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Use of Rotavirus Virus-Like Particles as Surrogates To Evaluate Virus Persistence in Shellfish

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Rotavirus virus-like particles (VLPs) and MS2 bacteriophages were bioaccumulated in bivalve mollusks to evaluate viral persistence in shellfish during depuration and relaying under natural conditions. Using this nonpathogenic surrogate virus, we were able to demonstrate that about 1 $\log_{10}$ of VLPs was depurated after 1 week in warm seawater (22°C). Phage MS2 was depurated more rapidly (about 2 $\log_{10}$ in 1 week) than were VLPs, as determined using a single-compartment model and linear regression analysis. After being relayed in the estuary under the influence of the tides, VLPs were detected in oysters for up to 82 days following seeding with high levels of VLPs (concentration range between 10^{10} and 10^{9} particles per g of pancreatic tissue) and for 37 days for lower contamination levels (10^{5} particles per g of pancreatic tissue). These data suggest that viral particles may persist in shellfish tissues for several weeks.

Bivalve mollusks may accumulate viral contaminants within their tissues during feeding, and outbreaks of viral gastroenteritis and hepatitis have been associated with oyster consumption (19, 21, 34) and even with depurated shellfish (15, 16, 30). Virus uptake by shellfish occurs rapidly (less than 24 h of exposure) following contact with sewage or seeded contaminated waters (3, 4, 11, 12, 19, 31, 33). However, little is known about viral persistence following depuration or relaying. Depuration is a dynamic process whereby shellfish are allowed to purge themselves of contaminants either in a natural setting or in land-based facilities, whereas relaying is the practice of transferring shellfish harvested from contaminated areas to clean shellfish-growing waters (31). Data on virus persistence are needed to address how long viruses may persist in contaminated oyster beds so that early reopening of beds that might pose a risk to the health of consumers can be prevented.

Working with live bivalve mollusks under laboratory conditions may introduce artificial parameters that do not adequately account for environmental factors (i.e., nutriments, temperature, or suspended matter, which can modify filtration rates) important for interpretation of results (8, 17, 27, 31, 33, 35). The use of large depuration tanks and professional equipment allows better reliability and reproducibility of different environmental parameters (aeration, UV, water quality, speed of water recycling, temperature, disinfection of wastewater). However, under such conditions, the potential environmental impact of live enteric viruses precludes their use, even when the viruses are of animal origin.

Coexpression of viral capsid proteins in the baculovirus expression systems results in the assembly of virus-like particles (VLPs) that maintain the structural and functional characteristics of the native particles: they resemble a real virus, but they are noninfectious (9). These VLPs have been helpful in fundamental research (7, 13) but also in environmental persistence and inactivation studies (5, 22) and in monitoring the fate and transport of viruses in subsurface water (28, 29).

The purpose of the study was to inoculate rotavirus VLPs containing full-length VP2 and VP6 (VLP2/6) and MS2 bacteriophage into bivalve mollusks and subsequently evaluate the effectiveness of seeded rotavirus VLPs to act as a surrogate in oysters over a prolonged period of time in an oyster farm.

MATERIALS AND METHODS

Rotavirus VLPs. VLP2/6 were produced as previously described (6). The VLP suspension was quantitated by estimation of the protein concentration by the method of Bradford using bovine serum albumin as a standard (6). Briefly, protein solution was mixed with Coomassie brilliant blue G250 (Pierce), and optical density at a lambda of 595 nm (OD_{595}) was read with a spectrophotometer. Protein concentration was determined using a standard curve established with a known amount of bovine serum albumin.

Phage. To produce the F^+ RNA bacteriophage strain MS2, 0.25 ml of an overnight culture of *Escherichia coli* K-12 Hfr (NCTC 12486) was inoculated into 25 ml of a tryptone-yeast extract-glucose medium and incubated for 1.5 h at 37°C. The phage MS2 (ATCC 15597-BI) was then added to a final concentration of 10^7 PFU/ml and incubated for 5 h at 37°C before addition of 2.5 ml of chloroform (Sigma). After centrifugation for 20 min at 3,000 × *g*, the supernatant was collected and used directly or frozen at −80°C.

