Development of A Monoclonal Antibody-Coated Immunomagnetic Beads for Separation and Detection of Norovirus (Genogroup II) in Oysters

Lin YAO, Yanhua JIANG, Wei JIANG, Fengling LI, Yuxiu ZHAI and Lianzhu WANG*

Key Laboratory of Test and Evaluation on Quality and Safety of Aquatic Products, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China.

Received February 26, 2013; Accepted March 30, 2013

The present study aimed to develop and evaluate a reverse transcription- polymerase chain reaction (RT-PCR) assay that incorporated immunomagnetic beads (IMS/RT-PCR) coated with a monoclonal antibody (Mab) for the separation and detection of noroviruses (NVs, genogroup II) in oysters. Mab-coated beads and polyclonal antibody (Pab)-coated beads were added to oyster extracts containing a series of tenfold dilution of NVs-positive fecal extracts. The oyster extracts were also treated with Proteinase K method. RNA was extracted and used as template for RT-PCR. Mab-based IMS/RT-PCR could detect 10 RT PCR unit in oyster homogenate, it was 10 times more sensitive than Pab-based IMS/RT-PCR, and 10² times more sensitive than proteinase K/RT-PCR. Analysis of 473 oysters showed that Mab-based IMS/RT-PCR has the highest percentage of positive samples (5.29%), followed by Pab-based IMS/RT-PCR (3.81%), and proteinase K/RT-PCR (2.11%). Mab-based IMS/RT-PCR is a sensitive method for the detection of NVs in oysters and could be a good candidate for routine screening and risk assessment.

Keywords: norovirus, oyster, immunomagnetic beads, separation, monoclonal antibody, RT-PCR

Introduction

Noroviruses (NVs), previously called “Norwalk-like viruses” or “small round-structured viruses,” are a type of human calicivirus (HuCV) (Hardy and Estes, 1996; Jiang *et al.*, 1990; Lambden *et al.*, 1993). The NVs genus is subdivided further into five genogroups (GI to GV) based on sequence information for the genes encoding the viral RNA-dependent RNA polymerase and the capsid protein (Zheng *et al.*, 2006). Three of these, genogroups I, II, and IV, are found in humans (Ando *et al.*, 2000; Karst *et al.*, 2003), with genogroup II viruses (more precisely, genogroup II.4 NVs) being the most prevalent (Atmar *et al.*, 2006; Kroneman *et al.*, 2008; Svraka *et al.*, 2007).

At present, NVs are considered the most common cause of outbreaks of acute nonbacterial gastroenteritis worldwide (Clark *et al.*, 2004; Lopman *et al.*, 2003), as well as an important cause of sporadic gastroenteritis in many countries (Marshall and Bruggink, 2006). The infectious dose of NVs is relatively low: as few as 10 to 100 infectious particles can

initiate an infection (Caul, 1996).

Viral gastroenteritis has often been related to the consumption of oysters harvested in fecal-polluted areas (Lees, 2000; Le Guyader *et al.*, 1996). In Europe and especially in Nordic countries, shellfish-borne gastrointestinal NVs have caused several large outbreaks (Lees, 2000).

Oysters are filter feeders, so NVs can become concentrated in them, thereby giving rise to human infections. In several studies, NVs were detected in approximately 5% to 20% of the oysters and clams examined (Formiga-Cruz *et al.*, 2002; Le Guyader *et al.*, 2000). Some of the oysters contained relatively large amounts of NVs genomes, with more than 100 copies of the capsid gene (Nishida *et al.*, 2003).

A practical test for viral contamination of oysters is thus clearly needed. Detection of NVs in oysters is currently based on molecular procedures, such as reverse transcription PCR (RT-PCR) and real-time RT-PCR (Baert *et al.*, 2007; Jothikumar *et al.*, 2005). Identification of NVs from oysters implicated in gastrointestinal outbreaks can be difficult, partly due to insufficient viral recovery and the presence of PCR inhibitors such as proteoglycans, polysaccharides, and lipids (Groppe and Morse, 1993; Richards, 1999; Schultz *et*

*To whom correspondence should be addressed.

