DETECTION BY DUPLEX RT-COUPLED NESTED PCR OF HEPATITIS A AND ROTAVIRUS IN OYSTERS FROM THAILAND EAST COAST

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Abstract. An efficient and rapid virus detection method is required for routine monitoring and risk assessment in food products. A duplex RT-coupled nested PCR method was developed to detect the simultaneous presence of hepatitis A virus (HAV) and rotavirus in commercial oysters from the eastern coast of Thailand. Primers were designed to amplify HAV VP4 and rotavirus VP7 genes. Although excess amounts of target template of one virus type interfered with RT-PCR amplification of the other, this was overcome by including a nested duplex PCR step. Detection limit for both types of virus of this technique in oyster samples was more than 1,000-fold lower than that of the equivalent monoplex method. Out of 41 oyster samples 63% were positive for either one or both viruses. All rotaviruses belonged to group A G1P[8]. The use of multiplex RT-coupled nested PCR technique provides a cost-effective, rapid, sensitive and efficient tool to detect a wide diversity of viral pathogens and to improve control of virus infection in oysters.

Keywords: duplex RT-coupled nested PCR, food safety, food-borne virus, virus detection

INTRODUCTION

Large outbreaks of hepatitis and gastroenteritis, suspected to have their origins in virus-infected food, have been reported (Oishi *et al*, 1994; Ponka *et al*, 1999; Sánchez *et al*, 2002; Hall *et al*, 2012). Hepatitis A virus (HAV) is a non-enveloped single-stranded positive sense RNA virus, family Picornaviridae, genus *Hepatovirus* (Nainan *et al*, 2006). Among the seven HAV genotypes, four (I, II, III, and VII) have been identified as human pathogens (Costa-Mattioli *et al*, 2002; Nainan *et al*, 2006). This has raised health concerns due to the extreme resistance of HAV to heat, drugs and other chemicals (Elikaei *et al*, 2008). In developing countries of Africa, East Asia and South America, HAV is a common endemic infection (Poovorawan *et al*, 2005), with the majority of children being seropositive by 6 years of age. Improved sanitary conditions in developed countries has reduced the prevalence of and increased susceptibility of the population to infection by HAV, resulting to a high probability of the development of severe symptomatic illness upon infection (Pintó *et al*, 2012).

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Rotavirus, family Reoviridae, contains 11 double-stranded RNA fragments enclosed within a triple-layered capsid (Bishop, 1996). Among the seven serogroups (A, B, C, D, E, F, G), only A, B and C are associated with human disease, with Group A rotavirus being major cause worldwide of infectious gastroenteritis in infants and children under five years of age (Oh *et al*, 2003; Santos and Hoshino, 2005; Parashar *et al*, 2006), but also has been linked to gastroenteritis in adults (Marrie *et al*, 1982; Hrdy, 1987; Timenetsky *et al*, 1996; Krishnan *et al*, 1999; Nilsson *et al*, 2000).

Generally, HAV is transmitted person-to-person but, increasingly, food-borne transmissions have been reported. Exposure to contaminated water, irrigated crops or cultured shellfish, has been linked to food-borne outbreaks (Kukkula *et al*, 1999; Costafreda *et al*, 2006; Le Guyader *et al*, 2006; Pintó *et al*, 2009; Le Guyader *et al*, 2010; Li *et al*, 2012). However, group A rotavirus has not been linked directly with infectious disease following seafood consumption. Transmission through person-to-person contact has been speculated because of the high rates of infection in the first 3 years of life regardless of sanitary conditions, probably from parents with asymptomatic infections to their non-immune children during food preparation (Parashar *et al*, 1998).

Patients with HAV and group A rotavirus infections may excrete viruses in large numbers in feces, 10^6-10^{11} HAV/g (Costafreda *et al*, 2006; Pintó *et al*, 2012) and > 10^{12} rotavirus/g (Gajardo *et al*, 1995; Dubois *et al*, 1997). Wastewater treatments are only partially effective at virus removal (Blatchley *et al*, 2007; El-Senousy *et al*, 2007). Fecal contamination in regions of oyster culture may be due to inadequate water treatment either of sewage or excessive volume from heavy rainfall (Westrell *et al*, 2010). Oysters filter large amounts of water across their gills and can concentrate viruses when grown in fecal-contaminated water. *In situ* studies in oysters have found virus accumulation of up to 99 times that in surrounding water (Burkhardt and Calci, 2000). Bivalve mollusks, especially oysters, consistently have proven to be an effective vehicle for the transmission of viral diseases (Lees, 2000). The association of incidents of infectious disease with oysters probably reflects their traditional consumption as raw or only lightly cooked whole animal including the viscera. Human health problems associated with shellfish consumption in concert with globalization of the food market and variable national standards in food production and safety practices can give rise to disease outbreaks (Falkenhorst *et al*, 2005; Pintó *et al*, 2009; Sarvikivi *et al*, 2012).

