

## Clinical features and molecular characterization of hepatitis A virus outbreak in a child care center in Thailand

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### Abstract

**Background:** As a result of declining hepatitis A endemicity in Thailand, an increasing number of children and adolescents have become susceptible to hepatitis A virus (HAV) infection. **Objective:** The present study was aimed at both investigating the clinical features and determining molecular characterization of HAV during an outbreak, which occurred in a childcare center located in a suburban area of Bangkok between November 2002 and February 2003. **Methods:** Serum samples obtained from all children in the center were tested for anti-HAV IgG and anti-HAV IgM. Testing for HAV-RNA was performed in sera, saliva and stool samples by the reverse transcription-polymerase chain reaction (RT-PCR) with primers located at the VP1-2A region. To further characterize the HAV genotype serum derived HAV-RNA-positive PCR products were sequenced. **Results:** Anti-HAV IgG and anti-HAV IgM were detected in 74 and 70 of 112 children in the center, respectively. Among those positive for anti-HAV IgM, 65 cases were asymptomatic, while five children had acute clinical hepatitis. The ratio between symptomatic and asymptomatic children was 1:13. Among the asymptomatic cases, 31 (47.7%) displayed biochemical hepatitis with elevated alanine aminotransferase (ALT) levels. All the isolates from this outbreak were found to be of subgenotype IA, which showed a high level of sequence homology with previous Thai isolates. HAV-RNA could not be detected in saliva, but was found in stool for at least 3 weeks after initial diagnosis of clinical or biochemical hepatitis. **Conclusion:** Because of the infection's characteristically asymptomatic spread, hepatitis A poses an increased risk to childcare centers. The presence of a single sub-genotype indicates that this HAV strain has been predominantly circulating in Thailand.

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**Keywords:** Hepatitis A; HAV; Outbreak; Childcare center

### 1. Introduction

Hepatitis A virus (HAV) infection has been a major public health problem in many developing countries worldwide (Cuthbert, 2001). In Thailand during the previous two decades, HAV infection has undergone a remarkable regression from high to intermediate endemic levels. This shifting epidemiology of hepatitis A has been attributed to general improvements in hygiene, living standards and socioeconomic progress (Poovorawan et al., 2002). As a result, the proportion of children and adolescents susceptible

to the infection has increased and major outbreaks caused by contaminated water and food have been periodically reported (Poovorawan et al., 2000). For instance in 1992, an outbreak caused by contaminated drinking water occurred among schoolchildren in a province in southern Thailand (Sinlaparatsamee et al., 1995). Between September 2001 and April 2002, two more recent major community outbreaks occurred in another province in southern Thailand (Theamboonlers et al., 2002).

HAV is a 7.5 kb positive-stranded RNA virus belonging to the *Picornaviridae* family (Yokosuka, 2000). The virion comprises three functional regions namely, P1, P2 and P3. The P1 region encodes the structural proteins VP1, VP2, VP3, and putative VP4, while the P2 and P3 regions encode nonstructural proteins associated with viral replication

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(Cuthbert, 2001). Since the region spanning VP1-2A has demonstrated substantial sequence heterogeneity, it is suitable for differentiating between HAV strains (Jansen et al., 1990). Based on VP1-2A sequencing, different HAV strains have been classified into seven genotypes designated I–VII, which are distinguishable by 15–25% sequence diversity (Robertson et al., 1992). Genotype I is the most common strain in humans worldwide, with genotype III as the second most prevalent. Genotypes I and III have been further divided into subgenotypes A and B. Sub-genotype IA is the major genotype in America and Asia, whereas subgenotype IB appears to predominate in Europe and the Mediterranean region (Robertson et al., 1992). In Thailand, all isolates obtained from recent outbreaks were found to be of subgenotype IA (Theamboonlers et al., 2002) showing a high level of sequence homology with those previously described (Robertson et al., 1992).

Between November 2002 and February 2003, an outbreak of hepatitis A occurred in a childcare center located in a suburban area of Bangkok. We conducted an investigation to determine the clinical and biochemical features of children infected during this outbreak. We further analyzed the phylogenetic relations between HAV isolates obtained from this outbreak and recent epidemics in order to clarify HAV genotypes that have been circulating among Thai populations. Thus, this study provides valuable information on the clinical aspects of hepatitis A and particularly, the molecular epidemiology of the virus in Thailand.