E-mail: Lianzhu_wang@126.com

al., 2007). Therefore, the most crucial step in viral detection in oysters is the sample treatment process for concentrating the virus. The use of different elution buffers, including acid adsorption (Mullendore *et al.*, 2001), alkaline (Myrmel *et al.*, 2004) and neutral buffers (Atmar *et al.*, 1995) have been proposed. Protein K digestion of shellfish tissue has also been used to release virus particles (Jothikumar *et al.*, 2005). Virus particles can be concentrated by precipitation using polyethylene glycol (PEG) (Traore *et al.*, 1998) or by ultracentrifugation (Muniain-Mujika *et al.*, 2000). Freon, chloroform (Muniain-Mujika *et al.*, 2000) and chloroform/butanol (Atmar *et al.*, 1995) have been used to purify viruses from shellfish concentrates. These methods are inconvenient due to the multitude of time-consuming and fastidious steps needed for virus isolation from shellfish. Some methods involve the use of environmentally harmful chemicals such as Freon and chloroform (Atmar *et al.*, 1995; Muniain-Mujika *et al.*, 2000). Consequently, they are not easily adaptable for routine diagnosis of NVs contamination in oysters.

Immunomagnetic separation (IMS) is a useful tool for virus separation and isolation. As an additional advantage, potential RT-PCR inhibitors are also effectively removed during IMS (Arnal *et al.*, 1999). IMS has been used for the recovery of NVs from fecal extracts using polyclonal antibodies (Pab) (Gilpatrick *et al.*, 2000; Mette *et al.*, 2000). However, the use of immunomagnetic beads coated with monoclonal antibodies (Mab) for NVs recovery in oysters has not yet been tested. Therefore, the aim of the current study is to develop an IMS/RT-PCR assay using Mab-coated immunomagnetic beads for the detection of NVs in oysters.

Materials and Methods

Virus and antibody A positive fecal specimen containing norovirus GII-4 was collected from the Guangdong Women and Children's Hospital in China. The NVs was identified by RT-PCR, and the amplicon of the RT-PCR was confirmed by sequencing and the concentration of NVs was 1×10^6 RT-PCR unit (Wang *et al.*, 2008). The fecal sample was diluted 1:10 in PBS (pH 7.2, prepared with Diethylenetriamine (DEPC, Sigma, Germany) treated water and cleared by centrifugation for 30 min at 1000 g and 4°C. The supernatant (fecal extract) was stored at -80°C until use.

Monoclonal antibody 12F4 (Mab), which could recognize GII-4 NVs specifically, was kindly provided by Dr. Wang (Wang *et al.*, 2008), polyclonal antibody (Pab) was prepared in our previously study (Yao *et al.*, 2009).

Oyster homogenate Twenty live oysters (*Crassostrea ariakensis*) purchased at a local market were tested to be negative for noroviruses and shucked. The stomach and digestive diverticula were recovered by dissection, cut into

small portions, mixed, and then divided into 1.5 g portions. These portions were then ground in 1.5 mL Tris-ethylenediamine tetraacetic acid buffer (pH 8.5) containing 100 µg mL⁻¹ proteinase K (Roche Molecular Diagnostics, Germany) with a sterile tissue grinder. The homogenate was shaken at 37°C for 1 h, placed in a heating block at 65°C for 10 min, and cleared by centrifugation for 10 min at 3000 g and 4°C. A 900 µL portion of the supernatant (oyster extract) was removed and stored at 4°C until further use.

Contamination studies were initiated by the addition of 50 µL fecal extract containing NVs or its tenfold dilutions (from 10⁻¹ to 10⁻⁵) to 450 µL of oyster extract as described previously (De Medici *et al.*, 2004). The final dilution of virus particles in the oyster extract ranged from 10⁻² to 10⁻⁶, the RT-PCR unit ranged from 10⁴ to 1, and incubation was carried out at room temperature for 1 h with constant rotation.

Naturally contaminated oysters A total of 473 live oysters were collected randomly from a local seafood market in Qingdao demonstrate that IMS/RT-PCR could be performed on live oysters. These oysters were shipped to the laboratory in cold storage within 1 h and were processed immediately. The preparation of oyster extract followed the steps described above.

Preparation of immunomagnetic beads M-280 Sheep anti-Rabbit IgG Dynabeads® (Invitrogen, USA) were coated with rabbit anti-NVs IgG following the manufacturer's protocol.

