DETECTION OF HEPATITIS A VIRUS AND BACTERIAL CONTAMINATION IN RAW OYSTERS IN THAILAND

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Abstract. This study was conducted to determine the presence of hepatitis A virus (HAV) in raw oysters (*Crassostrea belcheri*) using a virus concentration method and reverse transcription-nested polymerase chain reaction (RT-nested PCR). A total of 220 oyster samples were collected from oyster farms and local markets in Thailand. HAV was found in three oyster samples. Nested PCR products of HAV detected in oysters were characterized further by DNA sequencing of the VP1/2A region and subjected to phylogenetic analysis. All HAV sequences (168 basepairs) were associated with human HAV subgenotype IB (GIB). Fecal coliforms and *Escherichia coli* were determined using the multiple tube fermentation method, to assess the microbiological quality of collected oysters. Among oyster samples tested, 65% had fecal coliforms higher than the standard level for raw shellfish [<20 Most Probable Numbers (MPN)/g]; MPN values in the range of 21.0-4.6 x 10^4/g. Most oyster samples (85%) were contaminated with *E. coli* in the range of 3.0-4.6 x 10^4 MPN/g. One oyster sample with an acceptable level of fecal coliforms contained HAV GIB. *E. coli* was found in all HAV-positive oyster samples. The results suggest a significant presence of HAV and bacterial indicators of fecal contamination in raw oysters, which are a health risk for consumers and a source of gastrointestinal illness. Enteric viruses should also be tested to assess the microbiological quality of oysters.

Key words: hepatitis A virus, MPN, oysters, virus concentration, RT-nested PCR

INTRODUCTION

Hepatitis A virus (HAV) is one of the leading causes of foodborne illness (Fiore, 2004; Koopmans and Duizer, 2004). A great number of epidemiological studies indicate HAV infection is associated with consumption of improperly cooked or raw shellfish which have been subjected to fecal contamination. Foodborne outbreaks occurring in developed countries increase awareness that bivalve shellfish can transmit hepatitis A (Bosch *et al*, 2001; Romalde *et al*, 2001; Sanchez *et al*, 2002; Shieh *et al*, 2007; Pontrelli *et al*, 2008). In contrast, limited data is available from developing countries since molecular techniques for identification of enteric viruses in food have not been undertaken routinely. HAV, a member of the Hepatovirus genus, belongs to the *Picornaviridae* family. The virus is transmitted mainly via fecal-oral

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route by person-to-person contact and contaminated water and foods (Hollinger and Emerson, 2007). There is only one serotype of HAV and six genotypes (formerly seven genotypes) which have been identified by molecular methods based on the putative VP1/2A junction encoded on the RNA genome. Genotypes I, II and III cause acute hepatitis in humans; whereas, genotypes IV, V and VI are associated with infections in simians (Cristina and Costa-Mattioli, 2007).

Hepatitis A remains an important public health problem in Thailand. Outbreaks of hepatitis A have been reported (Poovorawan *et al*, 2005; Wattanasri *et al*, 2005; Barameechai *et al*, 2008) and a decline in the seroprevalence of HAV during the past decade has increased the susceptibility to infection among adolescents and adults (Poovorawan *et al*, 1997; Chatproedprai *et al*, 2007). HAV has been identified in environmental water in Thailand (Kittigul *et al*, 2000, 2006). However, there is no data regarding HAV in food in Thailand. In the present study, an established method for concentrating rotavirus from oysters in our laboratory (Kittigul *et al*, 2008) was used to detect HAV in oyster samples. Molecular characterization of the HAV present in oysters was carried out. Bacterial indicators (fecal coliforms and *E. coli*) for fecal contamination were also identified for determination of the microbiological food safety of these bivalve shellfish.

MATERIALS AND METHODS

Collection of oyster samples

From August 2005 to February 2006, 220 oyster samples (*Crassostrea belcheri*) were collected from local markets (110 samples) in Bangkok and oyster farms (110 samples) in Surat Thani Province, Thailand. Each sample was composed of three live oysters (with the shell) and transported in an ice box to the laboratory. Immediately after arrival, the oysters were washed, scrubbed and the shells opened with a sterile shucking knife. The liquor or mantle fluid was drained into a discard container. The oyster meat, except the adductor muscle that was left attached to the shell, was collected, cut into small pieces, and trimmed to 25 g each to analyze for HAV and bacterial indicators.

