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Research Note

Norovirus Monitoring in Bivalve Molluscs Harvested and Commercialized in Southern Italy

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ABSTRACT

Norovirus (NoV) is the main cause of human nonbacterial gastroenteritis throughout the world. NoVs are classified into five genogroups: GI, GII, GIII, GIV, and GV. NoVs from GI and GII are the most commonly reported NoVs associated with human infections, and raw or undercooked shellfish have been identified as the main potential infection vehicle. European Commission Regulation 2073/2005 defines only bacteriological parameters for use as safety criteria for shellfish because reference methods for detection of viruses are lacking. From July 2007 to April 2010, 163 shellfish samples were collected in southern Italy from harvesting areas, authorized or nonauthorized retailers, and a restaurant after an outbreak of human gastroenteritis. The shellfish were analyzed for the presence of NoVs from GI and GII using the one-step real-time reverse transcription PCR protocol. A total of 94 shellfish samples (57.7%) were positive for the presence of NoV, and GII was the most frequently identified genogroup.

Noroviruses (NoVs) are recognized as the main cause of nonbacterial human gastroenteritis in developed countries *(5, 31, 33)*. These viruses have a small positive sense single-stranded RNA genome (7.5 kb) organized in three open reading frames (ORFs). ORF1 encodes a nonstructural protein, ORF2 encodes VP1 (the major capsid protein), and ORF3 encodes VP2 (a small capsid protein). On the basis of their genetic complexity, NoVs are classified into five genogroups *(4, 10, 35)*. NoVs in genogroups GI, GII, and GIV cause human infections, and those in genogroups GI and GII currently are the most commonly reported. Genogroups are further subdivided into genotypes. In phylogenetic studies, NoV GI and NoV GII genogroups were divided into 14 and 17 genotypes, respectively *(14, 25, 32)*. Although various genotypes may cocirculate in the same area, the GII NoVs are responsible for 92% of NoV-caused human diseases *(7, 34)*.

The majority of NoV-related gastroenteritis outbreaks are due to the consumption of contaminated shellfish. Shellfish filtering activity can result in accumulation of wastewater pathogens to high levels. Shellfish also are often consumed raw or undercooked *(26, 27)*. Since January 2010, 334 cases of NoV-associated human gastroenteritis have been reported in various European countries, and most cases were linked to consumption of live bivalve molluscs *(3, 8, 24, 28)*. According to European Commission (EC) Regulation 2073/2005 *(19)*, only bacteriological parameters (*Salmonella* and *Escherichia coli* counts) are used as safety criteria for live bivalve molluscs. Conventional fecal indicators can be unreliable for demonstrating the presence of NoVs, and the reliance on absence of fecal bacterial indicators for determining shellfish depuration times may be an unsafe practice. However, criteria for determining the presence of NoVs in food have not been established because analytical methods are not sufficiently developed. Data on the incidence of enteric viruses in European shellfish harvesting areas are still limited because of the lack of international and local standard reference methods for the detection of these viruses.

In the past decade, molecular approaches have led to the development of a large number of methods (conventional and real-time PCR) *(2, 11–13)* particularly for the detection of viruses, including hepatitis A virus and NoV, that grow poorly or not at all in cell culture *(15)*. The aim of the present study was to determine the prevalence of NoV in shellfish collected from harvesting areas and retailers in southern Italy. Samples collected from a restaurant after a human gastroenteritis outbreak also were examined. Monitoring, in association with epidemiological data, is a necessary step for the evaluation of the risk linked to the consumption of foods contaminated by NoV as a cause of foodborne disease. The analyses were carried out using a one-step real-time reverse transcription (RT)–PCR protocol, using two separate reactions that allow the qualitative detection of GI and GII NoVs *(12)*.

