Genetic Analysis of Hepatitis A Viruses Detected in an Outbreak at a Military Training Center, Chonburi Province : Plausibility of Contaminated Drinking Water

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ABSTRACT Hepatitis A virus (HAV) is a causative agent of food- and water - borne acute hepatitis that usually affects young adults. This study was to investigate possible agents of a viral hepatitis outbreak in 2008 at a military training center in Chonburi, Thailand. Of 388 serum samples of cases, 188 (48.8%) were positive for anti - HAV IgM, whereas all were negative for anti - HCV antibodies, anti-HBc IgM and anti HEV IgM. Furthermore, studies on randomly selected samples using RT - PCR at VP1/2A junction of HAV genome found that 30 anti - IgM - positive sera, 42 stool and 1 drinking water samples were positive for HAV RNA. Nucleotide sequence analysis of VP1/2A junction (234 base pairs) was further determined. We found that all 21 samples selected for phylogenetic analysis demonstrated identical sequences and were categorized into genotype IA. Our finding also suggested that the 2008 hepatitis A virus outbreak was possibly occurred from contamination of drinking water. Continuation on control measures for clean drinking water is, therefore, essential.

Keywords: hepatitis A, HAV, molecular epidemiology, phylogenetic analysis deleted drinking water

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Introduction

Transmission of hepatitis A virus (HAV) via fecal-oral route as well as person-to-person contact can cause acute viral hepatitis.[1] The symptoms of HAV infection are self-limiting within 1 - 2 months. HAV-infected individuals often require hospitalization and proper therapy.[2] Recent reports described a number of outbreaks of hepatitis A in both Asian and European countries.[3-6] HAV usually affects young adults, and their mortality rate is relatively high, that is 0.1% and 2.1% in age groups of <14 years and >40 years, respectively.[7] Improved hygienic and socioeconomic conditions may be resulted in a decrease in natural HAV immunity.[8]

HAV belongs to the genus *Hepatovirus* in the family *Picornaviridae.* Its genome is a 7.6-kb single-stranded positive-sense RNA molecule. Owing to the fact that no practical cell culture systems for HAV isolation has been developed, nucleotide sequence analysis of VP1/2A region of HAV genome, followed by phylogenetic analyses, has then used to categorization of HAV isolates from various countries. Currently, there are seven HAV genotypes; genotype I (GI) to VII (GVII).[9-11] GI and GIII, are further divided into subgenotypes, IA and IB, and IIIA and IIIB, respectively.[11]

According to a nationwide surveillance system of viral hepatitis in Thailand, several HAV outbreaks were reported from many provinces including Suphanburi, Chantaburi, Chiangrai and Lampang in 2001, 2002, 2005 and 2005, respectively.[12,14] Wattanasri N et. al firstly reported that HAV isolates in Thailand were classified into two genetic groups, namely, GIA and GIB.[12]

The hepatitis outbreak in 2008 was reported to occur at a military training center in Chonburi Province. The present study was to serologically and genetically determine causative agent(s), as well as to identify possible source(s) of infection.

Materials and Methods

Patient sera

A total of 388 serum specimens were collected from patients with clinically diagnosed as having acute viral hepatitis during June 19th to July 7th in 2008. All, attending a training course during April 2nd to June 3rd, 2008 at a military training center in Chonburi, were recruits from Songkhla (n = 98), Chonburi (n = 228) and Chantaburi (n = 62) provinces. All sera were tested for Anti-HAV IgM antibody, anti-HAV total antibody, anti-hepatitis B core (HBc) IgM antibody, anti-hepatitis C virus (HCV), and anti-hepatitis E virus (HEV) IgM antibody using commercially available reagents; VIDAS HAV IgM (BioMerieux, Marcy-l'Étoile, France), VIDAS HAV total (BioMerieux), Mini VIDAS HBc IgM (BioMerieux), ETI-AB-HCVK-4 (DiaSorin, Saluggia, Italy), and HEV-IgM ELISA (MP Diagnostic, Singapore), respectively.

