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Reverse Transcriptase PCR detection of Hepatitis A virus (HAV) in cultured and wild shellfish from the Peninsular of Malaysia

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Abstract: Hepatitis A is a liver infection caused by the hepatitis A virus (HAV). Outbreaks of hepatitis A have been linked to the consumption of both raw and cooked shellfish. These outbreaks could induce a public confidence problem over shellfish safety and may result in important economic losses for the seafood industry. The work presented in this study investigated the presence of HAV in shellfish from Peninsular Malaysia. A total of 365 of cultured and wild shellfish from 36 sampling locations located throughout Peninsular Malaysia were examined using a commercial nucleic acid extraction and reverse transcription -polymerase chain reaction (RT-PCR) kit. HAV was not detected in almost all of the shellfish samples examined. Only one cockle sample from Changkat, Seberang Perai was positive for HAV. The results suggest the absence of HAV or very low amount of HAV viral particles in most of the shellfish examined.

Keywords: Hepatitis A virus, wild, cultured, shellfish, Peninsular Malaysia

Introduction

Shellfish are readily contaminated with viruses and bacteria present in water because of the concentrating effect of their filter feeding nature. As a consequence, they have been implicated as important vectors in the transmission of many enteric diseases (Lees, 2000). Hepatitis, which is caused by Hepatitis-A virus (HAV), is one of the most serious infectious diseases epidemiologically associated with shellfish consumption (Koopmans *et al*., 2002). Hepatitis-A virus (HAV) is an environmentally stable, positive single-stranded RNA virus belonging to the Hepatovirus group of the *Picornaviridae* family and is transmitted by the fecal-oral route. The outbreaks of hepatitis A have been linked to the consumption of both raw and cooked shellfish (Rippey, 1994). These outbreaks could induce a public confidence problem over shellfish safety and may result in important economic losses for the seafood industry.

The fact that shellfish can serve as vectors of important viral pathogens including HAV, has led to a recognized need for improvement of the sanitary control measures of this product. Current regulations of the sanitary quality of shellfish and shellfish harvesting waters are based on bacterial indicators namely fecal coliforms and *Escherichia coli*. However, it has been documented that such indicators are not correlated with the presence of viral pathogens (Doré *et al*., 1998) and shellfish meeting bacterial sanitary quality has been involved in disease outbreaks (Le Guyader *et al*., 2000). Furthermore, enteric viruses are very resistant to physical and chemical inactivation and may persist in shellfish tissue after depuration. Owing to these, the development of methods for rapid and reliable detection of human specific viral pathogen has become an important research goal over the last decade. Nucleic acid-based techniques, especially reverse transcriptase-polymerase chain reaction (RT-PCR), have emerged as methods of choice for sensitive and specific detection of enteric virus.

In Malaysia, the monitoring of shellfish and shellfish waters relies on fecal coliforms and *E. coli* counts according to the European Committee regulation (Directives 91/492/EC) (Wan Norhana and Nor Ainy, 2004; Wan Norhana *et al*., 2006). To our knowledge, very limited study has been carried out to detect the presence of viral pathogens in local shellfish. Hence, this study was carried out to detect the presence of HAV in wild and cultured shellfish of Peninsular Malaysia, using a commercially available viral extraction and detection kit.

Materials and Methods

Virus

Hepatitis A Virus (Enterovirus 72), cytopathic HM 175 (Clone 2) in infected cell lysates was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Virus stock titer was 10^6 $TCID_{50}$ (50% tissue culture infective dose).

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Table 1. Sample types and sampling locations