## 2. Materials and methods

### 2.1. Study population

This outbreak occurred in a childcare center located in a suburban area of Bangkok. During the course of the study, there were 112 children aged between 1 and 6 years (mean  $3.2 \pm 1.5$  years), 77 boys and 35 girls. The first case developed acute icteric hepatitis A in mid November 2002, and another four children had clinical symptoms of acute hepatitis 1–2 months later. As part of an investigation of the outbreak performed by the staff of the local hospital, serum specimens were collected and stored at  $-70^\circ\text{C}$  until tested. Stool and saliva samples were also collected from 19 patients with evidence of HAV infection to perform transmission studies for three additional weeks. Formal written consent to participate was obtained from both the director of the children's institution and the director of Chulalongkorn Memorial Hospital. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

### 2.2. Biochemistry tests

Liver function test for alanine aminotransferase (ALT) was performed using an automated analyzer (Hitachi 912). The normal value of the enzyme in children was 0–40 IU/l.

### 2.3. Serological study

The serum specimens were tested for anti-HAV IgG and IgM antibodies by enzyme-linked immunosorbent assay using commercially available ELISA kits (Abbott Laboratories, North Chicago, Ill). The cut-off levels for immunity to anti-HAV IgG and IgM were calculated, as specified by the manufacturer.

### 2.4. HAV-RNA detection

Fecal specimens were diluted 1:10 in phosphate buffered saline (PBS). After thorough mixing, each fecal suspension was centrifuged ( $250 \times g$ , 10 min). Small sub samples ( $50 \mu\text{l}$ ) of the supernatant thus generated and of the saliva, sera from subjects found positive for anti-HAV IgM were then analyzed for the presence of HAV-RNA by nested reverse transcriptase-polymerase chain reaction (RT-PCR), as described previously (Theamboonlers et al., 2002).

RNA was extracted from each test sample and reverse transcribed into cDNA using the BR-9b primer (5'-AGT CAC ACC TCT CCA GGA AAA CTT-3'). The cDNA was then amplified by nested PCR, with BR-5b (5'-TTG TCT GTC ACA GAA CAA TCAG-3') as the outer sense primer, BR-9b as the outer anti-sense primer, RJ-3c (5'-TCC CAG AGC TCC ATT GAA-3') as the inner sense primer and BR-6b (5'-AGG AGG TGG AAG CAC TTC ATT TGA-3') as the inner anti-sense primer (Bruisten et al., 2001). Both amplification reactions were performed in a 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, CT) set to run for 2 min at  $95^\circ\text{C}$  (denaturation), 1 min at  $55^\circ\text{C}$  (primer annealing), and 1 min at  $72^\circ\text{C}$  (extension) for 35 cycles, with a final extension step at  $72^\circ\text{C}$  for 10 min. After electrophoresis in a 2% agarose gel (Research Organics, Cleveland, OH) stained with ethidium bromide on preparation, an ultra-violet trans-illuminator (Gel Doc 1000, Bio-Rad, Hercules, CA) was used to check for the expected 234 bp-band.

### 2.5. HAV sequencing and genotype characterization

The target PCR products within the agarose gel were purified for sequencing using the Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, New York), as described previously (Theamboonlers et al., 2002). A sub-sample of each purified sample was once more subjected to electrophoresis in a 2% agarose gel, to ascertain its purity, and the absorbance at 260 nm of another sub-sample was spectrophotometrically determined (UV 160 A; Shimadzu, Tokyo). The absorbance values were used to estimate the DNA concentrations. Between 10 and 30 ng of each DNA sample were subjected to cycle sequencing, using a commercially available kit (Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction; Foster City, CA) in a 9600 thermocycler, with each  $20 \mu\text{l}$  reaction mix containing  $8 \mu\text{l}$  of dye terminator from the kit and  $3.2 \text{ pmol}$  of the specific primer (RJ-3c). The extension products were subsequently purified from excess, unincorporated

dye terminators by ethanol precipitation and subjected to sequence analysis in an ABI-Prism 310 Genetic Analyzer (Perkin-Elmer, Norwalk, CT) following the manufacturer's recommendations. The reverse primer BR-6b was used for sequence confirmation.