Water samples

Eleven water samples suspected of contamination were collected from each of 11 water tanks at the military training center. The water was generally used for both drinking and commercial purposes. Concentrations of HAV particles were measured as described.[13] Briefly, one liter of water was collected in a sterile bottle from each tank and stored in an icebox until analysis. The water was dechlorinated with sodium thiosulfate ($Na_2S_2O_3$) (Merck, Darmstadt, Germany) to obtain a final concentration of 50 mg/l. Aluminum chloride hexahydrate ($AlCl_3.6H_2O$) (Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 120 mg/l, and pH was adjusted to 3.5 with 1.0 N HCl. Each sample was filtrated with a 0.22 - μm filter (Millipore, Bedford, MA), and the filter was washed with 0.14 N NaCl for 5 min to remove excess Al^+. For the elution of the virus, the filter was soaked for 3 min in 300 μl of 3% Beef Extracts (BD, Beckon Dickinson, Lepontdeclaix, France) at pH 9.0. Antibiotic (Streptomycin and Penicillin) and 10 × MEM was added and the samples were kept at -70°C until further processing. A positive control, used for estimation of HAV recovery during concentration, was 1 ml of HAV vaccine antigens derived from the HM - 175 strain (Havrix1440, GlaxoSmithKline Biological, Rixensart, Belgium). All samples were tested in triplicate.

Stool samples

A total of 48 stool specimens were collected from the patients who were clinically diagnosed acute viral hepatitis in Chonburi Province. One gram of stool was used to prepare a 10% suspension (w/v) with phosphate-buffered saline (PBS), pH 7.4. The suspension was clarified by centrifugation for 30 min at 10,000 g at 4°C, and the supernatant was kept at -70°C until use.

Viral RNA extraction

Viral RNA was extracted from 100 μl of 10% suspension (w/v) of serum and stool samples, and the eluent from each water sample using Trizol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

RT-PCR and sequencing

RNA was dissolved with 20 μl RNase-free distilled water containing 40 U ribonuclease inhibitor (RNasin, Promega, Madison, WI). For cDNA synthesis and the first polymerase chain reaction (PCR), 10 μl of the RNA was incubated for 45 min at 42°C with 15 pmol of reverse primer BR-9 (3310 - 3286 nt, 5′AGT CAC ACC TCT CAA GGA AAA CTT 3′). For the RNA sample, 40 μl of the reaction mixture containing 1 × AMV buffer (10 mM Tris - HCl [pH 8.3], .5 mM $MgSO_4$, 50 mM KCl, and 0.001% gelatin), 5 U of AMV reverse transcriptase, 5 U Tfl, 10 mM deoxynucleoside triphosphates (dNTPs) (Access RT-PCR system kit, Promega), and 15 pmol of forward primer BR-5 (2950 - 2972 nt, 5′ TTG TCT GTC ACA GAA CAA TCA

G 3′) were added. After an initial denaturation at 94°C for 2 min, 35 cycles of amplification were performed using the GeneAmp PCR System 2400 (PE-Applied Biosystems, Foster City, CA). Each cycle consisted of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and an extension reaction for 1 min at 72°C followed by a final extension for 7 min at 72°C. The positions of the primers were numbered according to the complete nucleotide sequence of HAV HM175 strain.

A nested PCR was performed in 20 µl of reaction mixture containing 2 µl of the first PCR product, 15 pmol of forward primer RJ-3 (2984 - 3002 nt, 5′ TCC CAG AGC TCC ATT GAA 3′) 15 pmol of reverse primer BR-6 (3217 - 3193 nt. 5′AGG AGG TGG AAG CAC TTC ATT TGA 3′), 1 × Taq buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCL_2$, 50 mM KCl, and 0.001% gelatin), 2.5 mM deoxynucleoside triphosphate (dNTPs), and 1U Taq DNA polymerase (Promega). The PCR was performed under the same conditions as the first PCR. The nested PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). A nucleotide sequence was determined with an ABI 3110 Genetic Analyzer (PE-Applied Biosystems) using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE-Applied Biosystems) as described.[12] RJ3 and BR6 primers were used for sequencing. The nucleotide sequences were then aligned with the corresponding HAV genomic region using MacVector software version 4.5.3 (Kodak Scientific Imaging System, Oxford, UK).