MATERIALS AND METHODS

Shellfish sampling. From July 2007 to April 2010, 163 samples of bivalve molluscs were collected in the Campania region

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TABLE 1. *Shellfish samples collected*

Shellfish species	No. of samples	Origin: Harvesting area		Retailer		
		Class A	Class B	Registered retailers	Street vendors	Restaurant
Mytilus galloprovincialis	152	58	59	19	16	
Ensis minor	4			4		
Chamelea gallina	3			1	2	
Tapes philippinarum	2				2	
Ostrea edulis	1			1		
Glycymeris glycymeris	1					1
Total	163	58	59	25	20	1

of southern Italy. One hundred seventeen samples of *Mytilus galloprovincialis* were obtained from 15 harvesting areas situated along the Campania region coastline; 7 harvesting areas were in class A production areas (product suitable for direct consumption), and 8 were in class B areas (product suitable for consumption after a period of depuration) in accordance with EC Regulation 854/2004 *(18)*. During sampling, some weather and environmental conditions (wind, sea conditions, date of last storm, seawater temperature, and date of last rain day) were recorded. When heavy storms occurred, samples were not collected until the third day after the storm.

Mussels were collected from rows within four corners of the harvesting area and in the central region. Each sample was a pool of about 20 individuals taken randomly from the upper, middle, and lower parts of each row. Forty-five shellfish samples were purchased from retailers in provinces in the Campania region. Twenty-five samples were purchased in shops regularly registered in accordance with the EC Regulation 852/2004 *(17)*, and 16 samples of *M. galloprovincialis*, 2 samples of *Tapes philippinarum*, and 2 samples of *Chamelea gallina* were obtained from nonregistered retailers (street vendors) that sold unpacked mussels. One sample of *Glycymeris glycymeris* was obtained from a restaurant after the occurrence of an outbreak of gastroenteritis that was associated with that restaurant. The numbers of pooled shellfish samples differed based on the species examined (Table 1).

Preparation of the samples. Shellfish samples were immediately transferred to the laboratory in insulated boxes and were examined for NoV contamination on the same day of sampling. Fifteen to 20 individuals were randomly selected for each analysis. Before opening, the shellfish were washed with clean water to remove mud and debris present on the valves. Shellfish were aseptically opened using a sterile blade, and digestive tissue was dissected, cleaned, and finely chopped with a sterile razor. This tissue was used for the nucleic acid extraction.

Nucleic acid extraction. Aliquots of 2.0 g of chopped digestive tissue were transferred to a centrifuge tube, and 2 ml of proteinase K (0.1 mg/ml) was added for lysis. Suspensions were vortexed for about 1 min, incubated at 37°C in a shaking incubator for 60 min to allow digestion, and then placed in a waterbath at 60°C for 15 min to inactivate the enzyme. Samples were then centrifuged at 3,000 × *g* for 5 min, and the supernatant was collected and transferred into a new tube. The volume of the supernatant was recorded for each sample (2.3 to 3.0 ml, median = 2.7 ml) and was normalized to 3.0 ml by addition of sterile phosphate-buffered saline (PBS). Viral RNA extraction and purification was performed using the Nuclisens extraction kit (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The method is based on guanidinium thiocyanate lysis coupled with the nucleic acid–binding properties of silica particles *(6)*. For each sample, 500 μl of supernatant was subjected to lysis with the appropriate buffer, binding to magnetic silica, and washing with the specific solutions of the kit to remove amplification reaction inhibitors. Nucleic acids were recovered in 100 μl of elution buffer and stored at −80°C until they were subjected to real-time RT-PCR analysis.

Process control. The efficiency of the extraction procedure was determined by addition to each sample of 10 μl of a titrated suspension of Mengo virus, used as a process control *(11, 27)*. Recovery of Mengo virus was determined with the real-time RT-PCR assay by comparing *Ct* values obtained for the shellfish sample extracts with those for the virus control, taking into account the dilution factor for the extraction procedure and the volume of sample subjected to analysis. Real-time RT-PCR for Mengo virus and calculations were performed according to Costafreda et al. *(11)*. Recovery was considered acceptable at ≥1%. Samples failing to meet this criterion were reextracted.