The production of healthy oysters is important to Thailand's shellfish industry and the national economy (Fishery Information Technology Center, 2012). In general, food contamination by viruses is not detected due to a lack of appropriate methods. Nevertheless, it is important to be able to detect the presence of food-borne pathogens rapidly, accurately and economically. Culture methods are not practical for routine applications as contaminated food can be expected to contain low levels of virus that still constitute an infection hazard. HAV and rotavirus as low as 10-100 virions can cause infection (Gray, 2011), thus requiring sensitive detection methods. RT-PCR is currently the most sensitive and widely used method for their detection (Chironna *et al*, 2002; Calder *et al*, 2003). Conventional RT-PCR is monoplex, but multiplex RT-PCR is more efficient and cost-effective. The

detection limit of RT-PCR depends on various factors, *viz*, virus type, food matrix, dryness of sample, sample size and presence of enzyme inhibiting substances (Cheong *et al*, 2009). Although processing of samples prior to RT-PCR analysis in order to concentrate viral particles and eliminate RT-PCR inhibitors appears to be useful, in fact it seems to reduce virus levels (El-Senousy *et al*, 2013). Residual low virus levels that still pose a health hazard are difficult to detect by conventional single round RT-PCR (Lees *et al*, 1995), and nested RT-PCR technique is able to overcome this problem (Inoue *et al*, 2006; Jung *et al*, 2007; Fukuda *et al*, 2008; Kitajima *et al*, 2011). In addition, post-PCR characterization by nucleotide sequencing also provides major benefit for epidemiological investigations (Dupinay *et al*, 2014).

The objective of the present study was to evaluate the presence of HAV and rotavirus in oyster sharvested along the east coast of Thailand using nested duplex RT-PCR technique.

MATERIALS AND METHODS

Viral preparation and cell culture

HAV, HM 175 (ATCC 1402), was propagated in epithelial BSC-1cell line derived from the African green monkey, *Cercopithecus aethiops* (ATCC CCL26) in complete Eagle's minimum medium (MEM) (Gibco BRL/Life technologies, Gaitherburg, MD) as previously described (Intamaso and Ketkhunthod, 2014). In brief, after viral adsorption for 90 minutes at room temperature on a platform shaker (Labnet International, Woodbridge, NJ) at 40 rpm, uninfected virus in suspension was replaced with 5 ml of maintenance medium (cell growth medium with 2% fetal bovine serum). Cell cultures were grown until a cytopathic effect (CPE) >70% of the monolayer was observed at approximately 9-10 days post-infection.

Human rotavirus A strain Wa (ATCC 2018) was propagated in a Rhesus monkey kidney MA 104 cell line (ATCC CRL2378.1) in complete MEM (Gibco BRL/Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), 100 mM HEPES, 100 U/ml penicillin-streptomycin (Gibco BRL) and 1 μg/ml fungizone (Gibco BRL) (Arnold *et al*, 2009). Rotavirus was activated by incubating at 37°C for one hour with 10 μg/ml porcine pancreatic type IX trypsin (Sigma Aldrich, St Louis, MO). The inoculum subsequently was diluted 1:10 in 0.5 ml of serum-free MEM supplemented with 1.8 μg/ml porcine pancreatic type IX trypsin (Sigma Aldrich). Cell monolayer was washed twice with 5 ml of pre-warmed serum-free MEM, and added with the virus inoculum. Cells (in flask) were incubated at 37°C under an atmosphere of 5% CO_2 with gentle rocking for 1 hour, then the monolayer was washed once with 5 ml pre-warmed serum-free MEM and added with 5 ml 0.5 μg/ml porcine pancreatic type IX trypsin (Sigma Aldrich). Cells were cultured at 37°C for 9-10 days or until lysis of the monolayer was complete. HAV or rotavirus infected cells were subjected to freezing and thawing 3-4 times and virus suspension was centrifuged at 435*g*, 4°C for 30 minutes and supernatant stored as 0.5 ml aliquots at -80°C for further experiments.

Oyster collection and processing

Sydney rock oysters *(Saccostrea commercialis (glomerata)* cultured along the coast of Chon Buri, Chanthaburi and Rayong Provinces, Thailand were obtained at different locations during November 2012 to February 2013. Oysters were scrubbed,

washed and opened with a sterile knife. Digestive tissues from 3-4 oysters were grounded and pooled as a 1.5 g sample to avoid individual variability in virus infection. A total of 41 samples (31, 5 and 5 grounded samples from Chon Buri, Chanthaburi and Rayong, respectively) were stored at -80°C until analyzed for natural contamination.

For determination of the assay's detection limit, 1.5 g of ground oyster samples were spiked with 10 μl aliquot of a stock RNA template ($2.70x10^{13}$ molecules /ml of HAV or $5.54x10^{12}$ molecules /ml of rotavirus) (see below for preparation of artificial RNA templates) or 10 μl of 10-fold serial dilutions of each template stock. Viral RNA template-spiked samples were processed similarly to naturally contaminated samples as previously described (Intamaso and Ketkhunthod, 2014) but RNAs in oyster tissue homogenates were precipitated with 3% cetyltrimethylammonium bromide (CTAB)-0.4 M NaCl solution.

RT-PCR assays

Viral RNAs from 200 μl of oyster tissue homogenate were dissolved in absolute ethanol and purified using the High Pure Viral Nucleic Acid kit (Roche, Mannheim, Germany). Primers were designed to correspond to specific highly conserved regions of HAV VP4 (GenBank accession no. M14707.1) and rotavirus VP7 (GenBank accession no X99126.1) genes. Primer design was carried out by selecting 18-20 bp segments that contained 40%-60% of GC content and annealing temperature between 55ºC and 60°C for each amplicon (Table 1). Primers were also analyzed for formation of dimers, hairpins and secondary structures using Oligos 9.1 by Ruslan Kalendar (Institute of Biotechnology, University of Helsinki, Finland). As multiplex RT- PCR requires specific amplicons from several strains or organisms to be generated simultaneously, each primer pair has to have its annealing temperature within a small range and yields amplicon of different size. The specificity of each primer was evaluated using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against other food-borne viruses to prevent the amplification of non-specific products. All oligonucleotides were synthesized by Sigma-Aldrich (St Louis, MO).

