The Journal of Microbiology, August 2008, p. 436-440 Vol. 46, No. 4
DOI 10.1007/s12275-008-0131-1

Detection of Hepatitis A Virus from Oyster by Nested PCR Using Efficient Extraction and Concentration Method

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(Received May 27, 2008 / Accepted June 16, 2008)

The molecular methods using polymerase chain reaction have been proposed as useful tools for the identification of viral pathogens in food and water. However, the PCR-based methods are highly dependent on the methods of virus concentration and nucleic acid purification due to the low sensitivity of PCR in the presence of PCR inhibitors. We developed TPTT [tris elution buffer-PEG-TRIzol-poly(dT) magnetic bead] protocol in order to detect hepatitis A virus (HAV) inoculated in oyster digestive glands. The detection limit of HAV precipitated with zirconium hydroxide was 10^5 fold less sensitive in a nested PCR than that precipitated the HAV supernatant twice with PEG/NaCl (16% polyethylene glycol 6,000, 0.525 M NaCl) in a 1:2 (v/v) ratio, which provided an efficient detection of 0.0148 PFU/g from approximately 0.05 g of oyster homogenate. This method is efficient for potential use in the detection of HAV from shellfish and is more sensitive than most currently published tests.

***Keywords*:** Hepatitis A virus, RT-PCR, oyster, nested PCR

Hepatitis A virus (HAV) is a positive single-stranded RNA virus of 7.5 kb that is the prototype of the *Hepatovirus* genus within the *Picornaviridae* family (Costafreda *et al*., 2006; Sanchez *et al*., 2007). HAV is a pathogen responsible for acute viral hepatitis and foodborne diseases associated with the consumption of virus-contaminated filter-feeding bivalves (oysters, clams, cockles, and mussels), fruits, and salads (Sanchez *et al*., 2007). The incidence of fecal contamination of foods has been monitored by counting fecal coliform bacteria to ensure the food hygiene quality (Gerba and Goyal, 1978). However, human pathogenic viruses have been isolated from sampling sites with acceptable fecal coliform counts, indicating coliform standard is unreliable for monitoring foodborne viral pathogens from fecal contamination of food (Gerba and Goyal, 1978; Lopez-Sabater *et al*., 1997; Fong *et al*., 2005).

Over the years the molecular methods using nucleic acid amplification by PCR have been proposed as useful tools for the identification of viral pathogens in food and water (Lopez-Sabater *et al*., 1997; Lipp *et al*., 2001; Dubois *et al*., 2004; Ribao *et al*., 2004; Fong *et al*., 2005). However, the PCR-based methods are highly dependent on the methods of virus concentration and nucleic acid purification such as polyethylene glycol 8000 (PEG), zircodium chloride, and Freon (trichlorotrifluoroethane) due to the low sensitivity of PCR in the presence of PCR inhibitors such as polysaccharides and humic acids, particularly abundant in food samples (Lewis and Metcalf, 1988; Dix and Jaykus, 1998; D'Souza and Jaykus, 2002). In order to detect HAV inoculated in oyster digestive glands, we developed TPTT [tris elution buffer-PEG-TRIzol-poly(dT) magnetic bead] protocol modified from the GPTT procedure (Kingsley and Richards, 2001). This procedures included tris elution buffer and polyethylene glycol for elution and concentration steps and TRIzol reagent and poly (dT) bead for rapid extraction and purification steps.

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Materials and Methods

Virus stocks

Hepatitis A virus (HAV) strain HM-175/18f (VR-1402) was obtained from the American Type Culture Collection (Lemon *et al*., 1991). Virus titration was carried out on fetal rhesus monkey kidney cells (FRhK-4) in microtitration plates and expressed as 50%-tissue culture infectious dose ($TCID_{50}$) per volume unit. The titer of the HAV stock was 10^4 $TCID_{50}$/ml. Based on the equivalence of 1 $TCID_{50}$/ml to 0.69 PFU/ml, the titer was estimated to be about 6.9×10^3 PFU/ml (Dubois *et al*., 2004).

