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RAPID DETECTION OF HEPATITIS A VIRUS IN OYSTERS IN THE EAST COAST OF THAILAND

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ABSTRACT

Hepatitis A virus (HAV) is one of the major infectious diseases transmitted by the fecal-oral route. Oyster consumption, especially oysters grown in human discharged areas or touched by infected food handlers with poor sanitation, is well recognized as route of entry for HAV. Due to the habit of consuming raw or partially cooked oysters, the detection of fresh oysters before selling to consumers is considered to provide protection against diseases. In this study, RT-PCR was performed to detect HAV in fresh oysters cultured along the coast of Chanthaburi and Chon Buri Provinces in eastern Thailand. Nucleic acid of the virus was extracted by acid-adsorption alkaline elution and amplified with a pair of specific primers. Result showed only 1 cDNA band at 242 bp nucleotide lengths as expected but not in the other enteric viruses. Detection of HAV in oyster tissue or gut harvested over six months displayed cDNA bands but no hybridization signal. These results indicate that while RT-PCR is a sensitive method to detect HAV, their correct interpretation requires much care. Thus, RT-PCR protocol requires a hybridization step for the detection of HAV contamination in oysters.

Keywords: Hepatitis A virus, HAV, oysters, RT-PCR, hybridization, food contamination, food safety.

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INTRODUCTION

Hepatitis A virus (HAV) is a major infectious disease transmitted by the fecal-oral route associated with the consumption of bivalve mollusks, particularly when raw or undercooked oysters are consumed (Centers for disease control (CDC), 1990; Densenclos et al., 1991; Halliday et al., 1991; Rippey, 1994). Human sewage discharged from oyster-harvesting vessels was the probable cause identified in a major 'HAV outbreak' (Lees, 2000). Moreover, infected food handlers with poor sanitation may spread HAV when touching food without proper sanitation (Fiore, 2004). HAV is classified as a member of the family Picornaviridae. It is a small, non-enveloped spherical virus containing a single positive infectious RNA genome (Cuthbert, 2001). HAV have caused outbreaks in several countries such as the United States, China, Spain, and Italy (Potasman et al., 2002; Sanchez et al., 2002; Chironna et al., 2003; Chironna et al., 2004).

A low viral load in oysters is sufficient to pose an appreciable health risk (Sair et al., 2002). Traditional methods for the detection of enteric viruses in food based on cell culture assay have a low sensitivity, slow, expensive, and labor-intensive. Moreover, propagation of wild-type (wt) HAV strain in cell culture is not useful according to its slow replication (Flehmig, 1980) and non-cytopathic effect (Gauss-Muller and Deinhardt, 1984). Thus, a sensitive method to detect low levels of HAV in contaminated oysters is needed to prevent the viral disease.

Molecular approaches, particularly the reverse transcription-polymerase chain reaction (RT-PCR) technique has been proposed as an improved method due to its specificity, sensitivity and short time-consuming (Pina et al., 1998; LeGuyader et al., 2000; Casas and Suñen, 2001). RT-PCR assay has previously proved to detect purified HAV genome as low as 1 copy/μl of HAV RNA (Legeay et al., 2000). The detection of this virus in food samples usually requires a two step-process RT-PCR followed by oligoprobe hybridization that generally permits an endpoint detection of HAV-positive PCR signal in oyster tissues (Coelho et al., 2003; Di Pinto et al., 2004; Lewis et al., 2000; Romalde et al., 2004). However, this technique requires the processing of oyster samples prior to RNA amplification not only to eliminate inhibitory substances from oyster tissues but also to concentrate the viral pathogen because of the low concentration of virus particles usually present in oyster tissues. The methods commonly used to concentrate viruses from whole oysters include direct alkaline elution (De Medici et al., 2001) and acid adsorption-neutral elution (Mullendore et al., 2001). The acid adsorption-alkaline elution process performed by Kittigul et al. (2008) gave the highest sensitivity for rotavirus detection (3.13×10^3 PFU/ml or 125 PFU/g oysters) compared to the acid adsorption-neutral elution and the direct alkaline elution (1.25×10^4 PFU/25 g and 2.5×10^4 PFU/25 g, respectively).

The aim of the study was an evaluation of RT-PCR assay to assess the incidence of HAV contamination in raw oysters harvested along the east coast of Thailand.

