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Comparison of Hepatitis A and E Virus Infections Among Healthy Children in Mongolia: Evidence for Infection With a Subgenotype IA HAV in Children

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To compare the epidemiologic profiles of hepatitis A virus (HAV) and hepatitis E virus (HEV) infections in children in Mongolia, the prevalence of HAV and HEV infections was investigated serologically and molecularly among 717 apparently healthy individuals of 0–20 years of age (mean ± standard deviation, 8.6 ± 4.9 years) using serum samples obtained between October 2005 and January 2006. Total antibody against HAV (anti-HAV [total]) was detected in 494 (68.9%) of the 717 subjects, while IgG antibody against HEV (anti-HEV IgG) was detected in only five subjects (0.7%) ($P < 0.0001$). All five subjects who had anti-HEV IgG, were negative for anti-HEV IgM and HEV RNA. Anti-HAV was detectable in 24 (75.0%) of the 32 infants aged 7 days to 6 months, but not in any of the 8 infants aged 7 to <12 months. The prevalence of anti-HAV was 19.5% (17/87) in the age group of 1–3 years, and it increased to 50.0% (69/138) in the age group of 4–6 years, and further to 81.4% (105/129) in the age group of 7–9 years. Of note, 97.2% of the subjects in the age group of 16–20 years had anti-HAV. The presence of HAV RNA was tested in all 717 subjects, and three children of 1, 4, or 8 years of age were found to have detectable HAV RNA (subgenotype IA). No subject had a history of hepatitis or jaundice. In conclusion, HEV infection was uncommon, but HAV infection lacking overt clinical features was prevalent among children in Mongolia. ***J. Med. Virol. 79:18–25, 2007.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis A virus; hepatitis E virus; subclinical infection; children; Mongolia

INTRODUCTION

Hepatitis A virus (HAV) circulates in most regions in the world and HAV infection is a serious worldwide public health problem. In industrialized countries, the general population has a decreasing exposure rate to HAV due to improvements in hygiene and sanitation conditions [Jacobsen and Koopman, 2004]. In contrast, in developing countries where HAV infection is endemic, the majority of individuals are exposed to HAV during childhood. HAV infection is often asymptomatic in young children, whereas in older children and adults there is a wide range of clinical manifestations from mild, anicteric infection to severe, fulminant hepatic failure [Hollinger and Emerson, 2001]. HAV is the only member of the genus *Hepatovirus* within the family *Picornaviridae* [Melnick, 1992], and its viral genome consists of a 7.5-kilobases (kb) positive-stranded RNA. The HAV strains recovered from different parts of the world have been classified into six genotypes (I–VI), of which genotypes I, II, and III are found in humans: genotypes I, II, and III are further divided into subgenotypes IA and IB, IIA and IIB, and IIIA and IIIB, respectively [Robertson et al., 1992; Lu et al., 2004].

Hepatitis E virus (HEV) causes water-borne outbreaks and sporadic cases of acute hepatitis in many developing countries [Purcell and Emerson, 2001a]. Similar to hepatitis A, there is no evidence of chronic HEV infection in humans. Apart from imported cases of hepatitis E, domestically acquired hepatitis E has now

The nucleotide sequence data reported in this study have been assigned GenBank/EMBL/DDBJ accession numbers AB259811–AB259816.

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been found in many industrialized countries [Harrison, 1999; Smith, 2001; Okamoto et al., 2003; Mansuy et al., 2004; Ijaz et al., 2005]. Accumulating lines of evidence have indicated that hepatitis E is a zoonosis [Meng et al., 1997; Erker et al., 1999; Harrison, 1999; Meng, 2003; Tei et al., 2003; Yazaki et al., 2003]. HEV is a non-enveloped, single-stranded, positive-sense RNA virus, and its genome is approximately 7.2 kb long [Tam et al., 1991]. HEV was classified recently as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004].

Mongolia, a developing country in the northern part of Central Asia, is bordered by Russia to the north and China to the south. Mongolia has a population of 2.5 million as of 2004 and a total area of 1.6 million square kilometers, and has a climate that is classified as semi-arid continental with long, severe winter seasons. Many Mongolians are semi-nomadic herders of sheep, goats, cattle, horses, and camels, but more than half of the Mongolian population now live in urban areas (with 36% residing in Ulaanbaatar, the capital city of Mongolia) [http://www.moh.mn/]. Mongolia is confronting many communicable diseases including viral hepatitis [Ebright et al., 2003]. In our previous study [Takahashi et al., 2004], antibodies against HAV (anti-HAV) and HEV (anti-HEV) were detected in 249 (100%) and 28 (11%), respectively, of the 249 adults aged 23–86 years living in Ulaanbaatar, suggesting the presence of high prevalences of HAV and HEV infections in the Mongolian adult population. However, the prevalence of HAV and HEV infections in children in Mongolia has yet to be explored. In the present study, the prevalence of HAV and HEV infections was surveyed serologically and molecularly among 717 apparently healthy individuals of 0–20 years of age in Mongolia, stratified by age, gender, residence, and lifestyle, in order to understand better the molecular epidemiology of these two hepatitis viruses transmitted enterically.

MATERIALS AND METHODS

Serum Samples

Serum samples were collected from a total of 717 apparently healthy individuals aged 0–20 years (339 males and 378 females; age, mean $\pm$ standard deviation [SD], 8.6 ± 4.9 years) who resided in or around Ulaanbaatar, the capital city of Mongolia, between October 2005 and January 2006. Serum samples were stored at −40°C or below until testing for serological and molecular markers of HAV and HEV infections. After an explanation of this study was provided, signed informed consent was obtained from each subject (or, in subjects of 0–17 years of age, from his/her parent), and a questionnaire exploring sociodemographic factors and previous exposure and illnesses was administered. Ethical approval of this investigation was obtained from the ethical committees of the Ministry of Health of Mongolia and the Jichi Medical University in Tochigi, Japan.

Serological Testing

Total antibody against HAV (anti-HAV [total]) was assayed by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (HAT-EIA, Denkaseiken, Tokyo, Japan). IgM antibody against HAV (anti-HAV IgM) was assayed by ELISA (AxSYM HAVAB-M 2.0, Abbott Japan, Tokyo, Japan). IgG, IgM, and IgA classes of antibodies against HEV (anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA, respectively) were assayed by an "in-house" ELISA as described previously [Takahashi et al., 2005]. The OD values of 0.175, 0.440, and 0.642 were used as the cutoff value for anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA, respectively. The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe. If the OD value of the tested sample was reduced by $\geq$70% in the anti-HEV assay after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HAV RNA and Determination of HAV Genotype

Ten microliters each from 10 serum samples were pooled, and each pool was tested for HAV RNA by reverse transcription (RT)-polymerase chain reaction (PCR) with nested primers derived from the 5′ untranslated region (UTR) of the HAV genome as described previously [Tsatsralt-Od et al., 2006]. Briefly, the first-round PCR for 35 cycles was carried out with primers HA018 (5′-GAT ACC TCA CCG CCG TTT GC-3′) and HA009 (5′-TCA ATG CAT CCA CTG GAT GAG-3′), and the second-round PCR for 25 cycles was carried out with primers HA019 (5′-CGT TTG CCT AGG CTA TAG GCT-3′) and HA020 (5′-CAG TCC TYC GGC GTT GAA TGG-3′ [Y = T or C]). The amplification product of the first-round PCR was 474 base pairs (bp) (nt 62–535), and that of the second-round PCR was 437 bp (nt 75–511): nucleotide numbers are in accordance with the prototype HAV isolate of genotype IA (accession no. K02990). The PCR product of the second-round PCR was subjected to electrophoresis on an agarose gel, and a sample with a visible band at 437 bp was considered to be positive for HAV RNA. If a pool was positive for HAV RNA, the 10 serum samples of that pool were individually tested for the presence of HAV RNA, and the amplicons derived from HAV RNA-positive samples were subjected to sequence analysis. The HAV genotype was determined by phylogenetic analysis of the amplified HAV sequence (395 nt: primer sequences at both ends excluded).

To confirm the presence of HAV RNA, the VP1-2B region, which contains the 2A region, of the HAV genome, was amplified and sequenced as described previously [Mitsui et al., 2006]. In brief, the RNAs extracted from 10–50 μl of serum were subjected to cDNA synthesis with primer HA022 (5′-TTR TCA TCY TTC ATT TCT GTC C-3′ [R = A or G]). The cDNAs thus obtained were subjected to first-round PCR with

primers HA021 (5′-ATT GCA AAT TAY AAY CAY TCT GAT G-3′) and HA022. The second-round PCR was carried out with primers HA023 (5′-CAT TCT GAT GAA TAY TTG TC-3′) and HA024 (5′-CAT TTC TGT CCA TTT YTC ATC-3′). The amplification product of the first-round PCR was 548 bp (nt 2,903–3,450), and that of the second-round PCR was 522 bp (nt 2,918–3,439).

Detection of HEV RNA

For detection of HEV RNA in the serum samples, nested RT-PCR with primers targeting the ORF2 region of HEV RNA was performed as described previously [Mizuo et al., 2002].