Virus extraction and concentration

Oysters (*Crassostrea virginica*) were obtained from local seafood markets. Viral extraction and concentration were determined by a procedure modified from the method previously described by Kingsley and Richards (Kingsley and Richards, 2001). Briefly, 10 g of digestive glands obtained from oysters were homogenized in 190 ml of glycine elution buffer (0.1 M glycine, 0.3 M NaCl, pH 9.5) or tris elution buffer (100 mM Tris-HCl, 50 mM glycine, 1% beef extract, pH 9.5) at 20°C using a homogenizer (Omni Macro Homogenizer, USA) at the high setting for 2 min. One milliliter of shellfish extract was seeded with serial 10-fold dilutions of virus

ranging from 0.00069 to 6.9×10^3 PFU/ml. The seeded extract was then incubated for 30 min at 37°C. After centrifugation at 15,000×g at 4°C, the HAV of supernatant was mixed with an equal or two volumes of concentration buffer [16% polyethylene glycol 6000 (PEG, Sigma Chemical Co., USA) and 0.525 M NaCl] and incubated for 1 h on ice. HAV was centrifuged at 10,000×g for 5 min at 4°C. The virus was then resuspended in 300 µl of RNase-free H_2O. This process was repeated twice.

Extraction of viral RNA

Total RNA was extracted by a protocol modified from the method previously described by Kingsley and Richards (Kingsley and Richards, 2001). Briefly, 150 µl of HAV resuspended in 300 µl of DEPC-treated water were mixed with 1 ml of TRIzol reagent (Invitrogen, USA) and incubated at 20°C for 5 min. After adding 0.2 ml of chloroform, the sample was incubated at 20°C for 3 min and then centrifuged at 12,000×g for 15 min. The top aqueous layer, containing total RNA, was precipitated by adding 0.5 volume of isopropanol for 10 min at 20°C, followed by centrifuging at 12,000×g for 15 min. The resulting white pellets were washed with 1 ml of cold 75% ethanol and then centrifuged at 7,500×g for 5 min. The pellet was then resuspended in 100 µl of RNase-free water. One hundred microliters of 1× RNA binding buffer (20 mM Tris-HCl; pH 7.5, 1.0 M LiCl, 2 mM EDTA) was added, and the samples were heated to 65°C for 2 min after vortex-mixing for 30 sec and placed on ice. After the addition of 100 µl of Dynabeads-oligo$(dT)_{25}$ (Dynal, Norway) to the resuspended pellet, the sample was rotated at 8 rpm using a rotating shaker (Rose Scientific Ltd., Canada) for 5 min at a room