MATERIALS AND METHODS

Viral preparation and cell culture

A cytopathic strain of HAV HM 175 (ATCC 1402) was grown and assayed in a continuous cell line of monkey epithelial cells (BSC-1) derived from the African green monkey, *Cercopithecus aethiops*, followed the method described by Hopps et al. (1963). BSC-1 cells (kindly provided by Department of Virology, Armed forces Research Institute of Medical Sciences (AFRIMS), Thailand) were grown to form a confluence monolayer in 75-cm^2 plastic flasks (Corning, USA) in Eagle's Minimum Medium (MEM, Gibco BRL/Life technologies, Gaitherburg, MD) supplemented with 10% heat-inactivated fatal bovine serum (FBS) (Gibco BRL), 100mM HEPES, 0.01 μg/ml gentamicin, 100 μg/ml penicillin, and 1 μg/ml fungizone (Gibco BRL). The virus was allowed to propagate in a confluent monolayer of BSC-1 cells grown in maintenance medium (the same components as the medium used for cell growth but only 2% of FBS was used instead of 10%) in a 25-cm^2 plastic flask (Corning, USA). Cell cultures were observed daily until cytopathic effect

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was observed more than 70% of the monolayer, which was approximately 9-10 days post-infection. The virus was harvested and the infected cell suspension was freeze-thawed 3-4 times to release the virus particles. After centrifugation to remove residual debris, viral suspension was divided into 0.5 ml aliquots and stored at -80 °C for further experiments.

Plaque assay

The HAV concentration in the stock suspension was determined by plaque assay in a 6.8x10^5 BSC-1 cell monolayer grown in six-well culture plates. Wells of six cluster plates (Corning, USA) were inoculated with 0.5 ml of sequential dilutions of HAV in MEM medium supplemented with100 mM HEPES, 0.1% sodium bicarbonate, 0.01 μg/ml gentamicin, 100 μg/ml penicillin and 1 μg/ml fungizone (Gibco BRL). The virus was allowed to absorb for 90 minutes at room temperature on a Platform Shaker (Labnet, USA), set at 40 rpm. Each well was overlaid with 2 ml of overlay medium containing 1X MEM, 2% FBS and 1.2% gum tragacanth. The plate was incubated at 37 °C in 5% CO_2 incubator for nine days. During this incubation period, pH of the medium was adjusted to 6.8-7.0 with 0.5% sodium bicarbonate where the color of the medium was equilibrated to orange color. Plaques were stained by immersing the plates for 30 minutes in a solution containing 10% formaldehyde and 1.25% crystal violet, washed in tap water, dried, and subsequently examined for the characteristic of plaques resulting from the cytopathic effect. The virus quantity was estimated and expressed as plaque forming units per milliliter (PFU/ml). Each PFU refers to a transparent plaque against the dense background of stained uninfected cells.

Oyster sampling

Oysters, especially species *Saccostrea commercialis,* were collected from Tha Chalaeb district, Chanthaburi province, and Ang Sila district, Chon Buri province, located on the east coast of Thailand. Fifteen oysters were collected monthly and analyzed over a 6-month period (from May to October 2009). Oysters were washed, scrubbed, and opened with a sterile knife. Oyster digestive and flesh tissues were separately isolated and each tissue was pooled into 25g aliquots. Each aliquot contained flesh from 10-15 oysters or digestive tissue from 5-8 oysters. Oyster tissue was stored at -80 °C for further analysis.

Oyster processing and viral RNA extraction

A detection limit of HAV was determined by the addition of HAV particles ranging from 2.4x10^3 to 2.4x10^5 PFU to samples of oyster tissue for 60 minutes at room temperature, then homogenized and processed with the acid adsorption-alkaline elution as described by Kittigul et al. (2008). RNA was extracted from concentrated HAV using High Pure Viral Nucleic Acid kit (Roche Diagnostics, Germany). In brief, 200 μl of concentrated HAV was lysed and RNA was subsequently purified in a silica-based column according to manufacturer's protocol. RNA bound to the membrane in the column was eluted in 50 μl of RNAse-free water and aliquots were saved for further RT-PCR analysis.

