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Rapid Detection of Noroviruses in Fecal Samples and Shellfish by Nucleic Acid Sequence-based Amplification

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The purpose of this study was to determine the efficacy of a nucleic acid sequence-based amplification (NASBA) method of detecting noroviruses in artificially and naturally contaminated shellfish. We used 58 fecal samples that tested positive for noroviruses with electron microscopy (EM) to develop an NASBA assay for these viruses. Oligonucleotide primers targeting the polymerase coding region were used to amplify the viral RNA in an isothermal process that resulted in the accumulation of RNA amplicons. These amplicons were detected by hybridization with digoxigenin-labeled oligonucleotide probes that were highly specific for genogroup I (GI) and genogroup II (GII) of noroviruses. The expected band of 327 bp appeared in denaturing agarose gel without any nonspecific band. The specific signal for each amplicon was obtained through Northern blotting in many repeats. All fecal samples of which 46 (79.3%) belonged to GII and 12 (20.6%) belonged to GI were positive for noroviruses by EM and by NASBA. Target RNA concentrations as low as 5 pg/ml were detected in fecal specimens using NASBA. When the assay was applied to artificially contaminated shellfish, the sensitivity to nucleic acid was 100 pg/1.5 g shellfish tissue. The potential use of this assay was also confirmed in naturally contaminated shellfish collected from different ponds in Guangzhou city of China, of which 24 (18.76%) out of 128 samples were positive for noroviruses; of these, 19 (79.6%) belonged to GII and 5 (20.4%) belonged to GI. The NASBA assay provided a more rapid and efficient way of detecting noroviruses in fecal samples and demonstrated its potential for detecting noroviruses in food and environmental samples with high specificity and sensitivity.

Keywords: detection, noroviruses, NASBA, shellfish

Noroviruses belong to a group of single-stranded RNA viruses that have been classified as members of the family Caliciviridae (Pringle, 1999). Based on comparisons with genetic sequences of viral RNA-dependent RNA polymerase and the capsid protein, noroviruses have been subdivided into five genogroups (GI-GV). The two largest genogroups, GI and GII, include most of the diverse and common noroviruses (Ando *et al.*, 2000; Pang *et al.*, 2005; Zheng *et al.*, 2006). These viruses are now recognized worldwide as a major cause of nonbacterial gastroenteritis (Fankhauser *et al.*, 1998; Wheeler *et al.*, 1999; Johansson *et al.*, 2002). They are readily transmitted through the fecal-oral route from person-to-person but also in foodstuff (Hedberg and Dsterholm, 1993; Mead *et al.*, 1999). A large amount of epidemiological data indicate that shellfish comprise a major vector for the transmission of important human viral pathogens (Le Guyader *et al.*, 1996b, 2000; Lipp and Rose, 1997; Lees, 2000). Although viruses are strict intracellular parasites and cannot replicate in shellfish tissue, common seafood processing procedures used for icing and freezing are likely to enhance noroviruses survival (Lees, 2000). Noroviruses are also highly resistant to the effects of disinfectants, heat, pressure, and temperature, and just a few particles can cause an infection (Cliver, 1997). The consumption of virus-contaminated shellfish is a significant health problem for shellfish consumers as well as an economic threat to the seafood industry. Thus, there is an urgent need

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to develop practical, time-effective, and cost-saving methods to detect this virus in contaminated shellfish. To date, noroviruses have not been cultivated *in vitro.* Laboratory detection methods depend primarily on electron microscopy (EM) and reverse transcription-polymerase chain reaction (RT-PCR). Unfortunately, the EM method is cumbersome and insensitive, requiring the presence of a minimum of approximately 10^5–10^6 particles per milliliter stool sample and thus cannot be used to detect virus concentrations in environmental samples containing a low number of viruses (Sair *et al.*, 2002). Because of its high specificity and sensitivity, RT-PCR is the only published method that offers the possibility of direct detection of noroviruses in environmental samples. However, RT-PCR is vulnerable to contamination from DNA products. Moreover, the use of PCR is limited because of a lack of essential equipment in routine laboratories. A potential alternative to RT-PCR is nucleic acid sequence-based amplification (NASBA) (Compton, 1991). NASBA has several advantages over RT-PCR and has already been used successfully to detect rotaviruses (Jean *et al.*, 2002a, 2002b), hepatitis A virus (Jean *et al.*, 2001), noroviruses (Moore *et al.*, 2004), and astrovirus (Jennifer *et al.*, 2003) in clinical samples. However, in all of these studies, the investigators were mainly interested in detecting these viruses in human tissue; few attempts have been made to detect them in shellfish or other environmental samples. The major goals of this study were to (a) develop a NASBA assay for noroviruses and (b) validate the assay in artificially and naturally contaminated shellfish.