Depuration pilot. A pilot tank was built on the Prat-Ar-Coum premises (Etablissements Y. Madec, Lannilis, France), as previously described (27). Briefly, each independent calibrated tank was equipped with pumps to provide effective aeration and seawater recycling (5 to 30 m^3/h) and a heater/cooler to work at constant temperatures (8 to 40°C). Each tank had a volume capacity of 6 m^3 and a maximum oyster weight of 600 kg. The pilot used seawater that was disinfected through a large sand filter and with UV. This pilot system provided all facilities necessary to assess shellfish depuration according to professional practices.

Oyster contamination. A homogeneous group of oysters (*Crassostrea gigas*) was selected for study to limit the effects of shellfish size and age on other physiological parameters (filtration rate, growth status, risk of death) that could impact the success of depuration.

Contamination with VLP2/6. Three different concentrations of VLP2/6 were tested: suspensions of 5×10^{14} (experiment A), 5×10^{12} (experiment B), or 5×10^{8} (experiment C) VLP2/6 were diluted in 1 liter of seawater and then added to a tank containing 3 m^3 of seawater. The final concentrations were 1.7×10^{11}, 1.7

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$\times 10^9$, and 1.7×10^5 particles per liter, respectively, for experiments A, B, and C.

Contamination with VLP2/6 and phages. A suspension of VLP2/6 (5×10^8 particles) and a suspension of MS2 phages (2×10^9 PFU) were diluted in 1 liter of seawater and then added to a tank containing 3 m^3 of seawater (experiment D). Final concentrations were 1.7×10^5 particles and 6.7×10^5 PFU per liter.

For all contamination experiments, VLPs were dispersed in the water of the tank for 20 min by pumping air through the water, and then 10 kg of oysters (representing 100 to 120 individuals) was immersed into the contaminated water for 24 h. After 24 h, 1 liter of water and 10 oysters were collected for assay (day 0). The water in the tank was evacuated, and remaining oysters were placed under depuration conditions.

Oyster depuration and relaying. Depuration experiments were conducted in identical clean tanks with clean seawater. Seawater (final volume = 3 m^3) was pumped from the middle of the estuary and was cleaned by passage through a sand filter and exposure to UV-C at a 25.75-mJ/cm^2 minimal dose as used by professional producers. The seawater was changed twice a day for 7 days at 22°C and was under aeration. Twelve oysters were sampled at days 0, 1, 2, 3, 5, and 7 and kept at 4°C during transportation to the laboratory and until analysis.

Relaying experiments were performed using the remaining oysters that had been depurated for 7 days under the conditions described above. The oysters were placed in the estuary in front of the shellfish farm for a natural depuration under the influence of the tides. Depending on the tide, oysters were out of the water twice a day for 2 hours, 20 days per month.

Oysters were sampled every week until the VLP2/6 analysis gave negative results.

Water analysis. Ten milliliters of a polyethylene glycol 6000 (Sigma, St. Quentin, France; 50% [wt/vol])-sodium chloride (1.2 M) solution was added to 40 ml of seawater and rocked for 1 hour at 4°C. After centrifugation for 90 min at $11,000 \times g$, the pellet was suspended in 200 μl of sterile water and analyzed in duplicate by enzyme-linked immunosorbent assay (ELISA).

Oyster analysis. For analysis, the oysters were washed, shucked, and weighed. The stomach and digestive diverticula were removed by dissection. All analyses were performed in duplicate. VLP2/6 analysis was performed using 1.5 g of stomach and digestive diverticulum (representing about 1.5 oysters), as previously described (20). Briefly, the dissected tissues were homogenized with glycine buffer, pH 9.6, and the VLPs were eluted using chloroform-butanol and CatFLoc (Calgon, Elwood City, PA), followed by polyethylene glycol 6000 (Sigma, St. Quentin, France) precipitation. The pellet was then suspended in 500 μl of sterile water, and 100 μl was used to determine the VLP2/6 concentration by ELISA. The sensitivity limit of the ELISA methods was assessed by adding 100 μl of serial dilutions of VLP to 1.5 g of stomach and digestive diverticulum. Then the tissues were processed as described above.

