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Tulane Virus as a Potential Surrogate To Mimic Norovirus Behavior in Oysters

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Oyster contamination by noroviruses is an important health and economic problem. The present study aimed to compare the behaviors of Norwalk virus (the prototype genogroup I norovirus) and two culturable viruses: Tulane virus and mengovirus. After bioaccumulation, tissue distributions were quite similar for Norwalk virus and Tulane virus, with the majority of viral particles detected in digestive tissues, while mengovirus was detected in large amounts in the gills and mantle as well as in digestive tissues. The levels of persistence of all three viruses over 8 days were comparable, but clear differences were observed over longer periods, with Norwalk and Tulane viruses displaying rather similar half-lives, unlike mengovirus, which was cleared more rapidly. These results indicate that Tulane virus may be a good surrogate for studying norovirus behavior in oysters, and they confirm the prolonged persistence of Norwalk virus in oyster tissues.

Shellfish are filter feeders that can accumulate different types of pathogens from human fecal pollution and were identified as vectors for human enteric pathogen transmission more than a century ago. We have known for almost 40 years that bacteria and viruses show differences in terms of concentration and accumulation in and depuration from contaminated shellfish (1). Nowadays the problem of viral contamination has become dominant, and over the last 10 years about 40% of RASFF (Rapid Alert System for Food and Feed) notifications are related to the detection of norovirus (NoV) in oysters (2). Improvements in detection methods, increased epidemiological surveillance, and efforts by authorities to improve the quality of products put on the market have contributed to better recognition of viral contamination. These improvements have assisted in identifying that increases in human populations in coastal areas, as well as climate change, inducing heavy rainfall and associated sewage overflows, constitute risk factors for shellfish contamination (3, 4).

Among human enteric viruses, NoVs are recognized as the leading cause of epidemics and sporadic cases of gastroenteritis in all age groups of humans (5, 6). NoVs of human origin are excreted in large quantities by ill people, but they may also be present in asymptomatic, healthy individuals (7). As a consequence, they are discharged in large numbers into sewage, and due to their resistance to inactivation, they are frequently detected in wastewater treatment plant effluent and in surface waters (8–10). Sewage treatment which incorporates new technologies, such as membrane filtration, contributes to decreasing the numbers of microorganisms discharged into the coastal environment (11, 12), but this does not prevent accidental contamination. Depuration of shellfish, which was developed to eliminate bacteria, does not efficiently eliminate viruses that persist for several weeks or months in bivalve tissues (13, 14). As a consequence, in most cases of contamination, the only risk management option to prevent consumer infections is the closure of production areas, with viral testing of shellfish to evaluate the level and type of contamination.

NoVs are nonenveloped, single-stranded, positive-sense RNA viruses belonging to the *Caliciviridae* family. The short genome, which is organized into three open reading frames (ORFs), is highly variable (15). Importantly, NoVs were the first viruses for which it was shown that there is genetic sensitivity to infection through the recognition of histo-blood group antigens (HBGAs) (16). HBGAs, which are complex glycans present on many cell types, are synthesized from a series of precursor structures by stepwise addition of monosaccharide units via a set of glycosyltransferases (17). Evidence accumulated from volunteer studies and from analysis of outbreaks indicates that binding to these carbohydrates is required for infection (18, 19). Moreover, various human NoV strains that bind to HBGAs present distinct specificities for HBGAs. As a result, most strains infect only a subset of the population, based on HBGA expression (20, 21).

Specific binding of NoVs to the oyster digestive tract through an A-like carbohydrate structure (which is indistinguishable from the human blood group A antigen) and other ligands has been described (22–24). It was also demonstrated that these ligands have an impact on bioaccumulation efficiency, and a seasonal effect was observed for some strains (25, 26). A field study confirmed the preferential selection of genogroup I (GI) NoVs over GII and GIII NoVs by Pacific oysters (27). These observations suggest that oysters have the ability to specifically accumulate and concentrate a human pathogen based on the presence of a shared ligand between the two species rather than through nonspecific interactions only (28). Since different NoV strains show different specificities for HBGAs in humans, all strains may not be captured equally well by oysters. We also hypothesize that such specific ligands have an impact on NoV persistence in oysters.

