Journal of Food Protection, Vol. 78, No. 11, 2015, Pages 2064–2069
doi:10.4315/0362-028X.JFP-15-191

Retention of Rotavirus Infectivity in Mussels Heated by Using the French Recipe *Moules Marinières*

DORIS SOBRAL MARQUES SOUZA,[1,2] TAKAYUKI MIURA,[1] CÉCILE LE MENNEC,[1] CÉLIA REGINA MONTE BARARDI,[2] AND FRANÇOISE S. LE GUYADER[1]*

[1]*Ifremer, Laboratoire de Microbiologie, LSEM/SG2M/RBE, BP21105, Nantes 44311, France; and* [2]*Universidade Federal de Santa Catarina, Centro de Ciências Biológicas, Departamento de Microbiologia, Imunologia e Parasitologia, Laboratório de Virologia Aplicada, Florianópolis, Santa Catarina, CEP: 88040-970, Brazil*

MS 15-191: Received 30 April 2015/Accepted 16 July 2015

ABSTRACT

To evaluate the persistence of infectious virus after heating, mussels contaminated with a rotavirus strain were prepared following the French recipe *moules marinières* (mariner's mussels). Rotavirus was then quantified by real-time quantitative PCR (RT-qPCR) and a cell culture infectivity assay. Results showed the persistence of infectious virus after 3 min of cooking. After 5 min, when no infectious virus could be detected, the RT-qPCR approach showed a 1-log decrease compared with concentrations detected after 1 min of cooking.

Bivalve molluscan shellfish, through their filtration activity, may accumulate microorganisms such as human enteric viruses. Shellfish are generally considered a high-risk food as they are often grown in coastal areas that may be contaminated with human sewage due to the high density of the population in some catchments, and they are frequently consumed raw, especially oysters. As a consequence, they have been implicated in gastroenteritis and hepatitis outbreaks worldwide. In the United States, shellfish are accountable for 3% of foodborne illnesses cases, with viruses being responsible for 2% *(36)*. In Europe, outbreaks have been attributed to both locally produced and imported shellfish *(19)*. In contrast to bacterial contamination, depuration is not efficient in reducing viral contamination; thus, when shellfish are contaminated with viruses, the control options are to either close the production area or to undertake shellfish processing *(42, 46)*. Valid control options for processing are limited because when viruses are inside the shellfish body, they seem to be protected. Variables that may influence virus inactivation are numerous (e.g., temperature, pressure, time), and the interaction of these variables needs to be taken into account when considering viral inactivation. High pressure processing has been found to be partly effective in preventing infection in humans, and electron beam irradiation decreases the risk, but does not eliminate infectious virus *(31, 39)*. Thus, when shellfish microbial quality is compromised, some competent authorities recommend labeling shellfish with "cooking required before consumption" *(45)*.

* Author for correspondence. Tel: (33) 240374052; Fax: (33) 240374073; E-mail: soizick.le.guyader@ifremer.fr.

A large variety of recipes exist worldwide for cooking shellfish, with each country having specialties. In France, oysters are frequently consumed raw, but mussels are usually cooked. In this study, we evaluated viral reductions in mussels when they were prepared using a typical French recipe that included different ingredients that may interfere with viral inactivation. As no simple assay exists to demonstrate the infectivity of norovirus, the virus most frequently implicated in shellfish-related outbreaks, we selected a cultivable rotavirus strain to contaminate the mussels. Rotavirus has a triple-layer capsid and was found to be very stable in groundwaters, it is highly resistant to UV irradiation, and it remains infectious in human feces when stored for 2 months at 30°C *(11, 16, 18, 21)*. The strain used in this study recognizes histo-blood group antigen type A, a ligand that we found to be important for viral bioaccumulation and persistence in shellfish tissues *(30)*. However finding an appropriate surrogate for norovirus is difficult due to the uniqueness of the virus and the variety of environmental stressors that may occur *(15, 41)*. Thus, the findings reported are specific to rotavirus infectivity, and they may not be extrapolated further at this stage. Viral particles were detected following different cooking times by using two approaches: real-time quantitative PCR (RT-qPCR) to detect the viral genome and cell culture to provide an estimate of viral infectivity.