Sequence Analysis of PCR Products

The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac version 12.2.6 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Phylogenetic trees were constructed by the neighbor-joining method [Saitou and Nei, 1987]. Bootstrap values were determined on 1,000 resamplings of the data sets [Felsenstein, 1985].

Statistical Analysis

Data are presented as the mean ± SD. Statistical analyses were carried out using the Mann–Whitney's U-test for comparison of continuous variables between two groups, and the chi-square test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Prevalence of Anti-HAV and Anti-HEV Among Healthy Persons Aged 0–20 Years

Serum samples obtained from 717 apparently healthy persons of 0–20 years of age between October 2005 and January 2006, were tested for the presence of anti-HAV (total) and anti-HEV IgG. Anti-HAV was detected in 494 (68.9%) of the 717 subjects, while anti-HEV IgG was detected in only 5 subjects (0.7%), the difference being statistically significant ($P < 0.0001$). The five subjects with anti-HEV IgG were aged 1 month, and 1, 3, 8, and 15 years, and four of the five subjects were males (Table I). Anti-HEV IgM, anti-HEV IgA, and HEV RNA, which are markers of present infection of HEV, were not detectable in any of the five children. Only one 8-year-old girl had both anti-HAV (total) and anti-HEV IgG.

There were no appreciable differences in the prevalence of anti-HAV between males and females (69.9% vs. 68.0%) (Table II), nor between the population of 479 subjects who lived in apartment houses in the center of Ulaanbaatar and the population of 238 subjects who lived in gers (traditional style of Mongolian lodging) around the capital city (68.3% vs. 70.2%). Anti-HAV was detectable in 24 (75.0%) of the 32 infants aged 7 days to 6 months old; when stratified by months of age, the prevalence of anti-HAV decreased gradually from 93.3% (14/15) in the age group of <1 month to 72.7% (8/11), 50.0% (1/2), and 25.0% (1/4) in the age groups of 1–2 months, 3–4 months, and 5–6 months, respectively. Of note, anti-HAV was not detectable in any of the infants aged 7–11 months, although the number of subjects was small (n = 8). The prevalence of anti-HAV increased to 19.5% (17/87) in the age group of 1–3 years, to 50.0% (69/138) in the age group of 4–6 years, and further to >80% in the age groups of ≥7 years. Nearly all subjects (97.2%) in the age group of 16–20 years had anti-HAV.

Detection of HAV RNA Among All 717 Healthy Persons

Among 71 10-sample pools and one 7-sample pool, three 10-sample pools were positive for HAV RNA by RT-PCR using primers targeting the 5′UTR of the HAV genome. The 30 serum samples of the 3 pools that had been positive for HAV RNA were tested individually for the presence of HAV RNA by RT-PCR using the 5′UTR primers, and three samples (nos. 490, 522, and 588) were found to be positive for HAV RNA (Table III). To confirm the presence of HAV RNA, the three serum samples were subjected to another RT-PCR assay using primers derived from the VP1-2B region. All three samples were

TABLE I. Characteristics of the Five Subjects who Were Positive for Anti-Hepatitis E Virus (HEV) IgG

Subject number	Age (years)/sex	Anti-HEV (OD_{450})[a] IgG-class	IgM-class	IgA-class	HEV RNA	Anti-HAV(total)
119	3/M	0.191 (79)[b] (+)	0.067 (−)	0.016 (−)	−	−
304	15/M	0.401 (85) (+)	0.076 (−)	0.054 (−)	−	−
488	1/M	0.203 (94) (+)	0.117 (−)	0.012 (−)	−	−
500	0.08/M	0.326 (86) (+)	0.231 (−)	0.086 (−)	−	−
537	8/F	0.538 (94) (+)	0.091 (−)	0.010 (−)	−	+

[a]IgG, IgM, and IgA classes of antibodies against HEV were assayed by in-house ELISA [Takahashi et al., 2005]. The OD values of 0.175, 0.440, and 0.642 were used as the cutoff value for anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA, respectively.
[b]The number in parentheses is the percentage reduction in OD value of the tested sample in the anti-HEV IgG assay after absorption with the recombinant ORF2 protein.

TABLE II. Age-Specific Prevalence of Anti-Hepatitis A Virus (HAV) (Total) and Anti-Hepatitis E Virus (HEV) IgG Among 717 Healthy Individuals Aged 0–20 Years in Mongolia

	Number of subjects with anti-HAV (total)			Number of subjects with anti-HEV IgG		
Age (years)	Total	Male	Female	Total	Male	Female
0–0.5	24/32 (75.0%)	14/19 (73.7%)	10/13 (76.9%)	1/32 (3.1%)	1/19 (5.3%)	0/13
0.6–0.9	0/8	0/1	0/7	0/8	0/1	0/7
1–3	17/87 (19.5%)	6/40 (15.0%)	11/47 (23.4%)	2/87 (2.3%)	2/40 (5.0%)	0/47
4–6	69/138 (50.0%)	38/69 (55.1%)	31/69 (44.9%)	0/138	0/69	0/69
7–9	105/129 (81.4%)	51/65 (78.5%)	54/64 (84.4%)	1/129 (0.8%)	0/65	1/64 (1.6%)
10–12	156/187 (83.4%)	74/86 (86.0%)	82/101 (81.2%)	0/187	0/86	0/101
13–15	88/100 (88.0%)	38/43 (88.4%)	50/57 (87.7%)	1/100 (1.0%)	1/43 (2.3%)	0/57
16–20	35/36 (97.2%)	16/16 (100%)	19/20 (95.0%)	0/36	0/16	0/20
Total	494/717 (68.9%)	237/339 (69.9%)	257/378 (68.0%)	5/717 (0.7%)	4/339 (1.2%)	1/378 (0.3%)

also positive for HAV RNA by the VP1-2B PCR. Subject no. 490 was a 1-year-old girl and subject no. 588 was a 4-year-old girl, both of whom had anti-HAV (total) and anti-HAV IgM, whereas subject no. 522 (an 8-year-old boy) was negative for both anti-HAV (total) and anti-HAV IgM.

Phylogenetic Analysis of HAV RNA

The 395-nt partial nucleotide sequence within the 5′UTR of three HAV isolates obtained from the HAV-viremic children were determined and deposited in the GenBank/EMBL/DDBJ databases (AB259811 to AB259813). They differed by 98.2%–99.7% from each other, and were most closely related to reported HAV isolates of subgenotype IA with nucleotide differences of only 0%–2.5% in comparison with 18 Mongolian isolates obtained from patients with hepatitis A [Tsatsralt-Od et al., 2006] and of up to 5.8% in comparison with non-Mongolian isolates of this subgenotype. The phylogenetic tree constructed by the neighbor-joining method based on the 395-nt sequence within the 5′UTR of the HAV genome confirmed that the three HAV isolates (MNA06-490, MNA06-522, and MNA06-588) obtained in the present study segregated into subgenotype IA (Fig. 1A). The VP1-2B region sequence of 481 nt was determined for the three HAV isolates obtained in the current study (AB259814 to AB259816). Upon comparison of the 481-nt sequence in the VP1-2B region, the MNA06-490, MNA06-522, and MNA06-588 isolates were 94.2%–98.1% identical to known subgenotype IA isolates (retrievable from the GenBank/EMBL/DDBJ databases as of May 2006) and 77.8%–90.6% identical to 15 known human HAV isolates of subgenotypes IB, IIA, IIB, and IIIA whose common 481-nt sequence is known. The phylogenetic tree constructed based on the 481-nt sequence in the VP1-2B region of the HAV genome also confirmed that the three HAV isolates (MNA06-490, MNA06-522, and MNA06-588) are classifiable into subgenotype IA (Fig. 1B).

Age-Specific Prevalence of Anti-HAV and Anti-HEV IgG in the General Population of Mongolia

The age-specific prevalence of anti-HAV was compared with that of anti-HEV IgG among 966 apparently healthy individuals aged 7 days to 86 years in Mongolia (465 males and 501 females; age, 18.9 ± 19.3 years) (Fig. 2): the individuals included the 717 children/young adults enrolled in the present study and the 249 adults in our previous study [Takahashi et al., 2004]. The prevalence of anti-HAV increased rapidly from 0% among the infants aged 7–11 months to around 80% among the 9-year-old children. Anti-HAV was detected in 85.9% of the 313 subjects aged 10–19 years and, of note, in all 259 individuals (100%) aged 20–86 years. In contrast, the prevalence of anti-HEV IgG was 3.4% (33/966) among all individuals, and it was significantly lower among the persons aged 0–29 years (0.8% or 6/744) than among the population older than 30 years (12.2% or 27/222) ($P < 0.0001$).