Genetic analysis

Phylogenetic analysis was performed with the Phylogenetic Inference Package (PHYLIP) 3.5.7.2 (University of Washington, Seattle, WA). An evolutionary distance was estimated using DNADIST (UPGMA), and unrooted phylogenetic trees were constructed using the neighbor-joining (NJ) method. A phylogenetic tree was visualized and edited with TREE VIEW ver. 1.5.

Results

Determination of HAV Infection

Serological examination

All sera were tested for Hepatitis A, B, C and E infection. None were detected positive for Hepatitis B, C and E. Among 388 serum specimens, 188 were positive for anti-HAV IgM antibody. Of these, 51, 114 and 23 were specimens obtained from those coming from Songkhla, Chonburi and Chantaburi, respectively (Table 1). The results indicated that HAV was the causative agent of the 2008 hepatitis outbreak. In addition, clinical symptoms were correlated with an incubation time of HAV infection (data not shown).

Genetic analysis of HAV genotypes

An outbreak investigation team was further collected stool samples of some HAV-positive cases as well as possible drinking water as source(s) of contamination. Using RT-PCR, 42 out of 48 stool specimens and 1 out of 11 drinking tank water were positive for HAV RNA. Additionally, detection of HAV RNA was found in 30 out of 45 anti-HAV IgM positive sera (Table1).

Due to a limitation in the amount of specimens collected, nucleotide sequencing at VP1/2A junction was performed in 15, 5 and 1 of serum, stool and water specimens, respectivel Sequencing and phylogenetic analysis was shown that each NT sequences of each samples were identical and classified into genotype 1A (Fig. 1). According to the genetic analysis, it could indicate that the 2008 HAV outbreak was due to genotype IA and suggested that the possible source of HAV contamination was one of the drinking tanks in Chonburi training center.

Table 1 Detection of Hepatitis A virus in various specimens during the 2008 HAV outbreak at the military training center, Chonburi Province, Thailand

Province of samples collection	Type of sample	Duration of Samples collection	Positive for anti-HAV IgM antibody/Total (%)	HAV-RNA positive/ Anti HAV-IgM positive (%)	No. of Sequencing/ HAV-RNA positive	Genotype
Songkhla	Serum	June 19-July 4	51/98 (52.0)	11/19 (57.9)*	5/11**	IA
Chonburi	Serum	June 21-July 7	114/228 (50.0)	12/19 (63.2)*	5/11**	IA
	Stool	June 21-July 7	nd	42/48 (87.5)	5/42**	IA
	(Drinking tank water)	June 21-July 7	nd	1/11 (9.1)	1/1	IA
Chanthaburi	Serum	July 1	23/62 (37.0)	7/7 (100)	5/7	IA
Total			188/388 (48.8)	73/94 (77.7)	21/73 (28.8)	