Date of sampling	Sampling locations States		Common name/local name (sources)	No. of samples (n)
2/8/06	Penang	Georgetown	Cockles (retail)	15
11/8/06	Seberang Perai	Tok Kramat	Cockles (cultured)	2
		Bukit Tambun		2
		Kuala Juru		2
		Changkat		2
		Batu Kawan		2
		Sg. Belanak		2
		Sg. Udang		2
		Sg, Acheh		2
22/8/06	Perak	Manjong	Cockles (cultured)	6
		Kuala Trong		6
		Kuala Sangga		6
3/10/06		Sg. Jarum Mas		30
12/9/06	Kelantan	Laguna Semarak	Oysters (wild)	6
			Clams (wild)	6
			Tijah (wild)	6
			Lala (wild)	6
26/11/06		Pantai Seri Tujuh	Clams (wild)	6
		Sg. Semarak	Oysters (cultured)	6
		Pasir Mas	Tijah (wild)	4
			Lala (wild)	4
			Clams (wild)	4
		Sg. Setiu	Cockles (wild)	4
			Lala (wild)	4
		Pantai Seri Tujuh	Etak (wild/freshwater)	12
			Tijah ((wild)	6
			Clams (wild)	6
27/11/06		Sg. Ketereh	Etak (wild/freshwater)	24
21/9/06	Selangor	Pasir Penambang	Cockles (cultured)	6
		Sungai Buloh	Anakdara dua sebilik (wild)	6
			Lala (wild)	6
		Bagan Sungai Besar	Siput belitung (wild)	2
			Cockles (wild)	2
		Sungai Nibong	Cockles (wild)	2
		Sungai Lemau	Mentarang (wild)	2
		Bagan Nakhoda Omar	Cockles (wild)	2
		Pantai Tok Muda	Cockles (wild)	6
2/11/06	Johor	Kg. Sungai Melayu, Johor Bharu	Mussels (cultured)	16
		Sungai Muar	Mussels (cultured)	16
		Batu Pahat	Oysters (cultured)	10
5/11/06	Terengganu	Merchang	Oysters (cultured)	16
		Setiu		16
15/11/06	Melaka	Telok Mas	Mussels (cultured)	16
		Jasin		16
17/11/06	Kedah	Kuala Muda	Cockles (cultured)	24
28/11/06	Pahang	Rompin	Mussels (cultured)	24
TOTAL				365

Viral RNA extraction and RT-PCR kits

Viral RNA extraction was carried out using the RNeasy® kit (Qiagen, Hilden, Germany) while the Qiagen® One-Step RT-PCR kit (Qiagen, Hilden, Germany) was used for the detection of HAV in shellfish tissue.

Shellfish samples

A total of 365 of wild and cultured shellfish including blood cockles (*Anadara granosa*, *Anadara* sp.), oysters (*Crassostrea* sp.), clams (*Geloina cloaxans*), green mussels (*Perna viridis*), lala (*Paphia* sp.), tijah, mentarang, siput belitung as well as freshwater mollusc, etak (*Corbiculla fluemicea*), from 36 sampling stations collected from July until November 2006 were employed in this study (Table 1). The samples were transported in ice-cooled insulated box to the laboratory and processed immediately.

Sensitivity of the commercial kit

The first stage of this study was to determine the sensitivity of the one-step RT-PCR kit. With this aim, undiluted and serially diluted HAV stocks (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were prepared with and without Q solution. Q solution is an innovative additive by the manufacturer that facilitates amplification of difficult templates by modifying the melting behavior of nucleic acids. However, the components of Q solution were not revealed by the manufacturer. RNA was extracted by heat treatment.

Viral RNA extraction

At the laboratory, the shellfish were washed and scrubbed thoroughly in running water and opened aseptically. Shellfish flesh was shucked and the stomach and digestive diverticula were dissected. Viral RNA extraction was carried out according to the protocol supplied with the kit. Briefly, 30 mg of shellfish digestive tissue was homogenized with a homogenizer (IKA Ultra Turrax T25, Staufen, Germany) for 1 min in lysis buffer (contains guanidine thiocyanate) supplemented with 1% B-mercaptoethanol (Sigma, St. Luis, MO, USA). After a brief incubation at 4°C, the mixture was centrifuged at 10, 000 x g for 4 min at 4°C. RNA was extracted from the supernatant by a spin column method and finally eluted in 50 µl of RNAse-free water. Total RNA yield (µg/ml) and purity (A_{260}/A_{280}) were determined spectrophotometrically (Biophotometer, Eppendorf, Germany). To act as a positive control, 30 mg of shellfish tissue was spiked with 10 µl of corresponding serial dilution (10^{-1} and 10^{-4}) of the HAV stock and subjected to the same extraction procedure.

RT-PCR analysis of viral RNA

The sequence of the RT-PCR primers used in this study was based on the sequence of wild type HAV (strain HM-175). This primer amplified 489 bp regions (nucleotides 6256 to 6744) (Goswami *et al*., 1993). The sequence of the forward primer was 5' ATGC TATCAACATGGATTCATCTCCTGG 3' while the sequence of reverse primer was 5' CACTCATGATTCTACCTGCTTCTCTAATC 3'. The primers were synthesized by Research Biolabs Sdn. Bhd., Ayer Rajah Crescent, Singapore and stored at -20°C.