Briefly, 5 µg of rabbit anti-NVs IgG was used to coat 10⁷ beads for 2 h at room temperature with constant rotation. The coated beads were washed four times with PBS (pH 7.2, 0.02% NaN₃, 0.1% BSA) to remove unbound IgG, then stored at 4°C in washing buffer (6×10^8 beads mL⁻¹) until use.

The preparation of M-280 Sheep anti-Mouse IgG Dynabeads® (Invitrogen, USA) coated with Mab 12F4 followed the same steps described above.

Immunomagnetic separation The sensitivity of the Mab-based and Pab-based IMS/RT-PCR was tested by adding 20 µL beads to a series of oyster extracts (500 µL) artificially contaminated with NVs. After 1 h incubation at room temperature with constant rotation, the beads were collected by magnetic separation and washed four times with 1 mL washing buffer (PBS, pH 7.2, 0.02% NaN₃, 0.1% BSA). The beads were then suspended in 100 µL PBS (pH 7.2, prepared with DEPC-treated water).

Extraction of NVs RNA Viral RNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche, Germany) following the manufacturer's instructions.

Primers and RT-PCR The primer set JV12 (5'-ATACCACTATGATGCAGATTA-3', sense primer, genome location: 4552-4572) and JV13 (5'-TCATCATCAC-

CATAGAAAGAG-3', antisense primer, genome location: 4858-4878) was designed according to the RNA-dependent RNA polymerase gene, as previously described (Vinjé and Koopmans, 1996). A 327 bp portion of the RNA-dependent RNA polymerase gene was amplified by this protocol.

RT-PCR was performed according to the protocol described previously (Wang *et al.*, 2008), with some modifications. The first strand of cDNA was synthesized using the first strand cDNA synthesis kit (Fermentas, Canada). The Reverse Transcription mixture contained 2.0 μ L crude RNA extraction, 5.0 μ L 5 $\times$ reaction buffer, 200 U Moloney murine leukemia virus reverse transcriptase, 20 U RNase inhibitor, 1.0 μ L 10.0 mmol L⁻¹ dNTPs, and 2.0 μ L 10.0 μ mol L⁻¹ primer JV13. DEPC-treated water was added to bring the final volume to 25.0 μ L. The mixture was incubated for 60 min at 42°C. Reverse transcriptase was then inactivated by heating to 95°C for 5 min, followed by immediate chilling in ice.

The PCR mixture contained 5.0 μ L first strand cDNA, 2.0 U *Taq* DNA Polymerase (Fermentas, Canada), 5.0 μ L 10 $\times$ *Taq* buffer (with Mg²⁺), 1.0 μ L 10.0 mmol L⁻¹ dNTPs, and 1.0 μ L 10.0 mmol L⁻¹ primers (JV12 and JV13). Deionized water was also added to bring the final volume to 50.0 μ L. The PCR conditions were as follows: initial denaturation at 94°C for 5 min; denaturation for 30 s at 94°C; annealing for 45 s at 37°C; and extension for 30 s at 72°C for 35 cycles with an additional 7 min of extension at 72°C. The amplified 327 bp product was visualized by UV light after electrophoresis in 1.5% agarose gel (SBS Genetech Co., Ltd., China) in the presence of GeneFinder™ nucleic acid stain (Biov Genetech Co., Ltd., China) and sequenced in an automated sequencer (3730XL ABI, Shanghai Sangon Co., Ltd., China).

All experiments were repeated at least three times, and a negative control sample (deionized water) was run with each test.

Results and Discussion

Comparison of the sensitivities of IMS/RT-PCR and Proteinase K/RT-PCR A series of tenfold dilutions of fecal extract were added to oyster extracts to determine the

sensitivities of Mab-based and Pab-based IMS/RT-PCR and the Proteinase K-RT-PCR assays. The respective sensitivities of the three assays are shown in Table 1. IMS/RT-PCR using 12F4 showed an endpoint in the 10⁻⁵ dilution (10 RT PCR unit), and was at least 10 times more sensitive than incubation with Pab-coated beads, indicating that Mab has a higher sensitivity than Pab. The Proteinase K method consistently showed an endpoint in the 10⁻³ dilution (10³ RT PCR unit), indicating that Mab-based IMS/RT-PCR is at least 10² times more sensitive than this method.