DISCUSSION

Infection with HAV is one of the common causes of liver disease of great public health importance in the world. However, the level of endemicity, median age at time of infection, and the frequency of clinically apparent hepatitis caused by HAV vary by population [Jacobsen and Koopman, 2004]. The seroprevalence rate of HAV is highly correlated with the socioeconomic status and access to clean water and sanitation. Mongolia, one of the developing countries in Asia, has

TABLE III. Characteristics of Three Subjects who had Detectable Hepatitis A Virus (HAV) RNA in Serum

Subject number	Age (years)/sex	Anti-HAV (total)	Anti-HAV IgM	HAV genotype	Anti-HEV IgG (OD_{450})
490	1/F	+	+	IA	0.017 (−)
522	8/M	−	−	IA	0.023 (−)
588	4/F	+	+	IA	0.004 (−)

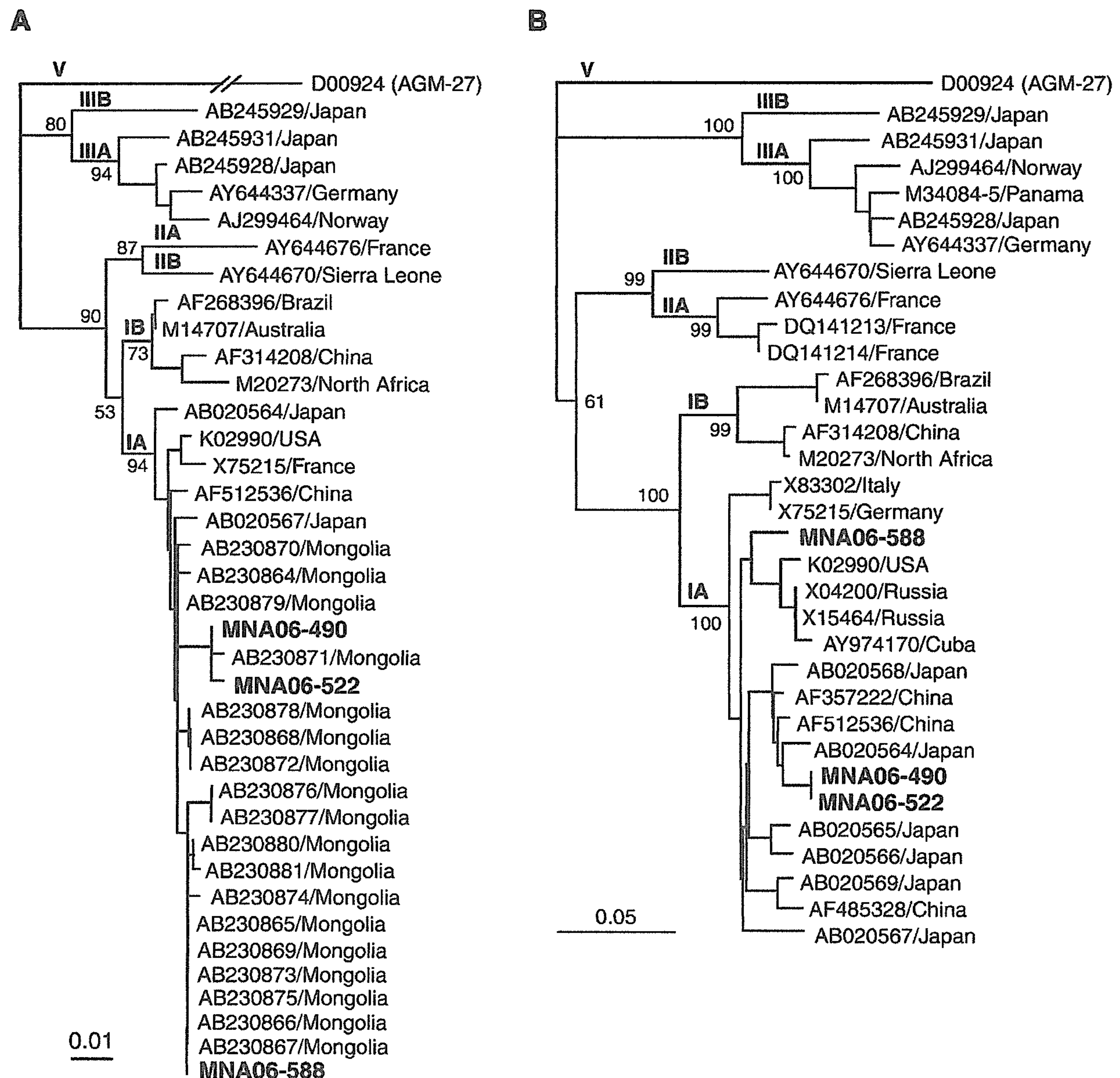

Fig. 1. Phylogenetic trees constructed by the neighbor-joining method based on the partial nucleotide sequence of the 5′UTR (395 nt) of 38 HAV isolates (A) and the VP1-2B region (481 nt) of 33 HAV isolates (B). A: In addition to the 3 HAV isolates found in the present study which are indicated with the prefix MNA followed by the year of isolation and the number of the patient, and with bold type for visual clarity, 18 HAV isolates obtained from patients with hepatitis A in Mongolia in our previous study [Tsatsralt-Od et al., 2006] and 17 reported HAV isolates of genotypes IA, IB, IIA, IIB, IIIA, IIIB, and V whose common 395-nt sequence in the 5′UTR is known were included for comparison. B: In addition to the 3 HAV isolates found in the present study which are indicated with bold type, 30 reported HAV isolates of genotypes/subgenotypes IA, IB, IIA, IIB, IIIA, IIIB, and V whose common 481-nt sequence in the VP1-2B region is known, were included for comparison. The reported isolates are indicated with the accession number followed by the name of the country where it was isolated. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings.

been considered to be highly endemic for HAV infection. However, there have been little or no data on the serological and molecular epidemiology of HAV infection among children in Mongolia. In the present study, the overall prevalence of anti-HAV among apparently healthy persons aged 0–20 years was found to be 68.9%, and age dependency of HAV seroprevalence was noted. All but one of the 15 neonates of 7–28 days of age had anti-HAV. The mother of the infant who was negative for anti-HAV was 19 years old, and the absence of anti-HAV in this infant may have been due to the lack of anti-HAV in the maternal antibodies (i.e., lack of anti-HAV in cord blood and colostrum). The prevalence of anti-HAV decreased to 72.7% in the age group of 1–2 months, 50% in the age group of 3–4 months, and 25.0% in the age group of 5–6 months, and reached 0% in the infants aged 7–11 months, suggesting the gradual disappearance of passively transferred

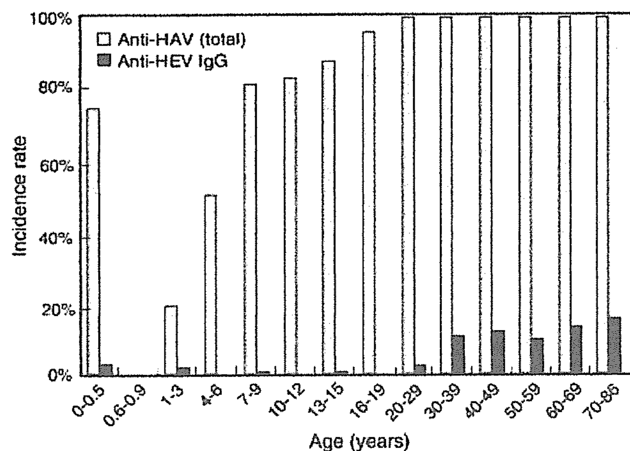

Fig. 2. Age-dependent prevalence of anti-HAV (total) and anti-HEV IgG in the general population of Mongolia (n = 966). Data on the 717 apparently healthy individuals aged 0–20 years in the present study and the 249 healthy adults aged 23–86 years in the study of Takahashi et al. [2004], are included.

maternal antibody against HAV within 6 months of life. Then, the prevalence of anti-HAV increased from 1 to 20 years of age, being 19.5% in the age group of 1–3 years, 50% in the age group of 4–6 years, and >80% in the age group of 7–9 years. Nearly all subjects (97.2%) in the age group of 16–20 years had anti-HAV. This is in agreement with our previous observation that all 249 apparently healthy adults aged 23–86 years were immune against HAV [Takahashi et al., 2004].

As a result of improvements in socioeconomic and hygiene conditions, there has been a marked reduction in HAV endemicity in industrialized countries including Australia, Canada, Japan, New Zealand, the United States, and most European countries [Shapiro and Margolis, 1993; Melnick, 1995; Sagnelli et al., 2001; Jacobsen and Koopman, 2004]. Clinically apparent hepatitis A is more likely with increasing age, and a considerable potential public health burden is generated as the distribution of HAV infection shifts to older ages [Lednar et al., 1985; Lemon, 1985; Forbes and Williams, 1990; Mele et al., 1997]. However, it is known that, in developing countries, where endemic infection with HAV occurs early in life, the agent is generally not a cause of significant morbidity. In the present study, although the medical history was sought carefully, all 717 subjects including those with anti-HAV had no history of hepatitis or jaundice, confirming that HAV infection in childhood is generally asymptomatic. Furthermore, upon testing serum samples obtained from all 717 subjects for HAV RNA, three HAV-viremic children with no signs or symptoms of hepatitis were found. Due to the limited amount of serum sample obtained from each subject, particularly from the neonates, we first screened for markers of HAV infection by testing 10-sample minipools (each pool contained 10-μl serum each from 10 subjects); then, in pools that were positive for HAV RNA, the individual serum samples were tested for the presence of HAV RNA. Since the anti-HAV IgM assay in commercially available kits requires 150 μl or more of serum for each test, only serum samples with HAV RNA were tested for anti-HAV IgM. Despite these limitations, current subclinical HAV infection was recognized molecularly in three children in this study. These three children with subclinical HAV infection were 1, 4, and 8 years of age. In contrast, in a previous study [Tsatsralt-Od et al., 2006], 18 patients were identified with clinical HAV infection of higher age, ranging from 16 to 20 years, among the 110 acute hepatitis patients of 16–48 years of age. Of interest, all but one of the 56 21–48-year-old patients who were diagnosed with acute hepatitis of a non-A category, were immune to HAV at the time of examination. These results suggest that most people in Mongolia acquire subclinical HAV infection, and the remaining minority of non-immune persons are likely to acquire clinical HAV infection between the ages of 10 and 20 years. Reflecting the extremely high prevalence of HAV infection, there were no appreciable differences in the prevalence of anti-HAV in relation to gender, residence, and lifestyle. It is assumed that the low levels of hygiene of the water supply and sewage systems in Mongolia increase significantly the likelihood of water-borne infection. To confirm this suggestion, it would be helpful if sewage sludge could be examined for HAV and the HAV RNA from such samples could be compared with the virus found in the sera of the HAV-viremic children.