Tulane virus (TV) is the prototype strain of the genus *Recovirus*

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(ReCV) within the *Caliciviridae* family. The ReCV genome is organized into three ORFs, like the genome of NoV, and also exhibits large genetic variability. Similar to the case for NoVs, the route of ReCV transmission is fecal-oral, and ReCVs are shed in large quantities in the stools of infected animals. Moreover, ReCVs also recognize HBGAs and can be grown to high titers in cultured cells, making them a valuable surrogate for the uncultivable human NoVs (29). Considering these features, in this study, we selected TV as a potential NoV surrogate and mengovirus (MgV) as a virus control to investigate NoV persistence in oysters. MgV, a member of the *Picornaviridae* family, is very stable in the environment, can be propagated in cell culture, and recognizes some sialic acids on red blood cells. This may contribute to the efficient bioaccumulation of MgV in oysters, which are rich in sialic acid (30–33).

The objectives of the present study were to evaluate the persistence of Norwalk virus (NV; the NoV GI prototype) in oysters and to compare it with those of two potential surrogates: TV and MgV.

MATERIALS AND METHODS

Viruses and cell culture. TV strain M033 was propagated in confluent monolayers of LLC-MK2 cells (ATCC CCL-7; ATCC, Manassas, VA) as previously described (34). MgV strain pMC0 (kindly provided by A. Bosch, University of Barcelona) was propagated in HeLa cells as previously described (30). When cytopathic effects (CPE) were complete, cultures were frozen and then thawed (−20°C) three times, and cell debris was removed by centrifugation at 1,000 × *g* for 30 min. The supernatant, which contained viral particles, was stored at −80°C in aliquots.

Aliquots of a fecal sample containing the Norwalk virus 8FIIa strain (GI.1) were kept at −20°C.

Virus stability in seawater. One-milliliter samples of TV and MgV were diluted in 9 ml of seawater and kept at 12°C. One-milliliter samples were collected at 1 h, day 1, and day 8.

Oysters. Live oysters (*Crassostrea gigas*) were purchased directly from a producer. Environmental data were recorded, including water temperature and salinity. Upon arrival at the laboratory, oysters were immediately rinsed and transferred to large seawater aquariums equipped with constant aeration. After 24 h of immersion at the designated temperature, oysters were individually checked, and only live oysters showing filtration activity were included in the experiments. Before all bioaccumulation experiments, oysters were tested for preexisting GI and GII NoVs, MgV, and TV.

Biometry parameters, such as total weight and flesh weight, were recorded. In addition, for the virus persistence experiments, the dry weights of 30 individual oysters per aquarium (including the control aquarium) were measured on day 0, day 28, and day 56 (35).

Bioaccumulation experiments. Bioaccumulation of the three different viruses was evaluated in both small and large batches, in separate aquariums. Natural seawater, collected from a single clean area in which turbidity and ammonium, nitrate, phosphate, and chlorophyll A concentrations were measured (data not shown), was used for all bioaccumulation experiments.

Small-batch bioaccumulation experiments were conducted between January and April 2013, with the seawater temperature adjusted according to the season (8 to 10°C). Four aquariums were filled with 20 liters of seawater each, and 3 of them were seeded with the virus suspensions, at $7.2 \pm 0.5 \log_{10}$ RNA copies/aquarium for NV, $8.5 \pm 0.2 \log_{10}$ RNA copies/aquarium for TV, and $9.1 \pm 0.4 \log_{10}$ RNA copies/aquarium for MgV. The remaining aquarium was not seeded and served as a control. Forty-five oysters were added to each aquarium, including the control one, yielding a ratio of 5 liters of water/kg of oysters (including the shell weight) (35). Fifteen oysters were collected from each aquarium at 1 and 24 h postseeding and were immediately dissected and frozen. At 24 h postseeding, the remaining oysters were transferred to clean aquariums, in which the seawater was changed daily for 8 days. The experiments for all viruses were conducted at the same time and were repeated five times.

Large-batch bioaccumulation experiments were conducted separately for each of the three viruses, using 500 to 600 oysters that were contaminated under the same conditions as those described for the small batches. After 24 h of bioaccumulation, virus concentrations were checked by randomly selecting and testing six oysters; if the concentration was less than 5×10^4 RNA copies/g digestive tissue (DT), more virus was added to the seawater (the amount added was estimated based on the concentration detected in DT), and bioaccumulation was continued for an additional 12 h.