RT-PCR assay and Southern blot hybridization

Viral RNA was performed in a 50 μl reaction mixture with 5 μl of extracted RNA using SuperScript III one-step RT-PCR with Platinum Taq (Gibco, BRL) according to the procedure recommended by the manufacturer. The oligonucleotide primers –forward (5′TTGCTGTTCAAGGG-3′) and HAV Reverse Primer (5′AAAGTGGTAAGCAC-3′) were designed based upon the external capsid protein-coding region (VP2) (Genbank accession number 14707) for amplification of a 242 bp product. Amplified products were visualized under UV light after electrophoresis onto 1.2% agarose gel in the presence of 1:10,000 the sybergold solution (Invitrogen, USA). Fragment sizes were compared with commercially available size standards (100 bp DNA ladder, TrackIt, Invitrogen, USA).

The RT-PCR product was confirmed by Southern blot hybridization using an internal oligonucleotide probe labeled with DIG at 3′(5′GATTGATCTGTGCTATGGTTCCTGGTG-DIG 3′) (Jansen et al.,

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1990) following the method described by Brassard et al. (2005). Hybridized probes were detected by chemiluminescence using the peroxidase-based ECL detection system (Amersham BioScience, UK) according to manufacturer's instructions.

RESULTS

Specificity of the RT-PCR product

The specificity of RT-PCR product was determined by using the pair of PCR primers designed for amplification of the VP2 region of purified HAV genome. The amplification product for RT-PCR was then analyzed on 1.2% agarose gel electrophoresis. As shown in Fig. 1 A, a unique band corresponding to the expected size of 242 nucleotides was detected. The specificity of the strong amplified band of 242 nucleotides was confirmed by Southern blot hybridization using a DIG-labeled oligoprobe (Fig. 1 B). The specificity of RT-PCR was also confirmed with non-target nucleic acids from different enteric viruses such as poliovirus and rotavirus. No band was observed (data not shown). The result showed that primers designed for RT-PCR were specific for only HAV nucleic acid sequence.

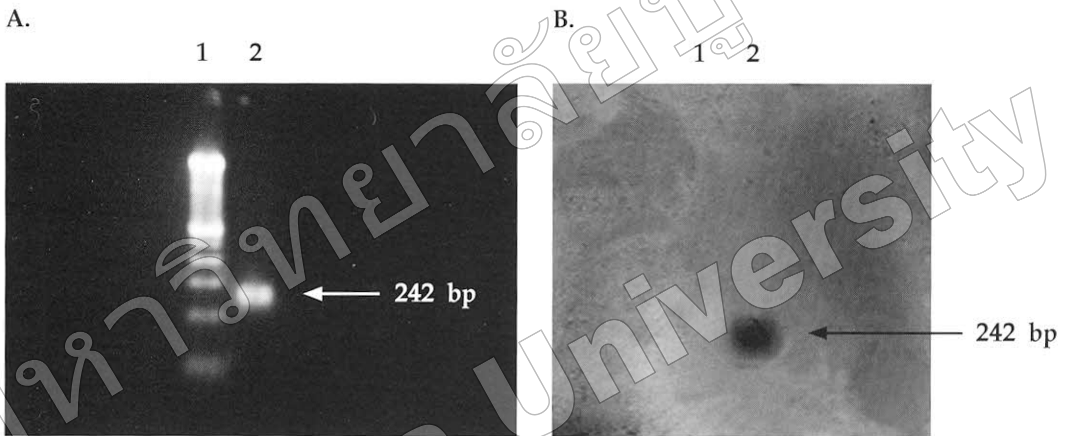

Figure 1. Detection of purified HAV genome by RT-PCR and Southern blot analysis.
A: Analysis of a RT-PCR amplification product on a 1.2% agarose gel electrophoresis.
B: Its corresponding Southern blot hybridized with DIG-labeled oligonucleotide probe targeting gene VP2 of HAV
Lane 1: 100 bp molecular marker
Lane 2: HAV amplicon in the presence of 10 ng purified HAV genome/reaction

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Detection of RT-PCR from experiments with inoculated oyster samples

The quantity of HAV HM175 stock suspension was determined by plaque assay before adding into oyster samples. Viral quantity was estimated as 2.4×10^6 PFU/ml. Tissue from oysters not-augmented with HAV was considered as controls. All RNA samples were then reverse transcribed and amplified. PCR products were analyzed by 1.2% agarose gel. As shown in Fig. 2, viral sequences were readily detected in all samples inoculated with HAV where the bands appeared at the expected size of 242 nucleotides but were not detected in uninoculated samples (Fig. 2 A, lane 3-4). However, when HAV was inoculated at 2.4×10^2 PFU in 25 g of oyster flesh (or about 9.6 PFU/g) RT-PCR could not detect the viral sequence (data not shown). The specificity of bands was confirmed by Southern blot hybridization using a DIG-labeled oligoprobe. All amplification products generated strong hybridization signals indicating a molecular size corresponding to the expected value of 242 nucleotides (Fig. 2B).