Materials and Methods

Fecal specimens

We collected 58 fecal specimens that tested positive for noroviruses by EM (O'Neill *et al.*, 2000) from patients who had developed gastroenteritis during an outbreak or as a sporadic occurrence between September 2003 and December 2004 in Guangzhou, China. Each specimen was prepared as a 10% suspension in phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 12,000 × g for 15 min at 4°C. The clarified and ultracentrifuged fecal extracts were stored at –20°C.

RNA extraction

Viral RNA was extracted from the fecal specimens using Trizol-LS reagent (Gibco BRL, USA) according to the manufacturer's protocol. The RNA pellets obtained at the end of the procedure were all dissolved in 50 μl of diethyl pyrocarbonate (DEPC)-treated water. RNA yields were measured based on the absorbance at 260 nm for a 1:100 dilution of each sample that was compared with an appropriate blank. The RNA solution was stored at –80°C until use.

Primers and probes

The oligonucleotide primer and probe sequences for noroviruses are shown in Table 1. The primer set of JV12 and JV13 was designed according to the RNA-dependent RNA polymerase gene, as described previously (Vinje and Koopmans, 1996). The reverse primer (JV13) bears the bacteriophage T7 RNA polymerase promoter region, which appears in italics. Digoxigenin (DIG)-labeled probes of PI and PII, corresponding to an internal region defined by the primers pairs, were synthesized to hybridize with the NASBA RNA products (Green *et al.*, 1998).

NASBA

Optimum NASBA amplification was achieved using a total reaction volume of 20 μl that contained 5 μl of the nucleic acid extract in 40 mM Tris, pH 8.5; 12 mM $MgCl_2$; 70 mM KCl; 5 mM dithiothriotol; 1 mM each of dATP, dCTP, dGTP, and dTTP; 2.0 mM each of ATP, CTP, and UTP; 1.5 mM GTP; 0.5 mM MITP; and 0.2 μM of each oligonucleotide (JV12 and JV13). The mixture was incubated at 65°C for 5 min to destabilize secondary RNA structures and then cooled to 42°C for 5 min for primer annealing. The enzyme mixture–which included 0.1 U RNase H (Promega, USA), 32 U T7 RNA polymerase (Pharmacia, Sweden),

Table 1. Primers and Probes of Noroviruses

Noro-virus	Primer/Probe	Sequence (5'-3')	Location	Product size (bp)
GI	JV12	AGCCAGTGGGCGATGGAATTC	4552-4572	327
	JV13[a]	*AATTCTAATACGACTCACTATAGGGAA*TCATCATCACCATAGAAAGAG	4858-4878	
	PI[b]	TCNGAAATGGATGTTGG	4691-4707	
GII	JV12	AGCCAGTGGGCGATGGAATTC	4279-4299	327
	JV13[a]	*AATTCTAATACGACTCACTATAGGGAA*TCATCATCACCATAGAAAGAG	4585-4605	
	PII	AGCCAGTGGGCGATGGAATTC	4495-4515	

[a] Letters in italics show the sequence of the bacteriophage T7 RNA polymerase promoter which bears the reverse primer JV13.
[b] Mixed bases in probes: N=A/T/C/G.

and 8 U AMV (Promega, USA)–was added immediately. The reaction mixture was incubated at 42°C for 90 min then stored at −80°C to stop the reaction. The NASBA products were analyzed by denaturing agarose gel electrophoresis and Northern blotting, as described previously (Jean *et al.*, 2001).

Denaturing agarose gel electrophoresis

Briefly, amplified RNA and an RNA molecular weight marker (Takara, Japan) were first prepared in a loading buffer consisting of 10 μl of formamide, 4.5 μl of formalin, and 2 μl of 10X running buffer (0.2 M borate buffer; pH 8.3, containing 2 mM EDTA). Samples were denatured at 65°C for 2 min and cooled on ice, then a 2 μl volume of tracking dye (bromophenol blue and xylene cyanol in 50% glycerol) was added. The NASBA products were separated on a 1.2% denaturing agarose gel in 1X running buffer containing 6% formalin. After several washes, staining with ethidium bromide, and destaining overnight in water, the separated bands were examined under ultraviolet (UV) light for gel electrophoresis then transferred onto a positively charged nylon membrane (Roche Diagnostics, Switzerland) for confirmation using Northern blotting.