Persistence experiments. Following bioaccumulation, the oysters were rinsed and transported to an experimental farm located on the coast, with direct access to natural seawater and facilities to maintain oysters for prolonged periods. Oysters were placed in clean seawater in large tanks (500 liters) located in a temperature-controlled room (11 ± 1°C) and supplied with constantly circulating (200 liters/h/tank) aerated and filtered natural seawater. Oysters were fed the phytoplankton *Skeletonema costatum* (about 10^{12} cells/h/aquarium). Parameters such as temperature, salinity, and pH were routinely measured. Two control tanks, one with uncontaminated oysters and one without oysters, were included in all experiments.

Twenty to 30 oysters were collected for analysis weekly from each tank, for up to 8 weeks. During sampling, the tanks were cleaned and particles that had settled onto the bottom of the tanks were removed. Prior to release into the environment, seawater was treated according to the safety rules of the experimental farm (which included acidification, filtration, and UV treatment). These persistence experiments were repeated three times between 13 November 2013 and 4 April 2014.

Shellfish processing. Harvested oysters were shucked immediately after collection, and the oyster bodies were weighed. To avoid any delays or differences with subsequent assays, oysters were dissected at the same time for each virus treatment group, by different laboratory members. To determine viral distribution, the DT, gills, and mantle were collected from at least 10 oysters per assay. Corresponding tissues were pooled, finely chopped using a scalpel, and mixed before preparation of 1.5-g aliquots that were stored at −20°C. For the persistence studies, only DT were processed as described above.

Virus recovery. Samples of each tissue type were extracted together (e.g., all gills together), and all tissues were extracted in triplicate as described previously (25, 36). Briefly, MgV (2×10^6 RNA copies) was added as an extraction efficiency control to each dissected tissue (1.5 g) before homogenization, except to oysters contaminated with MgV. Tissues were homogenized, extracted with equal volumes of chloroform-butanol for 30 s, and subjected to Cat-Floc T (173 μl per tube; Calgon, Ellwood City, PA) flocculation before centrifugation for 15 min at 13,500 × *g*. The resulting supernatant was precipitated with polyethylene glycol 6000 (PEG 6000) (Sigma, St. Quentin, France) for 1 h at 4°C and centrifuged for 20 min at 11,000 × *g* at 4°C as described previously (33).

NA extraction and purification. Nucleic acids (NAs) from oyster samples and viral inocula, including cell culture supernatant and 10% stool suspensions, were extracted using a NucliSens extraction kit and an automatic easyMAG extractor (bioMérieux, Lyon, France) according to the manufacturer's instructions, with minor modifications, as described previously (33). NAs were recovered in 100 μl of elution buffer (bioMérieux) and analyzed immediately or stored at −80°C.

Primers, probes, and rRT-PCR. For NoV and MgV, real-time reverse transcription-PCR (rRT-PCR) was conducted as previously described (37). Primers TVIFf (5′-CTGGGATACCCACAACATC-3′) and TVIFr (5′-GCCAGTTAACAGCTTCAGC-3′) and probe TVIFp (6-carboxyfluorescein [FAM]-TGTGTGTGCCACTGGATAGCTAGCACC-BHQ) were used to amplify the region from nucleotides 3775 to 3884 of the TV genome (GenBank accession number EU391643.1).

rRT-PCR was carried out by using an UltraSense One-Step quantitative RT-PCR system (Life Technologies, France) with 5 μl of extracted NA

per well (in a 25-μl reaction mixture) as described previously (33). Amplifications were performed in an Mx3000P quantitative PCR (qPCR) system (Agilent Technologies, France). All samples were analyzed undiluted and after 10-fold dilution. Undiluted NA extracts were analyzed in triplicate.

rRT-PCR controls and quantification. Filter tips and dedicated rooms were used to prevent sample contamination. Two negative controls (sterile, RNase-free water) were included in each amplification series.