Firstly, monoplex RT-PCR assays were performed in a 25-μl reaction mixture composed of 1X reaction buffer, 2 mM $MgSO_4$, 0.4 mM dNTPs, 0.4 μM each primer pair (rotavirus: VP7-F1 and VP7-R952; HAV: HAV-F230 and HAV-R991), 2 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 2 μl of RNA template. Duplex RT-PCR was performed as described above except that 0.2 μM HAV primer pair and 1 μl of HAV RNA template were used. Monoplex and duplex RT-PCR assays were carried out using the same thermocycling (T100™ Thermal Cycler, Bio-Rad, Hercules, CA) conditions as follows: 50°C for 30 minutes; 94°C for 2 minutes; 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 68°C for 1 minute; with a final heating at 68°C for 5 minutes. Amplicons were separated by 1.5% agarose gel-electrophoresis, stained with 10,000X SYBR® Gold nucleic acid gel stain (Invitrogen, Carlsbad, CA). Amplicons were extracted from gel using PureLink™ Quick PCR purification kit (Invitrogen, Carlsbad, CA) and stored at -20°C until used.

Nested PCR

A 1 μl aliquot of purified amplicon was transferred to a new batch of The 25-μl PCR mixture contained 1X*Taq* buffer (minus $MgCl_2$), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 μM primer pair of rotavirus

(VP7-F and VP7-R397) or HAV primer pair (HAV-F354 and HAV-R674), and 2.5 U *Taq* polymerase (Thermo-Scientific Fermentas, Rockford, IL). Thermocycling (carried out in T100™ Thermal Cycler; Bio-Rad, Hercules, CA) conditions were as follows: 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 25 seconds; with a final step of 72°C for 15 minutes. Amplicons were analyzed as described above. Samples were analyzed twice in independent experiments to avoid false-positive results.

Construction of viral RNA templates

For construction of HAV RNA template, firstly amplicon generated by RT-PCR using HAV-F354 and HAV-R674 primers was extended with dA at 3′ terminus in a 50-µl reaction mixture containing 15 µl of amplicon, 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dATP, and 1 U *Taq* polymerase (Thermo-Scientific Fermentas, Rockford, IL), which was incubated at 72ºC for 20 minutes. Reaction product was purified using Hiyield™ PCR DNA fragments extraction kit (RBC Bioscience, Taipei, Taiwan) and ligated with RBC TA cloning vector (RBC Bioscience) following the manufacturer's protocol. After incubation at 4°C overnight, 5 µl aliquot of the reaction solution was transfected into *E. coli* JM109 and transformants were selected on 100 µg/ml ampicillin and 20 µg/ml X-Gal supplemented agar plate. Plasmid DNA from transformed bacterial colonies were extracted using Hiyield™ Plasmid mini kit (RBC Bioscience) and sequenced (see below) to confirm the identity of the HAV inserts. The recombinant plasmid, which contains T7 promoter at 5′ terminus was amplified using M13 forward and reverse primers (a 25-µl PCR reaction mixture containing 1X *Taq* buffer (without $MgCl_2$), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 µM each primer, and 2.5 U *Taq* polymerase (Thermo-Scientific Fermentas). The amplicon was purified using PureLink™ Quick PCR Purification Kit (Invitrogen, Carlsbad, CA). Owing to technical difficulties, DNA template for rotavirus RNA preparation was constructed by PCR using rota-T7 forward primer containing T7 promoter sequence at 5′ terminus and VP7-R952 primer (Table 1) with thermocycling conditions as follows: 95ºC for 3 minutes; 30 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 1 minute; with a final step of 72ºC for 15 minutes. Amplicon was analyzed by agarose gel-electrophoresis as described above and purified using PureLink™ Quick PCR Purification Kit (Invitrogen). *In vitro* transcription was carried out in a 50-µl reaction solution composed of 1X transcription buffer (USB, Cleveland, OH), 4 mM NTPs (Promega), 10 U RNase inhibitor (Promega), 3 µl of purified HAV or rotavirus DNA template, and 4 U T7 RNA polymerase (USB, Cleveland, OH). After incubation for 2 hours at 37°C, 5 U DNAse (Sigma-Aldrich) were added, then the solution was incubated for a further 15 minutes at 37°C and reaction terminated at 75°C for 10 minutes. RNA transcript was purified using High Pure Viral Nucleic Acid kit (Roche, Mannheim, Germany) according to manufacturer's protocol but omitting the denaturation step. RNA concentration (µg/ml) was determined spectrophotometrically (1 $A_{260\ nm}$ = 40 µg/ml) and converted to molecules/ml using a MW of HAV and rotavirus RNA of 2.70×10^{13}molecules/ml and 5.54×10^{12}molecules/ml, respectively (Morales-Rayas *et al*, 2010).

Test for interfering factors of duplex RT-PCR assay

A series of serially ten-fold dilutions (10^0, 10^{-1}, 10^{-2}, and10^{-3}) of HAV or rotavirus samples (initial amount = 12.42 ng) were

employed to evaluate their effects on the detection efficiency of duplex RT-PCR.