MATERIALS AND METHODS

Virus multiplication. Human rotavirus (RV) strain HAL1166 (provided by M. K. Estes, Baylor College of Medicine, Houston, TX) was propagated using MA104 (African green monkey kidney cells; provided by D. Poncet, CNRS-INRA, Gif sur Yvette, France). Cells were cultured in a CO_2 atmosphere at 37°C

in Eagle's minimal essential medium that was supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, penicillin-streptomycin solution, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (all products from Gibco-Life Technologies, Saint Aubin, France). For cultivation, RV was treated with 10 μg/ml trypsin from porcine pancreas (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 30 min at 37°C and then inoculated onto confluent cells in Eagle's minimal essential medium without fetal bovine serum, but with 0.5 μg/ml trypsin, and incubated for 2 days at 37°C. Virus particles were recovered through three freeze-thaw cycles (–20 to 25°C) to release cell-associated virus. Cellular debris was removed by centrifugation for 10 min at 4,500 × *g*. After filtration (0.20-μm pore size), supernatant containing RV was stored at –80°C. Following the protocols described here, the mean ± standard deviation level was 9.3 ± 0.17 log RNA copies per ml and 5.3 ± 0.18 log PFU/ml. For all experiments, the same RV suspension was used; the suspension was kept at 4°C in aliquots to avoid variation in virus titer.

Mussel contamination and cooking. Fresh mussels were purchased from a local producer and kept at 4°C for <24 h. A volume of 10 μl of RV inoculum was spiked into the digestive tissue (DT) of each mussel, representing ~7.3 ± 0.17 log RNA copies and 3.3 ± 0.18 log PFU per mussel. Considering an average weight of 0.3 g of DT per mussel, the expected concentration was ~7.8 log RNA copies per g of DT.

Some preliminary tests were performed to optimize the contamination procedure, including spiking studies (whole tissue or DT) and investigations regarding the contact time (immediate extraction or 30 min of contact). All these assays were repeated three times before considering the final protocol, and a contact time of 30 min was selected before mussels were dissected, for both the control and cooked batches. Mussels were opened to conduct the spiking measurements (with the shell kept attached) that were performed by several persons to adhere to the delay of 30 min.

Next, 20 spiked mussels with shells on were cooked according to the typical French recipe *moules marinières* (mariner's mussels) with olive oil, white wine, and onions. In brief, 10 ml of olive oil was added to a stainless steel pot (20 cm in diameter) on medium heat (power level 6 on a halogen hotplate; HT1, Bibby Sterilin Ltd., Staffordshire, UK) for 30 s, and then one chopped onion, 20 mussels, and 100 ml of white wine were added. After the liquid (i.e., the oil and wine) started to simmer, cooking times of 1, 3, and 5 min were evaluated. Each experiment was repeated five times. The temperatures of the cooking liquid and the mussel tissue surface were measured using two extra-long-stem Ultra thermometers (23609-174, VWR International, Pessac, France) every 30 s during the 5-min cooking experiment. The mussel tissue surface temperature was measured by placing the thermometer on the surface of the mussel tissue of each of two mussels that were randomly selected in the pot; the temperatures were measured sequentially. A third identical thermometer was used to measure the liquid temperature. The mean temperature was reported.

RV recovery from DT. After cooking, mussels were dissected and 1.5 g of the DT was homogenized in 2.5 ml of glycine–NaOH buffer (pH 9.5) by using a tissue grinder (Fisher, Illkirch, France) for 30 s. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4°C. The viral eluate was collected and immediately neutralized with 1 M HCl.

Plaque assay. The infectious RV titer was determined by plaque assay using triplicate wells for each mussel sample. Cells were grown to obtain 90% confluent monolayers in six-well plates. Growth medium was then removed, and the cells were washed twice with phosphate-buffered saline. One milliliter of viral eluate was incubated with penicillin-streptomycin solution and 10 μg/ml trypsin for 30 min at 37°C. The solution was then diluted 1:5 in Eagle's minimal essential medium without fetal bovine serum to eliminate toxic effects to the cells. One milliliter of this dilution was added to each well, and the cells were incubated for 1 h at 37°C, with gentle agitation every 15 min. The inoculum was then removed, and 3 ml of overlay Eagle's minimal essential medium containing 0.6% agarose and 0.5 μg/ml trypsin was added. The plates were incubated for 48 h at 37°C in 5% CO_2, and the cells were stained with 0.03% neutral red solution for 3 h at 37°C. Thereafter, the neutral red solution was removed and the plaques were counted. Based on this protocol, the limit of detection (LOD) is 3.3 PFU/g of DT. For experiments involving 3 min of cooking, a concentration of 1.6 PFU/g of DT (half of the LOD) was assigned to calculate the infectious titer, because the three replicates gave no infectious plaques. A control consisting of uncontaminated mussel tissues was included in each series to exclude tissue impacts (such as cytotoxic effects) on cells.