nd = not done

* = randomly selected sera depended on the limited amount of serum

** = randomly selected sequencing samples depended on PCR product concentration

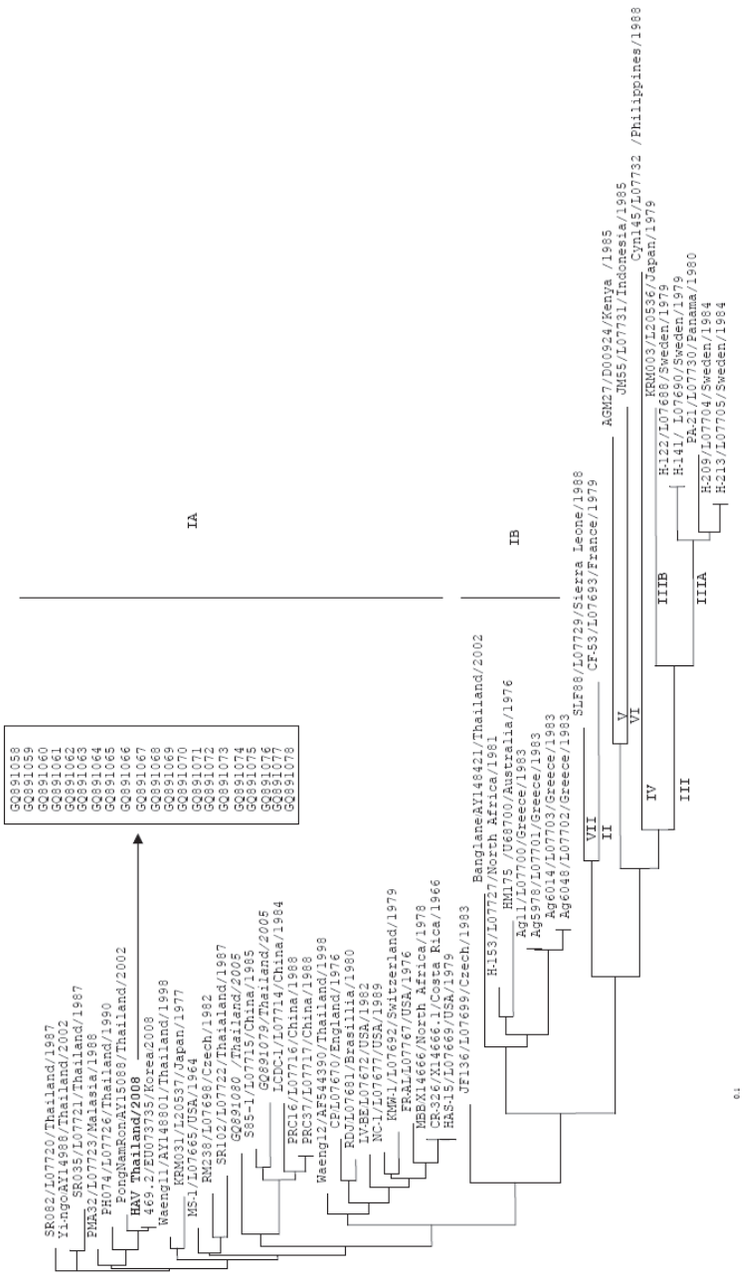

Fig. 1 Phylogenetic tree of HAV detected in outbreaks in Thailand in 2008. The nucleotide sequences of the VP1/2A region of the genome (168 bp) from 21 isolates were analyzed by the neighbor-joining (NJ) method along with 45 reference strains from GenBank. Two strains isolated previously in Rayong and Chiangrai provinces (GQ891079 and GQ891080, respectively) in Thailand are shown in italics. The HAV strains analyzed in this study are one Chonburi strain from water (CW), five Chonburi strains from stools (CS), five Chantaburi strains (B), five Chonburi strains (C), and five Songkhla strains (S). The nucleotide sequences determined in this study were deposited in GenBank under accession numbers GQ891058, GQ891059 - 63, GQ891064 - 68, GQ891069 - 73, and GQ891074 - 78.

Discussion

Acute hepatitis mainly occurs by eating and/or drinking of food and/or water contaminated with Hepatitis A virus. Anti-HAV and HAV RNA assays are used to determine HAV infection. Anti-HAV IgM, in particular, is used to detect acute or recent infection. A number of HAV outbreaks in Thailand were reported in the past 10 years.[12,14]

In this study, almost 50% of suspected cases were found positive for anti-HAV IgM. In addition, duration of specimens collection was in accordance with an incubation time known for HAV infection. Our findings could indicate that the 2008 outbreak of acute hepatitis among recruits attending the training course during June 19th to July 7th was caused by Hepatitis A virus.