Sensitivity test of monoplex and duplex RT-PCR assays

Oyster samples were spiked with 10 µl of rotavirus RNA (ranging from 8.31 to 8.31×10^8 molecules) and HAV (ranging from 4.05×10^1 to 4.05×10^9 molecules) to evaluate sensitivity of monoplex and duplex RT-PCR assays. For the latter assay, a mixture of HAV and rotavirus RNA templates were employed. Nested PCR assay also was performed to confirm the specificity of RT-PCR products as described above. Negative control contained no spiked viral RNA. Experiments were conducted in duplicate.

Nested duplex RT-PCR assay of collected oysters

Nested duplex RT-PCR assay was performed on collected oysters as described above. All virus-negative samples were diluted 10-fold and subjected to another round of nested duplex RT-PCR to avoid false-negative results caused by inhibitors. In addition, virus-negative samples were subjected to PCR amplification of 18S rDNA as previously described (Yishuai *et al*, 2013).

DNA sequencing

Nested PCR amplicons (and DNA inserts of recombinant plasmids) were sequenced to verify positive results and evaluate the variability of detected HAV and rotavirus strains. Nested PCR amplicons were extracted from agarose gel and purified using PureLink Quick PCR Purification kit (Invitrogen). Sense strands were sequenced using VP7-F primer and HAV-F354 primer for rotavirus and HAV, respectively (First BASE Laboratories SdnBhd, Selangor, Malaysia). Sequences were compared with those deposited with GenBank and EMBL using PubMed NCBI BLAST program (Rotavirus GenBank accession no.EU984109.1 and EF179194.1; HAVGenBank accession no. M14707).

RESULTS

Sensitivity of monoplex and duplex RT-PCR assays for HAV and rotavirus

Monoplex RT-PCR generated amplicon of 762 and 952 bp from RNA of HAV and rotavirus, respectively (data not shown). No reaction was obtained against non-target virus (data not shown). Nested PCR assay verified that correct RT-PCR was produced, namely, 321 and 397 bp for HAV and rotavirus, respectively (data not shown).The limit of detection for HAV and rotavirus was12.42 pg (2.89×10^7molecules) and 66 pg (1.23×10^8 molecules), respectively (data not shown). Employing duplex RT-PCR assay, the same two amplicons as in monoplex RT-PCR were obtained (Fig 1a). Detection limit of duplex RT-PCR assay was 12.42 and 120 pg for HAV and rotavirus, respectively, being 2-fold higher in the latter case (Fig 1a, lane 4). RT-PCR amplicons were not generated when template RNAs were absent from the assay (Fig 1a).

Interference test of duplex RT-PCR assay for HAV and rotavirus

In duplex RT-PCR assay, if the concentration of one template is much higher than the other template, there is the possibility that amplification of the latter may be compromised. Rotavirus concentration of at least 1,000-fold higher than that of HAV affected amplification of the latter, whereas only a 10-fold excess of HAV was sufficient to interfere with amplification of rotavirus template (Fig 1b).

Sensitivity of monoplex and duplex RT-PCR in detection of HAV and rotavirus in spiked oyster samples

Oyster samples (1.5 g) were spiked

Table 1
Primers used in the study.

Name	Sequence (5′-3′)	Amplicon size (bp)	Tm (°C)	Nucleotide position
VP7-F	ATGTA GGTATTGAATATACCAC	952	60	1 - 23
VP7-R952	CTAACGATCTCGATCTTTTGG		60	932 - 952
HAV-F230	TGTAGGAGTCTAAATTGGGGA	762	60	230 - 249
HAV-R991	CTTCATGGAAAAGAGCATGTG		60	974 - 991
VP7-F	ATGTATGGTATTGAATATACCAC	397	60	1 - 23
VP7-R397	ACTGATCCTGTTGGCCAWCC		62	378 - 397
HAV-F354	5′-GCTACGGGTGAAACCTCTTA-3′	321	60	354 - 373
HAV-R674	GGAAAAACCTAAATGCCCCTG		62	654 - 674
RS18-F	GCCATCAAGGGTATCGGTAGAC	168	68	116 - 137
RS18-R	CTGCCTGTTAAGGAACCAGTCAG		70	261 - 283
Rota-T7	TAATACGACTCACTATAGGGATGTAT GGTATTGAATATACC	952	110	-20 - +21
VP7-R952	CTAACGATCTCGATCTTTTGG		60	932 - 952
M13R	TGTAAA \ ACGACGGCCAGT	-	54	-
M13F	CAGGAAACAGCTATGACC	-	52	-

Tm, melting temperature; W, A or T; underlined sequence,T7 promoter.

with *in vitro* generated HAV and rotavirus RNA templates, either individually or combined for monoplex or multiplex RT-PCR assay, respectively. Limit of detection of monoplex RT-PCR assay was 405 HAV RNA molecules (Fig 2a), whereas no amplifications of rotavirus RNA templates were apparent (Fig 2c). Authenticity of RT-PCR amplicons was verified by nested PCR, which produced the expected 321 bp HAV amplicon (Fig 2b), but, unexpectedly, that of 397 bp rotavirus amplicon, with a detection limit of 8 RNA molecules (Fig 2d). However, the band intensity obtained from nested PCR amplification did not decrease in concordance with RT-PCR amplicon band intensities, indicating saturation in nested PCR amplification capacity and suggesting that the detection limit for HAV had not been reached. Theoretically, PCR has potential to amplify one molecule of DNA template, but with the large amounts of DNA template, nested PCR further amplifies them, reaching a plateau phase of PCR amplification. Thus the band intensity of nested PCR amplicon quickly becomes saturated and does not decrease in concordance with the serial dilution template. Likewise, nested PCR further enhanced duplex RT-PCR reaction for HAV and rotavirus RNA templates to 4.05×10^6 and 8.31×10^5 molecules, respectively (Fig 3).