The immunomagnetic separation method has several advantages over conventional methods. For example, manipulations are simple, special equipment is not required, the processing time is shorter, and RT-PCR interfering substances are effectively removed (Kobayashi *et al.*, 2004). In addition, many samples can be tested simultaneously, the method requires no organic reagents that are environmentally unsafe (such as Freon), and the possibility of detecting non-infectious virus particles or free RNA by subsequent RT-PCR is substantially reduced.

Recent research indicated that NVs can specifically bind to antigens that are similar to human histo-blood group antigens (HBGAs) in the oyster gut (Le Guyader *et al.*, 2006; Tian *et al.*, 2006). The function of Proteinase K is to release virus particles adequately from oyster gut (Jothikumar *et al.*, 2005). Furthermore, after proteinase K treatment, oyster extracts are no longer sticky, which facilitates bead separation from the oyster extract by magnet. Compared with Pab, Mab has more advantages with regard to sensitivity. In a detection limit study, the Mab-coated immunomagnetic beads successfully detected the virus sample in a 10⁻⁵ dilution (10 RT-PCR unit) in oyster extract, whereas the Pab-coated beads only detected a 10⁻⁴ dilution (10² RT-PCR unit) of the same virus sample. The concentration of RT-PCR inhibitors in these samples was assumed to be very high, but the Mab-coated beads were able to separate the virus from the inhibitors efficiently because of the strong sensitivity for the virus. However, in a previous research, when IMS/RT-PCR was performed using human fecal extract, both Mab-based and Pab-based IMS/RT-PCR detected the same virus sample in

Table 1. The detection limits for NV in artificially contaminated oyster extracts using two IMS/RT-PCR and proteinase K/RT-PCR assays.

Different Assays	Volume(mL)	RT-PCR unit				
		10 ^{4a}	10 ³	10 ²	10 ¹	1
Mab-coated beads	0.5	+ ^b (3/3 ^f)	+(3 ^g /3)	+(3/3)	+(3/3)	- ^c (0/3)
Pab ^d -coated beads	0.5	+(3/3)	+(3/3)	+(3/3)	- ^c (0/3)	-(0/3)
Proteinase K/RT-PCR	0.5	+(3/3)	+(3/3)	-(0/3)	-(0/3)	Np ^e

(a Different RT-PCR unit, b Positive RT-PCR, c Negative RT-PCR, d Polyclonal antibody, e Not performed, f the data was representative of 3 independent experiments, g the number of positive result was 3)

a higher dilution (10^{-7}) (Yao *et al.*, 2009). The inhibitors in oysters are probably more complicated than in fecal extract, which may affect the detection limits of the same methods when performed in different samples. Overall, the Mab-coated immunomagnetic beads are more sensitive than the Pab-coated beads for the separation of NVs.

Evaluation of Mab-based IMS/RT-PCR in live oysters

The three methods were also performed on 473 live oysters obtained from the local seafood market. All samples determined to be positive by Proteinase K/RT-PCR or Pab-based IMS/RT-PCR were also tested to be positive by Mab-based IMS/RT-PCR. Table 2 shows that Mab-based IMS/RT-PCR has the highest percentage of positive samples (5.29%, 25/473), followed by Pab-based IMS/RT-PCR (3.81%, 18/473) and Proteinase K/RT-PCR (2.54%, 12/473).

The RT-PCR products were visualized by UV light after electrophoresis in a 1.5% agarose gel with the presence of GeneFinder™ nucleic acid stain. The amplicon showed a sharp band at the expected size of 327 bp.

When sequenced, the positive amplicons proved to be fragments of the RNA-dependent RNA polymerase gene of the NVs. All of them were proved to belong to GII-4 NVs after the phylogenetic analysis (data not shown). No band was observed in the negative control.