NA extraction and RT-qPCR. Nucleic acids (NA) were extracted and purified using the NucliSens extraction kit (bioMérieux, Inc., Marcy l'Etoile, France), according to the manufacturer's instructions, directly from 100 μl of viral eluate. For RT-qPCR, double-stranded RNA was denatured for 5 min by heating at 95°C, and then the sample was immediately chilled on ice, and 5 μl of the NA extract was added to the RNA UltraSense One-Step Quantitative RT-PCR System (Gibco-Life Technologies), with adjusted concentrations of primers (900 nM for the reverse primer and 500 nM for the forward primer) and TaqMan probe (250 nM final concentration) *(29)*. Primers and probe used were as previously described, except that the probe was shortened and was labeled with minor groove binder *(37)*. The absence of inhibitors was verified by amplification of the undiluted and 10-fold-diluted NA extract. The number of RNA copies present in each positive sample was estimated by comparing the quantification cycle (C_q) values of samples to the standard curve that was generated by amplifying a dilution series of an in vitro transcription plasmid containing the complete NSP3 gene of strain RRV07 (GenBank AY065842, kindly provided by D. Poncet). The RNA concentration was then adjusted based on the volume of NA extract analyzed and reported per gram of DT.

Statistical analysis. A Student's *t* test was performed by using SPSS Statistics Version 19 (IBM, Bois-Colombes, France) to compare virus concentrations after of the three cooking times.

RESULTS

Experimental conditions. Mussels used for this study were of commercial size, as usually consumed in France. Before each experiment, the absence of RV and norovirus contamination was verified *(29)*. The mean weight of one raw mussel with the shell on was 7 ± 2 g, and the flesh of one individual mussel was 1.6 ± 0.4 g. No statistical difference was found between the weight of raw and cooked mussels ($P = 0.24$), or for the weight of DT for raw mussels (0.3 ± 0.06 g) compared with cooked mussels (0.26 ± 0.06 g) ($P = 0.26$).

After different techniques were trialed (data not shown), the most reproducible method to ensure similar levels of

virus within the different individual mussels was found to be spiking a small volume of RV directly into the DT. This method also had the advantage of being highly reproducible compared with bioaccumulation, thereby enabling the experiments to be accurately repeated. The temperature was measured in the liquid and in two mussels randomly chosen in the pan, and the experiment was repeated twice. The temperature of the mussels increased to reach 85°C after 2 min of cooking, 90°C after 3 min of cooking, and 95°C after 4 and 5 min of cooking.

RNA detection. Mengovirus or any other viral extraction controls were not added to tissue to control the extraction efficiency as they may interfere with the cell culture method. Nonetheless, we carefully checked the different steps of detection by repeating the extraction several times and including NA dilution to evaluate inhibitor removal. The RV extraction efficiency and RNA recovery from raw mussel tissue were surprisingly low. This finding was unexpected as the virus was spiked and then eluted with glycine solution before NA extraction by using the NucliSENS kit. The removal of inhibitors was verified and the same kit was used for all NA extractions, excluding the impact of inhibition or kit-to-kit variation. The impact of 30-min contact time with the mussel tissue made no difference to the virus detection (4.6 ± 0.5 compared with 4.4 ± 0.6 log RNA copies per g of DT when no contact time was included). When the virus was added directly to dissected DT and left for 30 min, the concentrations were 4.9 ± 0.2 log RNA copies per g of DT; this suggests that some RV may migrate to other tissues when the virus is spiked into the DT of whole mussels, or that RV is lost during the dissection. Finally, if the virus was added after the glycine buffer elution concentrations were ~5.9 ± 0.2 log RNA copies per g of DT.

Although recovery was low from raw mussels, the RNA recovery from cooked mussels was more efficient (2×10^6 RNA copies per g of DT after 1 min of cooking) and is closer to the expected amount of RV spiked (expected 6×10^7 RNA copies per g of DT) (Table 1). All the cooking experiments were repeated five times, and RT-qPCR amplifications were undertaken in triplicate; thus, the average C_q values presented in Table 1 were calculated using 15 values. The standard deviations observed were low except for raw DT, presumably due to the virus recovery problem. To lower the variability, when possible, all the amplifications were performed within the same run and within a short time. This approach allowed us to be confident in the quantitation of RV RNA copies in the cooked mussels. Given this, we found that RV RNA copies were significantly lower in mussels after cooking for 5 min than in mussels cooked for 1 min ($P < 0.01$).