Molecular epidemiological studies of HAV have identified geographic sources of HAV isolates. Most of the human strains cluster in genotype I (subgenotype IA), which accounts for 80% (67%) of all human HAV infections worldwide [Robertson et al., 1992]. In the present study, subgenotype IA HAV was recovered from all three children with current infection with HAV, confirming previous study that found that subgenotype IA was prevalent among patients with hepatitis A in Mongolia [Tsatsralt-Od et al., 2006]. Although further studies are needed to elucidate the exact distribution of HAV genotypes/subgenotypes and the range of genomic heterogeneity of HAV in Mongolia, the previous and present studies suggest that subgenotype IA strains of HAV with minor sequence variations (nucleotide difference of up to 2.5% within the 5′UTR sequence) may be responsible for clinical and subclinical HAV infections in Mongolia.

In this study, the seroprevalence of two hepatitis viruses, HAV and HEV transmitted enterically were compared. HAV and HEV had different epidemiological patterns in the Mongolian children, and only five children (0.7%) were found to be positive for anti-HEV IgG. The very low prevalence of HEV infection among children found in this study is in contrast to a study in the adult population in which as high as 11% were positive for anti-HEV IgG [Takahashi et al., 2004], in accordance with earlier observations of predilection of HEV in young adults [Purcell and Emerson, 2001a]. Whether the low prevalence of anti-HEV in children is attributable to lack of exposure to or replication of the virus remains unanswered [Arankalle et al., 2001]. Even in

the adult population, the prevalence of HEV infection was markedly lower than that of HAV infection (100%) in Mongolia [Takahashi et al., 2004]. This could be due to the lower infectivity, stability, and transmissibility of HEV than HAV, as indicated by previous observations that person-to-person transmission of HEV is uncommon not only in sporadic settings but also in outbreaks [Aggarwal and Krawczynski, 2000; Somani et al., 2003], and may be explained by the notion that transmission of HEV would not be influenced only by sanitary conditions [Purcell and Emerson, 2001a]. Increasing evidence indicates that pigs are animal reservoirs of HEV and that zoonosis is involved in the transmission of HEV [Harrison, 1999; Smith, 2001; Meng, 2003; Takahashi et al., 2003]. Recently, transmission of HEV from domestic pigs, wild deer, and boars to human beings, most likely by the ingestion of uncooked or undercooked meat or viscera from infected animals, has been reported in Japan [Tei et al., 2003; Yazaki et al., 2003; Li et al., 2005]. In Mongolia, approximately 30 million domestic animals including sheep, goats, cattle, horses, camels, and pigs are raised [http://www.discovermongolia.mn/country/nomad.lifestyle.html]. Although the prevalence of HEV infection has not yet been studied in these domestic animals in Mongolia, numerous animals have serological evidence of prior infection of HEV [Purcell and Emerson, 2001b]. Therefore, in addition to transmission by the fecal-oral route, zoonotic transmission of HEV from infected animals to humans should also be considered in Mongolia.

In conclusion, the present results show that infection with HAV (subgenotype IA), unaccompanied by overt clinical features, is very prevalent in children in Mongolia: approximately 50% of children are infected with HAV by the age of 5 years, 85% by the age of 9 years, and virtually everyone by the age of 19 years. In contrast, it appears that HEV infection is less common than HAV infection in Mongolia. However, it is likely that HEV infection will become epidemic in that country after gross contamination of the drinking water supply, and that zoonotic transmission of HEV will occur because many domestic animals are raised all over the country, although whether or not these animals are infected naturally with HEV and can serve as reservoirs for HEV infection in humans must be clarified. In this context, public health measures such as the preparation of drinking water with good quality, improvement of sanitation, and mass education in personal and public hygiene, are needed to control the hepatitis viruses, transmitted enterically and other enteric infections.

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Distinct Changing Profiles of Hepatitis A and E Virus Infection Among Patients With Acute Hepatitis, Patients on Maintenance Hemodialysis and Healthy Individuals in Japan

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To compare the epidemiologic profiles of hepatitis A virus (HAV) and hepatitis E virus (HEV) infections in Japan, the prevalence of clinical or subclinical HAV and HEV infections was investigated serologically and molecularly among 128 consecutive patients (age, mean ± standard deviation, 37.5 ± 14.7 years) who contracted acute hepatitis between 1989 and 2005 in a city hospital, and among 416 hemodialysis patients (60.1 ± 12.6 years) and 266 medical staff members (34.6 ± 11.4 years) at the same hospital, using stored periodic serum samples collected since the start of hemodialysis or employment, respectively. Between 1989 and 1995, among 93 patients with acute hepatitis, 51 (54.8%) were diagnosed with hepatitis A and only one patient with hepatitis E. Between 1996 and 2005, however, among 35 patients, only 3 (8.6%) were diagnosed with hepatitis A and 2 (5.7%) with hepatitis E. Although subclinical HEV infection was recognized in four hemodialysis patients (one each in 1979, 1980, 1988, and 2003) and two medical staff members (1978 and 2003) in previous studies, none of the 191 hemodialysis patients who had been negative for anti-HAV at the start of hemodialysis contracted HAV infection during the observation period of 7.6 ± 6.4 years. Only one (0.4%) of the 246 medical staff members who had been negative for anti-HAV at the start of employment acquired hepatitis A during the observation period of 7.9 ± 8.0 years: none had subclinical HAV infection. Clinical or subclinical HEV infection has occurred rarely during the last three decades, while HAV infection has markedly decreased at least since 1996. ***J. Med. Virol. 78:1015–1024, 2006.*** © 2006 Wiley-Liss, Inc.

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KEY WORDS: acute hepatitis; hepatitis A virus; hepatitis E virus; PCR; subclinical infection

INTRODUCTION

The clinical features of hepatitis A virus (HAV) infection are similar to those of hepatitis E virus (HEV) infection, in that they may both be transmitted by the fecal-oral route and both cause an acute self-limited illness that does not lead to a chronic state. However, previous studies suggested that the principal transmission route of these two viruses may differ and that HEV infection would not be prevented only by improved sanitary conditions [Purcell and Emerson, 2001].

Hepatitis A is a worldwide disease. Infection with HAV is endemic in developing countries and the majority of individuals in these countries are exposed to HAV during childhood. In contrast, the adult population in industrialized countries including Japan

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB245874-AB245927 (54 HAV isolates [5′UTR]), AB245928-AB245931 (4 HAV isolates [VP1/2A]) and AB245932 (one HEV isolate).

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has a decreasing rate of exposure to HAV due to improvements in hygiene and sanitation conditions [Jacobsen and Koopman, 2004]. In young children, HAV infection is often asymptomatic, whereas in older children and adults there is a wide range of clinical manifestations from mild, anicteric infection to severe, fulminant hepatic failure [Hollinger and Emerson, 2001]. HAV is the only member of the genus *Hepatovirus* within the family *Picornaviridae* [Melnick, 1992], and its viral genome consists of a 7.5-kilobases (kb) positive-stranded RNA. The HAV strains recovered from different parts of the world have been classified into six genotypes (I to VI), of which genotypes I, II, and III are found in humans: genotypes I, II, and III are further divided into subgenotypes IA and IB, IIA and IIB, and IIIA and IIIB, respectively [Robertson et al., 1992; Lu et al., 2004].