Northern blotting and dot blotting

The nylon membrane was fixed for 5 min by UV crosslinking, prehybridized for 30 min at 65°C in RNase-free hybridization solution 5X SSC (750 mM NaCl and 75 mM Na-citrate), 0.1% (w/v) *N*-laurylsarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS), and 1% (w/v) protein blocking reagent (Roche Diagnostics, Switzerland) and then placed in 5 ml of a hybridization solution containing 50 nM of the DIG-labeled oligonucleotide probe (PI and PII). After hybridization for 2 h at 65°C, the membrane was thoroughly washed twice in 2X SSC containing 0.1% (w/v) SDS for 5 min at room temperature and twice at 55°C for 15 min in 0.1X SSC containing 0.1% (w/v) SDS. The membrane was then incubated for 30 min in 0.1 M maleic acid and 0.15 M NaCl (pH 7.5) blocking solution containing 1% (w/v) blocking reagent prior to immunoenzymatic detection of the bound probe. Hybridization was detected in the membrane using an anti-DIG-peroxidase conjugate (Roche Diagnostics, Switzerland) at a concentration of 1:10,000 in the blocking solution for 30 min at room temperature and a colorimetric substrate–3, 3', 5, 5'-tetramethylbenzidine (TMB)–followed by washings five times in PBST (0.01 mM phosphate buffer; pH 7.2, 0.85% NaCl, and 0.05% Tween 20). For dot blotting, 10 μl of the NASBA product was spotted directly onto the positively charged nylon membrane using the same procedure as in Northern blotting.

Artificially contaminated shellfish

A total of 96 shellfish obtained from the local marine market and found to be negative for noroviruses were used to evaluate the efficacy of NASBA. The shellfish were shucked, and the stomach and digestive diverticuli were removed by dissection, cut into small portions, mixed, divided into 1.5 g portions, and frozen. Frozen aliquots of pancreatic tissue were used for artificial contamination with 200 μl of the stool suspension. The samples were homogenized for 1 minute by vortexing before the virus was concentrated and nucleic acids were extracted. The shellfish were processed for virus concentration and recovery using a previously described method (Atmar *et al.*, 1995; Le Guyader *et al.*, 2000).

Naturally contaminated shellfish

Field shellfish were collected between December 2004 and January 2005. We collected 128 oyster samples randomly from 4 oyster ponds in Guangzhou, China and shipped them in cold storage directly to the laboratory, where they were processed within a 24 h period. The shellfish were washed, scrubbed under clean running water, and opened using a sterile shucking knife. The stomach and digestive diverticuli were removed by dissection, cut into small portions, mixed, and divided into 1.5 g portions. Naturally contaminated oysters were treated for virus recovery, as described above.

Evaluation of the NASBA system

Ten-fold serial dilutions (10^{-1}-10^{-6}) of RNA extracted from feces and artificially contaminated shellfish specimens were prepared and underwent NASBA testing and dot blotting to determine the sensitivity of this test.

Controls and interpretation

All precautions were taken to prevent false-positive or false-negative results. Amplifications were performed in different rooms, and filter-equipped pipette tips were used throughout the assay. A sample was considered positive only if amplicons were detected by hybridization. All experiments were repeated at least twice, and a negative control sample (i.e., containing no nucleic acid) was run with each test.

Results

Specificity of NASBA reaction

The separation of NASBA products by denaturing agarose gel electrophoresis resulted in a very sharp band corresponding to the expected size for the RNA product (327 bp) (Fig. 1A). No band was observed in the negative control. The specificity of the band was confirmed by Northern blotting using two DIG-labeled oligoprobes that were highly specific for GI