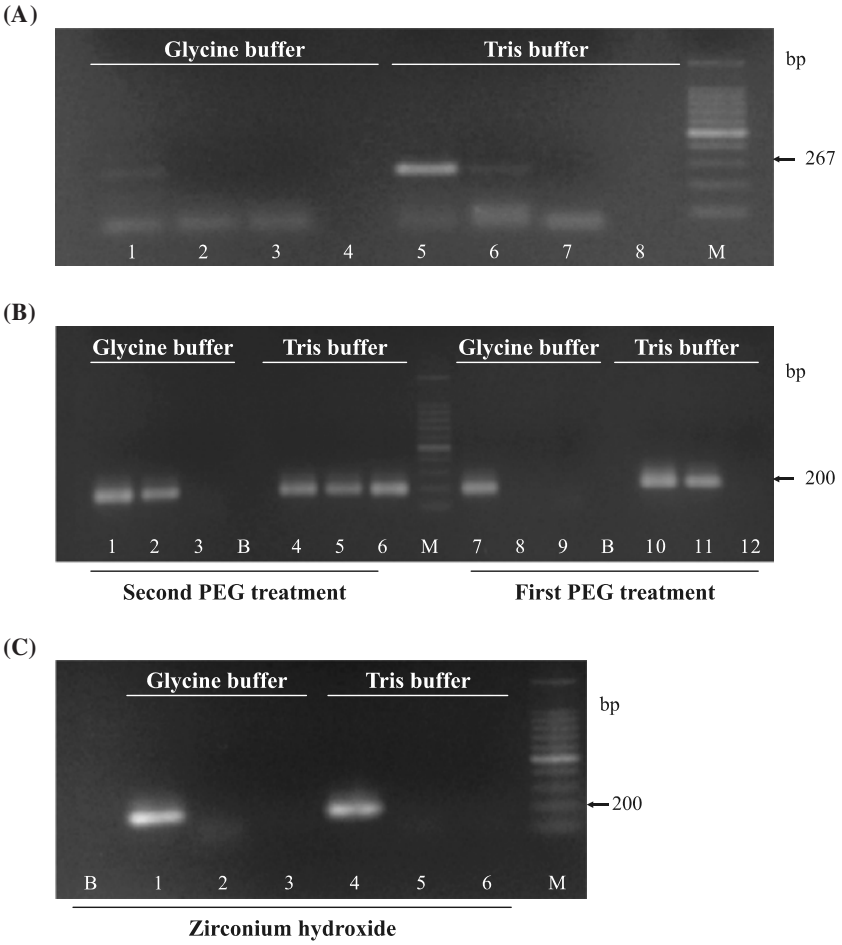

Fig. 1. Effect of the elution buffer (glycine and tris buffer) and concentration solutions (polyethylene glycol and zirconium hydroxide) for the detection of the hepatitis A virus (HAV) inoculated in oyster digestive glands by RT-PCR and nested PCR. (A) The homogenate was seeded with dilutions of HAV. Viral RNA was extracted by the glycine buffer and tris buffer, precipitated by polyethylene glycol, purified by oligo(dT) magnetic beads, and followed by one-step RT-PCR. (A) Lanes 1 and 5, 100 µl of 6.9×10^3 PFU/ml; 2 and 6, 100 µl of 1:10 dilutions (6.9×10^2 PFU/ml); 3 and 7, 100 µl of 1:100 dilutions (69 PFU/ml); 4 and 8, uninoculated oyster used as a control; M, 100-bp molecular size ladder. (B and C) 200-base amplified nested PCR products were obtained from reamplification using the primers (dka24 and dka25) and templates amplified in RT-PCR. (B) Lanes: 1, 4, 7 and 10, 100 µl of 1:10 dilutions (6.9×10^3 PFU/ml); 2, 5, 8, and 11, 100 µl of 1:10 dilutions (6.9×10^2 PFU/ml); 3, 6, 9, and 12, 100 µl of 1:100 dilutions (69 PFU/ml); (B) uninoculated oyster used as a control; M, 100-bp molecular size ladder. (C) Lanes 1 and 4, 100 µl of 1:10 dilutions (6.9×10^3 PFU/ml); 2 and 5, 100 µl of 1:10 dilutions (6.9×10^2 PFU/ml); 3 and 6, 100 µl of 1:100 dilutions (69 PFU/ml); (B) uninoculated oyster used as a control; M, 100-bp molecular size ladder.

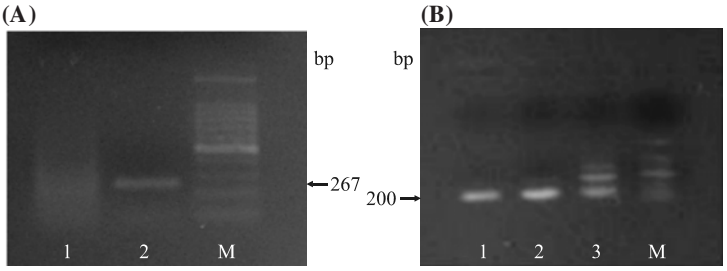

Fig. 2. Comparison of the RNA purification methods (A) and $MgSO_4$ concentration (B) for the detection of the hepatitis A virus. The homogenate was seeded with hepatitis A virus (100 μl of 6.9×10^3 PFU/ml). (A) Viral RNA was purified by commercial RNA Kit without magnetic beads (lane 1) and poly(dT) magnetic beads (lane 2), followed by one-step RT-PCR. (B) Viral RNA was purified by poly(dT) magnetic beads and followed by nested PCR using different concentrations of $MgSO_4$. Lanes 1, 0.25 mM; 2, 0.5 mM; 3, 0.75 mM $MgSO_4$; M, 100-bp molecular size ladder.

temperature. The samples were placed on the magnetic bead attractor for 1 min, and then the supernatant was removed. The samples were resuspended in a washing buffer (10 mM Tris-HCl; pH 7.5, 0.15 M LiCl, 1 mM EDTA). The samples were then resuspended in 100 μl of RNase-free H_2O and heated to 90°C for 2 min to liberate the viral RNA from the Dynabeads, followed by magnetic extraction to pellet the Dynabeads. RT-PCR was performed with 10-μl aliquots of the eluate.