ELISA. Falcon 3915 (Probind assay plate; Becton-Dickinson, France) microtiter plates were coated with 200 μl of rabbit antirotavirus serum 8148 diluted 1:2,500 in carbonate-bicarbonate buffer (pH 9). The plates were incubated overnight at 37°C and then washed five times. All washes were performed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Sigma Aldrich, France). Then the plates were blocked with 200 μl of PBS–4% nonfat dry milk for 1 h 30 min at 37°C and washed five times. Serial dilutions of VLP2/6 ranging from 10^3 to 10^8 particles/ml of shellfish extracts were made in PBS (100 μl) and then incubated in the microtiter plates for 1 h 30 min at 37°C. The plates were washed five times, and 100 μl of peroxidase-conjugated remi-2 antibody (Argène Biosoft, France) diluted 1:100 in PBS was added and incubated for 1 h at 37°C. After five additional washes, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma Aldrich, France) was added and incubated at room temperature in the dark for 15 min. The reaction was stopped by the addition of 100 μl of 1 M H_2SO_4, and optical density was read at 450 nm with a spectrophotometer (Safire, Tecan, France) (22).

MS2 phage analysis was performed following homogenization of the dissected tissues with a Waring blender (Bioblock) in the presence of 2 volumes of sterile peptone water and centrifugation for 15 min at $1,500 \times g$ at 4°C. Phages were counted, as previously described, according to the protocol ISO 10705-1 (2). Briefly, 2.5 ml of molten 1% tryptone-yeast extract-glucose agar, held at 45°C, was added to 1-ml volumes of appropriately diluted shellfish tissue homogenates and 1 ml of WG49 host culture. This mixture was stirred and poured onto a previously prepared 2% tryptone-yeast extract-glucose agar base in a 90-mm-diameter petri dish. The overlays were inverted and incubated overnight at 37°C.

RT-PCR. To check for rotavirus contamination from the estuary during the relaying experiments performed with experiments A, B, and C, nucleic acids were extracted as previously described (20). Briefly, viral nucleic acid was extracted and purified from the suspended polyethylene glycol pellet by digestion with 0.2 mg of proteinase K (Amresco, Solon, Ohio) per milliliter, phenol-chloroform (Applied Biosystems, Foster City, CA) extraction, ethanol precipitation, 1.4% (wt/vol) cetyltrimethylamonium bromide (Sigma) precipitation, and a final ethanol precipitation. Viral nucleic acid was suspended in 100 μl of RNase-free H_2O. Reverse transcription-PCR (RT-PCR) was performed using primers targeting the VP6 gene and consisted of 40 cycles of amplification. Negative results were confirmed to be negative by hybridization (20, 36).

Statistical analysis. The rate of VLP depuration was assumed to be linear (single-compartment model). Linear regression analysis was performed using the StatGraphic software. The probability for a difference between the slopes of the linear regression curves for experiments A, B, and C was calculated and tested for significance at the 0.05 confidence level.

RESULTS

Assay limits of detection. In a seeding experiment using purified VLPs, the limit of detection of the rotavirus VLP ELISA was 10^6 particles per liter and 10^4 particles per g of pancreatic and digestive tissues. For phages, the limit of detection was 1.1 PFU per g of pancreatic and digestive tissues.

Contamination of oysters with VLPs and phage. Oysters were exposed to aerated seawater contaminated with VLPs (experiments A to D) and phage (experiment D only) for 1 day, and samples were taken to evaluate the contamination. Almost all (>99%) of the VLPs added to the tank were concentrated in the oysters for each of the VLP2/6 quantities seeded into the seawater. For experiment A, the concentration in the oyster was 10^{12} particles/g of dissected tissue and in the water was 2.5×10^8 particles per liter. For experiment B, the concentration in the oyster was 10^{10} particles/g of dissected tissue and in the water was 2.5×10^6 particles per liter. For experiment C, the concentration in the oyster was 10^6 particles/g of dissected tissue and could not be detected in water (below the sensitivity limit of 10^6 particles per liter). For experiment D, the concentrations in the oyster were 10^6 particles/g of dissected tissue and 2.6×10^3 PFU/g of dissected tissue. For this experiment, no detection was attempted in the water.

Oyster depuration. The salinity during the experiments was equivalent to the seawater salinity in the estuary (33 to 34.5‰), and oxygen was up to 90% of saturation in all experiments.

As shown by the mean value calculated per day, the initial titer of 10^{12} particles/oyster at day 0 (experiment A) was reduced by 1.5 $\log_{10}$ units after 7 days at 22°C; for experiments B and C, a reduction of 1 $\log_{10}$ unit was observed after 7 days (Fig. 1). The rates of decline for experiments A, B, and C were similar, as determined by the linear regression analysis (−0.236 [95% confidence interval {CI}, −0.317 to −0.154], −0.147 [95% CI, −0.236 to −0.06], and −0.161 [95% CI, −0.240 to −0.08], respectively, for experiments A, B, and C) using a single-compartment model.