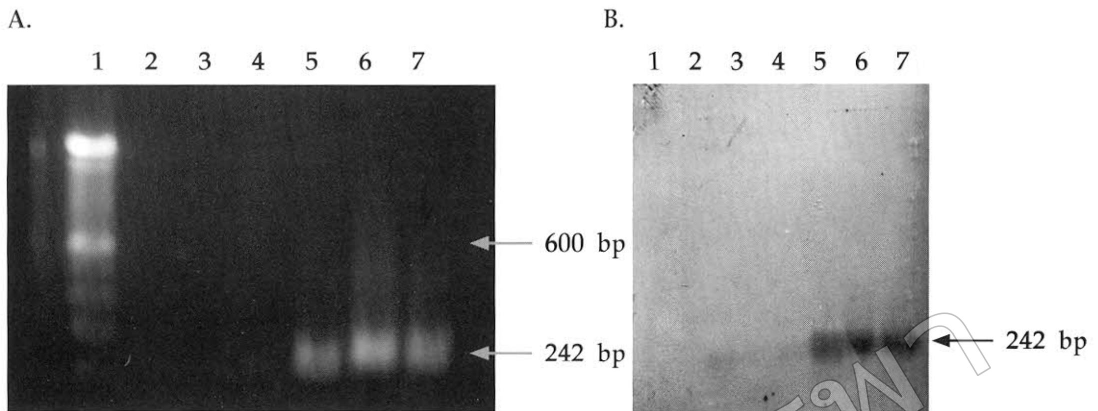

Figure 2. Detection of HAV extracted from inoculated oyster samples by RT-PCR and Southern blot analysis.
A: Analysis of RT-PCR amplification products by 1.2% agarose gel electrophoresis.
B: Its corresponding Southern blot hybridized with DIG-labeled oligonucleotide probe targeting gene VP2 of HAV
Lane 1: 100 bp molecular marker
Lane 2: negative control (dH_2O)
Lane 3-4: negative control (uninoculated oyster extracts)
Lane 5-7: HAV amplicon of oyster extracts inoculated with HAV titer corresponding to as $2.4x10^3$, $2.4x10^4$ and $2.4x10^5$ PFU, respectively

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Examination of hepatitis A virus in oyster samples

It was shown that some tissue extractions contained bands close in size to the positive control. However, bands were fainter and smeared on the agarose gel electrophoresis (Fig. 3A). There were no signals detected following Southern blot transfer and oligoprobing, as opposed to the positive control, clearly indicating that bands observed in the gel were not specific (Fig. 3B). These results were similar to those of digestive tissues (Fig. 4) and also to those from other farms in Chon Buri province (data not shown).

DISCUSSION

RT-PCR has offered the best alternative to *in vitro* cell culture technique for developing sensitive and specific tests for detection of food pathogens especially enteric viruses (RNA viruses) in shellfish (Atmar et al., 1993; Chung,et al, 1996). However, the successful application of RT-PCR to detect HAV in oysters requires the extraction and concentration of the small numbers of viruses present and the elimination of inhibitory substances in oysters. Using a processing procedure for virus concentration from oysters, acid absorption alkaline elusion, before RNA extraction allowed detection of only single band corresponding to the expected size of 242 nucleotides in all samples inoculated with HAV (Fig. 2), and that was similar to the RT-PCR product amplified from purified genome (Fig 1). These results have demonstrated the efficiency of the virus concentration and nucleic acid extraction procedure to exclude the natural inhibitors from oyster tissues and could be efficiently extracted viral RNA from oyster tissues contaminated in natural conditions. The detection limit is considered to be more sensitive than the process performed by Kittigul et al. (2008), in which we can detect $2.4x10^3$ PFU in 25 g or equivalent to 96 PFU/g and $3.12x10^3$ PFU/ml in 25 g or equivalent to 125 PFU/g of oysters, respectively. The differences in