Infectious virus detection. The absence of toxicity of the mussel DT to cells was checked for each experiment by including one negative control sample. The dilution of viral eluate in cell culture media and the contact time of 1 h were apparently sufficient to avoid cell damage. Based on the infectious titer of the inoculum, we expected to detect up to 7.4×10^3 PFU/g of DT. The raw mussel samples were close to this theoretical titer, with at least one replicate giving a titer of 1.7×10^2 PFU/g (Table 1), showing the good efficiency of the glycine method to recover infectious virus. After 1 min of cooking, a decrease in the infectious titer was observed, with the concentration being significantly lower compared with the raw mussels ($P < 0.01$, t test) (Table 1). After 3 min, infectious RV was detected in two of the five replicates and the concentration was low. After 5 min of cooking, no infectious virus was detected.

TABLE 1. *RV detection in mussel samples that were cooked for various times*

Cooking time (min)	RNA detection: Avg C_q ± SD	RNA detection: Geometric mean concn/g of DT	Infectious titer (PFU/g of DT)[a]
0	33.7 ± 1.7	4.47×10^4	34 (18–168)
1	28.1 ± 1.1	$1.78 \times 10^{6\,b}$	6.7 (8.3–5.6)
3	28.7 ± 0.7	$1.12 \times 10^{6\,b}$	3.0 (<LOD–7.7)
5	30.8 ± 1.0	$2.36 \times 10^{5\,b}$	<LOD

[a] Values in parentheses represent the minimum and maximum concentration, respectively, observed among the five experiments performed. LOD, limit of detection (3.3 PFU/g of DT).
[b] $P < 0.01$ (t test).

DISCUSSION

Human enteric viruses are known to be resistant to various disinfectants and other treatments, such as UV; therefore, cooking is recognized as one of the most effective methods to reduce viruses from food products *(43)*. Cooking is especially important for shellfish as depuration does not efficiently eliminate viruses; thus, cooking is a control option used to lower the human health risks *(12, 20, 35)*. However, some of the current cooking processes used for shellfish may not result in inactivation of all viruses present inside the shellfish tissue, as demonstrated by outbreaks linked to cooked oysters and clams *(2, 33, 38)*. One important factor to consider is that 90% of viral contamination is localized in the DTs, which are protected by surrounding connective tissue *(4)*. This may explain the variable results obtained in studies to date, as different shellfish species, different virus types, and different cooking procedures have been used *(1, 14, 25, 34)*. Similarly, the impact of the food matrix has been shown to be important for thermal resistance of viruses in milk, sausages, and berries *(5, 6, 10)*.

Considering studies on viral contamination of different shellfish species, mussels have been reported to be more frequently contaminated than other species, such as oysters or clams *(7, 17, 47)*. However, they are frequently consumed cooked or marinated *(14, 24)*. For example, in France, mussels are rarely consumed raw, but rather as moules marinières—a popular dish. The traditional recipe includes onions and white wine, and once the liquid is boiling, it usually involves waiting for 1 or 2 min after the shell opens before serving and consumption. After this time, all the mussels usually are open.