Hepatitis E is also found in many parts of the world [Emerson and Purcell, 2004]. HEV causes waterborne outbreaks and sporadic cases of acute hepatitis in many developing countries where sanitation is suboptimal. Besides imported cases of hepatitis E, domestically acquired hepatitis E has now been found in many industrialized countries including the United States, countries of the European Union and Japan [Harrison, 1999; Purcell and Emerson, 2001; Smith, 2001; Okamoto et al., 2003; Mansuy et al., 2004; Ijaz et al., 2005; Mizuo et al., 2005]. HEV has been characterized as a non-enveloped, single-stranded, positive-sense RNA virus. Its genome is approximately 7.2 kb long, and possesses three partially overlapping open reading frames (ORFs: ORF1, ORF2, and ORF3) [Tam et al., 1991; Wang et al., 2000]. HEV was recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004]. HEV sequences have been classified into four genotypes (genotypes 1–4) [Schlauder and Mushahwar, 2001; Emerson et al., 2004]. Accumulating lines of evidence have indicated that hepatitis E is a zoonosis [Meng et al., 1997, 1998; Erker et al., 1999; Harrison, 1999; Meng, 2000, 2003; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003]. In Japan, recent studies have indicated that polyphyletic HEV strains of genotypes 3 and 4 are circulating in this country [Mizuo et al., 2002; Takahashi et al., 2003], and that the zoonotic food-borne mode of transmission of HEV from various animals to humans may play an important role in the occurrence of domestic HEV infection [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004; Li et al., 2005].

However, the changing profiles of clinical or subclinical HAV and HEV infections in industrialized countries including Japan have not been fully explored. Therefore, in the present study, serological and molecular markers of HAV and HEV infections were determined and compared among consecutive patients with acute hepatitis at a city hospital in Japan between 1989 and 2005, and among hemodialysis patients and healthy individuals who had been working as medical staff members at the same hospital, using stored serum samples that had been periodically collected since the start of hemodialysis or employment, respectively.

MATERIALS AND METHODS

Serum Samples

A total of 128 consecutive Japanese patients (69 males and 59 females; age, mean ± standard deviation [SD], 37.5 ± 14.7 years; range 6–78 years) who were diagnosed clinically with acute hepatitis during the period from January 1989 to December 2005, at Masuko Memorial Hospital in Nagoya, Aichi Prefecture, of Japan were studied. All 128 patients lived in or around Nagoya City and had no history of traveling abroad within 6 months before the onset of acute hepatitis, except for one patient (JNG08-92) who contracted HAV infection while traveling in Madagascar in 1992. This study included patients with an acute illness, presenting with clinical signs or symptoms such as jaundice, dark urine, general fatigue, anorexia, nausea, vomiting and fever, and who had a serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) level that was >200 IU/L. Excluded from this study were patients with alcoholic liver disease, autoimmune liver disease, biliary tract disorders, or metabolic diseases such as fatty liver and those with a history of exposure to hepatotoxic drugs or chemicals. Serum samples obtained at the first visit and follow-up serum samples were stored at −40°C or below until testing for serological and molecular markers of HAV and HEV infections.

Serum samples that had been collected in January 2003 from 416 hemodialysis patients (274 males and 142 females; age, mean ± SD, 60.1 ± 12.6 years; range 23–91 years) and those that had been collected from the same 416 patients at the start of hemodialysis were used in the present study. Aliquots of the same 416 pairs of serum samples had been used for detection of IgG class antibodies against HEV (anti-HEV IgG) in a previous study [Mitsui et al., 2004]. In the present study, the 416 serum samples obtained at the screening and the 416 serum samples obtained at the start of hemodialysis were tested for the presence of antibodies against HAV (anti-HAV [total antibody]). The 416 hemodialysis patients had been receiving maintenance hemodialysis in the dialysis unit of Masuko Memorial Hospital for longer than 3 months (7.6 ± 6.3 [range 0.3–26.0] years).

Serum samples that had been collected from a total of 266 medical staff members (35 males and 231 females; age, mean ± SD, 34.6 ± 11.4 years; range 18–62 years) between March and April 2004 (or just before retirement or maternity leave), who had been working at Masuko Memorial Hospital for longer than 3 months (8.8 ± 8.5 [range 0.3–35.1] years), were used in the present study. Aliquots of the same 266 serum samples had been used for detection of anti-HEV IgG in a previous study [Mitsui et al., 2005]. In the present study, the 266 serum samples were tested for the presence of anti-HAV (total antibody). Additionally, stored serum samples that had been obtained from the 266 medical staff members at the

start of employment were tested for the presence of anti-HAV and anti-HEV IgG in the present study. This study conforms to the ethical guidelines and was approved by the ethics committee of Masuko Memorial Hospital. Informed consent was obtained from each patient and medical staff member.

Serological Testing

IgM antibody against HAV (anti-HAV IgM) was assayed by enzyme-linked immunosorbent assay (ELISA) (AXSYM HAVAB-M 2.0; Abbott Japan, Tokyo, Japan, or HAM(N)-EIA; Denkaseiken, Tokyo, Japan). Anti-HAV antibodies (total) were assayed by ELISA using a commercially available kit (HAT-EIA Denkaseiken). IgG, IgM, and IgA classes of antibodies to HEV (anti-HEV IgG, anti-HEV IgM, and anti-HEV IgA, respectively) were assayed by in-house ELISA as described previously [Takahashi et al., 2005].

Detection of HAV RNA and Determination of HAV Genotype

Sera from individuals with anti-HAV IgM were assayed for HAV RNA by reverse transcription (RT)-polymerase chain reaction (PCR) with nested primers derived from the 5′ untranslated region (UTR) of the HAV genome as described previously [Tsatsralt-Od et al., 2006]. Briefly, the first-round PCR for 35 cycles was carried out with primers HA018 (5′-GAT ACC TCA CCG CCG TTT GC-3′) and HA009 (5′-TCA ATG CAT CCA CTG GAT GAG-3′), and the second-round PCR for 25 cycles was carried out with primers HA019 (5′-CGT TTG CCT AGG CTA TAG GCT-3′) and HA020 (5′-CAG TCC TYC GGC GTT GAA TGG-3′ [Y = T or C]). The amplification product of the first-round PCR was 474 base pairs (bp) (nt 62–535), and that of the second-round PCR was 437 bp (nt 75–511): nucleotide numbers are in accordance with the prototype HAV isolate of genotype IA (accession no. K02990). The PCR product of the second-round PCR was subjected to electrophoresis on an agarose gel, and a sample with a visible band at 437 bp was considered to be positive for HAV RNA. The HAV genotype was determined by phylogenetic analysis of the amplified HAV sequence (395 nt: primer sequences at both ends excluded).

To confirm the HAV genotype/subgenotype, the VP1/2A junctional region of the HAV genome covering the 168-nt sequence, which has previously been used for genotyping [Robertson et al., 1992], was amplified and sequenced. The RNAs extracted from 100 μl of serum were subjected to cDNA synthesis with reverse transcriptase (Superscript II; Invitrogen, Tokyo, Japan) and primer HA022 (5′-TTR TCA TCY TTC ATT TCT GTC C-3′). The cDNAs were subjected to first-round PCR with *TaKaRa* Ex Taq (TaKaRa Bio, Shiga, Japan) and primers HA021 (5′-ATT GCA AAT TAY AAY CAY TCT GAT G-3′) and HA022 for 35 cycles (94°C for 2 min before the start of cycling: 94°C, 30 sec; 53°C, 30 sec; 72°C, 60 sec [additional 7 min in the last cycle]). The second-round PCR for 25 cycles was carried out under the same conditions as the first-round PCR with primers HA023 (5′-CAT TCT GAT GAA TAY TTG TC-3′) and HA024 (5′-CAT TTC TGT CCA TTT YTC ATC-3′). The amplification product of the first-round PCR was 548 bp (nt 2,903–3,450), and that of the second-round PCR was 522 bp (nt 2,918–3,439).

The sequences of the primers mentioned above were chosen from well-conserved areas of the entire HAV genome by comparing 22 known human and one known simian HAV sequences, which were retrievable from the DDBJ/GenBank/EMBL databases in December, 2005, in order to develop two universal RT-PCR assays that are capable of detecting HAV strains with significant sequence variations.

Detection of HEV RNA and Determination of HEV Genotype

For detection of HEV RNA in the serum samples, nested RT-PCR with primers targeting the ORF2 region of HEV RNA was performed as described previously [Mizuo et al., 2002]. The size of the amplification product of the first-round PCR was 506 bp, and that of the second-round PCR was 457 bp. The nested RT-PCR assay that we used has the capability of amplifying all four known genotypes of HEV strains reported thus far [Mizuo et al., 2002; Takahashi et al., 2003; Yazaki et al., 2003]. The HEV genotype was determined by phylogenetic analysis of the ORF2 region of the HEV isolates.

Sequence Analysis of PCR Products

The amplification products were sequenced directly on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Genetyx-Mac version 12.2.6 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ: National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Phylogenetic trees were constructed by the neighbor-joining method [Saitou and Nei, 1987]. Bootstrap values were determined on 1,000 resamplings of the data sets [Felsenstein, 1985].

Statistical Analysis

Data are presented as mean ± SD. Statistical analyses were performed using the χ^2 test or Fisher's exact probability test for comparison of proportions between two groups. Differences were considered to be statistically significant when the P-value was <0.05.

RESULTS

Prevalence of Type A or E Acute Hepatitis Between 1989 and 2005

The prevalence of HAV and HEV infections among 128 patients with acute hepatitis who were seen in a city hospital in Japan between 1989 and 2005, was surveyed by testing serum samples for serological and molecular markers of HAV and HEV infections.