Concentration of HAV from oyster samples

HAV from oyster samples was concentrated following the acid adsorption-alkaline elution method described by Kittigul *et al* (2008). Briefly, 25 g oyster meat in seven volumes of chilled, sterilized distilled water was homogenized at high speed twice. The conductivity of the homogenate was reduced to less than 2,000 μS/cm by adding sterilized distilled water. The homogenate was then adjusted to pH 4.8 with 1 N HCl, shaken for 15 minutes, and centrifuged at 2,000 *g* for 20 minutes at 4ºC. The supernatant was discarded and the pellet was suspended in 25 ml of 2.9% tryptose phosphate broth (TPB) containing 6% glycine, pH 9.0 for elution of the virus, shaken for 15 minutes, and centrifuged at 10,000*g* for 15 minutes at 4ºC. The pellet was re-eluted with 25 ml of 0.5 M arginine-0.15 M NaCl, pH 7.5. The suspension was shaken for 15 minutes and centrifuged. The supernatant from the elution and re-elution steps was collected and adjusted to pH 7.2. The virus in the supernatant was precipitated by adding polyethylene glycol (PEG) 8,000 to give a final concentration of 12.5% and 0.3 M NaCl. The mixture was refrigerated overnight at 4ºC. The pellet was dissolved in 15 ml of 0.05 M phosphate-buffered saline (PBS), pH 7.5 and precipitated again with PEG-NaCl. The mixture was stirred for 2 hours,

then centrifuged at 10,000*g* for 10 minutes at 4ºC. The pellet was dissolved in 5 ml PBS. The virus was extracted with chloroform at a final concentration of 30%. After centrifugation at 3,000*g* for 10 minutes, the top layer of the aqueous phase was collected. The pellet at the interface between the solvent and the aqueous phase was re-extracted with a 0.5 volume of arginine-NaCl pH 7.5. After centrifugation, the top layer from the re-extraction step was collected. The sample was reconcentrated using speedVac centrifugation to reduce the volume of the concentrate to approximately 1 ml and stored at -80ºC until used.

RT-nested PCR for HAV

Oyster concentrate (200 μl) was extracted for RNA using the RNeasy® Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. The presence of HAV RNA in the extracted tissue from the oyster samples was determined using RT-nested PCR as described by Wattanasri *et al* (2005) with some modification. Primers BR-5 (5' TTG TCT GTC ACA GAA CAA TCA G 3'), BR-9 (5' AGT CAC ACC TCT CAA GGA AAA CTT 3'), RJ-3 (5' TCC CAG AGC TCC ATT GAA 3'), and BR-6 (5' AGG AGG TGG AAG CAC TTC ATT TGA 3') were used for the amplification of sequences from the VP1/2A of the HAV (Bruisten *et al*, 2001).

RT-PCR was performed with 50 μl of reaction volume. The RNA extract diluted two-fold (5 μl) was incubated at 95ºC for 5 minutes, spun briefly, and placed on ice for at least 10 minutes. The denatured RNA was mixed with PCR mixture (45 μl) consisting of 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton®X-100, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 15 pmol of each primer (BR-5 and BR-9), 15 U of avian myeloblastosis virus reverse transcriptase, 40 U of Recombinant RNAsin® (Ribonuclease inhibitor), 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) and nuclease-free water. The reaction was carried out with the following steps: RT at 48ºC for 45 minutes; PCR cycle, 94ºC for 2 minutes, 35 cycles: 94ºC for 1 minute, 55ºC for 1 minute, 72ºC for 1 minute; and final extension, 72ºC for 7 minutes. The PCR amplification product (5 μl) was added to the reaction mixture (45 μl): 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton®X-100, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 15 pmol of each primer (RJ-3 and BR-6), 2.5 U *Taq* DNA polymerase, and nuclease-free water. The nested PCR cycle was as follows: 94ºC for 2 minutes, 35 cycles: 94ºC for 1 minute, 55ºC for 1 minute, 72ºC for 1 minute; and final extension, 72ºC for 7 minutes. PCR products were analyzed by electrophoresis on 1.5% agarose gel and ethidium bromide staining. A DNA fragment of 234 bp was considered as HAV DNA.