The viruses most frequently detected in shellfish and implicated in outbreaks are noroviruses. These viruses are the main agents of acute gastroenteritis in humans, and they are shed in high quantities in sewage, especially during the winter *(3, 44)*. However, noroviruses cannot yet be easily cultured, preventing the detection of infectious particles *(27)*. To compensate for the lack of an in vitro system, several surrogates have been used, all of which have their different advantages and disadvantages *(15, 41)*. For this study, a rotavirus strain was selected based on the resistant characteristics of rotaviruses and their capacity to recognize histo-blood group antigens that are suggested to be important for the persistence of some viruses in shellfish; however, extrapolating observed data from one virus to another may be difficult *(16, 26, 30)*. Rotavirus can be bioaccumulated in different species of shellfish and has been detected in different countries *(1, 23, 40)*. In preparing contaminated mussels for these experiments, we considered allowing the mussels to bioaccumulate the virus; however, considerable variation was observed between individuals. Thus, as performed by other researchers, the virus was inoculated directly into the DT of each shellfish, rather than via bioaccumulation of the virus *(25, 31)*. Although we had to open the shells to inoculate the mussels and thus we cannot check the time of shell opening, we believe that this method is the most accurate way to ensure the same virus concentration was present in each mussel. We also consider that the method resulted in realistic localization of the virus within the body flesh and that it may be more representative of natural contamination events compared with tissue homogenate seeding *(9, 13)*. The inoculated mussels were left for 30 min before cooking for technical reasons (to allow time to inoculate individuals) and also to ensure some diffusion of the viral inoculate into the tissues. Following the comparison of different methods for the recovery of infectious virus (by seeding the virus at different steps) and considering issues relating to toxicity of the samples to the cultured cells (data not shown), the simplest and most efficient recovery method was the glycine elution method *(9)*. This method is based on a simple elution that provides a rationale for why an extraction efficiency control was not included (per the usual protocol). The extraction was found to be efficient, as the infectious titer of raw mussels was close to the expected concentration calculated from the inoculum. However, other controls were included to evaluate the absence of cell toxicity, and RT-qPCR was undertaken in triplicate on pure and diluted NA to exclude persistence inhibitors. Furthermore, the five replicates performed provide confidence in the results presented here.

One unexpected observation of this study was the impact of cooking on virus recovery: more RVs were detected in mussels after 1 min of cooking compared with raw mussels. As mentioned, no extraction efficiency control was used, but the analysis of triplicates and special care given to inhibitor removal make us confident in this finding. This observation has not been previously reported and suggests that the shellfish meat changes due to cooking and facilitates the release of more viruses; this point is important to consider for data interpretation. One hypothesis that may explain this observation could be the enhanced release of the virus by ligand disruption, but without additional ligand expression measurements, this speculation cannot be confirmed. To our knowledge, no studies have been performed on the impact of heat on viral binding in shellfish tissues, but temperature increase is used for their quantification *(32)*.

Our results showed that 5 min of cooking has an effect on genome detection by RT-qPCR, but more importantly, it had an effect on the RV infectivity as all the RV present was inactivated with this treatment. However, after 3 min of cooking some infectious particles were still able to be detected. Hepatitis A virus was inactivated after 3 min at 90°C, but not after 30 min at 60°C in mussel homogenates *(13)*. In mussel homogenates, hepatitis A virus was reduced by 1 log after heat treatment between 50 to 72°C for 1.07 to 37.91 min *(9)*. A study to determine viral heat inactivation kinetics in mussel tissues showed that feline calicivirus and murine norovirus were inactivated according to time–temperature interactions, by using a Weibull model *(9)*. In contrast, infectious virus levels decreased, but were still detected, in bioaccumulated mussels after they were cooked au gratin (topped with cheese) for 5 min or steamed for 9 min, thus showing the role that is played by the shell, the flesh, or both to protect the virus *(1, 14)*. More than 7 min of cooking was needed to inactivate bioaccumulated murine norovirus in clams, confirming the high resistance of the virus and the protective effect of shellfish tissue *(48)*. Immersion of mussels for 2 min in boiling water inactivated all F-specific RNA phage, but infectious phage was still detected after immersion in lightly simmering water for 6 or 10 min *(22)*. The recommendation of Flannery et al. *(22),* to immerse mussels in boiling water for a minimum of 3 min, may not satisfy shellfish consumers as it may adversely affect the sensory quality of the shellfish, as observed for high pressure treatment *(28, 31)*.

Human enteric viruses are highly resistant and may be protected by shellfish tissue after being bioaccumulated. Despite all the data published to date, it is still difficult to get precise information on the inactivation of viruses via cooking. This study adds new evidence that demonstrates that home cooking, typically carried out for 1 to 2 min for moules marinières, may not be sufficient to inactivate all viruses. This observation suggests that home cooking may not always be sufficient to eliminate all infectious viruses. However, this is a complex problem, and factors such as the food matrix and kinetics of inactivation need to be considered *(8, 9, 19)*. Indeed, parameters such as time and temperature to be used for cooking mussels should be included in the advisories, and these parameters may be elucidated through risk assessment, as proposed previously for hepatitis A virus in clams *(38)*. For shellfish, thorough cooking may change the organoleptic characteristics and make shellfish unpalatable, which is not likely to be acceptable for shellfish consumers. Given the foregoing information, improving the water quality of shellfish production areas is the best way to enhance consumer safety.