Primers and PCR

Using one-step RT-PCR Kit from QIAGEN (USA), RT-PCR was performed to produce a 267-bp amplicon with 50 μl PCR reaction mixture containing 0.2 μM of primer 2949F; 5'-TATTTGTCTGTCACAGAACAATCAG-3' and 0.2 μM of primer 3192R; 5' -AGGAGGTGGAAGCACTTCATTTGA-3', 5 mM of dNTP, 25 mM of $MgSO_4$ in accordance with the procedures recommended by the manufacturer. RT-PCR amplification was carried out at 50°C for 30 min, followed by a 15-min *Taq* activation step at 95°C. Forty cycles were performed by using a 60°C annealing temperature for 1 min, 1 min of extension at 72°C, and 30 sec of denaturation at 95°C. For the final cycle, the annealing time was extended to 2 min and the final extension was performed for 10 min. The PCR products were separated by electrophoresis at 70 volts for 70 min on 1% agarose gels using tris-acetate/ethylenediamine tetraacetic acid electrophoresis (TAE) buffer. Nested PCR was performed to verify the positive HAV using a Pre-mix PCR Kit (Bioneer, Korea) and primer dkA24; 5' CTTCCTGAGCATACTTGAGTC-3' and primer dkA25; 5'-CCAGAGCTCCATTGAACTC-3', which generated a 200-bp amplicon. Nested-PCR amplification was carried out at 95°C for an initial *Taq* activation step of 15 min, followed by 40 cycles of annealing at 50°C for 1 min, extension for 1 min at 72°C, and denaturation at 95°C for 30 sec (Kingsley and Richards, 2001).

Results

Extraction and concentration of HAV

We compared the ability of glycine and tris elution buffer in extracting hepatitis A virus from oyster digestive glands seeded with 10-fold serial dilution of HAV. Tris elution buffer showed a 10-fold higher RT-PCR detection limit on agarose gel electrophoresis (Fig. 1A). For the tris elution buffer, detectable amplicons were visible for undiluted HAV seeded samples as well as for 10-fold diluted HAV treated samples, while glycine elution buffer produced an amplicon only for undiluted HAV seeded samples in RT-PCR.

We evaluated the combined effects of PEG and glycine elution buffer or PEG and tris elution buffer on the elution and concentration of HAV inoculated in oyster digestive glands in nested-PCR (Fig. 1B and C). For the tris elution buffer and the second PEG treatment, detectable amplicons were visible for 100-fold diluted HAV inoculated samples while tris elution buffer and the first PEG treatment produced an amplicon for 10-fold diluted HAV seeded samples in nested-PCR. However, the combination of glycine elution buffer and the first PEG treatment showed detectable amplicon only for undiluted HAV seeded samples. Overall, regarding the effect of PEG and zirconium hydroxide treatment on the concentration of HAV, the second PEG treatment showed a 10-fold higher nested-PCR detection limit than first PEG treatment (Fig. 1B) and the second PEG treatment showed a 100-fold higher detection limit than zirconium hydroxide treatment (Fig. 1C).

RNA purification and optimal PCR condition

The comparison of two RNA purification methods has highlighted a different efficiency in extraction and removing the inhibitors interfering in PCR amplification. The result of RT-PCR demonstrated that RNA purification method using TRIzol and magnetic poly(dT) beads successfully produced a 267-bp amplicon from 100 μl of undiluted HAV while RNA purification methods using a commercial RNA Purification Kit showed no amplicon (Fig. 2A). Magnesium concentration had a significant impact on the amplification efficiency of PCR because it works as a necessary cofactor for enzyme activity. As shown in Fig. 2B, lane 1 and 2 on the agarose gel obviously produced the single 200-bp amplicon from the nested-PCR, while lane 3 showed a 200-bp amplicon with non-specific band. These results led to the conclusion that this nested-PCR requires 0.25~0.5 mM $MgSO_4$ for specific PCR amplification.

Nested PCR and sequencing

Nested PCR was performed to detect serial dilutions of HAV ranging from 0.00069 to 6.9×10^2 PFU/ml from artifi-