DNA sequencing and phylogenetic analysis

DNA products (234 bp) were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced at the Bioservice Unit of the National Science and Technology Development Agency, Bangkok. The nucleotide sequences (168 bp) were compared with those of HAV strains deposited in the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) program (Altschul *et al*, 1990). The phylogenetic relationship of HAV was determined by aligning sequences with the ClustalX program. A phylogenetic tree was constructed according to the neighbor-joining method using MEGA version 3.1 (Kumar *et al*, 2004).

Nucleotide sequence accession numbers

The nucleotide sequences of the HAV strains THOYS071/2005, THOYS072/2005, and THOYS087/2005 have been deposited in the GenBank under the accession numbers FJ489653-55.

Bacteriological analysis

The values of most probable number (MPN) fecal coliforms and *E. coli* in oyster samples were determined using a multiple tube fermentation method in a three tube series of five dilutions which ranged from 10^{-1} to 10^{-5}, according to the Bacteriological Analytical Manual of the Food and Drug Administration (Hitchins *et al*, 1998). Fecal coliform density was determined using lactose broth as a presumptive medium, and confirmed with EC broth. The number of gassing tubes was recorded for calculation of MPN fecal coliforms by means of a MPN table. The presence of *E. coli* in EC broth tube was examined by isolation and tested for biochemical reactions. The MPN for *E. coli* was interpreted from the number of EC broth tubes that contained *E. coli* by means of the MPN table.

RESULTS

Determination of HAV in oyster samples

A total of 220 oyster samples were collected, concentrated, and tested for HAV. HAV RNA was detected in three raw oyster samples. Duplicate experiments were carried out to detect HAV in each sample. HAV-positive oyster concentrates were re-extracted for RNA and amplified separately for confirmation of the results. All three HAV-positive oyster samples were collected in September, 2005. Two samples were collected from two sites in one market in Bangkok and one sample from an oyster farm in Surat Thani Province, Thailand.

HAV genotyping and molecular analysis

To characterize HAV-positive oyster samples, PCR products from the VP1/2A junction region were sequenced with both forward RJ-3 and reverse BR-6 primers. Using a BLAST program, sequence comparison of 168 bp among the three HAV-positive oyster samples showed 99% identity with HAV isolate Ll-18/12/95 (AF386850), HAV isolate Ll-15/11/94 (AF386847) and HAV isolate from Barcelona sewage 05-Feb-1999 (AY867864). Genotyping of HAV was determined with phylogenetic analysis in the VP1/2A region. A phylogenetic tree of HAV is shown in Fig 1, including reference isolates from Thailand and other geographic regions. All HAV strains found in the oysters were classified as genotype I, subgenotype IB. When compared with subgenotype IB strains, the oyster isolates clustered with a wild type reference strain HM-175/1976/Australia and environmental water isolates Ll-18/12/95.

Bacterial contamination of oyster samples

The MPN fecal coliforms and MPN for *E. coli* in 220 oyster samples were in the range of $<3.0\text{-}4.6 \times 10^4$/g. Fecal coliforms were found in 193 (87.7%) oyster samples and 143 (65.0%) contained unacceptable levels of fecal coliforms ($21.0\text{-}4.6 \times 10^4$ MPN/g) which is higher than the standard level for raw shellfish (National legislation in Thailand, <20 MPN/g). One hundred eighty-seven oyster samples (85.0%) were contaminated with *E. coli* ($3.0\text{-}4.6 \times 10^4$ MPN/g). The presence of fecal coliforms and *E. coli* in the oyster samples collected from the oyster farm were similar to the market samples (Table 1). Interestingly, 45/77 (58.4%) oyster samples with acceptable levels of fecal coliforms were contaminated with *E. coli* in the range of 3.6-16.0 MPN/g.