ACKNOWLEDGMENTS

This work was supported by the French government (Direction générale de l'Alimentation [DGAl], grant 2014-13/1210720/NF); by the

Japan Society for the Promotion of Science fellowships for young scientists (23-4434); and by Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES) Foundation, Ministry of Education of Brazil, Brasilia (Ph.D. fellowship). We thank C. McLeods, Seafood Safety Assessment, Ltd., for critical review of the manuscript.

REFERENCES

1. Abad, F. X., R. M. Pinto, R. Gajardo, and A. Bosch. 1997. Viruses in mussels: public health implications and depuration. *J. Food Prot.* 60:677–681.
2. Alfano-Sobsey, E., D. Sweat, A. Hall, F. Breedlove, R. Rodriguez, S. Greene, A. Pierce, M. Sobsey, M. Davies, and S. L. Ledford. 2012. Norovirus outbreak associated with undercooked oysters and secondary household transmission. *Epidemiol. Infect.* 140:276–282.
3. Atmar, R. L. 2010. Noroviruses: state of the art. *Food Environ. Virol.* 2:117–126.
4. Atmar, R. L., F. H. Neill, C. M. Woodley, R. Manger, G. S. Fout, W. Burkhardt, L. Leja, E. R. McGovern, F. Le Guyader, T. G. Metcalf, and M. K. Estes. 1996. Collaborative evaluation of a method for the detection of Norwalk virus in shellfish tissues by PCR. *Appl. Environ. Microbiol.* 62:254–258.
5. Barnaud, E., S. Rogee, P. Garry, N. Rose, and N. Pavio. 2012. Thermal inactivation of infectious hepatitis E virus in experimentally contaminated food. *Appl. Environ. Microbiol.* 78:5153–5159.
6. Bidawid, S., J. M. Farber, S. A. Sattar, and S. Hayward. 2000. Heat inactivation of hepatitis A virus in dairy foods. *J. Food Prot.* 63:522–528.
7. Boxman, I. L. A., J. J. H. C. Tilburg, N. A. J. M. te Loeke, H. Vennema, K. Jonker, E. de Boer, and M. Koopmans. 2006. Detection of noroviruses in shellfish in the Netherlands. *Int. J. Food Microbiol.* 108:391–396.
8. Bozkurt, H., D. H. D'Souza, and P. M. Davidson. 2014. Determination of thermal inactivation kinetics of hepatitis A virus in blue mussel (*Mytilus edulis*) homogenate. *Appl. Environ. Microbiol.* 80:3191–3197.
9. Bozkurt, H., S. Leiser, P. M. Davidson, and D. D'Souza. 2014. Thermal inactivation kinetic modeling of human norovirus surrogates in blue mussel (*Mytilus edulis*) homogenate. *Int. J. Food Microbiol.* 172:130–136.
10. Butot, S., T. Putallaz, and G. Sanchez. 2008. Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. *Int. J. Food Microbiol.* 126:30–35.
11. Caballero, S., F. X. Abad, F. Loisy, F. S. Le Guyader, J. Cohen, R. M. Pinto, and A. Bosch. 2004. Rotavirus virus-like particles as surrogates in environmental persistence and inactivation studies. *Appl. Environ. Microbiol.* 70:3904–3909.
12. Codex Alimentarius. 2012. Guidelines for the application of general principles of food hygiene to the control of viruses in food. CAC/GL 79-2012. Food and Agriculture Organization of the United Nations, Rome, and the World Health Organization, Geneva.
13. Croci, L., M. Ciccozzi, D. De Medici, S. Di Pasquale, A. Fiore, A. Mele, and L. Toti. 1999. Inactivation of hepatitis A virus in heat-treated mussels. *J. Appl. Microbiol.* 87:884–888.
14. Croci, L., D. De Medici, S. Di Pasquale, and L. Toti. 2005. Resistance of hepatitis A virus in mussels subjected to different domestic cooking. *Int. J. Food Microbiol.* 105:139–144.
15. Cromeans, T., G. W. Park, V. Costantini, D. Lee, Q. Wang, T. Farkas, A. Lee, and J. Vinjé. 2014. Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Appl. Environ. Microbiol.* 80:5743–5751.
16. Desselberger, U. 2014. Rotaviruses. *Virus Res.* 190:75–96.
17. Donia, D., M. C. Dell'Amico, A. R. Petrinca, I. Martinucci, M. Mazzei, F. Tolari, and M. Divizia. 2012. Presence of hepatitis E RNA in mussels used as bio-monitors of viral marine pollution. *J. Virol. Methods* 186:198–202.
18. Espinosa, A. C., M. Mazari-Hiriart, R. Espinosa, L. Maruri-Avidal, E. Méndez, and C. F. Arias. 2008. Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water. *Water Res.* 42:2618–2628.
19. European Food Safety Authority. 2011. Scientifc opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. *EFSA J.* 9:2190.