(i) Extraction efficiency. MgV was used to evaluate the extraction efficiency. The threshold cycle (C_T) value for the undiluted samples (seeded with 2×10^6 RNA copies of MgV) was compared to the C_T value for the positive control used in the extraction series and to a standard curve made by endpoint dilution. The difference in C_T values between the controls and samples (ΔC_T) was used to determine the extraction efficiency, using the formula $100e-0.6978\Delta C_T$, and the extraction efficiency was expressed as a percentage for each tissue. Only samples with extraction efficiencies above 10% were considered for quantification. Given that the oysters used in each experiment were from the same initial batch, the extraction efficiencies obtained for NoV, TV, and the negative control were used to estimate the extraction efficiency of oysters contaminated with MgV.

(ii) Quantification. The absence of inhibitors of virus detection (for NV, TV, or MgV) was verified for each sample by comparing the C_T values for undiluted and 10-fold-diluted extracts, which were corrected for the dilution factor by using the standard curve coefficient. A difference of <1 C_T unit indicated the absence of significant inhibition, and all extracts were checked by using this control. In the virus persistence experiments, in which triplicate amplifications were performed, all C_T values were compared. If a variation of >1 C_T unit was observed, then the amplification was repeated. If the difference persisted (for concentrations close to the limit of quantification), this was considered in the quantification step. Otherwise, all three C_T values were averaged. After these verification steps, the number of RNA copies in each positive sample was estimated by comparing the C_T value for the sample to standard curves derived from plasmids containing nucleotides 146 to 6935 of Norwalk virus (GenBank accession no. M87661), nucleotides 3300 to 4299 of Tulane virus M33 (GenBank accession no. EU391643.1), or nucleotides 209 to 1061 of mengovirus isolate M (GenBank accession no. L22089).

(iii) Standard curve validation. After completion of all rRT-PCR runs, all standard curves for one virus were compared, and quality criteria were applied (38). Only standard curves with amplification efficiencies of 85 to 110% were kept. Average values were calculated for each point of the standard curves and were used to estimate the uncertainty of quantification for TV (0.21 log), NV (0.14 log), and MgV (0.14 log).

(iv) Quantification. The final concentration was then back calculated based on the volume of NA and expressed per gram of tissue. Triplicate extractions were calculated separately, and then the geometric mean concentration was calculated. rRT-PCR runs showing aberrant standard curves were checked to see if the C_T values for the samples were comparable to those for other extractions from the same series (as all tissues were extracted three times). If the C_T values were in the same range, the concentration was calculated based on the average standard curve values. If not, the run was repeated.

Data calculation and statistical analysis. The geometric mean titer (GMT) was calculated for each sample. For statistical analysis, the Mann-Whitney test was applied to assess the impact of feeding, and a nonlinear regression with a two-phase decay was used to calculate the half-life, using Prism 5 (GraphPad Software, Inc.).

RESULTS

Controls and bioaccumulation conditions. The stability of TV and MgV in the seawater that was used for bioaccumulation was verified in two separate experiments. Over 8 days, the variation observed was 0.78 ± 0.9 C_T unit for TV and 1.39 ± 0.54 C_T units for MgV. The stability of NV was 0.43 ± 0.5 C_T unit, as determined in our previous study (26).

Subsamples of the same stool suspension for NV or cell culture supernatants for TV and MgV were frozen and used for the first five experiments. Because of the differences observed in the concentrations of the different viruses (up to 2 log), new subsamples were prepared for the three persistence experiments.

All experiments were performed during the colder months (December to the beginning of April), using adult oysters of commercial size. The allometric coefficient (body weight divided by DT weight) was 14 ± 1.79 for oysters used in the six experiments on tissue distribution and 15 ± 2.58 for the three experiments on persistence. For the three persistence experiments, because the oysters were fed plankton, oyster flesh weight was measured at the beginning of the experiment (day 1), on day 28 (week 4), and on day 56 (week 8). The body weight did not change for experiment 2 (4.9 ± 1.2, 5.6 ± 1.3, and 5.3 ± 1.5 g, respectively) or experiment 3 (4.5 ± 1, 5.4 ± 1.2, and 4.8 ± 1.2 g, respectively), but it changed for experiment 1 (6.3 ± 1.3, 7.8 ± 1.7, and 9.9 ± 2, respectively). The body weight increase during the last 3 weeks was correlated with plankton feeding, which was accidentally increased for 10 days.

