heat. Survival of the virus ranged from 7 to 13% in different home cooking procedures. Virus resistance to inactivation was influenced by the method of processing used. Thus, virus survival was greater in fried and baked oysters (13%) than in steamed and stewed shellfish (7%).

It has been proven that most of the virus in contaminated oysters is found in the digestive tract (11, 15). The heat penetration studies have shown that, at the processing times normally used to cook oysters, the internal temperature of the shellfish is not sufficient to inactivate all of the virus which may be present.

These findings are of great public health significance, since it is often assumed that cooked oysters are ipso facto safe foods. We believe that the possible accumulation by, and survival of, virus in marine food products is an area in which more research should be conducted.

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