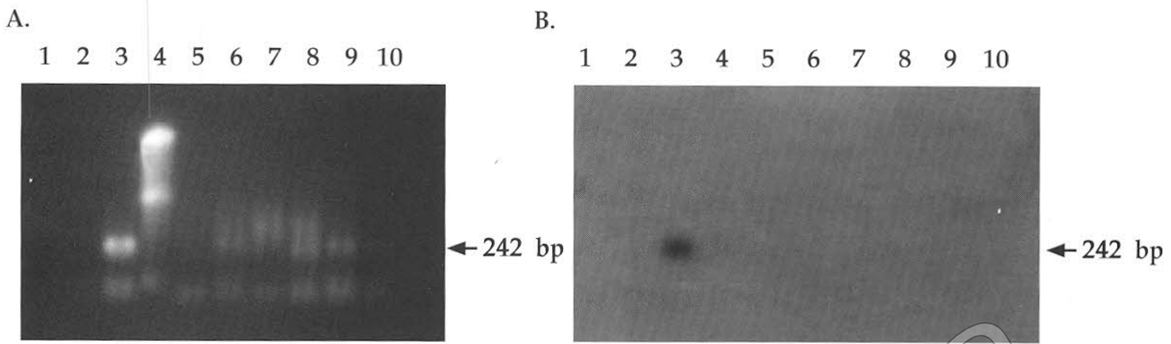

Figure 3. Detection of naturally contaminated HAV in oyster flesh tissues collected from oyster farms in Chanthaburi province, Thailand samples by RT-PCR and Southern blot analysis.

A: Analysis of RT-PCR amplification products by 1.2% agarose gel electrophoresis.

B: Its corresponding Southern blot hybridized with DIG-labeled oligonucleotide probe targeting gene VP2 of HAV

Lane 1: negative control (dH_2O)

Lane 2: negative control (uninoculated oyster extract)

Lane 3: HAV-positive control (oyster extract inoculated with 2.4×10^3 PFU)

Lane 4: 100 bp molecular marker

Lane 5-10: HAV amplicon of concentrates from oyster samples collected from May to October 2009

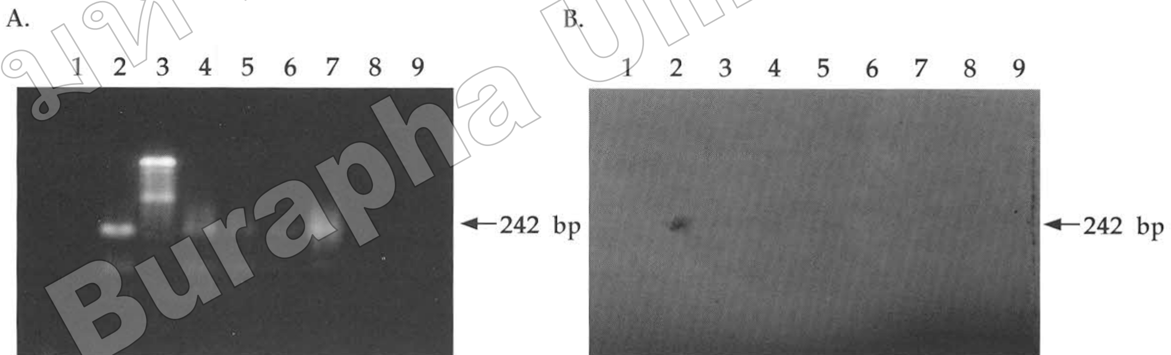

Figure 4. Detection of naturally contaminated HAV in oyster digestive tissues collected from oyster farms in Chanthaburi province, Thailand by RT-PCR and Southern blot analysis.

A: Analysis of RT-PCR amplification products by 1.2% agarose gel electrophoresis.

B: Its corresponding Southern blot hybridized with DIG-labeled oligonucleotide probe targeting gene VP2 of HAV

Lane 1; negative control (uninoculated oyster extract)

Lane 2: HAV-positive control (oyster extract inoculated with 96 PFU/g oyster or 2.4×10^3 PFU)

Lane 3: 100 bp molecular marker

Lane 4-9: HAV amplicon of concentrates from oyster samples collected from May to October 2009

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sensitivity between the two studies are probably due to using different commercial viral extraction and RT-PCR kits.