## 2.6. Phylogenetic analysis

The VP1-2A sequences obtained from the outbreak isolates were compared with the corresponding GenBank reference sequences for genotype I-VII (L20549, L20551, L20552, L20553, L07683, L07671, L07728, L07720, L07703, L07702, L07701, L07700, L07693, L07694, L07729, L07730, L07732, L20543, L07691, L20544, L20536, L20532, L20530, AJ299467, AJ299466, AJ299465, AJ299464, AJ296172.1, AL07668, L07689, AL07688 and L07731), and three sequence (AF509833–AF509835) of the previous outbreak in the southern part of Thailand. All of these sequences were multiply aligned, using Clustal X from version 3.75c of the PHYLIP software package (Prof. J. Felsenstein, Department of Genetics, University of Washington, Washington, DC). Version 1.5 of the Treeview programme (Dr. R.D.M. Page, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK) was then used to construct an un-rooted phylogenetic tree, as described previously (Theamboonlers et al., 2002).

## 2.7. Statistical analysis

The data were expressed as mean values  $\pm$  standard deviation. Statistical significance in mean values was evaluated by the Mann-Whitney test or the Student's *t*-test as appropriate. *P*-values below 0.05 were considered significant.

## 3. Results

Serological testing revealed that anti-HAV IgG and anti-HAV IgM were detected in 74 and 70 children, respectively. Of those positive for anti-HAV IgM, 65 cases were asymptomatic, while five children showed the typical symptoms of acute hepatitis including fever, nausea, vomiting, abdominal pain and jaundice. The ratio between symptomatic and asymptomatic was 1:13. The mean age of symptomatic cases was slightly higher than that of asymptomatic cases, but did not reach statistical significance ( $3.9 \pm 1.6$  and  $2.9 \pm 1.3$  years, respectively,  $P = 0.07$ ). Symptomatic children had higher ALT levels than asymptomatic cases ( $1074.8 \pm 225.1$  and  $182.4 \pm 370.5$  IU/l, respectively,  $P < 0.001$ ). Of the asymptomatic children, 31 (47.7%) had biochemical hepatitis with elevated ALT levels ranging from 41 to 1894 IU/l.

HAV-RNA was detected by PCR in 24 of 70 (34.3%) children whose sera had tested positive for anti-HAV IgM. No differences were discernible between HAV-RNA-positive

and -negative groups in terms of mean ALT levels ( $278.5 \pm 501.0$  and  $229.3 \pm 390.8$  IU/l, respectively). Similarly, the levels of anti-HAV IgM displayed no differences between HAV-RNA-positive and -negative groups.

Of the HAV-RNA-positive specimens (24), eight samples were randomly selected for direct sequencing. The obtained sequences were submitted to GenBank which made them accessible under accession number AY 352212, 352214, 352217, 352221–352222, 352224–352226. The obtained HAV VP1-2A region (168 bp) sequences were aligned with those of the isolates of known genotype and subjected to phylogenetic analysis (Fig. 1). All isolates from