Real-time RT-PCR. The real-time RT-PCR assay was carried out using the primers and the probes listed in Table 2. The two probes for NoVs GI and GII were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxytetramethylrhodamine at the 3′ end. The probe for Mengo virus was labeled with FAM at 5′ end and MGB (minor groove binder) at the 3′ end *(11)*. Reverse transcription and PCR were performed in one step on an ABI Prism 7700 SDS system (Applied Biosystems, Foster City, CA). Amplification was carried out with the Platinum Quantitative RT-PCR One-Step System (Invitrogen, Saint Aubin, France). Five microliters of nucleic acid extract was added to 20 μl of reaction mixture containing 1× thermoScript reaction mix, 900 nM reverse primer, 500 nM forward primer, 250 nM Taqman probe, 1× ROX reference dye, and 0.5 ThermoScript Plus/Platinum *Taq* enzyme mixture. The total volume was 25 μl for each reaction. Amplification conditions consisted of reverse transcription for 60 min at 55°C, 5 min at 95°C, and amplification for 45 cycles of 15 s at 95°C (denaturation), 1 min at 60°C (annealing), and 1 min at 65°C (extension).

All samples were tested undiluted and in 10-fold dilutions to evaluate the effect of RT-PCR inhibitors. The amplification efficiency (*E*) in an undiluted sample was calculated according to the formula $E = 2 - \Delta Ct$, where ΔCt is *Ct* undiluted minus (*Ct* 1:10 dilution − 3.3). Amplification tests were performed in triplicate using the described protocols. Two negative controls (molecular grade water) were added to each run. For positive

TABLE 2. *Primers and probes used for real-time RT-PCR*

Primer or probe	Sequence	Reference
Norovirus GI		
QNIF4 (forward)	CGC TGG ATG CGN TTC CAT	*11*
NV1LCR (reverse)	CCT TAG ACG CCA TCA TCA TTT AC	*12*
NVGG1p (probe)	TGG ACA GGA GAY CGC RAT CT	*12*
Norovirus GII		
QNIF2 (forward)	ATG TTC AGR TGG ATG AGR TTC TCW GA	*21*
COG2R (reverse)	TCG ACG CCA TCT TCA TTC ACA	*13*
QNIFS (probe)	AGC ACG TGG GAG GGC GAT CG	*21*

TABLE 3. Ct *values for the norovirus-positive samples*

NoV type in each sample	No. of samples in each *Ct* category				
	<30.0	30.1–35.0	35.1–40.0	40.1–45.0	Total
GI	0	27	18	3	48
GII	30	44	18	0	92
Total	30	71	36	3	140

TABLE 4. *Results from analysis for norovirus presence*

Virus type	No. (%) of positive samples	No. (%) of negative samples	Total
GI	2		
GII	46		
GI plus GII	46		
Total	94 (57.7)	69 (42.3)	163

controls, two fecal samples containing NoV (GI.4 and GII.1) were extracted using the Mini Mag Nuclisens Magnetic Extraction kit (bioMérieux), and 10-fold dilutions of the nucleic acid were tested in quadruplicate. Two dilutions ($Ct = 30.08 \pm 0.51$ for GI and 29.62 ± 0.76 for GII) were selected as positive controls. Runs containing the samples were considered acceptable when the *Ct* value for the positive controls was in the defined range and no amplification was detected in the negative controls.

RESULTS

The real-time RT-PCR method allowed detection of NoV GI and GII and amplification of the Mengo virus process control using the specific primers and probes *(12)*. Samples were considered positive when the *Ct* value was less than 44.0 in at least two replicates and no amplification was detected in the negative controls. *Ct* values in NoV-positive undiluted samples ranged from 31.9 to 39.5 (median, 34.6) for NoV GI and from 25.8 to 40.4 (median, 32.3) for NoV GII. Comparison of the results obtained for undiluted samples and their 10-fold dilution revealed no relevant amplification inhibition (amplification efficiency $\geq$ 95%). Extraction efficiency ranged from 1.0 to 9.3% (median, 3.1%); two samples failed to meet the recovery criterion on first extraction but met it on the second attempt. The *Ct* values are showed in Table 3.