Immunomagnetic beads used in IMS may exhibit non-specific binding to other pathogens (Tomoyasu, 1998), but this non-specific binding has not been observed to alter the detection limits of IMS/RT-PCR assays (Gilpatrick, 2000). In the current study, assay specificity was largely controlled by the primer set JV12-JV13, which has been proven specific for NVs detection in other studies (Vinjé and Koopmans, 1996; Kou *et al.*, 2006). Direct sequencing of the amplicons obtained would have been reasonable to confirm the specificity of the RT-PCR positive results. However, as the amounts of RT-PCR final product obtained were quite low, sequencing the amplicons directly was difficult. The first-round product of RT-PCR was reamplified with primers JV12 and JV13 for 30 further cycles under the same conditions (data not shown). The sequence results confirmed the specificity of the Mab-based IMS/RT-PCR. All the positive samples belonging to GII-4 NVs was an unexpected result for us. One explanation could be that GII-4 was epidemic strain in China, so the isolated strain from polluted oysters was also belonging to GII-4 NVs. Another explanation could be that the antibody against GII-4 NVs played an important role in specificity. However, positive samples of Proteinase K method, which did not use antibodies, were also belonging to GII-4 NVs, so the second explanation needs more experiment to confirm, such as using different genotype of NVs to prepare artificially contaminated oysters.

Table 2. Comparison of the performance of Mab-based or Pab-based IMS/RT-PCR and proteinase K/RT-PCR for the detection of NVs in 473 live oysters.

Method	Total number of positive samples	Positive rate (%)
Mab-coated beads	25	5.29
Pab-coated beads	18	3.81
Proteinase K/RT-PCR	10	2.11

An obstacle to the effective use of IMS in NVs detection is the wide antigenic variation in this virus. If common antigen epitopes were to be revealed by antigen mapping of NVs, then the use of high sensitivity antibodies against these epitopes, combined with the molecular or immunochromatographic test (Takanashi *et al.*, 2008), could facilitate the capture and separation of different antigenic variants. Another method was to prepare the mixture of several kinds of Mab against different NVs. Theoretically speaking, the development of IMS/RT-PCR assays with broader NVs specificity or with the mixture of Mabs will be possible when these antibodies become available and are used in conjunction with primers and probes that allow the detection of nearly all NVs types (Le Guyader *et al.*, 1996; Green *et al.*, 1995).

In summary, the Mab-based IMS/RT-PCR assay described in the present paper is a rapid, specific method for the detection of NVs in oysters. The method was proven more sensitive than the Pab-based IMS/RT-PCR or the Proteinase K/RT-PCR method, and is a good candidate for routine screening of oysters. Furthermore, this assay can serve as a model for future assays as additional broadly reactive, NVs-specific Mabs are developed.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 31101883), the Promotive Research Fund for Excellent Young and Middle-Aged Scientists of Shandong Province (BS2010SW041), the China and Australia Bilateral Research Program (2010DFA31720).

References

- Ando, T., Noel, J.S. and Fankhauser, R.L. (2000). Genetic classification of "Norwalk-like viruses". *J. Infect. Dis.*, **181**, 336-348.
- Arnal, C., Ferré-Aubineau, V., Besse, B., Mignotte, B., Schwartzbrod, L. and Billaudel, S. (1999). Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A virus amplification. *J. Virol. Methods.*, **77**, 17-26.
- Atmar, R.L. and Estes, M.K. (2006). The epidemiologic and clinical importance of norovirus infection. *Gastroenterol. Clin. N. Am.*, **35**, 275-290.
- Atmar, R.L., Neill, F.H., Romalde, J.L., Le Guyader, F., Woodley, C.M., Metcalf, T.G. and Estes, M.K. (1995). Detection of Nor-