RT-PCR was carried out in a volume of 50.0 µl reaction mixtures containing 10.0 µl 5X Qiagen One-Step RT-PCR buffer (12.5 mM $MgCl_2$), 2.0 µl dNTP Mix (10 mM of each dNTP), 10.0 µl Q solution, 0.3 µl of each forward and reverse primer (0.6 µM), 2.0 µl Qiagen One-Step RT-PCR Enzyme Mix, template RNA (1 pg-2 µg) and RNAse free water to make up to 50.0 µl. RT-PCR was performed with a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: reverse transcription at 50°C/30 min; initial PCR activation at 95°C/15 min; 3-step cycling-denaturation at 94°C/40 s, annealing at 49°C/40 s and extension at 72°C/60 s for 25-40 cycles and left at 4°C until next step. PCR activation at 95°C/15 min was included prior to the initiation of PCR cycles because Qiagen kit contained a hot-start *Taq* polymerase.

From each RT-PCR product, 10 µl was electrophoresed on 1% agarose gel stained with ethidium bromide and amplicons were visualized with gel-photodocumentation. Included in each run were a negative control (containing no nucleic acid) and a positive control (RNA from viral stocks). The sample was considered as positive or HAV was considered present when amplicons were detected by gel electrophoresis.

Results and Discussion

Several studies have demonstrated the successful application of PCR in the detection of HAV in laboratory. However, few studies have been reported on the application of molecular methods for HAV in naturally polluted shellfish (Chironna *et al*., 2002). Hence in this study, we attempted to detect the presence of HAV in wild and cultured shellfish of Peninsular Malaysia by RT-PCR technique.

The sensitivity of the optimized RT-PCR system employed in this study was determined by using 10-fold serial dilutions of HAV stock. The performance of the RT-PCR system was demonstrated with viral RNA of HAV stock solutions detectable up to a dilution of 10^{-3} (Figure 1). As indicated in Fig. 1, the incorporation of Q solution has proven to be effective in increasing the RT-PCR specificity. The performance time required to complete the RNA extraction per sample was 20-30 min while 4-5 hrs was required for the detection of HAV in shellfish tissue.

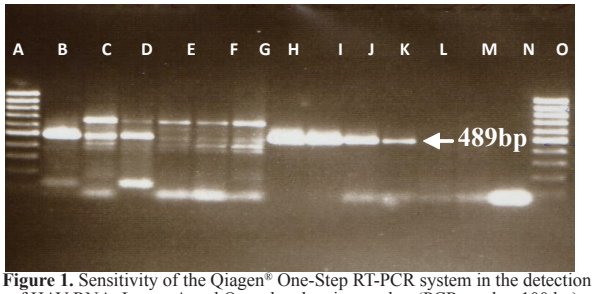

Figure 1. Sensitivity of the Qiagen® One-Step RT-PCR system in the detection of HAV RNA. Lanes A and O, molecular size marker (PCR marker 100 bp); Lanes B-G, amplification of RNA from dilutions of the HAV stock (without Q solution) at concentrations of undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},10^{-5}; Lanes H-M, amplification of RNA from dilutions of the HAV stock culture (with Q solution) at concentrations of undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},10^{-5}; Lane N, negative control. Arrows denote 489 bp HAV amplicon

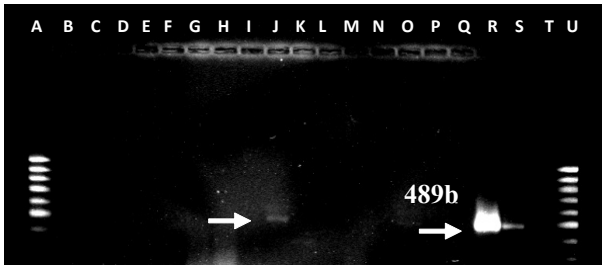

Figure 2. Detection of HAV-specific amplicon in cockle samples from Penang by RT-PCR. Lanes A & U, molecular size marker (PCR marker 100 bp); Lanes B & C, cockle samples from Kuala Juru; Lanes D & E, cockle samples from Sg. Batu; Lanes F & G, cockle samples from Bukit Tambun; Lanes H & I, cockle samples from Batu Kawan; Lanes J & K, cockle samples from Changkat; Lanes L & M, cockle samples from Sg. Udang; Lanes N & O, cockle samples from Sg. Acheh; Lanes P & Q, cockle samples from Teluk Kumbar; Lanes R & S, positive control (30 mg of shellfish tissue seeded with 10^{-1} and 10^{-3} of the HAV stock); Lane T, negative control. Arrows denote 489 bp HAV amplicon