20. European Food Safety Authority. 2012. Norovirus (NoV) in oysters: methods, limits and control options. *EFSA J.* 10:2500.
21. Fisher, T. K., H. Steinsland, and P. Valentiner-Branth. 2002. Rotavirus particles can survive storage in ambient tropical temperatures for more than 2 months. *J. Clin. Microbiol.* 40:4763–4764.
22. Flannery, J., P. Rajko-Nenow, J. B. Winetrbourn, S. K. Malham, and D. L. Jones. 2014. Effectiveness of cooking to reduce norovirus and infectious F-specific RNA bacteriophage concentrations in *Mytilus edulis*. *J. Appl. Microbiol.* 117:564–571.
23. Grodzki, M., J. Ollivier, J.-C. Le Saux, J.-C. Piquet, M. Noyer, and F. S. Le Guyader. 2012. Impact of xynthia tempest on viral contamination of shellfish. *Appl. Environ. Microbiol.* 78:3508–3511.
24. Hewitt, J., and G. E. Greening. 2004. Survival and persistence of norovirus, hepatitis A virus, and feline calicivirus in marinated mussels. *J. Food Prot.* 67:1743–1750.
25. Hewitt, J., and G. E. Greening. 2006. Effect of heat treatment on hepatitis A virus and norovirus in New Zealand Greenshell mussels (*Perna canaliculus*) by quantitative real-time reverse transcription PCR and cell culture. *J. Food Prot.* 69:2217–2223.
26. Hu, L., S. E. Crawford, R. Czako, N. W. Corted-Penfield, D. S. Smith, J. Le Pendu, M. K. Estes, and B. V. V. Prasad. 2012. Cell attachment protein VP8 of a human rotavirus specifically interacts with A-type histo-blood group antigen. *Nature* 485:256–260.
27. Jones, M., M. Watanabe, S. Zhu, C. L. Graves, L. R. Keyes, K. R. Grau, M. B. Gonzales-Hernandez, N. M. Iovine, C. E. Wobus, J. Vinjé, S. A. Tibbetts, S. M. Wallet, and S. M. Karst. 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science* 346:755–759.
28. Kingsley, D. H., D. D. Kuhn, G. J. Flick, J. Oh, L. S. Lawson, G. L. Meade, and C. C. Giesecke. 2014. Desirability of oysters treated by high pressure processing at different temperatures and elevated pressures. *Am. J. Food Technol.* 9:209–216.
29. Le Guyader, F., S. Parnaudeau, J. Schaeffer, A. Bosch, F. Loisy, M. Pommepuy, and R. L. Atmar. 2009. Detection and quantification of noroviruses in shellfish. *Appl. Environ. Microbiol.* 74:618–624.
30. Le Guyader, F. S., R. L. Atmar, and J. Le Pendu. 2012. Transmission of viruses through shellfish: when specific ligands come into play. *Curr. Opin. Virol.* 2:103–110.
31. Leon, J. S., D. H. Kingsley, J. S. Montes, G. P. Richards, G. M. Lyon, G. M. Abdulhafid, S. R. Seitz, M. L. Fernandez, P. F. Teunis, G. J. Flick, and C. L. Moe. 2011. Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing. *Appl. Environ. Microbiol.* 77:5476–5482.
32. Maalouf, H., M. Zakhour, J. Le Pendu, J.-C. Le Saux, R. L. Atmar, and F. S. Le Guyader. 2010. Norovirus genogroup I and II ligands in oysters: tissue distribution and seasonal variations. *Appl. Environ. Microbiol.* 76:5621–5630.
33. MacDonnel, S., K. B. Kirkland, W. G. Hlady, C. Aristeguita, R. S. Hopkins, S. S. Monroe, and R. I. Glass. 1997. Failure of cooking to prevent shellfish associated viral gastroenteritis. *Arch. Intern. Med.* 157:111–116.
34. Millard, J., H. Appleton, and J. V. Parry. 1987. Studies on heat inactivation of hepatitis A virus with special reference to shellfish. *Epidemiol. Infect.* 98:397–414.
35. National Advisory Committee on Microbiological Criteria for Foods. 2008. Response to the questions posed by the food and drug administration and the national marine fisheries service regarding determination of cooking parameters for safe seafood for consumers. *J. Food Prot.* 71:1287–1308.
36. Painter, J. A., R. M. Hoekstra, T. Ayers, R. V. Tauxe, C. R. Braden, F. J. Angulo, and P. M. Griffin. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerg. Infect. Dis.* 19:407–415.
37. Pang, X., M. Cao, M. Zhang, and B. Lee. 2011. Increased sensitivity for various rotavirus genotypes in stool specimens by amending three mismatched nucleotides in the forward primer of a real-time RT-PCR assay. *J. Virol. Methods* 172:85–87.