For the phage/VLP contamination experiment, the level of contamination per oyster was about 2.6×10^3 PFU for phages and 5×10^6 particles for VLP2/6. As shown by the mean value calculated per day, after 7 days at 22°C, the initial titer of phages was reduced by approximately 2 $\log_{10}$ units when a reduction of 1 $\log_{10}$ unit was observed for VLP2/6 (Fig. 2). Phages were depurated more rapidly than were VLP2/6, as determined using a single-compartment model and linear regression analysis (slopes −0.29 versus −0.18, respectively [$P < 0.05$]).

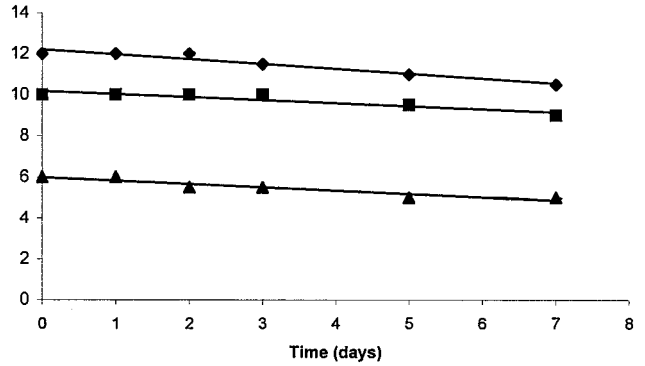

FIG. 1. VLP2/6 depuration kinetics for oysters in the pilot system during 7 days at 25°C. Oysters, contaminated to a final concentration of 10^{12} (experiment A; diamonds), 10^{10} (experiment B; squares), and 10^{6} (experiment C; triangles) VLP2/6 particles/per g of dissected shellfish tissue, were depurated for 7 days in clean seawater at 22°C under aeration. *x* axis, time in days; *y* axis, $\log_{10}$ number of particles per gram of dissected tissue.

Oyster relaying. The variation of the seawater temperature was 19°C to 22°C (July to September 2003), and the salinity varied from 33 to 34.5‰. The results of natural relaying for the three experiments are summarized in Table 1. For experiments A and B, with high concentrations of VLP2/6, the last positive results (more than 10^4 particles/oyster, the sensitivity threshold of our ELISA method) were obtained at days 82 and 70, respectively. When the initial concentration was lower (10^5 VLP per oyster in experiment C), VLP2/6 could be detected for up to 37 days. Controls examined for rotavirus by RT-PCR were negative, excluding the possibility of external virus contamination during relaying.

TABLE 1. Reductions of numbers of VLPs in oysters under relaying conditions[a]

No. of days of relaying	$\log_{10}$ of VLP concentration in oyster tissues for:		
	Expt A	Expt B	Expt C
0	10.5	9	5
7	10	9	5
14	10	8	4.5
21	8.5	8	ND
30	ND	ND	4
37	ND	ND	4
41	7.5	6.5	<4
49	7	6	ND
70	ND	5	ND
82	5	<4	ND
89	<4	ND	ND

[a] After 1 week of depuration, oysters were placed on the shore under the influence of tides for a natural relaying. Samples were collected and analyzed by ELISA in duplicates. ND, analysis not done.

DISCUSSION

VLPs were concentrated rapidly by oysters, and almost all the input was detected in dissected oyster tissues, whereas the concentration in the water was much lower after 24 h. It has been previously estimated that shellfish can concentrate virus up to 50-fold (17, 18, 24). After 1 week at 22°C in clean water, the initial VLP concentration was reduced by about 1 $\log_{10}$. Such a slow decrease has been previously described for hepatitis A virus in oysters at 18°C (17, 35), for rotavirus in mussels (1), and for enterovirus in oysters (7). Temperature affects the efficiency of shellfish depuration (10, 27, 33), and no decrease in norovirus concentration, as estimated by RT-PCR, was observed following depuration at 22°C for shorter periods of time (33). A 1-week purification time was selected in this study based on previously published data (17, 33, 35) but also to preserve oyster quality (to avoid weight loss). F-RNA bacteriophages have been proposed as an alternative viral indicator for shellfish, but they are depurated more rapidly than VLPs, raising questions about their adequacy as indicators of human enteric viruses. Phage has been shown to persist longer than *E. coli* under depuration conditions (12), but to our knowledge, no direct comparisons of depuration have been made to human enteric viruses. Differences in depuration rates have been noted in oysters contaminated by poliovirus type 1 or hepatitis A virus (35). These data raise the possibility that a noninfectious VLP of the same virus may be a better surrogate for depuration studies than are unrelated viruses.