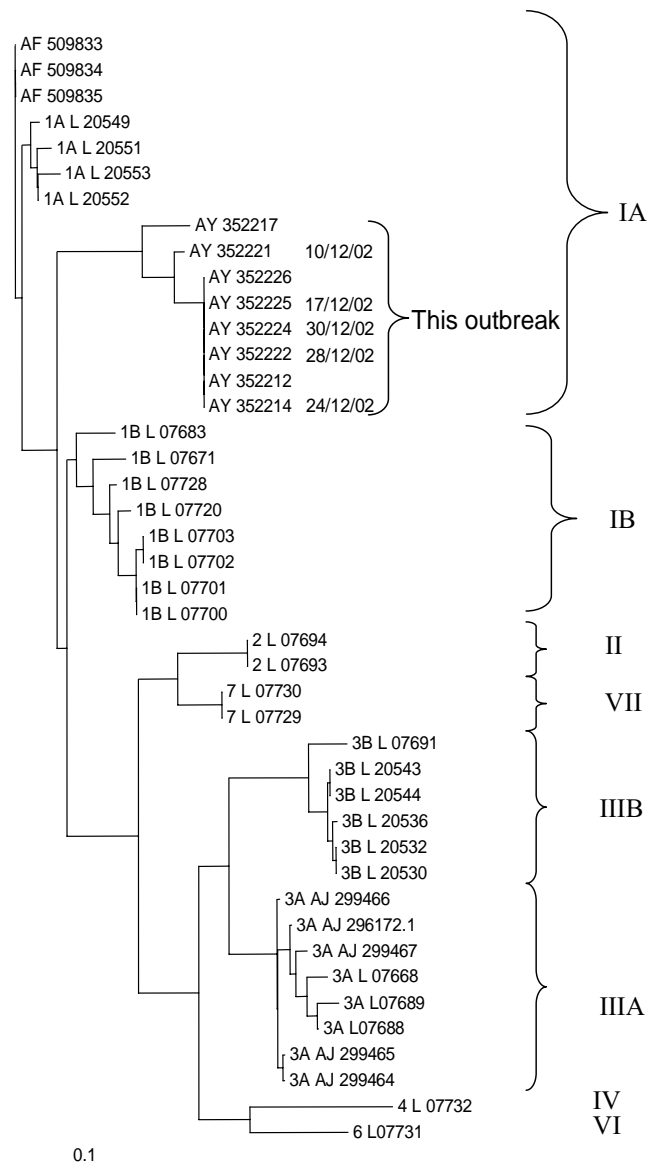


Fig. 1. Phylogram based on nucleotide sequences of the hepatitis A VP1-2A region. The genotype and GenBank accession numbers of reported sequences are indicated in the phylogenetic tree. Accession number AY 352212, 352214, 352217, 352221, 352224–352226 were isolated from the present study. Dates (day/month/year) shown in parenthesis represent dates of first symptoms of corresponding cases.

this outbreak clustered within subgenotype IA, displaying 90–100% sequence homology and 92–100% with the isolates obtained from previous outbreaks in the southern part of Thailand (Theamboonlers et al., 2002).

HAV–RNA could be detected in stool by RT-PCR for at least 3 weeks after clinical or biochemical hepatitis had been first diagnosed. However, HAV–RNA could not be detected in saliva of those patients with biochemical hepatitis.

Susceptible children, who were anti-HAV IgG negative, were immunized with inactivated hepatitis A vaccine. One of them developed clinical hepatitis 1 week after immunization. Two weeks after the first vaccine dose, no further case of hepatitis A occurred.

#### 4. Discussion

Although its incidence has declined over the past decade, sporadic outbreaks of hepatitis A continue to occur in Thailand. Our previous data as to the prevalence of HAV demonstrated a marked decrease in anti-HAV antibody among children and adolescents from 31% in 1987 to 13% in 1996 (Poovorawan et al., 1997). As a consequence, a large proportion of the young generations are susceptible to the infection thereby enhancing its impact, should an outbreak occur. In the present study, investigations of a hepatitis A outbreak in a childcare center defined the clinical features of the children who were infected with the virus. In addition, the genetic relatedness between isolates recovered from this epidemic and from recent outbreaks in Thailand was also examined. A better understanding of the HAV genotypes circulating in Thailand should facilitate studies on the epidemiological spread at the molecular level, and consequently improve prevention approaches. In fact, the importance of molecular characterization of HAV infections has been emphasized in recent reports by several authors (Chironna et al., 2003; Costa-Mattioli et al., 2001a, 2001b; Sanchez et al., 2002; Tallo et al., 2003).

HAV infection is usually self-limiting and the disease severity is age dependent. In children below the age of 6 years, the infection is generally asymptomatic or characterized by non-specific symptoms that are indistinguishable from other viral infections. In contrast, exposure of non-immune adolescents and adults results in a range of clinical manifestations that can range from mild, anicteric infection to fulminant hepatic failure. Asymptomatic infection can be further classified into two categories namely, subclinical and inapparent infections. In subclinical infections, only the biochemical features of hepatitis can be detected, whereas inapparent infections can be identified by serological studies (Ciocca, 2000). The present report confirms the characteristic asymptomatic spread of hepatitis A among children since the ratio between symptomatic and asymptomatic infection was approximately 1:13, and the fraction of inapparent infections exceeded 50%. On the contrary, in an outbreak of hepatitis A affecting school children aged 7–12

years, the ratio of symptomatic to asymptomatic hepatitis was approximately 1.3:1 (Sinlaparatsamee et al., 1995).