Detection in oyster samples of HAV and rotavirus by duplex-RT-coupled nested PCR Assay

The duplex RT-coupled nested PCR assay revealed that 14/41(34%) and 9 (22%) samples contained only HAV and rotavirus, respectively, and that 3 (7%) harbored both types of viruses (Fig 4). Ten samples were considered true negatives as they were positive for amplification of oyster 18S rDNA (data not shown). The remaining 5 samples were positive

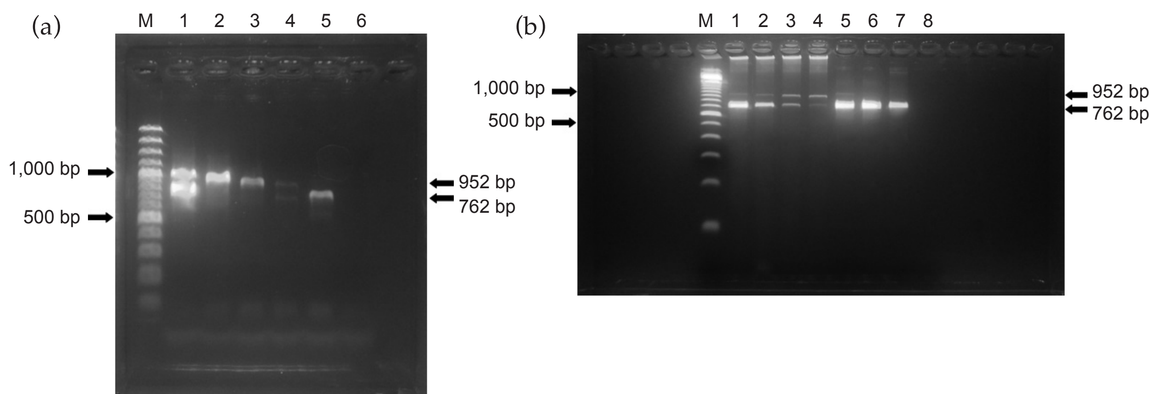

Fig 1–Determination of (a) sensitivity and (b) interference of duplex-RT-PC in detection of HAV and rotavirus. Experimental protocols are described in Materials and Methods, and primers used are listed in Table 1. (a) Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker (Thermo Scientific); lane 1, 12.42 ng of rotavirus RNA:12.42 ng of HAV RNA; lane 2, 12.42 ng of rotavirus RNA:0.12 pg of HAV RNA; lane 3, 1.24 ng of rotavirus RNA:1.24 pg of HAV RNA; lane 4, 0.12 ng of rotavirus RNA:12.42 pg of HAV RNA; lane 5, 12.42 pg of rotavirus RNA: 0.12 ng of HAV RNA; lane 6, reaction control **(**water**).** (b) Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker (Thermo Scientific); 1ane 1, 12.42 ng of HAV RNA:12.42 ng of rotavirus RNA; lane 2, 1.24 ng of HAV RNA:12.42 ng of rotavirus RNA; lane 3, 0.12 ng of HAV RNA:12.42 ng of rotavirus RNA; lane 4, 12.42 pg of HAV RNA:12.42 ng of rotavirus RNA; lane 5, 12.42 ng of HAV RNA:1.24 ng of rotavirus RNA; lane 6, 12.42 ng of HAV RNA:124 pg of rotavirus RNA; lane 7, 12.42 ng of HAV RNA:12.42 pg of rotavirus RNA; lane 8, negative control (water).

for amplification of oyster 18S rDNA but PCR negative for both types of viruses following 10-fold dilution to avoid false-negative results caused by inhibitors. Thus, overall, 26 samples were virus-positive. Sequence analysis of all nested PCR rotavirus amplicons showed 99% identity with group A rotavirus, genotype G1P[8] by typing at the VP7and VP4 regions (data not shown). However, HAV genotype could not be identified because amplicons were not from a HAV conserved region.

DISCUSSION

Multiplex RT-PCR has been used for routine simultaneous laboratory testing of various viral species in clinical samples in environmental samples and in food (Elnifro *et al*, 2000; Yan *et al*, 2003). Application of this technique in food samples is difficult due to lower levels of viral pathogens than those found in clinical samples. However, oysters accumulate viruses in their stomach, thus the dissecting of this organ could increase the opportunity to detect viral pathogens in bivalve samples (Le Guyader *et al*, 2000). Detection of food-borne virus requires the extraction of virus genome from food samples, which may contain as few as 10-100 viral particles, equivalent to an infectious dose, without compromising virus genome quality, especially RNA viruses. Several previous studies have used a combination of RT-PCR and nested PCR to enhance sensitivity compared to that of the conventional RT-PCR (Inoue *et al*, 2006; Jung *et al*, 2007).