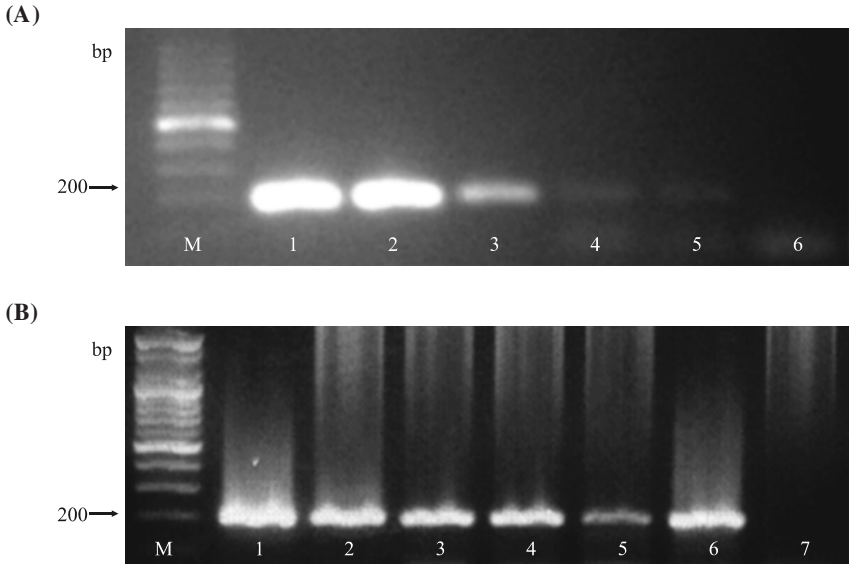

Fig. 3. Effect of concentration of polyethylene glycol (PEG) on the detection sensitivity of hepatitis A virus (HAV) inoculated in oyster digestive glands by nested PCR. The homogenate was seeded with dilutions of HAV. HAV was precipitated twice with PEG in a ratio of 1:1 (A) and 1:2 (B). Viral RNA was extracted by tris buffer, followed by one-step RT-PCR and nested PCR. Lanes 1, 100 μl of 1:10 dilutions (6.9×10^2 PFU/ml); 2, 100 μl of 1:100 dilutions (69 PFU/ml); 3, 100 μl of 1:1000 dilutions (6.9 PFU/ml); 4, 100 μl of 1:10,000 dilutions (0.69 PFU/ml); 5, 100 μl of 1:100,000 dilutions (0.069 PFU/ml); 6, 100 μl of 1:1,000,000 dilutions (0.0069 PFU/ml); 7, 100 μl of 1:10,000,000 dilutions (0.00069 PFU/ml); M, 100-bp molecular size ladder.

cially inoculated digestive glands. Nested PCR was used to evaluate the effectiveness of polyethylene glycol to precipitate HAV when the HAV of supernatant was mixed with an equal or two volume of PEG. After extracting HAV RNA using TPTT procedure, each template obtained from RT-PCR was used for the detection of a 200-bp amplicon by using nested PCR primers dkA24 and dkA25. This amplicon was sequenced and further confirmed as a strain of HAV. As shown in Fig. 3, the concentration method of HAV precipitated twice with PEG/NaCl in a 1:2 (v/v) ratio gave a 10-fold higher than the concentration method of HAV precipitated twice with an equal volume of PEG. The detection limit of HAV precipitated twice with PEG/NaCl in a 1:2 (v/v) ratio was 0.0069 PFU/ml.

Discussion

Numerous virus RNA extraction and detection methods from shellfish have been described (Lopez-Sabater *et al*., 1997; Kingsley and Richards, 2001; Lipp *et al*., 2001; Ribao *et al*., 2004; Fong *et al*., 2005; Dubois *et al*., 2007). We demonstrated a nested RT-PCR protocol for the detection of HAV from oyster digestive glands using an efficient RNA extraction, concentration, and purification procedures called TPTT procedure. The TPTT procedure involves homogenization of oyster tissues in tris-NaCl buffer at pH 9.5 to elute viruses from the solids. Following precipitation of virus by PEG, TRIzol Reagent, and chloroform were used to extract RNA. The HAV RNA layer in the aqueous phase was precipitated by the addition of isopropyl alcohol and then dissolved in RNase-free H_2O. The poly(dT) magnetic beads was used to remove RT-PCR inhibitors. The total time required to perform the TPTT extraction procedure is approximately 8 h when coupled with one-step RT-PCR and Trizol reagent and Dynabeads. In order to extract HAV efficiently, tris elution buffer was applied in artificially inoculated fruits and vegetable samples (Dubois *et al*., 2004). Glycine elution buffer was used as a virus elution buffer in shellfish samples (Kingsley and Richards, 2001). We evaluated elution efficiency using glycine and tris elution buffer after incubating HAV seeded oysters for 30 min at 37°C. Tris elution buffer showed a 10-fold higher RT-PCR detection limit on agarose gel electrophoresis than glycine elution buffer. The use of tris elution buffer and PEG improved detection limit of a nested PCR. The use of zirconium hydroxide facilitates the separation of food-borne bacteria and virus (hepatitis A virus and norovirus) using the interaction between ligands (free amino, hydroxyl, and carboxyl residues) on the virion surface and the hydroxyl groups of zirconium hydroxide, facilitating precipitation (D'Souza and Jaykus, 2002; Lucore *et al*., 2002). Zirconium hydroxide suspension was tested on the efficiency of HAV concentration, as described by D'Souza and Jaykus (2002). However, detection limit of HAV precipitated with zirconium hydroxide was 10^5 fold less sensitive in a nested PCR than that precipitated the HAV supernatant twice with PEG/NaCl (16% polyethylene glycol 6000, 0.525 M NaCl) in a 1:2 (v/v) ratio. In conclusion, TPTT procedure is efficient enough for potential use in the detection of HAV from shellfish and provides an efficient detection of 0.0148 PFU/g from approximately 0.05 g of oyster homogenate. This method is more sensitive than most currently published tests by Kingsley and Richards (2001) showing the detection of 0.04 PFU/g from 3.75 g of oyster tissue homogenate of and by Cromeans *et al.* (1997) providing the detection of 8 PFU of HAV per g of oyster meat.