Bacterial indicators of fecal contamination were identified in HAV-positive

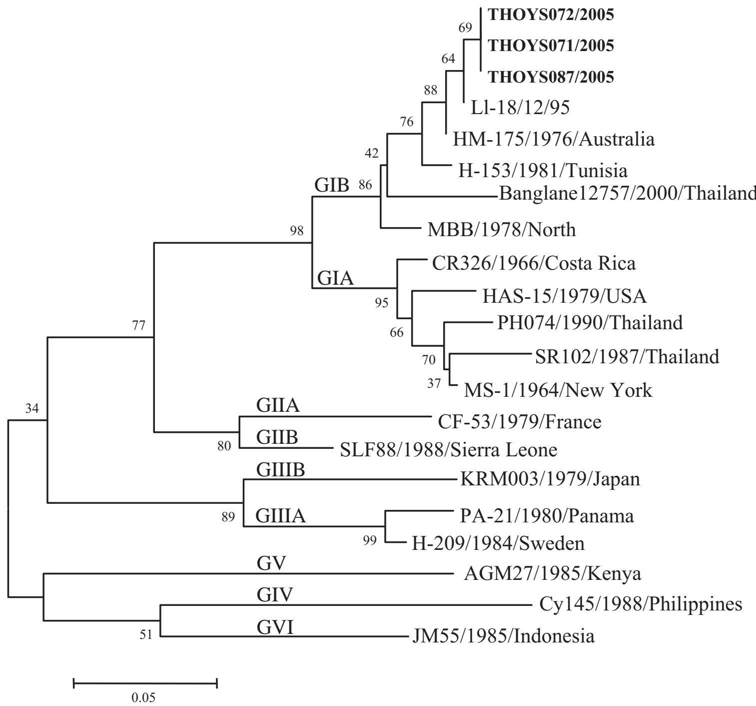

Fig 1–Phylogenetic analysis of VP1/2A junction nucleotide sequences of HAV detected in Thai oysters (THOYS071/2005, THOYS072/2005, and THOYS087/2005). The GenBank accession numbers for known HAV genotypes were: GIA; MS-1 (L07665), CR326 (M10033), HAS-15 (X15464), PH074 (L07726), SR102 (L07722), GIB; HM-175 (M14707), MBB (M20273), H-153 (L07727), Banglane12757 (AY148421), GIIA; CF-53 (L07693), GIIB; SLF88 (L07729), GIIIA; PA-21 (L07730), H-209 (L07704), GIIIB; KRM003 (L20536), GIV; Cy145 (L07732), GV; AGM27 (D00924), and GVI; JM55 (L07731). The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

oyster samples. Fecal coliforms were found to be higher than the standard level for raw shellfish in two oyster samples THOYS071/2005 and THOYS087/2005, at levels of 23 and 43 MPN/g, respectively. One oyster sample (THOYS072/2005) with an acceptable level of fecal coliforms (15.0 MPN/g) was positive for HAV. All HAV-positive oyster samples were contaminated with *E. coli* (15.0-23.0 MPN/g), as shown in Table 2.

DISCUSSION

This is the first report of HAV in Thai oysters. Three oyster samples were contaminated with HAV: two collected from a market and one from an oyster farm. Some oyster samples used in this study had been tested previously for rotaviruses, showing a presence (3.3%) (Kittigul *et al*, 2008). No oyster samples were positive for both HAV and rotaviruses. The oyster samples were positive for HAV were collected in September. Three HAV sequences were identical among 168 nucleotides. This result may indicate a potential point-source contamination. From interviews it was learned the oysters in local markets in Bangkok were from oyster farms located in Surat Thani Province where the oysters were collected in this study. It is possible that in September, HAV contamination occurred in oyster farms and a number of oysters were distributed to local markets in Bangkok.

Using the BLAST program, sequence comparison of 168 bp among the three HAV-positive oyster samples showed 99% identity with HAV detected in sewage and river water in Spain (Pina *et al*, 2001). Phylogenetic analysis of the VP1/2A region showed all HAV strains were genotype I subgenotype IB and related closely to HAV

Table 1
Detection rates and MPN values of bacterial indicators in oyster samples.

Source of oyster collection	No. of samples	Fecal coliforms		Fecal coliforms (≥20 MPN/g)[a]		*E. coli*	
		No. (%)	Range of MPN/g (geometric mean)	No. (%)	Range of MPN/g (geometric mean)	No. (%)	Range of MPN/g (geometric mean)
Local markets	110	99 (90.0)	3.6-2.1 x 10^4 (86.6)	71 (64.5)	21.0-2.1 x 10^4 (2.2 x 10^2)	95 (86.4)	3.6-2.1 x 10^4 (71.2)
Oyster farms	110	94 (85.5)	3.6-4.6 x 10^4 (1.0 x 10^2)	72 (65.5)	23.0-4 .6 x 10^4 (2.5 x 10^2)	92 (83.6)	3.0-4.6 x 10^4 (99.5)

[a]The standard level of fecal coliforms in raw shellfish is <20 MPN/g.