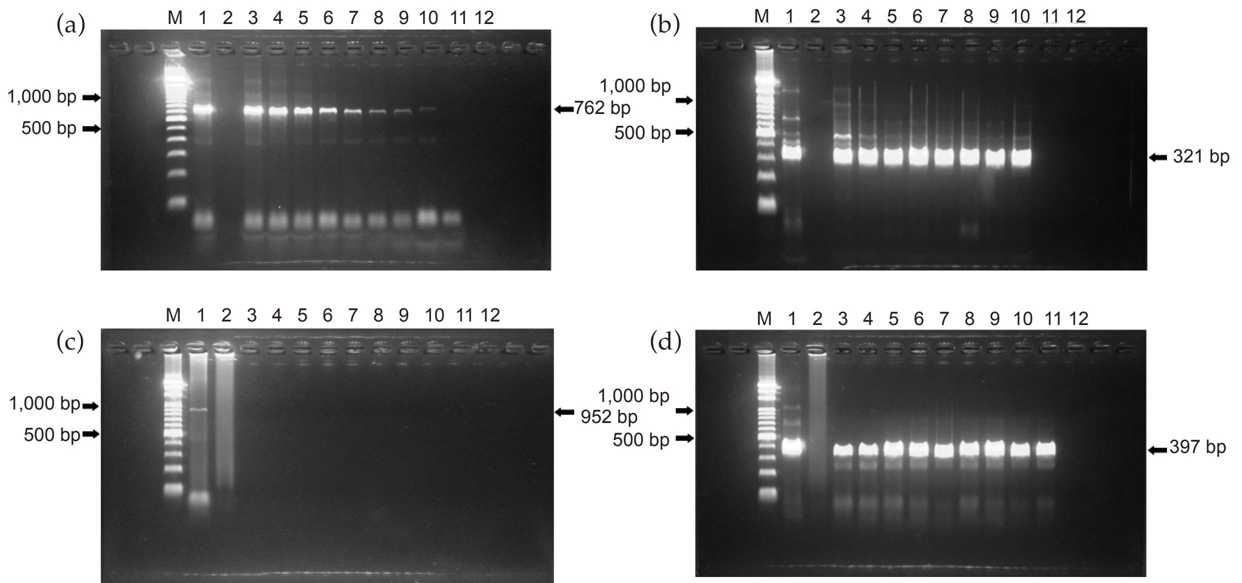

Fig 2 –Monoplex RT-PCR (a, c) followed by nested PCR (b, d) in detection of HAV and rotavirus RNA spiked in oyster samples. Experimental protocols are described in Materials and Methods, and primers used are listed in Table 1. (a) and (b) Lane M; 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with HAV; lane 2, non spiked; lane 3, spiked with 4.05×10^9 HAV RNA molecules; lane 4, spiked with 4.05×10^8 HAV RNA molecules; lane 5, spiked with 4.05×10^7 HAV RNA molecules; lane 6, spiked with 4.05×10^6 HAV RNA molecules; lane 7, spiked with 4.05×10^5 HAV RNA molecules; lane 8, spiked with 4.05×10^4 HAV RNA molecules; lane 9, spiked with 4.05×10^3 HAV RNA molecules; lane 10, spiked with 4.05×10^2 HAV RNA molecules; lane 11, spiked with 40 HAV RNA molecules; lane 12, negative control (water). (c) and (d) Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with rotavirus; lane 2, non spiked; lane 3, spiked with 8.31×10^8 rotavirus RNA molecules; lane 4, spiked with 8.31×10^7 rotavirus RNA molecules; lane 5, spiked with 8.31×10^6 rotavirus RNA molecules; lane 6, spiked with 8.31×10^5 rotavirus RNA molecules; lane 7, spiked with 8.31×10^4 rotavirus RNA molecules; lane 8, spiked with 8.31×10^3 rotavirus RNA molecules; lane 9, spiked with 8.31×10^2 rotavirus RNA molecules; lane 10, spiked with 8.31×10^1 rotavirus RNA molecules; lane 11, spiked with 8 rotavirus RNA molecules; lane 12, negative control (water).

In this study contamination of HAV and rotavirus in oysters obtained along the east coast of Thailand was successfully assessed simultaneously by duplex RT-coupled nested PCR technique. The development of multiplex RT-PCR poses a greater challenge than that of monoplex RT-PCR. In most cases, sensitivity of duplex PCR is about 10-100-folds lower than that of monoplex PCR, especially if one of the viruses is present in concentrations much lower than those of other viruses amplified in the same assay (Tsai *et al*, 1994; Jackson *et al*, 1996). For this reason, individual nested PCR assay is recommended for rapid detection of viruses, especially in environmental or food samples in which many different types of viruses