Acknowledgements

This research was supported by Korea Food and Drug Administration, Republic of Korea.

References

Costafreda, M.I., A. Bosch, and R.M. Pinto. 2006. Development, evaluation, and standardization of a real-time Taqman reverse transcription-PCR assay for quantification of Hepatitis A virus in clinical shellfish samples. *Appl. Environ. Microbiol.* 72, 3846-3855.

Cromeans, T.L., O.V. Nainan, and H.S. Margolis. 1997. Detection of hepatitis A virus RNA in oyster meat. *Appl. Environ. Microbiol.* 63, 2460-2463.

D'Souza, D.H. and L.A. Jaykus. 2002. Zirconium hydroxide effectively immobilizes and concentrates human enteric viruses. *Lett. Appl. Microbiol.* 35, 414-418.

Dix, A.B. and L.A. Jaykus. 1998. Virion concentration method for the detection of human enteric viruses in extracts of hard-shelled clams. *J. Food Prot.* 61, 458-465.

Dubois, E., C. Hennechart, G. Merle, C. Burger, N. Hmila, S. Ruelle, S. Perelle, and V. Ferre. 2007. Detection and quantification by real-time RT-PCR of hepatitis A virus from inoculated tap waters, salad vegetables, and soft fruits: Characterization of the method performances. *Int. J. Food Microbiol.* 117, 141-149.

Dubois, E., G. Merle, C. Roquier, A.L. Trompette, F.L. Guyader, C. Cruciere, and J.J. Chomel. 2004. Diversity of enterovirus sequences detected in oysters by RT-heminested PCR. *Int. J. Food Microbiol.* 92, 35-43.

Fong, T.T., D.W. Griffin, and E.K. Lipp. 2005. Molecular assays for targeting human and bovine enteric viruses in coastal waters and their application for library-independent source tracking. *Appl. Environ. Microbiol.* 71, 2070-2078.

Gerba, C.P. and S.M. Goyal. 1978. Detection and occurrence of enteric viruses in shellfish: a review. *J. Food Prot.* 41, 743-754.

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.

Lemon, S.M., P.C. Murphy, P.A. Shields, L.H. Ping, S.M. Feinstone, T. Cromeans, and R.W. Jansen. 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. *J. Virol.* 65, 2056-2065.

Lewis, G.D. and T.G. Metcalf. 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus from oyster, water and sediment samples. *Appl. Environ. Microbiol.* 54, 1983-1988.

Lipp, E.K., R. Kurz, R. Vincent, C. Rodriguez-Palacios, S.R. Farrah, and J.B. Rose. 2001. The effects of seasonal variability and weather on microbial fecal pollution and enteric pathogens in a subtropical estuary. *Estuaries* 24, 266-276.

Lopez-Sabater, E.I., M.Y. Deng, and D.O. Cliver. 1997. Magnetic immunoseparation PCR assay for detection of hepatitis A virus in American oyster (*Crassostrea virginica*). *Lett. Appl. Microbiol.* 24, 101-104.

Lucore, L.A., M.A. Cullison, and L.A. Jaykus. 2002. Immobilization with metal hydroxides as a means to concentrate foodborne bacteria for detection by cultural and molecular methods. *Appl. Environ. Microbiol.* 66, 1769-1776.

Ribao, C., I. Torrado, M.L. Vilarino, and J.L. Romalde. 2004. Assessment of different commercial RNA-extraction and RT-PCR kits for detection of hepatitis A virus in mussel tissues. *J. Virol. Methods* 115, 177-182.

Sanchez, G., A. Bosch, and R.M. Pinto. 2007. Hepatitis A virus detection in food: Current and future prospects. *Lett. Appl. Microbiol.* 45, 1-5.