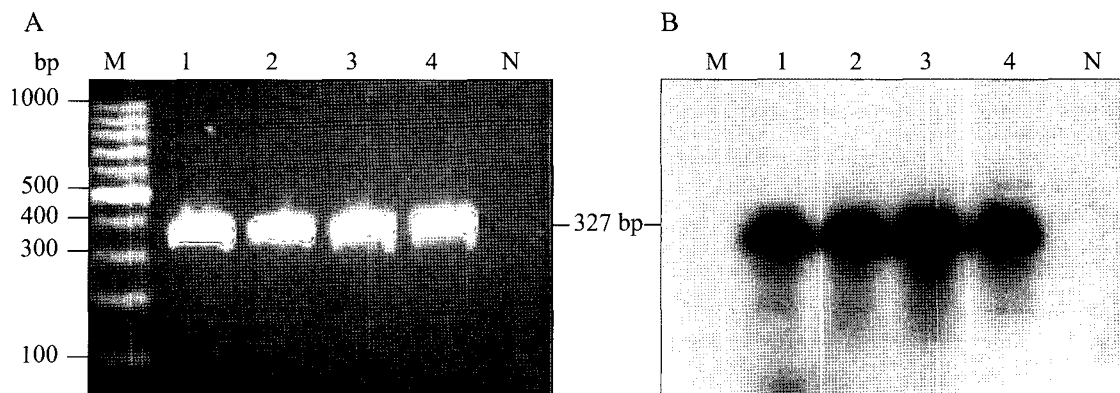

Fig. 1. (A) The amplification product of NASBA in denaturing agarose gel. M, RNA marker; Lane 1-4, RNA of noroviruses from fecal samples as template; N, negative control. (B) The specificity of NASBA was confirmed by Northern blotting. The positive hybridization signals correspond to those in the lanes in (A).

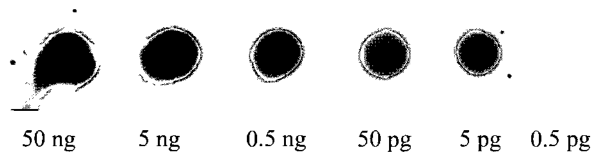

Fig. 2. The sensitivity of NASBA to noroviruses in fecal samples was evaluated using 10-fold serial dilutions of RNA that correspond to 50 ng, 5 ng, 0.5 ng, 50 pg, 5.0 pg, and 0.5 pg respectively, which were tested by dot blotting. The high detection limits exhibited as 5 pg RNA were amplified by NASBA.

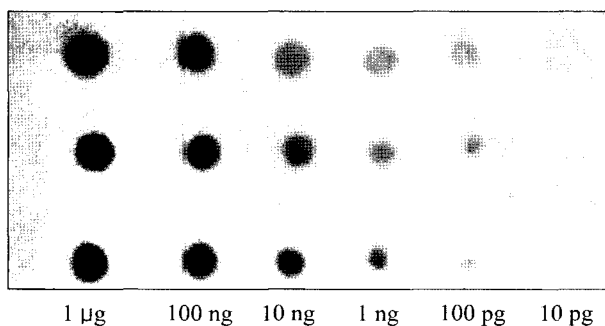

Fig. 3. The sensitivity of NASBA to noroviruses in artificially contaminated shellfish was evaluated by 10-fold serial dilutions of RNA, corresponding to 1 μg, 100 ng, 10 ng, 1 ng, 100 pg, and 10 pg, which were tested by dot blotting. The high detection limits exhibited as 100 pg RNA were amplified by NASBA.

and GII noroviruses. The amplification product generated a strong hybridization signal indicating a molecular size corresponding to the expected 327 bp band (Fig. 1B).

The 58 fecal specimens, which had been confirmed positive for noroviruses by EM, were detected by NASBA. None of the samples that were found to be positive by EM were found to be negative by NASBA. The genogroups of these specimens were distinguished by oligoprobes of GI and GII, with 12 (20.6%) samples belonging to GI and 46 (79.3%) to GII. These results confirm that the NASBA system using a primer pair and probes can effectively detect the target region of the norovirus genome.

Sensitivity of NASBA in fecal samples

To determine the sensitivity of the NASBA system to noroviruses in fecal samples, 10-fold serial dilutions of nucleic acid were made and detected by NASBA. The amplicons of each RNA dilution in NASBA were confirmed by dot blot assay. The positive signals exhibited during hybridization indicated that the highest detection limits could be determined clearly. RNA in concentrations as small as 5 pg/ml in the fecal samples was detected by NASBA (Fig. 2).

Sensitivity of NASBA in artificially contaminated shellfish

Artificially contaminated shellfish were used to determine whether the NASBA method could be carried out in live contaminated shellfish. Ten-fold serial dilutions of RNA from the shellfish extracts were tested. A positive signal was exhibited during dot blotting when the RNA preparations were adjusted to 100 pg per 1.5 g of contaminated shellfish tissue. A total of 96 experimentally contaminated shellfish were tested and similar results were obtained (Fig. 3).