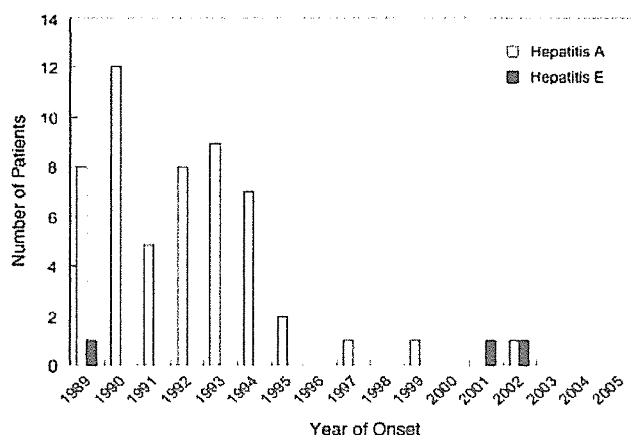

Fig. 1. Annual number of patients who were diagnosed as having hepatitis A or E at our hospital between 1989 and 2005.

Hepatitis A (anti-HAV IgM-positive) was diagnosed in 54 patients (42.2%), all of whom had detectable HAV RNA. The annual number of patients with hepatitis A decreased markedly starting in 1996 (Fig. 1). Three patients were positive for anti-HEV IgM and anti-HEV IgA as well as HEV RNA, and they were diagnosed as having hepatitis E: they acquired HEV infection in 1989, 2001, and 2002, respectively.

Between 1989 and 1995, among the 93 patients with acute hepatitis, 51 patients (54.8%) were diagnosed with hepatitis A; in contrast, only one patient (1.1%) was diagnosed with hepatitis E during this period. Interestingly, between 1996 and 2005, only three patients (8.6%) were diagnosed with hepatitis A and two patients (5.7%) with hepatitis E (Table I).

Prevalence of Anti-HAV Among Hemodialysis Patients and Medical Staff Members

At the time of screening, serum samples obtained from 416 patients on maintenance hemodialysis at a dialysis unit of a city hospital in January 2003 and serum samples obtained from 266 medical staff members at the same hospital in March and April 2004 (or just before retirement or maternity leave), were tested for the presence of anti-HAV (total). Anti-HAV was detected in 225 (54.1%) of the 416 hemodialysis patients and 21 (7.9%) of the 266 medical staff members, the difference being statistically significant ($P < 0.0001$) (Table II). However, anti-HAV was not detected in any of the six hemodialysis patients and 110 healthy individuals in the age group of 18–29 years, and the patterns of age-specific prevalence of anti-HAV were similar in the hemodialysis patients and the medical staff members in the age groups of 30–39, 40–49, 50–59, and 60–69 years. The prevalence of anti-HAV gradually increased with age until 50–59 years old, and the prevalence was >70% in the age groups greater than 60 years.

Comparison of the Age-Specific Prevalence of Anti-HAV and Anti-HEV IgG Among the Hemodialysis Patients or the Medical Staff Members

At the time of screening, among the 416 hemodialysis patients, 23 patients (5.5%) had both anti-HAV (total) and anti-HEV IgG, while 202 patients (48.6%) had anti-HAV alone and 16 (3.8%) had anti-HEV IgG alone. The age-specific prevalence of anti-HAV was compared with that of anti-HEV IgG among the 416 patients on maintenance hemodialysis (Table III). The prevalence of anti-HAV or anti-HEV IgG was each 3.0% in the age group of 23–39 years and 7.5% in the age group of 40–49 years. However, anti-HAV was significantly more prevalent than anti-HEV IgG in the age groups over 50 years. Although anti-HEV IgG was more prevalent among males than among females as described previously [Mitsui et al., 2004], the prevalence of anti-HAV was quite similar between males and females.

As for the medical staff members, none of the 266 subjects had both anti-HAV and anti-HEV IgG, while 21 subjects (7.9%) had anti-HAV only and 6 (2.3%) had anti-HEV IgG only. The age-specific prevalence of anti-HAV and anti-HEV IgG was comparable in the age groups of 18–29, 30–39, and 40–49 years. However, in the age group of 50–62 years, the prevalence of anti-HAV was significantly higher than that of anti-HEV IgG (38.6% [17/44] vs. 2.3% [1/44], $P = 0.0004$).

TABLE I. Demographic Features and Laboratory Findings in 128 Patients With Acute Hepatitis in a City Hospital Between 1989 and 2005

Feature	Total (n = 128)	Type A (n = 54)	Type E (n = 3)
Mean age ± SD (years)	37.5 ± 14.7	37.6 ± 14.7	49.3 ± 2.5
Male (%)	69 (53.9%)	23 (42.6%)	3 (100%)
Year of onset			
1989–1995	93	51 (54.8%)	1 (1.1%)
1996–2005	35	3 (8.6%)	2 (5.7%)
Laboratory data[a]			
Total bilirubin (mg/dl)	5.3 ± 5.9	6.1 ± 2.8	4.7 ± 4.5
ALT (IU/L)	1,741.5 ± 1,578.0	2,522.0 ± 1,944.6	1,609.3 ± 496.5
AST (IU/L)	1,412.4 ± 1,922.6	2,239.0 ± 2,592.5	868.7 ± 563.8

Normal range: total bilirubin (0.2–1.0 mg/dl); ALT, alanine aminotransferase (5–40 IU/L); AST, aspartate aminotransferase (10–40 IU/L).
[a]At the first examination.

TABLE II. Age-Specific Prevalence of Anti-Hepatitis A virus (HAV) Among 416 Patients on Maintenance Hemodialysis and 266 Healthy Individuals (Medical Staff Members at a City Hospital) at Screening

Age (years)	Hemodialysis patients[a] (n = 416)	Staff members[b] (n = 266)	*P*-value
18–29	0/6	0/110	
30–39	1/27 (3.7%)	1/75 (1.3%)	0.4613 (NS)
40–49	3/40 (7.5%)	3/37 (8.1%)	0.6244 (NS)
50–59	37/109 (33.9%)	16/43 (37.2%)	0.7172 (NS)
60–69	103/138 (74.6%)	1/1 (100%)	0.7482 (NS)
70–79	63/77 (81.8%)	0	
80–91	18/19 (94.7%)	0	
Total	225/416 (54.1%)	21/266 (7.9%)	<0.0001

[a]Serum samples collected from 416 hemodialysis patients in January 2003, were tested.
[b]Serum samples collected from 266 subjects in March and April 2004 (or just before retirement or maternity leave), were tested.

Anti-HAV Antibodies Among the Hemodialysis Patients and Medical Staff Members

The presence of anti-HAV was surveyed in stored serum samples that had been obtained from the 416 patients at the start of hemodialysis. All 225 patients who had anti-HAV in the serum samples obtained at the screening, tested positive for anti-HAV at the start of hemodialysis (Table IV), indicating that they all remained seropositive from the time that hemodialysis was started, and that none of the hemodialysis patients contracted de novo HAV infection. In other words, although subclinical HEV infection was recognized in four hemodialysis patients (1.1%) in the previous study [Mitsui et al., 2004], none of the 191 hemodialysis patients who had been negative for anti-HAV at the start of hemodialysis contracted clinical nor subclinical HAV infection during the observation period of 7.6 ± 6.4 years.

Among the 21 medical staff members who were positive for anti-HAV at the screening, 20 subjects (95.2%) tested positive for anti-HAV at the start of employment and the remaining one subject was negative for anti-HAV, indicating that the seroconverted subject contracted de novo HAV infection during the follow-up. Although subclinical HEV infection was noted in two medical staff members (0.8%) in a previous study [Mitsui et al., 2005], none of the 246 staff members who had been negative for anti-HAV at the start of employment contracted subclinical HAV infection during the observation period of 7.9 ± 8.0 years. Of note, only one (0.4%) of the 246 medical staff members who had been negative for anti-HAV at the start of employment acquired hepatitis A in 1991. The subject (Subject JNG04-91: see Fig. 2) was a 23-year-old nurse and was diagnosed with acute hepatitis A after she was found to have an elevated ALT level of 354 IU/L at a monthly health check-up conducted on June 13, 1991 and tested positive for anti-HAV IgM and HAV RNA. She had first complained of mild general fatigue and poor appetite 6 days before the health check-up.

Genetic Analysis of HAV and HEV Isolates

The 395-nt partial nucleotide sequence within the 5′UTR of 54 HAV isolates obtained from patients with acute hepatitis A were determined and deposited in the DDBJ/GenBank/EMBL databases (AB245874 to AB245927). They differed by up to 10.7% from each other, and 50 of the 54 isolates were most closely related to reported HAV isolates of subgenotype IA with nucleotide differences of up to 7.3%. The remaining four HAV isolates (HA-JNG04-90, HA-JNG06-90, HA-JNG08-90, and HA-JNG08-92) were most similar to the subgenotype IIIA isolates from Germany and Norway (AY644337 and AJ299464, respectively) with identities of 94.2–98.7%, suggesting that they are classifiable into genotype III. The phylogenetic tree constructed by the neighbor-joining method based on the 395-nt sequence within the 5′UTR of the HAV genome confirmed that the 54 HAV isolates obtained in the present study segregated into genotype IA or III (Fig. 2).