Transmission of HAV is usually by the fecal oral route through person-to-person contact, but contaminated water and food frequently cause epidemics (Massoudi et al., 1999). In this outbreak, although the sources of the infection were unclear various potential modes of transmission were possible. Person-to-person spread was the most likely source of the infection since the prolonged period over which cases presented argues against an outbreak due to a point source such as water or food contamination. Nonetheless, while the dynamics of the outbreak suggested that particular food- or water-borne sources were unlikely, food or water could still be the vehicle in person-to-person spread. The high rate of hepatitis transmission by these children was probably due both to the high proportion of asymptomatic infections and to the poorly developed hygiene and toilet habits of this age group. Specifically, children in this age group often touch objects orally and stool may spread easily to the immediate environment. In addition, centers with a large enrollment of children in which toilet-trained and non-toilet-trained children share rooms, as described in this report, have been shown to be associated with more frequent transmission of HAV infection (Hadler et al., 1982).

We detected HAV–RNA in sera and fecal samples, but not in saliva specimens. Serum samples are easier to handle and store compared with the possibly more hazardous fecal samples. Furthermore, recent data demonstrated that HAV viremia persisted longer than previously believed and could be detectable in the majority of cases in their early convalescent phase by using nested RT-PCR (Bower et al., 2000; Fujiwara et al., 1997; Kwon et al., 2000). Therefore, the detection of HAV–RNA in sera is practical and useful to study the molecular epidemiology of HAV infection. Nonetheless, HAV concentration in feces is usually higher than in sera and fecal shedding can persist for months after resolution of hepatitis (Yotsuyanagi et al., 1996). In fact, delayed fecal excretion of HAV is predominantly observed in neonates or young children, reflecting the importance of cell-mediated immunity in resolving the infection (Rosenblum et al., 1991). In this study, we showed that HAV–RNA was detectable in stool for several weeks after the patients had recovered from either clinical or biochemical hepatitis. The prolonged excretion of HAV may well explain the continued spreading of the virus for months by person-to-person contact in this outbreak. These observations reinforce the need for vaccination of children and staff in childcare facilities and rigorous personal hygiene for prevention of transmission, even after children with hepatitis have entered the recovery phase.

Unlike many other RNA viruses, HAV does not appear to exhibit a rapid accumulation of genetic changes. Within the subgenotypes of HAV (IA, IB, IIIA, or IIIB) there is less than 7.5% nucleotide diversity (Robertson et al., 1992). In the present study, we demonstrated that all isolates identified during this outbreak were clustered within subgenotype IA, the most prevalent subgenotype in Asia. Strains

isolated in this study also showed a high level of sequence homology with previous Thai isolates collected from different outbreaks and in different years (Robertson et al., 1992; Theamboonlers et al., 2002). These data indicate that subgenotype IA has been predominantly circulating in Thailand for more than a decade. Interestingly, it should be noted that although all isolates in this study belonged to subgenotype IA, the sequences were not 100% homologous among them. This is in agreement with previous data that genetic variability of HAV may occur in an outbreak originated from a common source (Sanchez et al., 2002). In addition, co-circulation of more than one subgenotype has been observed in some regions of the world. For example, recent reports revealed the concomitant circulation of subgenotypes IA and IB in some countries such as Brazil and the Netherlands (Bruisten et al., 2001; de Paula et al., 2002). Moreover, phylogenetic analysis of HAV infection during an outbreak in France showed an extensive genetic heterogeneity among strains belonging to subgenotypes IA, IB and IIIA, suggesting a mixture of strains imported from other geographical regions, as well as a continuous low level of transmission of endemic isolates among certain high-risk groups (Costa-Mattioli et al., 2001a, 2001b).

Based on our observations, we conclude that hepatitis A poses a high risk to childcare centers due to the characteristic asymptomatic spread of the infection and poor hygiene of the children. Comprehensive administration of hepatitis A vaccine to the children and staff of such facilities could improve control of similar outbreaks. Using molecular analysis, we confirmed that all isolates obtained during this outbreak were of subgenotype IA, displaying a high level of sequence homology with previous Thai isolates. Nonetheless, additional isolates recovered from other epidemics need to be analyzed in the future to ascertain whether the same or a similar subgenotype has been predominant in Thailand.

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