- walk virus and hepatitis A virus in shellfish tissues with the PCR. *Appl. Environ. Microbiol.*, **61**, 3014-3018.
- Baert, L., Uyttendaele, M. and Debevere, J. (2007). Evaluation of two viral extraction methods for the detection of human noroviruses in shellfish with conventional and real-time reverse transcriptase PCR. *Lett. Appl. Microbiol.*, **44**, 106-111.
- Caul, E.O. (1996). Viral gastroenteritis: small round structured viruses, caliciviruses and astroviruses. Part II. The epidemiological perspective. *J. Clin. Pathol.*, **49**, 959-964.
- Clark, B. and McKendrick, M. (2004). A review of viral gastroenteritis. *Curr. Opin. in Infect. Dis.*, **17**, 461-469.
- De Medici, D., Croci, L., Suffredini, E. and Toti, L. (2004). Reverse transcription-booster PCR for detection of Noroviruses in shellfish. *Appl. Environ. Microbiol.*, **70**, 6329-6332.
- Formiga-Cruz, M., Tofiño-Quesada, G., Bofill-Mas, S., Lees, D.N., Henshilwood, K., Allard, A.K., Conden-Hansson, A.C., Hernroth, B.E., Vantarakis, A., Tsibouxi, A., Papapetropoulou, M., Furones, M.D. and Girones, R. (2002). Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Appl. Environ. Microbiol.*, **68**, 5990-5998.
- Gilpatrick, S.G., Schwab, K.J., Estes, M.K. and Atmar, R.L. (2000). Development of an immunomagnetic capture reverse transcription-PCR assay for the detection of Norwalk virus. *J. Virol. Methods.*, **90**, 69-78.
- Green, J., Gallimore, C.I., Norcott, J.P., Lewis, D. and Brown, D.W.G. (1995). Broadly reactive reverse transcriptase polymerase chain reaction in the diagnosis of SRSV-associated gastroenteritis. *J. Med. Virol.*, **47**, 392-398.
- Groppe, J.C. and Morse, D.E. (1993). Isolation of full-length RNA templates for reverse transcription from tissues rich in RNase and proteoglycans. *Anal. Biochem.*, **210**, 337-343.
- Hardy, M.E. and Estes, M.K. (1996). Completion of the Norwalk virus genome sequence. *Virus. Genes.*, **12**, 287-290.
- Jiang, X., Graham, D.Y., Wang, K. and Estes, M.K. (1990). Norwalk virus genome cloning and characterization. *Science*, **250**, 1580-1583.
- Jothikumar, N., Lowther, J.A., Henshilwood, K., Lees, D.N., Hill, V.R. and Vinjé, J. (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl. Environ. Microbiol.*, **71**, 1870-1875.
- Karst, S.M., Wobus, C.E., Lay, M., Davidson, J. and Virgin, H.W.IV. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science*, **299**, 1575-1578.
- Kobayashi, S., Natori, K., Takeda, N. and Sakae, K. (2004). Immunomagnetic capture RT-PCR for detection of norovirus from foods implicated in a foodborne outbreak. *Microbiol Immunol.*, **48**, 201-204.
- Kou, X.X., Wu, Q.P., Zhang, J.M. and Fan, H.Y. (2006). Rapid detection of noroviruses in fecal samples and shellfish by nucleic acid sequence-based amplification. *J. Microbiol.*, **44**, 403-408.
- Kroneman, A., Harris, J., Vennema, H., Duizer, E., van Duynhoven, Y., Gray, J., Iturriza, M., Bottiger, B., Falkenhorst, G., Johnsen, C., von Bonsdorff, C.H., Maunula, L., Kuusi, M., Pothier, P., Galloway, A., Schreier, E., Koch, J., Szucs, G., Reuter, G., Krisztalovics, K., Lynch, M., McKeown, P., Foley, B., Coughlan, S., Ruggeri, F.M., Di Bartolo, I., Vainio, K., Isakbaeva, E., Poljsak-Prijatelj, M., Hocevar, Grom, A., Bosch, A., Buesa, J., Sanchez, Fauquier, A., Hernandez-Pezzi, G., Hedlund, K. O. and Koopmans, M. (2008). Data quality of 5 years of central norovirus outbreak reporting in the European Network for food-borne viruses. *J. Public. Health.*, **30**, 82-90.
- Lambden, P.R., Caul, E.O., Ashley, C.R. and Clarke, I.N. (1993). Sequence and genome organization of a human small round-structured (Norwalk-like) virus. *Science*, **259**, 516-519.
- Le Guyader, F., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., Doyle, A., Zidane, M., Suffredini, E., Kohli, E., Madadalo, F., Monini, M., Galloway, A., Pommepuy, M., Pothier, P. and Ruggeri, F.M. (2006). Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. *J. Clin. Microbiol.*, **44**, 3878-3882.
- Le Guyader, F., Haugarreau, L., Miossec, L., Dubois, E. and Pommepuy, M. (2000). Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.*, **66**, 3241-3248.
- Le Guyader, F., Neil, F.H., Estes, M.K., Monroe, S.S., Ando, T. and Atmar, R.L. (1996). Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl. Environ. Microbiol.*, **62**, 4268-4272.
- Lees, D. (2000). Viruses and bivalve shellfish. *Int. J. Food. Microbiol.*, **25**, 81-116.
- Lopman, B.A., Reacher, M.H., Van Duynhoven, Y., Hanon, F.X., Brown, D. and Koopmans, M. (2003). Viral gastroenteritis outbreaks in Europe, 1995-2000. *Emerg. Infect. Dis.*, **9**, 90-96.
- Marshall, J.A. and Bruggink, L.D. (2006). Laboratory diagnosis of norovirus. *Clin. Lab.*, **52**, 571-581.
- Mette, M., Espen, R. and Yngvild, W. (2000). Immunomagnetic separation of a Norwalk-like virus (genogroup I) in artificially contaminated environmental water. *Int. J. Food Microbiol.*, **62**, 17-26.
- Mullendore, J.L., Sobsey, M.D. and Shieh, Y.C. (2001). Improved method for the recovery of hepatitis A virus from oysters. *J. Virol. Methods.*, **94**, 25-35.
- Muniain-Mujika, I., Girones, R. and Lucena, F. (2000). Viral contamination of shellfish: evaluation of methods and analysis of bacteriophages and human viruses. *J. Virol. Methods.*, **89**, 109-118.
- Myrmel, M., Berg, E.M., Rimstad, E. and Grinde, B. (2004). Detection of enteric viruses in shellfish from the Norwegian coast. *Appl. Environ. Microbiol.*, **70**, 2678-2684.