Efficiency of NASBA in naturally contaminated shellfish

A total of 128 samples naturally contaminated shellfish tissue were analyzed by NASBA assay; of these 24 samples (18.7%) were positive for noroviruses. To determine the specificity of this test for the genogroup of each positive sample, we carried out a hybrid-

ization procedure using different probes for GI and GII. Amplicons with the correct sizes were observed for all of the positive samples. Out of the 24 positive samples, 19 (79.6%) belonged to the norovirus GII group and 5 (20.4%) belonged to the norovirus GI group.

Discussion

The fact that molluscan shellfish can serve as vectors of important human viral pathogens has led to a widely recognized need to improve sanitary control measures for the processing and distribution of these marine products. Noroviruses comprise the predominant type of gastrointestinal virus worldwide and have been found to be the primary etiological agents in infectious diseases associated with shellfish consumption (Koopmans *et al.*, 2002). Over the last few years, the development of methods for rapid and reliable detection of this viral pathogen that can overcome the flaw in conventional detection methods has become an important research goal (Atmar *et al.*, 2001; Kingsley and Richards, 2001). In this study, we developed an NASBA method to detect noroviruses and investigated its potential in determining the viral content of naturally contaminated shellfish. We found that noroviruses RNA could be extracted, amplified, and detected directly from clinical fecal samples and shellfish using this method.

To determine the specificity of this test, we hybridized amplicons of RNA extracted from fecal samples or shellfish using specific probes. Nonspecific signals did not appear in the agarose gel and hybridization. Thus, the study results demonstrated that the primer, probe, and reaction conditions required for this test were highly specific.

The sensitivity of such a test is significant in determining whether it can be applied to foods and environmental samples with complex components and many inhibitors. The initial application of some noroviruses detection methods is successful in clinical samples, in which the viral titers is expected to be high, but not in environmental samples, in which the viral titer is typically very low, or in food samples, which may have components that interfere with the detection of the virus; test specificity and sensitivity may be reduced in such samples. In our assay, every sample that was found by EM to be positive for noroviruses was also found to be positive by NASBA. In all, 46 samples were GII and 12 were GI. In the sensitivity test, we found that the lowest detection limits in fecal samples and 1.5 g shellfish tissues were 5 pg and 100 pg, respectively. The decrease in detection limits may be due to our failure to exclude the natural inhibitors in shellfish completely, despite our use of the method described by Atmar (Atmar *et al.*, 1995), which was proven efficient in other studies (e.g., Loisy *et al.*, 2005). In future studies, we will focus on finding a more efficient method for virus recovery and pretreatment to eliminate inhibitors of shellfish.

Although the detection limits were reduced, we were still able to screen noroviruses in shellfish, indicating the efficiency of the assay in detecting noroviruses in naturally contaminated shellfish. Out of a total of 128 shellfish tissue samples collected from ponds that were highly polluted with human sewage, we found 24 (18.7%) positive samples, 19 (79.6%) of which contained noroviruses GII and 5 (20.4%) of which contained noroviruses GI. In fecal samples or natural shellfish, the prevalent genotype was GII, which was the same as reported in other studies (Vinje *et al.*, 2004). Of course, the usefulness of the assay should be confirmed and more data on various genogroups collected by testing more shellfish in our future studies.

We conclude that the results of this study demonstrate that NASBA is a rapid and sensitive alternative to RT-PCR for detecting RNA of noroviruses and may prove useful in detecting noroviruses during routine monitoring and risk assessment, as well as for large-scale screening during disease outbreaks and in marine products.

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References

Ando, T., J.S. Noel, and R.L. Fankhauser. 2000. Genetic classification of "Norwalk-like viruses". *J. Infect. Dis.* 181, S336-S338.

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.

Atmar, R.L. and M.K. Estes. 2001. Diagnosis of nonculturable gastroenteritis viruses, the human caliciviruses. *Clin. Microbiol. Rev.* 14, 15-37.

Compton, J. 1991. Nucleic acid sequence-based amplification. *Nature* 350, 91-92.

Cliver, D.O. 1997. Virus transmission via food. *Food Technol.* 51, 71-78.

Fankhauser, R.L., J.S. Noel, S.S. Monroe, T. Ando, and R.I. Glass. 1998. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* 178, 1571-1578.

Hedberg, C.W. and M.T. Dsterholm. 1993. Outbreaks of foodborne and waterborne viral gastroenteritis. *Clin. Microbiol. Rev.* 6, 199-210.

Jean, J., B. Blais, A. Darveau, and I. Fliss. 2001. Detection of hepatitis A virus by the nucleic acid sequence-based amplification technique and comparison with reverse transcription-PCR. *Appl. Environ. Microbiol.* 67, 5593-5600.