For sensitive detection of viruses from food by RT-PCR, nucleic acid extracts from the food matrix must be pure from inhibitory substances such as polysaccharides, lipids and proteoglycans so as not to interfere with the reverse transcriptase activity. Results obtained indicates that the extraction kit used was effective in the removal of PCR inhibitors from the shellfish samples since HAV was clearly detected in two of the HAV positive samples (Figure 2).

HAV was not detected in majority of the shellfish samples examined although the number of samples analyzed was extensive (365 shellfish samples). After amplification, only one cockle sample harvested on 11/8/206 from Changkat, Seberang Perai was positive for HAV (Figure 2). A very vague HAV amplicon was noticed in cockles sample from nearby Sg. Acheh on the same date (Figure 2). The results suggest the absence of HAV or very low amount of HAV viral particles in most of the shellfish examined and most probably not due to technical problem as the efficiency of the RT-PCR was controlled by running the seeded HAV or positive control. Sampling period may also be an important factor in determining the outcomes of this kind of study. The decrease in the prevalence of HAV in warmer months for example is well known (Le Guyader *et al*., 2000) due to lower circulation and rapid degradation of viral particles at high temperatures and direct sunlight. Finally, it is also noteworthy that the kit used in this study was not designed to be applied to shellfish tissue. Currently, specific HAV extraction kit for shellfish is not readily available in the market, thus we have to use the general HAV detection kit.

The absence of HAV in 30 retailed cockles from Serdang Malaysia, analyzed using various extraction methods, RT-nested PCR and real time RT-PCR from Serdang, Malaysia has been reported (Tek, 2009). Similarly Vilariño *et al*. (2009) also failed to detect HAV in wild and cultured shellfish in France. Others, however, have reported much higher HAV prevalence such as 26% in mussels and clams from Tunisia (Elamri *et al*., 2006), 20-23% in mussels from Italy, (Chironna *et al*., 2002) and 27.4% in shellfish from Spain (Romalde *et al*., 2002). Comparison of percentage of HAV detected in all the studies is however, difficult as different conditions (sampling site, sample size, extraction method, detection method, primer used etc.) employed in each of them tends to yield varying results.

Surprisingly, HAV was not detected in cockles from nearby locations in Seberang Perai such as Kuala Juru and Bukit Tambun which have been continuously demonstrated to harbour much higher fecal coliform and *E. coli* counts (unpublished data) compared to Changkat. This observation supports previous reports indicating lack of correlation between fecal contamination and the presence of viral pathogens in shellfish (Lee *et al*., 1999; Croci *et al*., 2000; Romalde *et al*., 2002)

Previous studies of viral bioaccumulation in shellfish demonstrated that most enteric viruses localized in the stomach and digestive tissues (Romalde *et al*., 1994). Therefore we employed these organs for the virus extraction in order to increase the method's sensitivity and decrease the possible sample-associated inhibitors. We failed to distinguish any difference in the prevalence of HAV between wild and cultured shellfish although samples from wild had been demonstrated to be more frequently contaminated than those of cultured (Romalde *et al*., 2002; Vilariño *et al*., 2009). In the present study, HAV was detected in cockles rather than the other types of shellfish. Similarly, Le Guyader *et al*. (1993) demonstrated that cockles are indeed more contaminated with enteroviruses than mussels. These observations were supported by Lees (2000) who noted different retention of virus by different species of shellfish.

Conclusion

In conclusion, the sensitivity of the kit used in this study was demonstrated to be relatively weak in detecting HAV. Further research is needed especially on the use of different extraction and concentration methods in order to increase the recovery of viral RNA before proceeding with RT-PCR steps. The use of composite and larger sample weight should also be looked into. In addition, employing different primers targeting small portions of the polymerase region could also be attempted.

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