Detection and characterization of hepatitis A virus in water samples in Thailand

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Abstract
Aims: Outbreaks of hepatitis A in Thailand have been reported continually and associated with water supply. However, the genetic analysis of hepatitis A virus (HAV) in water is limited. This study described the application of virus concentration method and reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) to detect HAV RNA and analyse the genetic sequence of the virus in environmental water samples.

Methods and Results: The HAV from water samples was concentrated by using a developed virus concentration method (adsorption-elution and subsequent speedVac reconstitution) and the viral RNA was detected by RT-nested PCR followed by sequencing of the amplified DNA products. Detection limit of HAV determined by the RT-nested PCR was 1.29 radioimmunofocus assay (RIFA) units ml⁻¹. The DNA band appeared at 183 basepairs. No cross-reactivity was observed in the presence of other enteric viruses (poliovirus and rotavirus). A total of 180 water samples were collected, concentrated, and detected for HAV. The HAV was found in 6/40 (15%) of water samples collected from a swamp and 3/30 (10%) collected from a canal. Ten river samples and 100 tap water samples stored in containers for drinking and domestic uses were negative for HAV. In sequence analysis of the DNA products and alignment with the HAV sequence deposited in GenBank, six water samples showed the nucleotide sequence associated with HAV. The 120 nucleotides in the N-terminal VP1 region obtained from two swamp samples showed 95 and 96.7% identity to HAV genotype IA. In nearly all water samples where HAV was present bacterial indicators (faecal coliforms and Escherichia coli) were found for faecal contamination.

Conclusions: A coupled virus concentration method and RT-nested PCR was successfully applied to examine HAV in water samples collected from various sources. DNA sequencing of nested PCR products showed the genotype IA associated with HAV that is predominate in Thailand.

Significance and Impact of the Study: This research is the first study of genetic sequence of HAV in water samples in Thailand. The presence of naturally occurring HAV might pose a potential health risk for people.

Introduction
Hepatitis A virus (HAV) is the leading cause of acute hepatitis throughout the world. The decreasing prevalence of antibodies to HAV in Thailand poses a high risk of infection in nonimmunized individuals (Chatchatee et al. 2002). Routine childhood vaccination has not yet been recommended and hepatitis A vaccine is still costly in this developing country. The HAV is primarily transmitted via faecal-oral route by person-to-person contact or ingestion.
of contaminated food or water (Cuthbert 2001). Waterborne outbreaks of hepatitis A disease have been reported and associated with contaminated water supply in various countries including Thailand (Bloch et al. 1990; Poonawagul et al. 1995; De Serres et al. 1999; Utaipiboon et al. 2002). TheHAV has been detected in rivers (Pina et al. 2001; Taylor et al. 2001; Borchardt et al. 2004; Jiang and Chu 2004), dam (Taylor et al. 2001), canal (Griffin et al. 1999), groundwater (Borchardt et al. 2003) and drinking water (Grabow et al. 2001). Wild-type HAV replicates poorly in cell cultures and there is a low density of virus in water samples; however, nucleic acid amplification techniques such as polymerase chain reaction (PCR) give high sensitivity to detect HAV in water samples (Tsaia et al. 1993). Various versions of PCR for detection of HAV in environmental samples have been developed. These include conventional reverse transcriptase (RT)-PCR (Tsaia et al. 1994), antigen-capture PCR (Deng et al. 1994), Immunomagnetic capture PCR (Jothikumar et al. 1998), integrated cell culture/PCR (Reynolds et al. 2001), and real-time RT-PCR (Brooks et al. 2005). Moreover, virus concentration procedures have been alternative strategies to concentrate viruses from water samples and subsequently to use RT-PCR for detection. The adsorption-elution from microporous filters seems to be the most promising technique and has been recommended as a standard method for virus concentration from water and wastewater (American Public Health Association 1998). We have successfully developed and applied an adsorption-elution method followed by speedVac centrifugation to concentrate enteric viruses from water samples (Kittigul et al. 2001, 2005).

Although the genetic analysis of HAV isolates from human patients in Thailand has been reported (Poovorawan et al. 2005; Wattanarsi et al. 2005), the genetic variation of HAV in environmental waters has not yet been studied. Here, the potential virus concentration method published previously (Kittigul et al. 2005) was used with RT-nested PCR to detect HAV in environmental water samples collected from various sources. The presence of HAV in water samples collected from a swamp and a canal was compared with the presence of bacterial indicators (faecal coliforms/Escherichia coli). The sequences of nucleic acids in HAV-positive samples were analysed from the VP1 N-terminal region of HAV. This study provides a method of detecting HAV and defining the molecular characterization of HAV strains found in aquatic environments.

Materials and methods

Water samples

One hundred and eighty samples were collected from August to December, 2001 in Bangkok, Thailand. One-litre surface water samples were collected from a swamp (40 samples), a canal (30 samples) and a river (10 samples). Five-litre tap water samples were collected from storage containers for drinking (50 samples) and domestic uses (50 samples) in a congested community. All water samples were transported in ice boxes to the laboratory, and processed within 2 h after collection.