Tissue distribution. Five separate experiments were performed to compare NV, TV, and MgV concentrations in the DT, gills, and mantle following bioaccumulation periods of 1 and 24 h. The extraction efficiencies were acceptable and varied from 13 to 19% for DT, 55 to 67% for gills, and 51 to 61% for mantles. For comparison of the different viruses, the maximal theoretical bioaccumulation (MTB) was calculated for each virus, using the following assumptions: (i) 1 log of virus would be lost (by adsorption to aquarium walls, shell, etc.), as demonstrated using radioactive virus (39); (ii) 90% of the viruses would be concentrated in the DT, as observed during preliminary experiments and demonstrated for norovirus and hepatitis A virus (HAV) (40); and (iii) the level of bioaccumulation could be based on the weight of the DT recovered. For example, for NV, the dose seeded in the aquarium was 1.5×10^7 RNA copies, and 24 g of DT was recovered. Therefore, 1.5×10^6 RNA copies were available for uptake (assuming a 1-log loss), of which 90% were assumed to be present in the DT (1.35×10^6). Considering the weight of the DT recovered (24 g), this gave an MTB of 5.6×10^4 RNA copies/g of DT. The MTB was calculated for each virus and was found to be 1.2×10^6 RNA copies/g of DT for TV and 5×10^6 RNA copies/g of DT for MgV. These MTB values were useful for evaluating the efficiency of bioaccumulation in the DT for each virus (Table 1). However, as MgV is distributed equally in all tissues, the MTB for all tissues (MBT-AT) was calculated by considering the loss of 1 log of virus and then dividing this concentration by the total weight of the oyster tissues (oyster weight without shell).

For greater confidence in quantification, all tissues were extracted three times in independent extraction runs, and all NA extracts were amplified in duplicate; each data point represents a total of 7 to 10 oysters. Results presented are based on the geometric mean concentrations obtained for five separate experiments (each of which was quantified using three independent concentration values) (Table 1).

NV accumulated rapidly in the DT and in other tissues, such as the gills and mantle, and it reached a concentration of about 100 copies/g after 1 h. After a bioaccumulation period of 24 h, concentrations in the DT increased about 100 times, while they

TABLE 1 Tissue distributions of NV, TV, and MgV after 1 or 24 h of bioaccumulation[a]

Virus	Time (h)	Gills		Mantle		DT		Bioaccumulation efficiency in DT	
		Mean extraction efficiency (%)	Geometric mean concn (copies/g)	Mean extraction efficiency (%)	Geometric mean concn (copies/g)	Mean extraction efficiency (%)	Geometric mean concn (copies/g)	MTB-DT (%)	MTB-AT (%)
NoV	1	63	187	51	191	14	537	1	13
	24	55	245	61	161	19	1.4×10^4	25	350
TV	1	65	66	55	<QL	13	176	0.02	0.2
	24	67	280	46	181	13	3.0×10^3	0.5	7
MgV	1		1.7×10^3		810		2.5×10^3	0.05	0.8
	24		2.1×10^4		7.3×10^3		9.2×10^4	2	30

[a] The mean extraction efficiencies represent the average values for three extractions each from five experiments (so 15 values). The geometric mean concentrations were calculated for five experiments, with each concentration determined on the basis of three separate extractions. The bioaccumulation efficiency in DT was calculated based on the assumptions of a 1-log loss of virus and that 90% of the contamination was in DT (MTB-DT) or equally distributed in all tissues (MTB-AT). QL, quantitation limit.

decreased or remained stable in the other tissues, confirming previous observations (26) (Table 1). Considering the MBT, the bioaccumulation efficiency was about 1% after 1 h and reached 25% after 24 h. With the assumption that NV is equally distributed in all tissues, the bioaccumulation efficiency reached 13% after 1 h and 350% after 24 h, confirming that NV is indeed more concentrated in DT.

TV displayed slightly lower concentrations than those of NV in all tissues after 1 h of bioaccumulation, but the results showed that TV accumulated preferentially in DT. After 24 h, TV concentrations increased in the three tissues, but to a larger extent in the DT (the geometric mean concentration in the DT was 10 times greater than the concentration detected in the gills). Nevertheless, the bioaccumulation efficiency was poor (0.5%), even after 24 h. If we considered that TV was equally distributed in all tissues, the bioaccumulation efficiency reached 7% (Table 1).