NoV was detected in 94 (57.7%) of 163 analyzed samples (Table 4). Samples from 13 harvesting areas (86%) were positive for NoV. Of the seven harvesting areas in class A, five produced samples that were positive for NoV, and all harvesting areas in class B produced samples that were positive for NoV. Sixty (51.3%) of 117 samples from all the harvesting areas were positive for NoV. NoV GII was detected in 30 samples, and both NoV genogroups (GI and GII) were found in 28 samples (Table 5). Environmental and meteorological conditions apparently did not affect NoV contamination in samples from the various harvesting areas. Generally, samples positive for NoV were found during all seasons, with different seawater temperatures and wind and current directions. However, NoV presence did appear to be influenced by the presence of sewage drains near harvesting areas (Table 6).

Thirty-four samples collected from retailers (73.9%) were positive for NoV. Only eight samples of *M.*

TABLE 5. *Results for samples collected from harvesting areas*

Harvesting area type	No. of areas	Sampling period	No. of samples	No. (%) of positive samples			
				NoV GI	NoV GII	NoV GI + NoV GII	Total
Class A	7	Jul 2007–May 2008	34	0	2	4	6
	7	Sep 2008–Jun 2009	23	2	2	2	6
	7	Nov 2009–Mar 2010	1	0	1	0	1
Total			58	2 (3.4)	5 (8.6)	6 (10.3)	13 (22.4)
Class B	8	Jul 2007–May 2008	33	0	17	5	22
	8	Sep 2008–Jun 2009	16	0	5	11	16
	8	Nov 2009–Mar 2010	10	0	3	6	9
Total			59	0	25 (42.4)	22 (37.3)	47 (79.7)
Grand total			117	2 (1.7)	30 (25.6)	28 (23.9)	60 (51.3)

TABLE 6. *Results for norovirus presence in samples collected in harvesting areas under different environmental and meteorological conditions*

Harvesting area type	Area no.	Sampling time	Wind strength	Sea condition, time of last storm	Seawater temp (°C)	Last raining day	Notes	NoV detected
Class A	1	Jul 2007	Dead calm	Calm sea, 7 days before	20	NR[a]	Sea stream from southeast	No
		Sep 2008	Wind	Sligh sea, 7 days before	15.3	NR		No
	2	Jun 2008	NR	NR	NR	NR		No
		Jun 2009	Breeze	Smooth sea, 50 days before	25	15 days before		Yes
		Mar 2010	NR	NR, 20 days before	NR	20 days before		Yes
	3	Jan 2008	Breeze	Calm sea, 10 days before	14.2	10 days before	Sea stream from north	Yes
		Sep 2008	Moderate	Light sea, 3 days before	13.8	1 day before		No
	4	Feb 2008	Moderate	Calm sea, 15 days before	14.2	NR		Yes
	5	Feb 2008	Moderate	Calm sea, NR	14.4	NR		No
		Jun 2009	Dead calm	Smooth sea, 10 days before	24	15 days before	Outside temp 31°C	No
	6	Feb 2008	Moderate	Calm sea, NR	14.2	Drizzle during the sampling		Yes
	7	Feb 2008	Moderate	Calm sea, 7 days before	14.3	NR		No
		May 2009	Moderate	Smooth sea, 8 days before	8.2	3 days before		Yes
Class B	1	Apr 2008	Moderate	Calm sea, 7 days before	16.2	NR	Harvesting area sited on a lake and near to sewage drains	Yes
		Dec 2008	Dead calm	Calm sea, NR.	8	NR		Yes
		Nov 2009	Dead calm	NR, 7 days before	17	NR		Yes
	2	Apr 2008	Breeze	Calm sea, 7 days before	16.8	4 days before	Harvesting area sited near a seawater purification system	Yes
		May 2009	Dead calm	Smooth sea, 10 days before	20	15 days before		Yes
	3	May 2008	Breeze	Calm sea, 7 days before	17	NR		Yes
		Mar 2009	NR	Light sea, 3 days before	13.8	1 day before		Yes
		Sep 2010	Light wind	Calm sea, 7 days before	24.9	7 days before		Yes
	4	May 2008	Breeze	Calm sea, 7 days before	17	NR		Yes
		Mar 2009	NR	Light sea, 3 days before	13.8	1 day before		Yes
		Sep 2010	Light wind	Calm sea, 7 days before	24.9	7 days before		Yes
	5	Jan 2008	Moderate	Light sea, NR	16	NR		Yes
	6	May 2008	Breeze	Calm sea, 7 days before	17	NR		Yes
		Mar 2009	NR	Light sea, 3 days before	13.8	1 day before		Yes
		Sep 2010	Light wind	Calm sea, 7 days before	24.9	7 days before		Yes
	7	May 2008	Breeze	Calm sea, 7 days before	17	NR		No
		Mar 2009	NR	Light sea, 3 days before	13.8	1 day before		Yes
		Sep 2010	Light wind	Calm sea, 7 days before	24.9	7 days before		Yes
	8	May 2008	Breeze	Calm sea, 7 days before	17	NR		Yes
		Mar 2009	NR	Light sea, 3 days before	13.8	1 day before		Yes
		Sep 2010	Light wind	Calm sea, 7 days before	24.9	7 days before		Yes