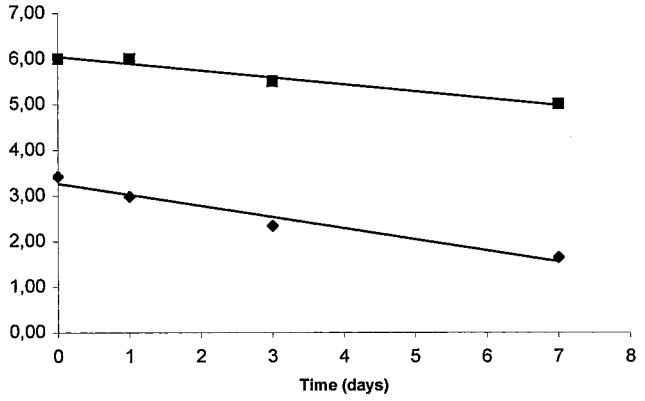

FIG. 2. VLP2/6 and MS2 phage depuration kinetics for oysters in the pilot system at 25°C during 7 days. Oysters, contaminated at a concentration of 5×10^6 VLP2/6 particles/oyster (squares) and 2.6×10^3 PFU for phages (diamonds), were depurated for 7 days in clean seawater at 22°C under aeration. *x* axis, time in days; *y* axis, $\log_{10}$ number of particles per gram of dissected tissue (VLP) or $\log_{10}$ PFU/g (phage).

In the natural environment (i.e., the estuary), the VLP concentration decreased slowly to reach undetectable levels after a few weeks for the low initial concentration in shellfish and after a few months for the high initial concentration. Recent quantitative data obtained using real-time RT-PCR and most-probable-number RT-PCR have shown virus concentrations of about 10^2 to 10^3 genome copies per oyster (21, 23, 26), which is lower than the sensitivity threshold of the procedure for VLP detection employed in this study. Moreover, to be able to follow a 4 $\log_{10}$ decrease in VLP concentration (as suggested for water treatment by Gerba et al. [14]), we had to increase the tracer concentration to be able to follow the relaying, and it is not clear whether the use of higher levels of VLPs may have affected our results. But this may be representative of contamination found in shellfish from prohibited areas close to sewage discharge.

This is the first study that clearly demonstrates that a virus

surrogate (virus-like particles) persists in shellfish for several weeks in a natural environment. However, this persistence was suggested as early as 1973 when an outbreak of hepatitis A was linked to shellfish consumption (25). The shellfish were likely to have been contaminated following a flood in the spring and retained infectious virus for 1 to 2 months before the outbreak. Virus carriage for an extended period of time has been suspected previously to have an important impact on public health (8, 16, 25), after detection of infectious hepatitis A virus for at least 3 weeks and for 6 weeks by RT-PCR in an oyster laboratory experiment (17). After a norovirus outbreak following oyster consumption, the follow-up of the harvest area for approximately 2 months showed the persistence of the virus strain contaminating shellfish at levels up to 1,000 RT-PCR units per oyster prior to depuration of the shellfish (21). However, in this case, the possibility of continued exposure of the shellfish could not be excluded. One advantage of the VLPs, as they contain no viral RNA, is that the possibility of a recurrent contamination by rotavirus from the environment can be evaluated by RT-PCR. Although we did not directly assess the physical integrity of the VLPs over the course of the study, previous studies have shown that the rates of decline of live rotavirus and rotavirus VLP2/6 are similar in seawater (22), and it is likely that nonparticulate antigen would be digested by the shellfish.

In summary, this study demonstrated that VLPs can be used in field experiments and that this virus surrogate can persist in shellfish for long periods of time. The data presented here also suggest that after contamination by human enteric viruses, shellfish may be unsafe for human consumption for quite a long period of time. Virus persistence may explain the number of outbreaks linked to depurated shellfish meeting coliform standards but still contaminated by viruses, and virus particles may be detected outside the digestive tract (11, 32, 33). Such a long persistence needs to be confirmed and validated for other enteric viruses such as norovirus and for shellfish exposed to contaminants over prolonged periods. For such studies, VLPs are very good surrogates, as they are available now for most enteric viruses.

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