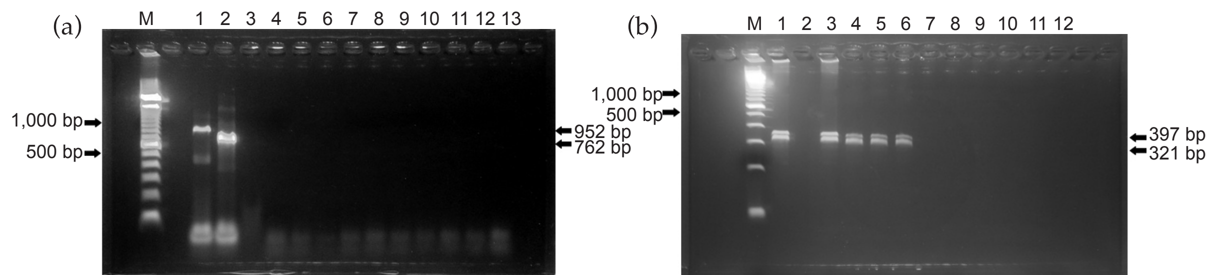

Fig 3–Duplex RT-PCR (a) followed by nested PCR (b) in detection of HAV and rotavirus RNA spiked in oyster samples. Experimental protocols are described in Materials and Methods, and primers used are listed in Table 1. (a) Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with rotavirus; lane 2, monoplex positive control with HAV; lane 3, non spiked; lane 4, 8.31×10^8 rotavirus RNA molecules:4.05×10^9HAVmolecules; lane 5, 8.31×10^7 rotavirus RNA molecules: 4.05×10^8 HAV molecules; lane 6, 8.31×10^6 rotavirus RNA molecules: 4.05×10^7HAV molecules; lane 7, 8.31×10^5rotavirus RNA molecules: 4.05×10^6 HAV molecules; lane 8, 8.31×10^4 rotavirus RNA molecules: 4.05×10^5 HAV molecules; lane 9, 8.31×10^3 rotavirus RNA molecules: 4.05×10^4 HAV molecules; lane 10, 8.31×10^2 rotavirus RNA molecules: 4.05×10^3 HAV molecules; lane 11, 8.31×10^1 rotavirus RNA molecules: 4.05×10^2 HAV molecules; lane 12, 8 rotavirus RNA molecules: 40 HAV molecules; lane 13, negative control (water). (b) Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, duplex positive control with HAV and rotavirus; lane 2, non spiked; lane 3 8.31×10^8 rotavirus RNA molecules: 4.05×10^9 HAV molecules; lane 3, 8.31×10^7 rotavirus RNA molecules: 4.05×10^8HAV molecules; lane 5, 8.31×10^6 rotavirus RNA molecules: 4.05×10^7 HAV molecules; lane 6, 8.31×10^5 rotavirus RNA molecules: 4.05×10^6 HAV molecules; lane 7, 8.31×10^4 rotavirus RNA molecules: 4.05×10^5 HAV molecules; lane 8, 8.31×10^3 rotavirus RNA molecules: 4.05×10^4 HAV molecules; lane 9, 8.31×10^2 rotavirus RNA molecules: 4.05×10^3 HAV molecules; lane 10, 8.31×10^1rotavirus RNA molecules: 4.05×10^2 HAV molecules; lane 11, 8 rotavirus RNA molecules:40 HAV molecules; lane 12, negative control (water).

occur (Lees, 2000; Mounts *et al*, 2000; Formiga-Cruz *et al*, 2002).We showed that nested duplex relative to monoplex RT-PCR was >1,000 less sensitive in the detection of target viruses in oyster extracts. However, with the correlation of HAV particles with genomic quantity, where 1 PFU = 10^5 genome copies (Coudray *et al*, 2013), nested duplex RT-PCR technique can detect approximately 40 PFU HAV, which is sensitive enough to detect viruses within the range of an infectious dose (Gray, 2011).

In this study, RT-PCR amplification of rotavirus was more sensitive to the greater concentration of HAV than the converse. These results were not consistent with the previous observation that the amounts of each virus did not affect the amplification of the other viruses at the optimal primer concentrations (Tsai *et al*, 1994).This could be rectified by the inclusion of a nested PCR step following the duplex RT-PCR procedure. In fact, nested PCR generated amplicons (visualized in agarose gel) from RT-PCR products not previously discernable.

Unfortunately, cytopathic effect (CPE) was unable to be observed with human rotavirus A strain Wa (ATCC 2018), the ref-