MgV was seeded at a higher concentration than those of the other two viruses, which explains the higher concentrations detected. However, the tissue distribution was clearly different from that for NV or TV. Concentrations were comparable in all three tissues analyzed after 1 h and increased in all tissues after 24 h. The bioaccumulation efficiency was in the same range as that observed for TV after 1 h and increased to 2% after 24 h. As MgV concentrations detected in the gills and mantle were in the same range as concentrations recorded in the DT, we calculated the bioaccumulation efficiency based on an equal distribution of MgV in all tissues. In this case, the bioaccumulation efficiency was less than 1% after 1 h and increased to 30% after 24 h.

Persistence over 8 days. The second step of this study was to evaluate the persistence of these three viruses in the DT over 8 days. Two different approaches were undertaken: oysters were kept in filtered seawater (with very low nutrient levels) or in filtered seawater supplemented with phytoplankton. Experiments were repeated five times for trials in which no food was added and three times for those in which plankton was added. A statistical difference was found for TV, with a more rapid decline in the concentration in the DT when oysters were fed ($P = 0.035$) (Table 2). For both NV and MgV, the impact of feeding was not significant over 8 days ($P = 0.25$ and $P > 0.99$, respectively).

Persistence over 2 months. Oysters contaminated with 4.58 ± 0.42 log RNA copies/g DT for NV, 4.83 ± 0.23 for TV, and 5.18 ± 0.34 for MgV were relocated to a scientific farm to closely mimic normal commercial environmental conditions, albeit in a secure facility. Seawater entering the farm was filtered and was treated following experiments to prevent contamination of the environment through seawater release. An uncontaminated batch of oysters was added for each experiment and was analyzed every week. None of the viruses were detected in the control oysters. Because the seawater used was filtered, we added plankton at a concentration normally found in seawater to feed the oysters. Unfortunately, during the first experiment, overfeeding occurred and led to an increase in oyster weight. Nevertheless, this had no impact on virus concentrations, and the results were comparable to those of the other two experiments, even for TV, for which a statistical difference was associated with feeding over 8 days.

NV concentrations decreased slowly in the DT and reached the limit of quantification of the method by week 8 (1.85 ± 0.48 log RNA copies/g DT) (Fig. 1). TV concentrations decreased faster, with the limit of quantification being reached by week 5 (1.78 ±

TABLE 2 Persistence of NV, TV, and MgV in oyster DT over 8 days[a]

Virus	No feeding			Feeding		
	Mean extraction efficiency (%)	Geometric mean concn (copies/g)		Mean extraction efficiency (%)	Geometric mean concn (copies/g)	
		1 day	8 days		1 day	8 days
NV	16	1.4 ± 10^4	4.6 ± 10^3	40	5.9 ± 10^4	7.6 ± 10^3
TV	13	3.0 ± 10^3	1.2 ± 10^3	59	6.9 ± 10^4	1.1 ± 10^4
MgV		9.2 ± 10^4	3.3 ± 10^4		1.6 ± 10^5	5.9 ± 10^4

[a] Concentrations expressed here are the geometric means for five experiments conducted under "no feeding" conditions and for three experiments conducted under "feeding" conditions. Extraction efficiencies are average values. No statistical difference was observed for NV ($P = 0.25$) and MgV ($P > 0.99$), but the difference was found to be statistically significantly different for TV ($P = 0.03$).