Since the 5′UTR sequence of subgenotype IIIB isolates is not available, the VP1/2A junctional region sequence of 481 nt was determined for the four genotype III isolates obtained in the current study. Upon comparison within the VP1/2A region sequence of 168–481 nt, the HA-JNG04-90 and HA-JNG08-92 isolates were 92.9–99.4% identical to 34 known genotype IIIA isolates

TABLE III. Age-Specific Prevalence of Anti-Hepatitis A Virus (HAV) (Total) and Anti-Hepatitis E Virus (HEV) IgG Among 416 Patients on Maintenance Hemodialysis at Screening in January 2003

	Number of patients with anti-HAV (total)			Number of patients with anti-HEV IgG[a]		
Age (years)	Total	Male	Female	Total	Male	Female
23–39	1/33 (3.0%)	1/21 (4.8%)	0/12	1/33 (3.0%)	0/21	1/12 (8.3%)
40–49	3/40 (7.5%)	3/29 (10.3%)	0/11	3/40 (7.5%)	3/29 (10.3%)	0/11
50–59	37/109 (33.9%)*	20/71 (28.2%)	17/38 (44.7%)	10/109 (9.2%)*	9/71 (12.7%)	1/38 (2.6%)
60–69	103/138 (74.6%)**	72/92 (78.3%)	31/46 (67.4%)	17/138 (12.3%)**	13/92 (14.1%)	4/46 (8.7%)
70–79	63/77 (81.8%)**	41/48 (85.4%)	22/29 (75.9%)	6/77 (7.8%)**	5/48 (10.4%)	1/29 (3.4%)
80–91	18/19 (94.7%)***	13/13 (100%)	5/6 (83.3%)	2/19 (10.5%)***	1/13 (7.7%)	1/6 (16.7%)
Total	225/416 (54.1%)**	150/274 (54.7%)	75/142 (52.8%)	39/416 (9.4%)**	31/274 (11.3%)	8/142 (5.6%)

[a]From Mitsui et al. [2004].
*$P = 0.0001$.
**$P < 0.0001$.
***$P = 0.0002$.

TABLE IV. Characteristics of 416 Hemodialysis Patients and 266 Healthy Individuals, Stratified by the Presence/Absence of Anti-Hepatitis A Virus (HAV) at the Start of Follow-Up and at Screening: Comparison With the Results of Anti-Hepatitis E Virus (HEV) IgG

Presence of anti-HAV or anti-HEV at the start of follow-up/at screening	Anti-HAV (total)			Anti-HEV IgG[a]		
	Number of subjects	Age (mean ± SD, years)[b]	Duration of follow-up (mean ± SD, years)	Number of subjects	Age (mean ± SD, years)[b]	Duration of follow-up (mean ± SD, years)
Hemodialysis patients (n = 416)						
No/Yes[c]	0	NA	NA	4 (1.0%)	40.0 ± 7.8	18.2 ± 6.4
No/No	191 (45.9%)	44.3 ± 12.7	7.6 ± 6.4	370 (88.9%)	52.3 ± 14.3	7.5 ± 6.1
Yes/Yes	225 (54.1%)	59.4 ± 11.4	7.5 ± 6.1	35 (8.4%)	56.6 ± 11.9	6.3 ± 5.9
Yes/No	0	NA	NA	7 (1.7%)	50.6 ± 10.9	13.0 ± 6.9
Healthy individuals (n = 266)						
No/Yes[c]	1 (0.4%)	21	14.7	2 (0.8%)	21.5 ± 2.5	17.7 ± 19.3
No/No	245 (92.1%)	25.1 ± 7.4	7.9 ± 8.0	258 (97.0%)	25.7 ± 7.8	8.7 ± 8.4
Yes/Yes	20 (7.5%)	34.4 ± 8.4	19.2 ± 7.2	4 (1.5%)	32.5 ± 10.2	4.4 ± 3.9
Yes/No	0	NA	NA	2 (0.8%)	35.0 ± 7.1	18.3 ± 4.0

[a]In part from Mitsui et al. [2004, 2005].
[b]At the start of hemodialysis or employment.
[c]de novo infection during the observation period.

(retrievable from the DDBJ/GenBank/EMBL databases as of January 2006) and 86.3–88.1% identical to six known genotype IIIB isolates, indicating that these two isolates segregate into subgenotype IIIA. On the other hand, the HA-JNG06-90 and HA-JNG08-90 isolates were 100% identical to each other and were more similar to the known IIIB isolates than to IIIA isolates (95.2–100% vs. 85.7–91.6%), indicating that these two HAV isolates are further classifiable into subgenotype IIIB. The HA-JNG04-91 isolate recovered from the serum sample that had been obtained from Subject JNG04-91 on June 13, 1991 was 100% identical in the 395-nt 5′UTR sequence to the HA-JNG11-90 isolate recovered from the serum sample that had been obtained from a hospitalized patient on December 29, 1990. However, considering the interval between the dates of disease onset of the two hepatitis cases, the possibility that Subject JNG04-91 contracted HAV infection from the hospitalized patient can be ruled out.

The 412-nt sequence of ORF2 of three HEV isolates (HE-JANGP89, HE-JANGP1, and HE-JANGP2) that were recovered from three patients who contracted clinical HEV infection in 1989, 2001, and 2002, respectively, were compared with each other and with reported HEV isolates. The HE-JANGP89, HE-JANGP1, and HE-JANGP2 isolates differed from each other by 7.5–11.7%, but were closely related to the prototype Japanese genotype 3 isolate (JRA1 [AP003430]) with nucleotide sequence identity of 86.8–93.0%, and were only 77.6–79.3%, 75.7–77.3%, and 77.8–80.0% similar to the SAR-55 isolate [M73218] of genotype 1, MEX-14 isolate [M74506] of genotype 2, and T1 isolate [AJ272108] of genotype 4, respectively, in the 412-nt ORF2 sequence. The phylogenetic tree constructed based on the common 412-nt sequence within the ORF2 region confirmed that the HE-JANGP89, HE-JANGP1, and HE-JANGP2 isolates belonged to genotype 3 (data not shown).

DISCUSSION

This study examined the changing profiles of clinical HAV and HEV infections during the last 17 years and subclinical HAV and HEV infections during the observation period of 0.3–27 years in a city hospital in Aichi Prefecture, which is located in the central part of Honshu Island of Japan. Although the results cannot simply be generalized for the whole country, previous studies on the age-specific prevalence of HAV and/or HEV infections revealed similar changing trends in other urban and rural areas including Metropolitan Tokyo, a small district of South Kiso town located between Aichi and Tokyo, and Okinawa in Japan [Furusyo et al., 1998; Tanaka et al., 2001, 2005; Malaty et al., 2003].

The prevalence of HAV infection is mainly influenced by general hygiene, especially by factors that reflect living standards and socioeconomic status [Hollinger and Emerson, 2001; Jacobsen and Koopman, 2004]. As a result of the improvement in socioeconomic and hygiene