Viruses

The HAV strain HM 175 was kindly provided by Dr. T. Endy, Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. Sequence data accessed from the National Centre for Biotechnology Information/GenBank has the accession number M14707 (HAV HM 175 wild type).

Virus concentration

All water samples were concentrated to 1–5 ml with an adsorption-elution technique followed by speedVac reconstitution (Kittigul et al. 2005). Briefly, the surface water samples and dechlorinated tap water samples were adjusted to pH 3.5 with 1 N HCl and aluminum chloride was added at a final concentration of 0.0015 N to flocculate viruses. The water samples were stirred gently at room temperature for at least 30 min. The water samples were passed through negatively charged membrane filters, 47 mm diameter, 0.45 µm pore size (Gelman, Ann Arbor, MI, USA). After washing the membranes with 0.14 NaCl, pH 3.5, the viruses were eluted by adding 2.9% tryptose phosphate broth containing 6% glycerine, pH 90. The eluates were adjusted to pH 7.0–7.4 with 4 N HCl and reconstituted using speedVac centrifugation.

Nucleic acid extraction and RT-nested PCR

The concentrated water samples or eluates were subjected to RNA extraction with QIAamp® Viral RNA extraction kit (QIAGEN, Basel, Switzerland). Amplicons were generated from the VP3 to VP1 junction of the capsid with primers HAV-2389 (5’ GGA AAT GTC TGA GGT ACT TTC TTT G 3’), HAV-2167 (5’ GTT TTT CTC CTC TTC ATC ATG CTA TG 3’) and HAV-2232 (5’TCA ACA ACA GGT TCT ACA GA 3’) previously described by Robertson et al. (1991). The amplification was performed with a RT-PCR kit (Promega, Madison, WI, USA) with some modifications. The RT-nested PCR method was carried out as described by Kittigul et al. (2000). Negative control was composed of the PCR mixture and nuclelease-free water. The HAV strain HM 175 was used for positive control. The nested PCR product (183 basepairs) was
identified by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 μg ml⁻¹).

Sequencing of purified PCR products

Nested PCR products of HAV with 183 basepairs were purified and sequenced at the BioService Unit of the National Science and Technology Development Agency, Bangkok using the same forward HAV-2232 primer. The sequences were aligned and searched for nearly identical sequences with the HAV sequence using the Basic Local Alignment Search Tool (BLAST) program available on the NCBI network server.

Bacterial examination

Bacterial indicators (faecal coliforms and E. coli) for faecal contamination were determined by a standard multiple tube fermentation method (American Public Health Association 1998).

Results

Sensitivity and specificity of RT-nested PCR

The sensitivities of RT-nested PCR for detection of HAV in phosphate-buffered saline (PBS) and in seeding experiments were evaluated. Various concentrations of HAV in PBS were determined by RT-nested PCR. The RT-nested PCR detected as little as 1.29 RIFA units ml⁻¹. Primers of HAV were specific; other enteric viruses (poliovirus type 1 and rotavirus) did not show any amplified product with the primer sets used for HAV (data not shown). In the seeding experiments, 1 ml of serially diluted HAV (2−2 × 10⁶ RIFA units) was inoculated into 1 l of HAV-negative tap water and concentrated according to the procedures described in materials and methods. The detection limit for HAV before concentration was 20 RIFA units (data not shown).

Presence of HAV in water samples

A total of 180 water samples were collected during this study: 100 tap water samples from storage containers and 80 environmental samples from a swamp and a canal in Bangkok, Thailand. Nine concentrated water samples were positive for HAV using RT-nested PCR. Positive signals for HAV were observed in water samples collected from a swamp (6/40 samples, 15%) and from a canal (3/30 samples, 10%). Negative results were found in river (10 samples) and tap water samples stored in containers for drinking and domestic uses (100 samples). Figure 1 shows agarose gel electrophoresis of the nested PCR amplification products derived from three swamp samples corresponding to DNA band of HAV. Two canal samples were negative for HAV. Although non-HAV-specific signal was found in nested PCR product amplified from RT-PCR product diluted 1 : 5, they appeared as very faint bands in RT-PCR product diluted 1 : 100 which was the condition we used throughout this study. Additional PCR products (greater than 500 basepairs) were observed in some environmental water samples. No inhibitory effects on RT-nested PCR were present when undiluted water concentrates with seeding HAV were used for testing (data not shown).

Nucleic acid sequence analysis

Of nine HAV-positive samples, six enabled sequencing of a portion of the VP3–VP1 region after nested PCR amplification. The nucleic acid sequences (149 nucleotides) of nested PCR amplicons were compared to the sequence of wild-type HAV deposited in the GenBank and showing association with HAV. The sequences of 120 nucleotides from the VP1 N-terminal region were aligned with the consensus sequence of HAV reported by Robertson et al. (1991). Two swamp samples (SW13 and SW33) showed identity of 95 and 96.7% in agreement with HAV genotype IA similar to HAV Thailand strains and quite different from HAV strain HM 175 (genotype IB), as shown in Fig. 2 indicating the presence of HAV variants that were closely related to genotype IA. The HAV-positive water samples collected from a swamp were similar to genotype IA whereas those obtained from a canal showed a great variety of nucleic acid sequences (data not shown).
Detection and characterization of hepatitis A virus

Characteristics of HAV-positive water samples

Nine surface water samples with the presence of HAV (six swamp samples and three canal samples) were collected during September-October, 2001. Temperature was in the range of 27.5-30.5°C and pH was in 6.8-8.1 range. Faecal coliforms and E. coli were found in eight water samples with high MPN values of $2 \times 10^3 - 7.9 \times 10^6$ and $2 \times 10^5 - 2.2 \times 10^9$ MPN/100 ml, respectively. One swamp sample (SW14) contained no bacterial indicators but was positive for HAV, as shown in Table 1.