Table 2
Characteristics of the oyster samples where hepatitis A virus was detected.

Oyster sample code	Date of collection	Source of collection	Hepatitis A virus genotype	Fecal coliforms MPN/g	*E. coli* MPN/g
THOYS071	10 Sep 2005	Local market	GIB	23.0	23.0
THOYS 072	10 Sep 2005	Local market	GIB	15.0[a]	15.0
THOYS 087	24 Sep 2005	Oyster farm	GIB	43.0	23.0

[a]Level of fecal coliforms is acceptable; the standard level in raw shellfish, <20 MPN/g.

isolate Ll-18/12/95 from river water in Spain and wild type HM-175/1976/Australia. Additionally, HAV subgenotype IB strains in oyster samples from Surat Thani Province in southern Thailand were distantly related to HAV subgenotype IB (Banglane12757/2000/Thailand) found in Nakhon Pathom Province in central Thailand suggesting HAV subgenotype IB has genetically diverged (Wattanasri *et al*, 2005). The presence of HAV subgenotype IA in sewage was reported previously (Kittigul *et al*, 2006). Although HAV subgenotype IA is common in outbreaks in Thailand (Poovorawan *et al*, 2005; Barameechai *et al*, 2008) HAV subgenotype IB has also been found in patients with acute hepatitis (Wattanasri *et al*, 2005). Taken together, HAV subgenotypes IA and IB cocirculate in the environment and among patients with hepatitis A in different regions of Thailand where there is high and intermediate endemicity for hepatitis A disease. Further studies of HAV prevalence in oysters and bivalve shellfish should evaluate the predominant HAV subgenotype in the environment.

HAV subgenotype IA and/ or subgenotype IB found in bivalve shellfish have been reported from various countries (Sánchez *et al*, 2002; Macaluso *et al*, 2006; Nenonen *et al*, 2006; Croci *et al*, 2007, Pontrelli *et al*, 2008). HAV subgenotype IA is the most prevalent in humans worldwide (Cristina and Costa-Mattioli, 2007). Cocirculation of HAV subgenotypes IA and IB has been recently reported from several countries (Reuter *et al*, 2006;

Davidkin *et al*, 2007; Ngui *et al*, 2008; Normann *et al*, 2008). These findings imply that both HAV subgenotype IA and subgenotype IB may be transmitted via contaminated foods, including bivalve shellfish, and cause acute hepatitis in humans.

Regarding the bacteriogical quality of collected oysters, most of the samples were contaminated with fecal coliforms and *E. coli*. These oysters were exposed to fecal contamination that may occur during harvesting, processing or transportation to the markets. The oyster samples from the oyster farm might have been harvested from an area with fecal pollution and/or processed under unsanitary conditions. The determination of only fecal coliforms in oysters may not guarantee microbiological food safety. The presence of HAV RNA in one oyster sample with an acceptable level of fecal coliforms (15.0 MPN/g) confirms this problem. Previous studies demonstrated no relation between bacterial indicators and the presence of viruses in shellfish (Le Guyader *et al*, 1994; Romalde *et al*, 2001; Chironna *et al*, 2002; Gabrieli *et al*, 2007). These findings suggest bivalve shellfish should be tested not only for bacterial indicators but also for enteric viruses, to evaluate the quality of shellfish products for human consumption and exportation, and to prevent outbreaks associated with raw consumption.

The present study highlights the importance of monitoring HAV in oysters by molecular methods. Comparison of nucleotide sequences in the VP1/2A region of the genome allows study of the genetic relation of detected HAV strains to known genotype strains, and thus expands the study of molecular epidemiology of HAV. The presence of HAV and *E. coli* in oysters otherwise meeting standard criteria poses a risk for gastrointestinal infection, and therefore determination of food safety by testing only fecal coliform bacteria should be interpreted with caution.

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