- Nishida, T., Kimura, H., Saitoh, M., Shinohara, M., Kato, M., Fukuda, S., Munemura, T., Mikami, T., Kawamoto, A., Akiyama, M., Kato, Y., Nishi, K., Kozawa, K. and Nishio, O. (2003). Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. *Appl. Environ. Microbiol.*, **69**, 5782-5786.
- Richards, G.P. (1999). Limitations of molecular biological techniques for assessing the virological safety of foods. *J. Food Protect.*, **62**, 691-697.
- Schultz, A.C., Saadbye, P., Hoorfar, J. and Nørrung, B. (2007). Comparison of methods for detection of norovirus in oysters. *Int. J. Food Microbiol.*, **114**, 352-356.
- Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteyn, B. and Koopmans, M. (2007). Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *J. Clin. Microbiol.*, **45**, 1389-1394.
- Takanashi, S., Okame, M., Shiota, T., Takagi, M., Yagyu, F., Tung, P.G., Nishimura, S., Katsumata, N., Igarashi, T., Okitsu, S. and Ushijima, H. (2008). Development of a rapid immunochromatographic test for noroviruses genogroups I and II. *J. Virol. Methods.*, **148**, 1-8.
- Tian, P., Bates, A.H., Jensen, H.M. and Mandrell, R.E. (2006). Norovirus binds to blood group A-like antigens in oyster gastrointestinal cells. *Lett. Appl. Microbiol.*, **43**, 645-651.
- Tomoyasu, T. (1998). Improvement of the immunomagnetic separation method selective for *Escherichia coli* O157 strains. *Appl. Environ. Microbiol.*, **64**, 376-382.
- Traore, O., Arnal, C., Mignotte, B., Maul, A., Laveran, H., Billau-del, S. and Schwartzbrod, L. (1998). Reverse transcriptase PCR detection of astrovirus, hepatitis A virus, and poliovirus in experimentally contaminated mussels: comparison of several extraction and concentration methods. *Appl. Environ. Microbiol.*, **64**, 3118-3122.
- Vinje, J. and Koopmans, M.P. (1996). Molecular detection and epidemiology of small round structured viruses in outbreaks of gastroenteritis in the Netherlands. *J. Infect. Dis.*, **174**, 610-615.
- Wang, D.P., Wu, Q.P., Kou, X.X., Yao, L. and Zhang, J.M. (2008). Distribution of norovirus in oyster tissues. *J. Appl. Microbiol.*, **105**, 1966-1972.
- Yao, L., Wu, Q., Wang, D., Kou, X. and Zhang, J. (2009). Development of monoclonal antibody-coated immunomagnetic beads for separation and detection of norovirus (genogroup II) in faecal extract samples. *Lett. Appl. Microbiol.*, **49**, 173-178.
- Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I. and Monroe, S.S. (2006). Norovirus classification and proposed strain nomenclature. *Virology*, **346**, 312-323.