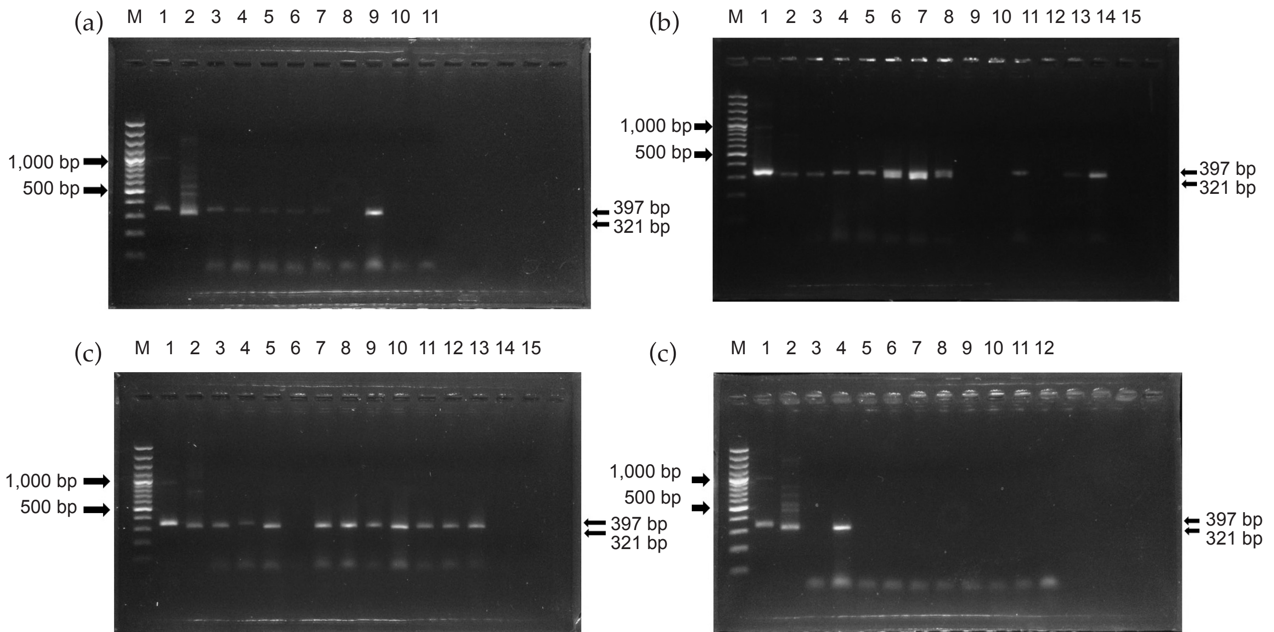

Fig 4–Duplex RT-coupled nested PCR in detection of HAV and rotavirus in oyster samples. Experimental protocols are described in Materials and Methods, and primers used are listed in Table 1. (a) Oyster samples, set 1, collected from Chon Buri Province, Thailand. Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with rotavirus; lane 2, monoplex positive control with HAV; lanes 3-10, oyster sample numbers 1-8; lane 11, negative control (water). (b) Oyster samples, set 2, collected from Nong Mon market, Chon Buri Province. Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with rotavirus; lane 2, monoplex positive control with HAV; lanes 3-14, oyster sample numbers 1-12; lane 15, negative control (water). (c) Oyster samples, set 3, collected from Aung Sira fish market, Chon Buri Province. Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with rotavirus; lane 2, monoplex positive control with HAV; lanes 3-14, oyster sample numbers 1-12; lane 15, negative control (water). (d) Oyster samples collected from Rayong and Chanthaburi Provinces. Lane M, 100 bp markers (GeneRuler 100 bp Plus DNA ladder marker, Thermo Scientific); lane 1, monoplex positive control with rotavirus; lane 2, monoplex positive control with HAV; lanes 3-6, oyster sample from Rayong numbers 1-4; lanes 7-11, oyster sample from Chanthaburi numbers 1-5; lane 12, negative control (water).

erence strain, and thus the concentration of virus particles could not be detected by plaque assay. Thus, sensitivity of the duplex RT-coupled nested PCR assay was assessed by spiking uncontaminated oyster samples with known amounts of *in vitro* transcribed and purified HAV and rotavirus RNA templates, demonstrating detection levels of 400 and less RNA molecules per assay. This is equivalent to < 1 virus particle/g oyster tissue. This is consistent with a study of norovirus, which found that nested RT-PCR is 10-folds more sensitive than conventional RT-PCR (Schultz *et al*, 2007).

It is important to demonstrate that

negative duplex RT-coupled nested PCR results are not due to inhibitor(s) in the oyster samples (Jung and Chae, 2005; Cheong *et al*, 2009; Intamaso and Kethkhunthod, 2014).This was tested by repeating the assay with diluted samples and including amplification of an internal control (oyster 18S rDNA). Another possible cause of false-negative results in PCR-based assays is nucleotide variations of strains in the primer-binding sites. In our study, HAV primers were designed to correspond to HAV VP4 gene, which is more highly conserved than rotavirus VP7 gene (GenBank accession no. M14707.1 and X99126.1, respectively). Thus, detection of rotavirus in oyster samples has a greater risk of false-negatives than that of HAV.

Oysters from the eastern coast of Thailand harbored HAV and rotavirus, either singly or both, with prevalence of HAV being higher. A double contamination of rotavirus and HAV has been reported in 8% of oysters from Galicia, Spain (Romalde *et al*, 2002). In Thailand, it has been reported that 3.8% of oysters, 2.9% of cockles and 6.5% of mussels collected from a culture farm along the coast of Surat Thani Province and two retailed markets in Bangkok are positive for HAV (Namsai *et al*, 2011). Noroviruses were also detected in 38% of oysters collected from local markets and oyster farms in southern Thailand (Kittigul *et al*, 2011). All rotavirus-positive samples were found to belong to group A G1P[8]. However, we cannot exclude the possibility that the primer set used was not sensitive enough to detect other rotavirus strains (Gabreili *et al*, 2007).

In Thailand, there is, as yet, no surveillance system to investigate the transmission of viruses among humans who have ingested contaminated seafood. Oysters may have been cultured in water contaminated with human waste from which they accumulate viruses in their digestive organs. As a consequence of market globalization, there is a distinct possibility of introducing new strains or non-indigenous enteric viruses that may cause outbreaks. Thus, food products must be carefully monitored for virus contamination. However, several shortcomings must be addressed before the inclusion of virus analysis of food products as a regular procedure, such as robustness of the techniques, cost of virus monitoring, harmonization and standardization of the assays and the ever changing nature of the target virus genomes.

In conclusion, the current study describes the development of duplex RT-coupled nested PCR for simultaneous detection of HAV and rotavirus in oyster with high specificity and sensitivity. This approach can be adapted for detecting other types of viruses co-existing in food. Multiplex RT-coupled nested PCR provides a significant advantage over that of monoplex RT-coupled nested PCR in providing simultaneous detection of multiple virus species or diverse viral strains and at a lower cost.The information obtained provides valuable data on the prevalence of single and mixed infections of HAV and rotavirus in oyster samples from the eastern coast of Thailand and emphasizes the need for simultaneous detection of multiple virus species. The technique provides an efficient tool to improve control of virus infections in oysters and for epidemiological investigations.

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