Due to low levels of virus contamination heterogeneous exposure of individual oyster to virus in natural conditions, tissues from several oysters were analyzed simultaneously to increase test sensitivity. The 25 g of flesh and digestive tissues aliquots used for analysis represents approximately 10-15 and 5-8 oysters, respectively. RT-PCR amplifications of flesh or digestive tissues could be observed on the agarose gel electrophoresis in size close to the positive control (Figs. 3A and 4A, respectively). The importance of confirmation of RT-PCR results by oligoprobing is evident when signals are not detected as of a positive control. It has been shown that the nonspecific RT-PCR amplified bands are also observed in negative control ham sample but disappeared following Southern transfer and oligoprobing (Schwab et al., 2000). These artifacts are produced because RT-PCR assay is very sensitive; hence, it needs to be coupled with hybridization for specificity. Besides specificity, hybridization is also increased the sensitivity of the RT-PCR assay approximately 100 folds higher than that of RT-PCR alone (Sincero, et al., 2006).

In conclusion, the RT-PCR assay is rapid and sensitive for detecting HAV in oysters that usually takes for 2-3 hours; however, it needs to be followed by hybridization to confirm its specificity. If it were considered to be used for routine screening and risk assessment, it would need to improve the extraction procedure due to time consuming and numerous steps for virus isolation from oysters that usually takes two days for tissue processing and virus concentration. Moreover, other improvements by the substitution of the Southern blot hybridization with a micro-titer plate hybridization assay, would allow further simplification of this procedure and assaying more samples (15 samples and 96 samples, respectively).

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REFERENCES

Atmar, R. L., Metcalf, T. G., Neill, F. H., and. Estes, M. K. 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. *Applied Environment Microbiology* 59: 631–635.

Brassard, J., Seyer, K., Houde, A., Simard, C., Trottier, Y-L. 2005. Concentration and detection of hepatitis A and rotavirus in spring water samples by reverse transcriptase-PCR. *Journal of Virological Methods* 123: 163-169.

Casas, N., and Suñen, E. 2001. Detection of enterovirus and hepatitis A virus RNA in mussels (*Mytilus* spp.) by reverse transcriptase-polymerase chain reaction. *Journal of Applied Microbiology* 90: 89–95.

Centers for Disease Control and Prevention (CDC). 1990. Food-borne hepatitis A-Alaska, Florida, North Carolina, Washington. *MMWR* 39: 228-232.

Chung, H., Jaykus, L. A. and Sobsey, M. D. 1996. Detection of human enteric viruses in oysters by in vivo and in vitro amplification of nucleic acids. *Applied Environment Microbiology*. 62:3772–3778.

Chironna, M., Grottola, A., Lanave, C., Villa, E., Barbuti, S., and Quarto, M. 2003. Genetic analysis of HAV strains recovered from patients with acute hepatitis from southern Italy. *Journal of Medical Virology* 70: 343–349.

Chironna, M., Lopalco, P., Prato, R., Germinario, C., Barbuti, S., Quarto, M. 2004. Outbreak of infection with hepatitis A virus (HAV) associated with a food handler and confirmed by sequence analysis reveals a new HAV genotype IB variant. *Journal of Clinical Microbiology* 42: 2825–2828.

Coelho, C., Heinert, A.P., Simoes, C.M., and Barardi, C.R. 2003. Hepatitis A virus detection in oysters (Crassostra gigas) in santa catarina state,

มหาวิทยาลัยบูรพา Burapha University

Brazil, by reverse transcription-polymerase chain reaction. *Journal of Food Protection* 66: 507-511.

Cuthbert, J.A. 2001. Hepatitis A: old and new. *Clinical Microbiological Reviews* 14: 38–58.

Densenclos, J-C-A., Klontz, K.C., Wilder, M.H., Nainan, O.V., Margolis, H.S., and Gumm, R.A. 1991. A multistate outbreak of hepatitis A caused by the consumption of raw oysters. *American Journal of Public Health* 81: 1268-1272.

De Medici, D., Croci, L., Di Pasquale, S., Fiore, A., Toti, L. 2001. Detecting the presence of infectious hepatitis A virus in Molluscs positive to RT-nested PCR. *Letters in Applied Microbiology* 33: 362-366.

Di Pinto, A., Conversano, M.C., Forte, V.T., La Salandra, G., Montervino, C., and Tantillo, G.M. 2004. A comparison of RT-PCR-based assays for the detection of HAV from shellfish. *New Microbiologica* 27: 119-124.