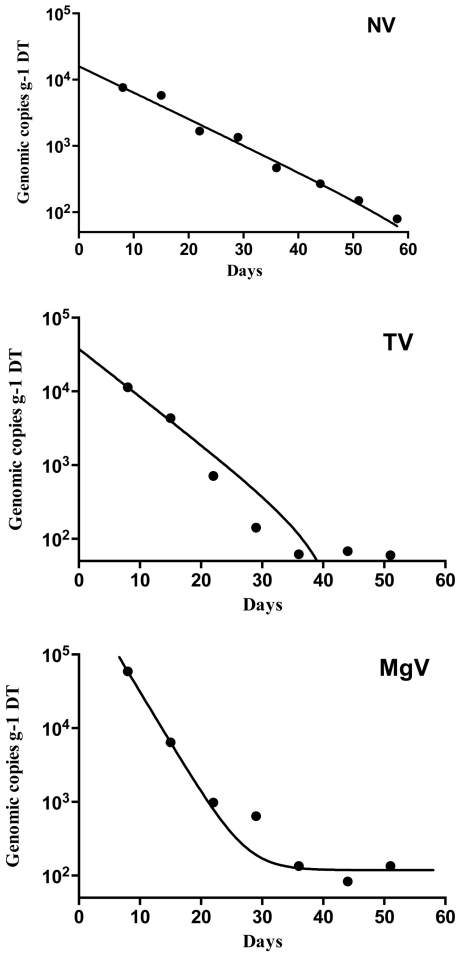


FIG 1 Decreases of NV, TV, and MgV concentrations in oyster DT over 8 weeks. NV, Norwalk virus (half-life, 7.56 days); TV, Tulane virus (half-life, 4.65 days); MgV, mengovirus (half-life, 2.17 days).

0.22 log RNA copies/g DT) (Fig. 1). MgV concentrations decreased quite rapidly and reached the limit of quantification by week 6 (1.90 ± 0.34 log RNA copies/g DT) (Fig. 1). The data allowed for calculations of half-lives, which were 7.56 days for NV, 4.65 days for TV, and 2.17 days for MgV.

DISCUSSION

Viral contamination of oysters is a challenging problem because classical indicators, such as *Escherichia coli* or fecal coliforms, are not satisfactory and depuration is not efficient. Human NoVs cannot be cultivated easily in cell culture systems (41, 42), but in recent years, our understanding of the prevalence and impact of NoV infections has greatly increased, with the advent of molecular diagnostics and by the use of some surrogate animal caliciviruses to improve the knowledge on basic virus biology (43). The combined use of molecular diagnostics and surrogate viruses is an approach that should also help to improve our understanding of NoV behavior, especially persistence, in shellfish.

One important point to consider when studying viral behavior in shellfish is the mode of contamination. For example, a virus that bioaccumulates in shellfish may be protected against heat by the surrounding tissue, in contrast to the same virus artificially seeded into a shellfish homogenate (44–46). However, large amounts of viruses are needed for bioaccumulation studies. Obtaining sufficient amounts of material containing a particular NoV strain is a significant challenge due to the genetic variability of NoVs and because the viruses can be isolated only from human stools. The search for an appropriate surrogate to study NoV behavior has been intensive (47). Phages have been used in some studies as potential indicators of human enteric viruses in shellfish, and they were shown to persist longer than bacteria (48, 49). More recently, bioaccumulation of an F-specific RNA bacteriophage and NoV in mussels demonstrated that a high temperature is needed to reduce phage infectivity and the amount of NoV RNA present (46). Culturable enteric viruses, such as hepatitis A virus (HAV), have also been used to study the effect of cooking on virus infectivity in shellfish (45). HAV was also compared to poliovirus (PV) in depuration experiments with artificially contaminated oysters over 5 days, and both viruses were very stable in the oyster DT (50). Oysters that had bioaccumulated HAV, NoV, and PV displayed almost no decrease in HAV or NoV over 1 day of depuration; in contrast, >90% of the PV was eliminated (51). Animal caliciviruses, including feline calicivirus (FCV) and murine NoV (MNV), have been used widely as human NoV surrogates. FCV was used to evaluate the stability of NoV in marinated mussels and following thermal inactivation (52, 53). A study comparing the bioaccumulation levels of MNV, a bacteriophage, and HAV in two oyster species reported the superiority of MNV over the other two surrogates in mimicking human NoV behavior (54). MNV offered the possibility of studying NoV infectivity in an animal model and provided information on the potential infection risks in electron beam-irradiated oysters (55). Previous studies using a mouse model demonstrated the inactivation of MNV in contaminated oysters after high-pressure treatment, while NoV stayed infectious, suggesting that MNV might not have been a good model for NoV in this case (56, 57).