HAV genotyping studies illustrated that all 21 strains were classified into GIA, one of the two genotypes previously reported in acute hepatitis patients during 1998 - 2002.[12] Previous investigations suggested that the HAV outbreaks were due to indigenous HAV, where HAV strains identified in the central part of Thailand were different from those in the eastern part of the country.[14] The present study also showed a strain indigenous to Chonburi province where the occurrence and the possible source of the outbreak were found. Interestingly, comparison of 168 bases in the VP1/2A region of the genome (168 bp) among sequences previously reported, it was shown that the sequences of the outbreak strains are identical to that of a strain detected in Korea in 2006 (EU073735.1)[15], however, the relationship between these two strains is unknown.

Using molecular technique, the possible source of the 2008 outbreak was subjected to be one of the drinking water tanks. Raising awareness on good hygiene to the public and continuation on control measures of food and drinking water are, therefore, important. Additionally, laboratory-based surveillance should be regularly performed as a part of an alert system on occurrence of possible outbreaks.

Conclusion

The 2008 hepatitis A outbreak at the military training center, Chonburi was potentially caused by a contaminated drinking water tank. We also found that nucleotide sequencing of HAV from all representative clinical specimens demonstrated identical sequences and were classified into genotype IA. Good practices on hygiene are recommended to prevent any outbreaks that might occur.

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สายพันธุ์เชื้อไวรัสตับอักเสบ เอ ที่ระบาดในศูนย์การฝึกอบรมทหารเรือ จังหวัดชลบุรี โดยสาเหตุจากแหล่งน้ำดื่ม

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บทคัดย่อ เชื้อไวรัสตับอักเสบ เอ สามารถติดต่อได้ทางการกิน การดื่ม จากอาหาร หรือน้ำที่มีการปนเปื้อนของเชื้อไวรัส ซึ่งเป็นปัญหาสาธารณสุขที่สำคัญของประเทศไทย โดยเฉพาะในกลุ่มวัยเริ่มทำงาน ได้ทำการตรวจตัวอย่างซีรั่มของผู้ที่มีอาการป่วยสงสัยว่าอาจเกิดจากเชื้อไวรัสตับอักเสบ จำนวน 388 ตัวอย่าง หลังจากเข้ารับการฝึกอบรมในปี พ.ศ. 2551 ที่ศูนย์การฝึกอบรมทหารเรือ จังหวัดชลบุรี ผลการตรวจด้วยเทคนิคซีโรโลยี พบว่าให้ผลบวกกับแอนติบอดีชนิดไอจีเอ็มต่อไวรัสตับอักเสบ เอ จำนวน 188 ตัวอย่าง โดยที่ไม่พบการบ่งชี้การติดเชื้อเฉียบพลันจากไวรัสตับอักเสบ ซี บี และอี จากนั้นได้สุ่มตัวอย่างซีรั่มที่ให้ผลบวกต่อไวรัสตับอักเสบ เอ จำนวน 45 ตัวอย่าง พร้อมกับตัวอย่างอุจจาระ 48 ตัวอย่าง และตัวอย่างน้ำจากแหล่งน้ำดื่ม 11 ตัวอย่าง นำมาตรวจสารพันธุกรรมของเชื้อไวรัสตับอักเสบ เอ โดยวิธี reversed-transcriptase polymerase chain reaction (RT-PCR) ในส่วนเชื่อมต่อ VP1/2A (234 เบส) พบว่า ให้ผลบวก 30, 42 และ 1 ตัวอย่าง ตามลำดับ เมื่อวิเคราะห์ลำดับเบสในส่วนเชื่อมต่อ VP1/2A ของเชื้อไวรัสตับอักเสบ เอ ที่ได้จากตัวอย่างแหล่งน้ำดื่ม และตัวอย่างผู้ป่วย พบว่าลำดับเบสของแต่ละตัวอย่างตรงกันทั้งหมด และจัดอยู่ในกลุ่มจีโนทัยป์ 1 เอ (Genotype 1A, GIA) การศึกษานี้แสดงว่าสาเหตุของการระบาดที่ศูนย์การฝึกอบรมทหารเรือครั้งนี้อาจเกิดจากการปนเปื้อนในแหล่งน้ำดื่ม ดังนั้นมาตรการป้องกัน และการตรวจสอบความสะอาดของแหล่งน้ำจึงเป็นสิ่งจำเป็น