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PRESENT EPIDEMIOLOGICAL PATTERN OF ANTIBODY TO HEPATITIS A VIRUS AMONG CHIANG MAI CHILDREN, NORTHERN THAILAND

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Abstract. Hepatitis A virus (HAV) infection is common in Southeast Asia, and most of the inhabitants acquire a lifelong immunity as a result of natural infection during childhood. However, the age-specific seroprevalence is changing with development of socioeconomic and hygiene status in this area and the infection is predicted to shift to adulthood with more severe clinical manifestations in the future. In this study, we report the present epidemiological pattern of antibody to HAV (anti-HAV) among schoolchildren in Chiang Mai, Northern Thailand. The overall prevalence rate of anti-HAV was 9.6% (11.4% in female and 7.5% in male children, and 10.8% in urban and 8.9% in rural schoolchildren, respectively). Our study, comparing with previous reports from other parts in Thailand, indicates a steady decline of anti-HAV prevalence among schoolchildren in Chiang Mai area, and discussed a possibility of an outbreak of HAV infection among urban schoolchildren.

INTRODUCTION

Hepatitis A virus (HAV) infection is highly endemic in Southeast Asian countries, including Thailand; however, it is not regarded as an important public health problem. HAV infection, which universally occurs during childhood in these developing countries, is often asymptomatic or mildly symptomatic and yields a lifelong immunity before adulthood (Burke *et al*, 1981; Echeverria *et al*, 1983). Over the past one or two decades, many countries in this area have experienced dramatic changes in age-specific seroprevalence of anti-HAV antibodies. With improvements in socioeconomic conditions, this infection has been shifting from childhood to adulthood.

HAV infection becomes more symptomatic and severity of the illness increases with advancing age. A review of the age-related seroprevalence of HAV in Southeast Asian countries showed HAV infection pattern has changed over recent years; Singapore, Thailand and Malaysia have all shown a marked decline in childhood and adolescent HAV seroprevalence (Kunasol *et al*, 1998). Although the data from previous studies in Thailand showed a significant decline in anti-HAV prevalence in children (Burke *et al*, 1981; Echeverria *et al*, 1983; Innis *et al*, 1991; Kosuwan *et al*, 1996; Poovorawan *et al*, 1991; 1993), there is little information concerning the seroprevalence of anti-HAV antibodies in the northern region of Thailand.

In this study we report the age-specific seroprevalence of anti-HAV antibodies among children aged 4-16 years in urban and rural schools in Chiang Mai Province, Northern Thailand. We compare these data with previous reports from various parts of Thailand and also discuss a possible outbreak of HAV in an urban school in this area.

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MATERIALS AND METHODS

Study population

During the period of July 1998 to August 2000, a total of 1,145 serum samples were randomly collected from children aged 4-16 years in Chiang Mai Province, Northern Thailand. These children attended one of 7 rural schools (720 children, with 340 males and 380 females) or one of 3 urban schools (425 children with 192 males and 233 females). All subjects or parents were previously informed about the goals of the project, and written consent was obtained from the parents and headmasters of the specific schools. Serum samples were stored at 4°C not more than 3 days or at -20°C until tested.

Serologic study

Sera were tested for the presence of anti-HAV antibodies by an ELISA method using a commercial kit HAV Total from Sanofi Diagnostic Pasteur (Manes la Coquette, France). Positive samples were retested; only repeatedly reactive sera were considered positive.

RESULTS

Among the 1,145 schoolchildren, 110 (9.6%) were positive for anti-HAV antibodies. Female children showed a higher rate of seropositivity (11.4%) than male children (7.5%). The prevalence rate of anti-HAV antibody positivity increased with age (Fig 1).

The average prevalence rate of anti-HAV seropositivity was 10.8% in urban schoolchildren, compared with 8.9% in rural children. The age-specific seroprevalence of anti-HAV antibodies showed a linear increase among rural children, while urban children revealed an irregular pattern of age-specific seroprevalence (Fig 2).