Considering the specificity issues associated with human NoV bioaccumulation, including HBGA binding, in this study we evaluated TV as a potential surrogate to mimic NoV persistence in oysters. MgV, a sialic acid-binding virus, was included in the study as a control. However, it is not permissible to place contaminated oysters into the open environment in France, even if the contamination is with animal viruses (TV infects monkeys not naturally present in France) or the avirulent strain of murine MgV (vMC0). To overcome this limitation, oysters were placed in an experimental farm where the quality of intake seawater was controlled and discharge was treated prior to release into the environment. The ability to control experimental conditions in the farm, including plankton feeding, enabled us to closely mimic conditions experienced by oysters in the natural environment. Despite the significant impact of feeding found for TV over 8 days, we chose to add phytoplankton because the seawater entering the farm was filtered. Starving was recently demonstrated to be efficient to eliminate viral particles through autophagy, but oysters have access to food under natural conditions (58). During the first experiment, accidental overfeeding occurred, but this did not affect the final virus concentrations. To avoid variations due to physiological differences between animals, all experiments were repeated over a short time and during the period of the year when the maximum contamination of oysters occurs in France and the HBGA-like ligand expression in oysters is the highest (25, 26).

Based on our experience in viral quantification, and following

steps to verify the efficiency of the extraction step, the concentrations expressed are based on triplicate extractions, with each extraction being analyzed in triplicate by rRT-PCR. We found this approach to be more accurate, as it minimized extraction and amplification variations (36). Based on quality criteria that were developed to validate standard curves for animal diseases, we validated or removed all standard curves that did not fit the criteria (38). For standard curves that were removed, the C_T values for replicate samples were compared to those for other extractions of the same sample, and eventually the rRT-PCR was repeated. This improved the quantitative aspects of the assays, as it allowed us to estimate the uncertainty of quantification, which was found to be higher for TV than for the other two viruses. This may suggest that the rRT-PCR assay needs to be optimized further for TV; however, the level of uncertainty was far lower than the difference observed in the tissue distributions of TV compared to NV and MgV, and also lower than the loss of TV observed in the persistence study.

The difference in ligand recognition may explain the variance observed in tissue distribution; for example, sialic acid residues are detected in all organs, including the gills, mantle, and DT, which is consistent with the poorly selective accumulation of MgV in these three tissues (59). In contrast, NV does not recognize sialic acid but binds to neutral carbohydrates of the HBGA type that are exclusively located in the DT, consistent with the selective bioaccumulation of NV in this organ. TV also recognizes neutral HBGAs and displayed a tissue distribution that was more closely aligned with that of NV than with that of MgV, although it did not bioaccumulate as efficiently as NV. Since there are significant differences in the tissue distribution and retention of different NoV strains in oysters (26), the availability of diverse, cell culture-adapted ReCVs representing different HBGA binding patterns makes this surrogate even more attractive for modeling NoV bioaccumulation in shellfish (29, 60). The TV bioaccumulation efficiency obtained here is more comparable to those obtained for GII NoVs in our previous study, where the bioaccumulation efficiency of a GII.3 NoV was 0.1 to 0.5% at 1 h and 0.9 to 4.1% at 24 h. Moreover, a GII.4 NoV strain (<0.1%) bioaccumulated very poorly in oysters over 24 h (26), consistent with the recent demonstration that TV recognizes the A type 3 and B HBGAs (61).

Previous studies investigating NoV persistence in oysters in the open environment are rare, and comparisons of data should be made with caution, as uncontrolled events may influence the outcome. After a sewage contamination event, we monitored the concentrations of GI and GII NoVs in the DT of oysters over 4 weeks, and the concentrations decreased from 8.2×10^3 RNA copies/g of DT to 4.2×10^3 RNA copies/g of DT (62). In a study combining relaying and depuration, naturally contaminated oysters at an initial concentration of around 2.9×10^3 RNA copies/g of DT displayed a decrease to 1.4×10^2 RNA copies/g of DT in 28 days (63). One study performed under laboratory conditions with artificial seawater reported the persistence of NoV, MNV, and HAV over a 1-month period (54). Our study, which was performed for a longer period, confirmed the persistence of NV, as concentration in the oyster DT decreased only 50% over an 8-day period.

In conclusion, this study demonstrated similar tissue distributions and half-lives of TV and NV in oysters, strongly indicating that TV is an adequate surrogate for studying NoV behavior during shellfish contamination. Although further studies are needed to improve our understanding of the comparative behavior of ReCVs and NoVs, it is important to emphasize that infectivity studies can be undertaken with cell culture-adapted ReCVs. Such studies may provide useful information for risk analysis and decision-making by authorities during shellfish contamination, lead to improved public safety, and help to identify the mechanisms of NoV persistence in shellfish.

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