38. Pinto, R. M., M. I. Costafreda, and A. Bosch. 2009. Risk assessment in shellfish-borne outbreaks of hepatitis A. *Appl. Environ. Microbiol.* 75:7350–7355.
39. Praveen, C., B. A. Dancho, D. H. Kingsley, K. R. Calci, G. K. Meade, K. D. Mena, and S. D. Pillai. 2013. Susceptibility of murine norovirus and hepatitis A virus to electron beam irradiation in oysters and quantifying the reduction in potential infection risks. *Appl. Environ. Microbiol.* 79:3796–3801.
40. Quiroz-Santiago, C., C. Vasquez-Salinas, I. Natividad-Bonifacio, B. L. Barron-Romero, and E. I. Quinones-Ramirez. 2014. Rotavirus G2P[4] detection in fresh vegetables and oysters in Mexico City. *J. Food Prot.* 77:1953–1959.
41. Richards, G. P. 2012. Critical review of norovirus surrogates in food safety research: rationale for considering volunteer studies. *Food Environ. Virol.* 4:6–13.
42. Richards, G. P., C. McLeod, and F. S. Le Guyader. 2010. Processing strategies to inactivate enteric viruses in shellfish. *Food Environ. Virol.* 2:183–193.
43. Rodríguez-Lázaro, D., N. Cook, F. M. Ruggeri, J. Sellwood, A. Nasser, M. S. J. Nascimento, M. D'Agostino, R. Santos, J. C. Saiz, A. Rzeżutka, A. Bosch, R. Gironés, A. Carducci, M. Muscillo, K. Kovač, M. Diez-Valcarce, A. Vantarakis, C.-H. von Bonsdorff, A.-M. de Roda-Husman, M. Hernández, and W. H. van der Poel. 2012. Virus hazards from food, water and other contaminated environments. *FEMS Microbiol. Rev.* 36:786–814.
44. Sima, L. C., J. Schaeffer, J.-C. Le Saux, S. Parnaudeau, M. Elimelech, and F. S. Le Guyader. 2011. Calicivirus removal in a membrane bioreactor wastewater treatment plant. *Appl. Environ. Microbiol.* 77:5170–5177.
45. Simmons, G., C. Garbutt, J. Hewitt, and G. Greening. 2007. A New Zealand outbreak of norovirus gastroenteritis linked to the consumption of imported raw Korean oysters. *N. Z. Med. J.* 120:U2773.
46. Souza Marques Sobral, D., R. S. Piazza, M. R. Pilotto, M. de Almedia do Nascimento, V. Moresco, S. Taniguchi, D. A. G. Leal, É. C. Schmidt, E. Cargin-Ferreira, M. C. Bícego, S. T. Sasaki, R. C. Montone, R. A. de Aeaujo, R. M. B. Franco, Z. L. Bouzon, A. C. D. Bainy, and C. R. M. Barardi. 2013. Virus, protozoa and organic compounds decay in depurated oysters. *Int. J. Food Microbiol.* 167:337–345.
47. Suffredini, E., L. Lanni, G. Arcangeli, T. Pepe, R. Mazzette, G. Ciccaglioni, and L. Croci. 2014. Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy. *Int. J. Food Microbiol.* 184:21–26.
48. Toffan, A. Brutti, A. De Pasquale, E. Cappellozza, F. Pascoli, M. Cigarini, M. Di Rocco, C. Terregino, and G. Arcangelo. 2013. The effectiveness of domestic cook on inactivation of murine norovirus in experimentally infected Manila clams (*Ruditapes philippinarum*). *J. Appl. Microbiol.* 116:191–198.