Jean, J., B. Blais, A. Darveau, and I. Fliss. 2002a. Rapid detection of human rotavirus using colorimetric nucleic acid sequence-based amplification (NASBA) enzyme-linked immunosorbent assay in sewage treatment effluent. *FEMS Microbiol. Lett.* 210, 143-147.

Jean, J., B. Blais, A. Darveau, and I. Fliss. 2002b. Simultaneous detection and identification of hepatitis A virus and rotavirus by multiplex nucleic acid sequence-based amplification (NASBA) and microtiter plate hybridization system. *J. Virol. Methods* 105, 123-132.

Jean, J., D. D'Souza, and L. Jaykus. 2003. Transcriptional enhancement of RT-PCR for rapid and sensitive detection of Noroviruses. *FEMS Microbiol. Lett.* 226, 339-345.

Jennifer, H.T., M.S. Ewert, G. Belliot, R.I. Glass, and S.S. Monroe. 2003. Development of a rapid method using nucleic acid sequence-based amplification for the detection of astrovirus. *J. Virol. Methods* 110, 119-127.

Johansson, P.J., M. Torven, A.C. Hammarlund, U. Bjorne, K.O. Hedlund, and L. Svensson. 2002. Food-borne outbreak of gastroenteritis associated with genogroup I calicivirus. *J. Clin. Microbiol.* 40, 794-798.

Kingsley, D.H. and G.P. Richards. 2001. Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. *Appl. Environ. Microbiol.* 67, 4152-4157.

Koopmans, M., C.H. Von Bonsdorff, J. Vinje, D. De Medici, and S. Monroe. 2002. Foodborne viruses. *FEMS Microbiol. Rev.* 26, 187-205.

Lees, D. 2000. Viruses and bivalve shellfish. *Int. J. Food Microbiol.* 59, 81-116.

Le Guyader, F., F.H. Neill, M.K. Estes, S.S. Monroe, T. Ando, and R.L. Atmar. 1996b. 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.

Le Guyader, F., L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy. 2000. Three-year study to assess human enteric viruses in shellfish. *Appl. Environ. Microbiol.* 66, 3241-3248.

Lipp, E.K. and J.B. Rose. 1997. The role of seafood in foodborne diseases in the United states of American. *Rev. Sci. Tech.* 16, 620-640.

Loisy, F., R.L. Atmar, P. Guillon, P. Le Cann, M. Pommepuy, and F.S. Le Guyader. 2005. Real-time RT-PCR for norovirus screening in shellfish. *J. Virol. Methods*. 123, 1-7.

Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food- related illness and death in the United States. *Emerg. Infect. Dis.* 5, 607-625.

Moore, C., E.M. Clark, C.I. Gallimore, S.A. Corden, J.J. Gray, and D. Westmoreland. 2004. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in fecal material. *J. Clin. Virol.* 29, 290-296.

O'Neill, H.J., C. McCaughey, P.V. Coyle, D.E. Wyatt, and F. Motchell. 2002. Clinical utility of nested multiplex-like viruses in acute viral gastroenteritis in children and adults. *J. Clin. Methods* 25, 335-343.

Pang, X.L., J.K. Preiksaitis, and B. Lee. 2005. Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. *J. Clin. Virol.* 33, 168-171.

Pringle, C.R. 1999. Virus taxonomy. *Arch. Virol.* 144, 423-429.

Sari, A.I., D.H. D'Souza, and L.A. Jaykus. 2002. Human enteric viruses as causes of foodborne diseases. *Compr. Rev. Food Sci. Food Saf.* 1, 73-89.

Vinje, J. and M. Koopmans. 1996. Molecular detection and epidemiology of small round structured viruses in outbreaks of gastroenteritis in the Netherlands. *J. Infect. Dis.* 174, 610-615.

Vinje, J., R.A. Hamidjaja, and M.D. Sobsey. 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. *J. Virol. Methods* 116, 109-117.

Wheeler, J.G., D. Sethi, J.M. Cowden, P.G. Wall, L.C. Rodrigues, D.S. Tompkins, M.J. Hudson, and P.J. Roderick. 1999. Study of infectious intestinal disease in England: rates in the community, presenting to general practice and reported to national surveillance. *Br. Med. J.* 318, 1046-1050.

Zheng, D.P., T. Ando, R.L. Frankhauser, R.S. Beard, R.I. Glass, and S.S. Monroe. 2006. Norovirus classification and proposed strain nomenclature. *Virol.* 346, 312-323.