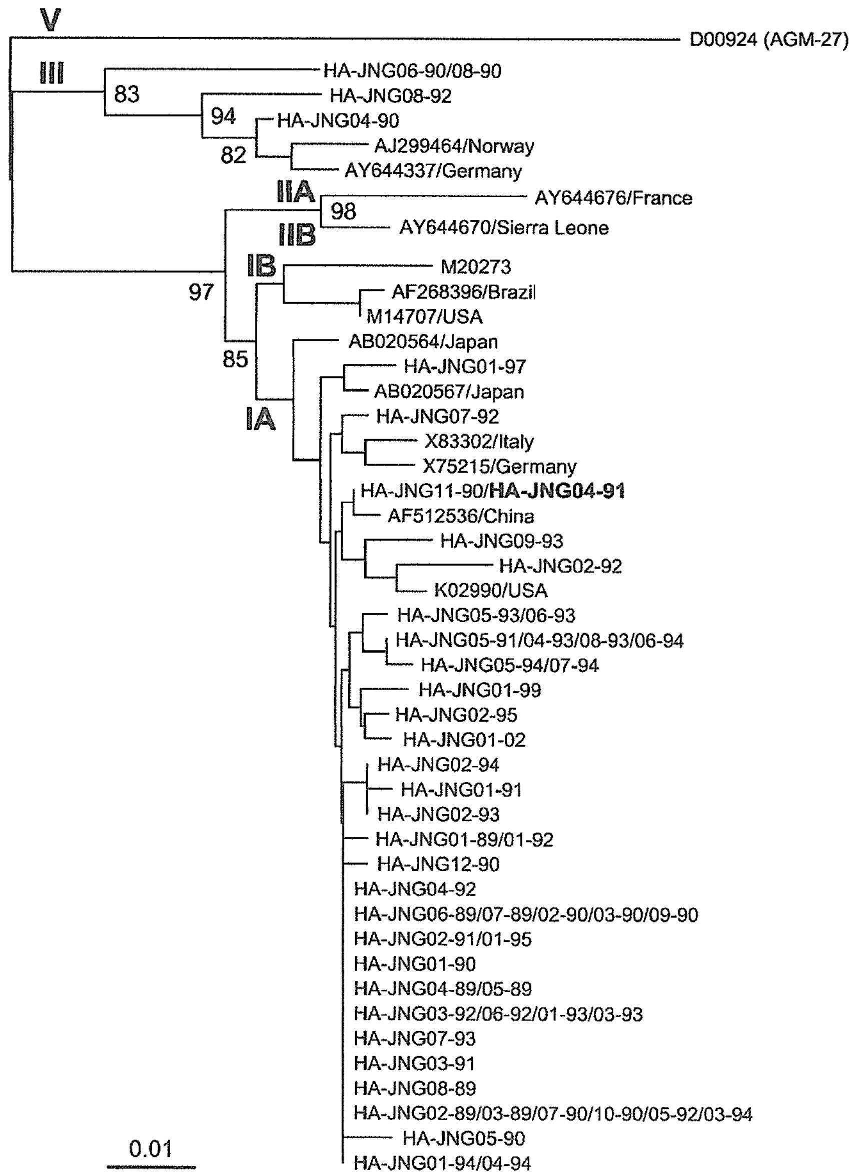

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the 5′UTR (395 nt) of 68 HAV isolates. In addition to the 54 HAV isolates found in the present study which are indicated with the prefix HA-JNG followed by the number of the patient and the year of isolation, 14 reported HAV isolates of genotypes IA, IB, IIA, IIB, III, and V whose entire sequence is known were included for comparison. The HA-JNG04-91 isolate that was recovered from a 23-year-old nurse who had contracted hepatitis A in 1991 and had been first diagnosed through a monthly health check-up, is indicated in bold type. The reported isolates are indicated with the accession number followed by the name of the country where it was isolated, when available. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings.

conditions, there has been a marked reduction in HAV endemicity in Western countries over the past two decades [Shapiro and Margolis, 1993; Melnick, 1995; Sagnelli et al., 2001], with a subsequent shift in the prevalence of the disease to older individuals and an increase in the number of cases with a symptomatic and severe illness [Mele et al., 1997]. In Japan, there was rapid improvement in sanitary conditions and standards of living after World War II, and clean public water systems were introduced in the 1950s. Surveys from across the country reported that the age at which more than half of the population had HAV antibodies was above 30 years in the 1970s [Moritsugu et al., 1978; Ichida et al., 1981] and has been increasing [Kiyohara et al., 1997; Furusyo et al., 1998]. The present study revealed that the age at which more than half of the hemodialysis patients and healthy individuals (medical staff members) who were positive for anti-HAV (total), was both above 60 years. Tanaka et al. [2005] observed a similar phenomenon; in the age-specific profiles of anti-HAV in healthy Japanese volunteers, the prevalence of anti-HAV started to increase sharply in the 20- to 29-year age group in 1974, in the 30- to 39-year age group in 1984, and in the 40- to 49-year age group in

1994, suggesting that HAV infection terminated in about 1974. In the present study which included consecutive patients who were diagnosed with acute hepatitis at a city hospital over a 17-year period (between 1989 and 2005), the prevalence of HAV infection among patients with acute hepatitis decreased markedly from 54.8% between 1989 and 1995 to 8.6% between 1996 and 2005: the number of newly diagnosed hepatitis A patients per year at the city hospital changed from 7.3 (51/7) to 0.3 (3/10), suggesting that de novo HAV infection has been declining continuously even during the past decade in Japan.

In industrialized countries including Japan, maintenance of good hygiene of the water supply and sewage systems now makes the likelihood of water-borne infection extremely low. However, it was reported that, based on the age-specific distribution of anti-HEV in 1974, 1984, and 1994, exposure to HEV has not decreased during the past 20 years in Japan, unlike that to HAV [Tanaka et al., 2005]. This is consistent with the notion that the principal transmission route of HEV may be different from that of HAV and that transmission of HEV would not be prevented by only improvement of sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [Purcell and Emerson, 2001]. In support of the previous observations, the HEV prevalence rate among patients with acute hepatitis between 1989 and 1995 was comparable with that between 1996 and 2005 in the present study (Table I). Increasing lines of evidence indicate that pigs are animal reservoirs of HEV and that zoonosis is involved in the transmission of HEV [Meng et al., 1997, 1998; Harrison, 1999; Meng, 2000, 2003; Okamoto et al., 2001; Smith, 2001; Nishizawa et al., 2003; Takahashi et al., 2003]. Recently, transmission of HEV from domestic pigs, wild deer and boars to human beings, most likely by ingestion of uncooked or undercooked meat or viscera from infected animals, has been reported in Japan [Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004; Li et al., 2005]. However, the mode of HEV transmission in the three patients with clinical HEV infection in the present study was unclear. Notably, however, all three patients were males with the age of 47, 49, or 52 years. Although there is a possibility that older people are more susceptible to infection or more likely to manifest symptomatic disease, it was reported that a substantial proportion of patients with domestically acquired hepatitis E were elderly men, not only in Japan [Mizuo et al., 2002, 2005; Okamoto et al., 2003] but also in France and the United Kingdom [Mansuy et al., 2004; Ijaz et al., 2005].

Infections with HAV and HEV have been extensively studied in various parts of the world. A number of cross-sectional studies have compared the seroepidemiologic patterns of HAV and HEV infection to address whether there is a common infection pathway(s) [Arankalle et al., 2001; Sidal et al., 2001; Murhekar et al., 2002; Wong et al., 2004; Tanaka et al., 2005; Lin et al., 2006]. These studies suffer from some limitations, because their designs only allowed examination of the seroprevalence pattern of both infections within a community at a single, or at most three time points, and sequential samples were not available from each of the seroconverted individuals. Therefore, longitudinal data on de novo infection of HAV or HEV in substantial numbers of susceptible individuals have not been collected. Taking advantage of stored serum samples that had been obtained periodically from 416 hemodialysis patients and 266 medical staff members at the same city hospital since the start of hemodialysis or employment, respectively, the current study was conducted to evaluate the presence of subclinical HAV and HEV infections among a total of 682 subjects. Although subclinical HEV infection, which is unaccompanied by ALT elevation, was recognized in four hemodialysis patients (1.1%) (one patient each in 1979, 1980, 1988, and 2003) [Mitsui et al., 2004], none of the 191 hemodialysis patients who had been negative for anti-HAV at the start of hemodialysis contracted subclinical (nor even clinical) HAV infection during the mean observation period of 7.6 years. Subclinical HEV infection was noted in two medical staff members (0.8%) (one subject each in 1978 and 2003) [Mitsui et al., 2005]; none of the 246 staff members who had been negative for anti-HAV at the start of employment contracted subclinical HAV infection during the mean observation period of 7.9 years. Of note, only one medical staff member (23-year-old nurse) who had been negative for anti-HAV at the start of employment (1989) acquired hepatitis A in 1991. Taken altogether, HAV infection tended to be less prevalent than HEV infection during a comparable observation period, irrespective of the manifestation of acute illness, with the difference falling short of being statistically significant (0.2% [1/437] vs. 0.9% [6/636]).

Molecular epidemiological studies on HAV have identified geographic sources of HAV isolates. Most of the human strains cluster in genotype I (subgenotype IA), which accounts for 80% (67%) of all human HAV infections worldwide [Robertson et al., 1992]. In the present study, subgenotype IA was also predominant (93% or 50/54), corroborating the previous study that found that subgenotype IA was the most predominant and subgenotypes IB and IIIB were rarely found in Japan, accounting for 91%, 1%, and 8%, respectively [Robertson et al., 1992]. Subgenotype IIIA HAV isolates have been isolated in India, Malaysia, Nepal, Panama, Sri Lanka, Sweden, and the USA, but not in Japan thus far. One (HA-JNG08-92) of the two subgenotype IIIA isolates obtained in the present study was isolated from a 26-year-old man who had developed acute hepatitis while traveling in Madagascar and was diagnosed with hepatitis A soon after returning to Japan in 1992, suggesting that he was infected with a IIIA HAV strain indigenous to Madagascar. However, the other isolate (HA-JNG04-90) of subgenotype IIIA was recovered from a 23-year-old woman who had never been abroad, suggesting that a subgenotype IIIA HAV strain is circulating in Japan.

In conclusion, the present results indicate that the prevalence patterns and changing trends of HAV and

HEV infections differ considerably, and suggest that clinical or subclinical HEV infection has rarely been occurring during the last three decades, while HAV infection has been continuously decreasing and markedly decreased at least since 1996. These results raise two points. Considering the worldwide distribution and perpetuation of HEV, closer attention to infection with HEV is required, especially because it can induce fulminant hepatitis not only in pregnant women in developing countries [Khuroo and Kamili, 2003], but also in sporadic cases, with predilection for elderly men, in industrialized countries [Suzuki et al., 2002]. Considering that, although HAV infection is often asymptomatic in children, most infected adults present with severe illness, adequate and timely measures need to be taken to eliminate infection of HAV in elderly persons. In this context, continuing monitoring of the seroprevalence of HAV and HEV infections is necessary to describe the most recent epidemiological pattern in order to develop public health interventions against HAV and HEV infections in Japan, a low-endemic country for these two viruses.

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