[a] NR, not recorded.

TABLE 7. *Results for shellfish collected from retailers and at a restaurant*

Species	No. of samples	No. (%) of positive samples			
		NoV GI	NoV GII	NoV GI + NoV GII	Total
Mytilus galloprovincialis	35	0	12	15	27
Ensis minor	4	0	0	0	0
Chamelea gallina	3	0	1	2	3
Tapes philippinarum	2	0	1	1	2
Ostrea edulis	1	0	1	0	1
Glycymeris glycymeris	1	0	0	1	1
Total	46	0	15 (32.6)	19 (41.3)	34 (73.9)

galloprovincialis and four samples of *Ensis minor* were negative for NoV. NoV GII was detected in all positive samples from retailers, and in 15 samples (32.6%), both NoV GI and NoV GII were present. The *G. glycymeris* sample that was collected from a restaurant associated with a suspected case of NoV gastroenteritis was contaminated with both genogroups (Table 7). Of the shellfish samples collected from authorized and/or regularly registered retailers and from street vendors that sold unpacked mussels, 19 (76%) of the 25 samples and 14 (87%) of the 16 samples, respectively, were positive for NoV.

DISCUSSION

The one-step real-time RT-PCR protocol used allowed the detection of NoV in shellfish harvested and sold in the Campania region of Italy. The presence of NoV in shellfish harvested in class A areas confirms that fecal bacteria cannot be considered reliable indicators for NoV in bivalves, and the shellfish safety criteria established by EC Regulation 2073/2005 *(19)* should be revised. In class B production areas, NoV contamination was detected in almost all samples, and no significant differences were observed among seasons. NoV infection has been called ''winter vomiting disease'' because epidemic peaks often occur during the winter months, probably because of the high resistance of this virus to low temperatures *(9, 20, 22)*. Presently, cases of gastroenteritis caused by NoV infection occur throughout the year. This pattern could be due to (i) higher temperature resistance of the emerging new variant NoV GII.4, as reported by recent epidemiological data, (ii) an increasing and constant release of NoV into the sea from sewage, and (iii) the high sensitivity of molecular methods that can detect NoV more effectively than could previous analytical methods *(23, 30)*.

A high frequency of NoV contamination (74% of samples) was found in shellfish purchased from retailers, and more than half of the samples from nonregistered street vendors that sold unpacked shellfish were contaminated with NoV. This second result is not unexpected, because the conditions of collecting, transport, storage, and marketing of shellfish are not always in line with good manufacturing practices and hygiene standards. NoV found in shellfish from registered retailers might have resulted from cross-contamination after immersion of the bivalves in unclean water to demonstrate their vitality to the consumers. The NoV found in the *G. glycymeris* sample (suspected as the source of a gastroenteritis outbreak associated with a restaurant) might have come from contaminated water, but the contamination may have been exacerbated by the storage conditions and the practices of the kitchen staff of the restaurant.

All except two of the NoV-positive samples in this study were contaminated with NoV GII. This result is in accordance with that of other researchers *(7, 34)*. However, in approximately one-half of the positive samples (46.7% of shellfish collected in harvesting areas and 55.9% of shellfish collected from retailers), NoV from both GI and GII genogroups were found, indicating high circulation of NoV GI in the environment. This finding is not consistent with data from human infections, which indicate an apparent worldwide dominance of NoV GII *(1, 29)*, and could be related to a greater resistance to breakdown during wastewater treatment of NoV GI strains *(12)*.