Fiore, A.E. 2004. Hepatitis A transmitted by food. *Clinical Infectious Diseases* 38:705-715

Flehmig, B. 1980. Hepatitis A virus in cell culture: I. Propagation of different hepatitis A virus isolates in a fetal rhesus monkey kidney cell line (Frhk-4). *Medical Microbiology and Immunology* 168:239-248.

Gauss-Muller, V., and Deinhardt, F. 1984. Effect of hepatitis A virus infection on cell metabolism in vitro. *Proceedings of the Society for Experimental Biology and Medicine* 175:10-15.

Halliday, M.L., Kang, L.-Y., Zhou, T-K., Hu, M-D., Pan, Q-C, Fu, T.Y., Huang, Y.S., and Hu, S-L., 1991. An epidemic of hepatitis A attributable to the ingestion of raw clams in Shanghai, China. *Journal of Infectious Diseases* 164: 852-859.

Hopps, H. E., Bernheim, B. C., Nisalak, A., Tjio, J. H., and Smadel, J. E. 1963. Biologic Characteristics of a Continuous Kidney Cell Line Derived from the African Green Monkey *Journal of Immunology* 91: 416-424.

Jansen, R.W., Siegl,G., and Lemon, S.M. 1990. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. *Proceedings of the National Academy of Sciences of the United States of America* 87: 2867-2871.

Kittigul, L., Pombubpa, K., Rattanatham, T., Diraphat, P., Utrarachkij, F., Pungchitton, S., Khamrin, P, and Ushijima, H. 2008. Development of a method for concentrating and detecting rotavirus in oysters. *International Journal of Food Microbiology* 122: 204-210.

Le Guyader, F.L., Haugarreau, L, Miossec, L., Dubois, E., and Pommepuy, M. 2000. Three-year study to access human enteric viruses in shellfish. *Applied and Enviromnetal Microbiology* 66: 3241-3248.

Lees, D. 2000. Viruses and bivalve shellfish. International *Journal of Food Microbiology* 59: 81–116.

Legeay, O, Caudrelier, Y., Cordevant, C., Rigottier-Gois, L., and Lange, M. 2000. Simplified procedure for detection of enteric pathogenic viruses in shellfish by RT-PCR. *Journal of Virological Methods* 90: 1-14.

Lewis, G.D., Molloy, S.L., Greening, G.E., and Dawson, J. 2000. Influences of environmental factors on virus detection by RT-PCR and cell culture. *Journal of Applied Microbiology* 88: 633-640.

Mullendore, J.L., Sobsey, M.D., and Shieh, Y-S.C. 2001. Improved method for the recovery of hepatitis A virus from oysters. *Journal of Virological Methods* 94: 25-35.

Pina, S., Puig, M., Lucena, F., Jofre, J., and Girones, R. 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. *Applied and Environmental Microbiology* 64: 3376–3382.

Potasman, I., Paz, A., and Odeh, M. 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clinical Infectious Diseases* 35: 921–928.

Rippey, S. R. 1994. Infectious diseases associated with molluscan shellfish consumption. *Clinical Microbiology Reviews* 7:419–425.

Romalde, J.L., Ribao, C., Luz Vilarino, M., and Barja, J.L. 2004. Comparison of different primer

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sets for the RT-PCR detection of hepatitis virus and astrovirus in mussel tissues. *Water Science and Technology* 50: 131-136.

Sair, A.I., D'Souza, D.H., and Jaykus, L.A. 2002. Human enteric viruses as causes of foodborne disease. *Comprehensive Reviews in Food Science and Food Safety* 1: 73–89.

Sanchez, G., Pinto, R.M., Vanaclocha, H., and Bosch, A. 2002. Molecular characterization of hepatitis a virus isolates from a transcontinental shellfish-borne outbreak. *Journal of Clinical Microbiology* 40: 4148–4155.

Schwab, K.J., Neill, F.H., Fankhauser, R.L., Daneils, N.A., Monroe, S.S., Bergmiire-Sweat, D.A., Ester, M.K., and Atmar, R.L. 2000. Development of methods to detect "Norwalk-like viruses" (NLVs) and hepatitis A virus in delicatessen foods" application to a foodborne NLV outbreak. *Applied And environmental Microbiology* 66: 213-218

Sincero, T.C.M., Levin, D.B., Simoes, C.M.O., and Barardi, C.R.M. 2006. Detection of hepatitis A virus (HAV) in oysters (*Crassostrea gigas*). *Water Research* 40: 895-902.

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