Discussion

The RT-PCR has proved to be a very sensitive tool for the detection of HAV in water samples because of the slow and non-cytopathic replication of wild-type HAV strains. Moreover, the low density of the virus in water makes it possible to improve the variety of concentration techniques for the isolation of HAV from water samples. In the present study, we adapted the improved concentration method and RT-nested PCR for detecting of HAV in water samples. The RT-nested PCR provided high sensitivity and specificity for the detection of HAV.

The adsorption-elution method that we used for concentration of HAV from water is the technique that yielded high recovery of enteric virus previously reported (Kittigul et al. 2005). The second step of concentration or recombination of primary eluate using speedVac centrifugation improved the recovery of the virus significantly (Kittigul et al. 2001). Although those studies focused on different enteric viruses such as rotavirus, the improved

Table 1 Characteristics and bacteriological analysis of water samples where HAV was detected

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Date of sampling</th>
<th>Temperature</th>
<th>$\text{pH}$</th>
<th>Faecal coliforms MPN/100 ml</th>
<th>$E. \text{coli}$ MPN/100 ml</th>
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<tr>
<td>SW 13</td>
<td>September 10</td>
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<td>6.8</td>
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<td>$7.8 \times 10^4$</td>
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<tr>
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<td>September 10</td>
<td>28</td>
<td>6.9</td>
<td>$7.8 \times 10^4$</td>
<td>$7.8 \times 10^4$</td>
</tr>
<tr>
<td>SW 29</td>
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<td>28</td>
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<tr>
<td>SW 33</td>
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<td>$3.5 \times 10^4$</td>
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<tr>
<td>SW 36</td>
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<td>28</td>
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<tr>
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<tr>
<td>Canal samples</td>
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<td></td>
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</tr>
<tr>
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<td>7.2</td>
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<td>$2.2 \times 10^6$</td>
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<td>October 17</td>
<td>30.5</td>
<td>7.2</td>
<td>$79 \times 10^6$</td>
<td>$2.2 \times 10^6$</td>
</tr>
<tr>
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<td>October 22</td>
<td>29</td>
<td>7.7</td>
<td>$79 \times 10^6$</td>
<td>$1.7 \times 10^5$</td>
</tr>
</tbody>
</table>

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method could detect HAV in canal samples (Kittigul et al. 2000).

This study showed the presence of HAV in swamp and canal samples which were collected from an area known to be impacted by human sewage. Sewage from households in a congested community drained into a swamp, which flowed through a nearby canal. Since HAV is transmitted by the faecal-oral route, the virus particles have been attributed to faecally contaminated food (Sanchez et al. 2002) and drinking water supply (Poonawal et al. 1995; Taylor et al. 2001). In Thailand, outbreaks of hepatitis A were traced to faecally contaminated water reservoir (Poonawal et al. 1995; Utaipiboone et al. 2002). The present study is the first report to define the sequence of HAV in environmental water samples in Thailand. Two swamp samples contained HAV with DNA sequence homology to the sequence of HAV and thus belonged to genotype IA. The few nucleotide differences of HAV observed in these two samples were probably because of the high conservation found in VP3-VP1 coding sequences. The high degree of HAV sequence variations were observed in water samples collected from a canal (data not shown). The study of genetic variation of HAV in water samples may provide potentially useful data for epidemiological study for HAV spread and pathogenicity. The VP1 amino terminus of HAV showing genetic variability could be used for genotyping (Costa-Mattiole et al. 2001; Pusch et al. 2005). In addition, nucleotide sequences in the VP1/2A junction region have been generally used to determine the genotype of HAV and the sequence analysis resulted in the designation of seven genotypes of HAV (Robertson et al. 1992). The study of HAV in acute hepatitis patients in Thailand by analysis of genetic sequence in the VP1/2A region showed the majority of genotype IA (Poonowaran et al. 2005; Wattanartri et al. 2005), which is the most dominant type worldwide.

Although the present RT-nested PCR does not directly test for viral infectivity, a positive result at least shows that the HAV RNA was contaminated in environmental water samples. The presence of HAV in water is therefore a potential health risk for the low socio-economic communities and also the children who swim in the swamp and canal. One swamp sample was positive for HAV where faecal coliforms and E. coli were absent. The relationship between the presence of HAV and bacterial indicators was not clear although no correlation in drinking water supply was mentioned (Taylor et al. 2001). This may be in part due to the difference in the source of water or the difference in the behaviour and stability of virus and bacterial indicators.

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References