According to the concepts of EC Regulation 178/2002, to ensure food safety it is necessary to consider the complete food chain, from primary production to sale or distribution to the consumer, because each step may entail new or additional hazards. This holistic approach is of particular concern for foods such as shellfish, which are often consumed raw or undercooked. Regulators and producers must recognize hazards, identify critical control points, and establish monitoring procedures at each production step *(16)*.

REFERENCES

1. Atmar, R. L., and M. K. Estes. 2006. The epidemiologic and clinical importance of norovirus infection. *Gastroenterol. Clin. North Am.* 35: 275–290.
2. Atmar, R. L., F. H. Neill, J. L. Romalde, F. Le Guyader, C. M. Woodley, T. G. Metcalf, and M. K. Estes. 1995. Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. *Appl. Environ. Microbiol.* 61:3014–3018.
3. Baker, K., J. Morris, N. McCarthy, L. Saldana, J. Lowther, A. Collinson, and M. Young. 2010. An outbreak of norovirus infection linked to oyster consumption at a UK restaurant, February 2010. *J. Public Health (Oxf.)* 33:205–211.
4. Bertolotti-Ciarlet, A., S. E. Crawford, A. M. Hutson, and M. K. Estes. 2003. The 3′ end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. *J. Virol.* 77:11603–11615.
5. Boccia, D., A. E. Tozzi, B. Cotter, C. Rizzo, T. Russo, G. Buttinelli, A. Caprioli, M. L. Marziano, and F. M. Ruggeri. 2002. Waterborne outbreak of Norwalk-like virus gastroenteritis at a tourist resort, Italy. *Emerg. Infect. Dis.* 8:563–568.

6. Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28:495–503.
7. Campos, G. S., V. H. Moreau, A. Bandeira, G. Barberino, P. F. Almeida, D. M. Amador, M. O. de Lima, and S. I. Sardi. 2008. Molecular detection and genetic diversity of norovirus in hospitalized young adults with acute gastroenteritis in Bahia, Brazil. *Arch. Virol.* 153:1125–1129.
8. Carter, M. J. 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. *J. Appl. Microbiol.* 98:1354–1380.
9. Cesarini, F. 2008. Zoonosi alimentari ad eziologia virale:aspetti epidemiologici e diagnostici dei norovirus. Webzine Sanità Pubblica Veterinaria, no. 48. Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy.
10. Chakravarty, S., A. M. Hutson, M. K. Estes, and B. V. Prasad. 2005. Evolutionary trace residues in noroviruses: importance in receptor binding, antigenicity, virion assembly, and strain diversity. *J. Virol.* 79:554–568.
11. Costafreda, M. I., A. Bosch, and R. M. Pintó. 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription–PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Appl. Environ. Microbiol.* 72:3846–3855.
12. da Silva, A., J. C. Le Saux, S. Parnaudeau, M. Pommepuy, M. Elimelech, and F. S. Le Guyader. 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription–PCR: different behaviors of genogroups I and II. *Appl. Environ. Microbiol.* 73: 7891–7897.
13. De Medici, D., L. Croci, E. Suffredini, and L. Toti. 2004. Reverse transcription–booster PCR for detection of noroviruses in shellfish. *Appl. Environ. Microbiol.* 70:6329–6332.
14. Donaldson, E. F., L. C. Lindesmith, A. D. Lobue, and R. S. Baric. 2010. Viral shape-shifting: norovirus evasion of the human immune system. *Nat. Rev. Microbiol.* 8:231–241.
15. Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. Koopmans, and M. K. Estes. 2004. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* 85:79–87.
16. European Commission. 2002. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Off. J. Eur. Union* L 31:1–24.
17. European Commission. 2004. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. *Off. J. Eur. Union* L 226:3–21.
18. European Commission. 2004. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Off. J. Eur. Union* L 226:83–127.
19. European Commission. 2005. Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Union* L 338:1–26.
20. Fretz, R., D. Schmid, S. Jelovcan, R. Tschertou, E. Krassnitzer, M. Schirmer, M. Hell, and F. Allerberger. 2009. An outbreak of norovirus gastroenteritis in an Austrian hospital, winter 2006–2007. *Wien. Klin. Wochenschr.* 121:137–143.
21. Fuhrman, J. A., X. Liang, and R. T. Noble. 2005. Rapid detection of enteroviruses in small volumes of natural waters by real-time quantitative reverse transcriptase PCR. *Appl. Environ. Microbiol.* 71:4523–4530.
22. Greer, A. L., S. J. Drews, and D. N. Fisman. 2009. Why "winter" vomiting disease? Seasonality, hydrology, and norovirus epidemiology in Toronto, Canada. *EcoHealth* 6:192–199.
23. Ho, E. C., P. K. Cheng, A. W. Lau, A. H. Wong, and W. W. Lim. 2007. Atypical norovirus epidemic in Hong Kong during summer of 2006 caused by a new genogroup II/4 variant. *J. Clin. Microbiol.* 45: 2205–2211.
24. Iizuka, S., T. Oka, K. Tabara, T. Omura, K. Katayama, N. Takeda, and M. Noda. 2010. Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish. *J. Med. Virol.* 82:1247–1254.
25. Kageyama, T., M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, S. Kojima, R. Takai, T. Oka, N. Takeda, and K. Katayama. 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. *J. Clin. Microbiol.* 42:2988–2995.
26. Koopmans, M., and E. Duizer. 2004. Foodborne viruses: an emerging problem. *Int. J. Food Microbiol.* 90:23–41.
27. Le Guyader, F. S., J. C. Le Saux, K. Ambert-Balay, J. Krol, O. Serais, S. Parnaudeau, H. Giraudon, G. Delmas, M. Pommepuy, P. Pothier, and R. L. Atmar. 2008. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *J. Clin. Microbiol.* 46:4011–4017.
28. Lopman, B., H. Vennema, E. Kohli, P. Pothier, A. Sanchez, A. Negredo, J. Buesa, E. Schreier, M. Reacher, D. Brown, J. Gray, M. Iturriza, C. Gallimore, B. Bottiger, K. O. Hedlund, M. Torvén, C. H. von Bonsdorff, L. Maunula, M. Poljsak-Prijatelj, J. Zimsek, G. Reuter, G. Szücs, B. Melegh, L. Svennson, Y. van Duijnhoven, and M. Koopmans. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet* 363:682–688.
29. Lopman, B., M. Zambon, and D. W. Brown. 2008. The evolution of norovirus, the "gastric flu." *PLoS Med.* 5:e42.
30. Lopman, B. A., M. Reacher, C. Gallimore, G. K. Adak, J. J. Gray, and D. W. Brown. 2003. A summertime peak of "winter vomiting disease": surveillance of noroviruses in England and Wales, 1995 to 2002. *BMC Public Health* 3:13.
31. Marks, P. J., I. B. Vipond, D. Carlisle, D. Deakin, R. E. Fey, and E. Q. Caul. 2000. Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant. *Epidemiol. Infect.* 124:481–487.
32. Vinje, J., H. Vennema, L. Maunula, C. H. von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S. S. Beard, S. S. Monroe, E. de Bruin, L. Svensson, and M. P. Koopmans. 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. *J. Clin. Microbiol.* 41:1423–1433.
33. Westrell, T., V. Dusch, S. Ethelberg, J. Harris, M. Hjertqvist, N. Jourdan-da Silva, A. Koller, A. Lenglet, M. Lisby, and L. Vold. 2010. Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark. *Euro Surveill.* 15 pii:19524.
34. Widdowson, M. A., A. Sulka, S. N. Bulens, R. S. Beard, S. S. Chaves, R. Hammond, E. D. Salehi, E. Swanson, J. Totaro, R. Woron, P. S. Mead, J. S. Bresee, S. S. Monroe, and R. I. Glass. 2005. Norovirus and foodborne disease, United States, 1991–2000. *Emerg. Infect. Dis.* 11:95–102.
35. Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe. 2006. Norovirus classification and proposed strain nomenclature. *Virology* 346:312–323.