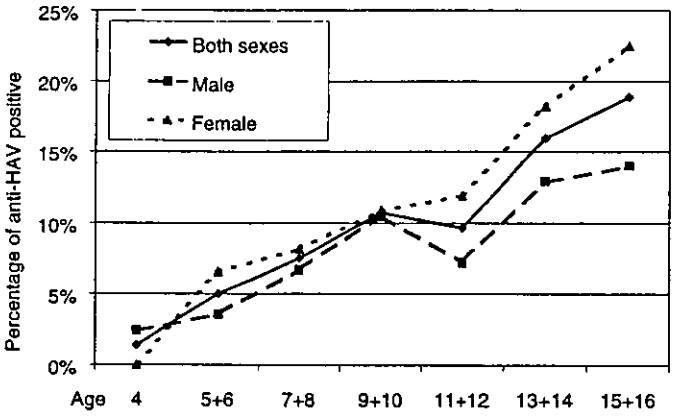

Fig 1–Age-specific seroprevalence of anti-HAV antibodies in Chiang Mai children.

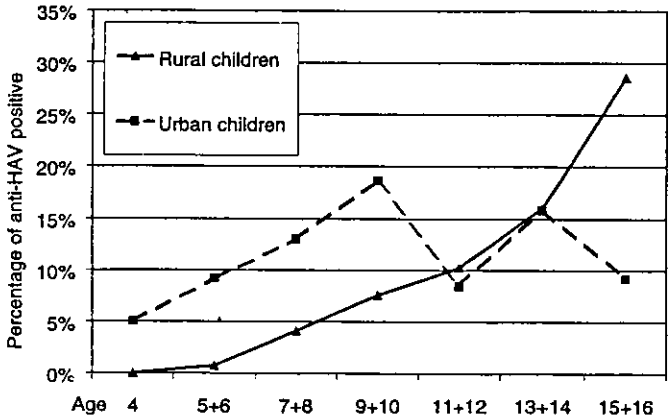

Fig 2–Age-specific seroprevalence of anti-HAV antibodies among rural and urban schoolchildren in Chiang Mai.

DISCUSSION

Hepatitis A virus (HAV) is present in most parts of the world and is responsible for both epidemic and sporadic infections. Serological surveys in the 1970s demonstrated that hepatitis A was very common in Thailand. A nearly 50% prevalence rate was found in 8-9 year old rural children and 10-11 year old children in Bangkok, while almost all (97%) of those older than 16 years were positive for anti-HAV antibodies (Burke *et al*, 1981). For the last ten years, the seroprevalence of anti-HAV antibodies has been decreasing and the pattern of age-specific seroprevalence has changed. These changes have been brought about mainly by improvement in the socioeconomic situation, including living standards, education and personal hygiene (Innis *et al*, 1991; Kosuwan *et al*, 1996; Poovorawan *et al*, 1993, 1997). Several

reports have shown significant reductions in seroprevalence of anti-HAV antibodies among children and adolescents in Thailand over the last two decades (Kunasol *et al*, 1998; Poovorawan *et al*, 1993). In the early 1980s, there was a reported 65.7% seroprevalence in Bangkok children aged 2 months to 5 years (Viranuvatti *et al*, 1982) and 50% in children aged 6-7 years in an isolated rural community in Northern Thailand (Echeverria *et al*, 1983). By around 1990, these values had dropped to 46.7% in children aged 11 years of middle socioeconomic classes in Bangkok (Poovorawan *et al*, 1989) and 33.3% in children aged 11-12 years in a rural area of eastern Thailand (Poovorawan *et al*, 1991). A further decrease was reported more recently with a seroprevalence of only 1.1% in children under 5 years of age (Kalayanarooj *et al*, 1995) and 5.6-22.7% for children aged 15-18 years in Bangkok (Poovorawan *et al*, 1997; 2000). The level of ~50% seroprevalence, previously reached around age 5, is now reached closer to age 30 (Pramoolsinsap *et al*, 1999). These results parallel those obtained by us in the present study; we found the seroprevalence rate of anti-HAV antibodies to be 18.9% for children aged 15-16 years in Chiang Mai.

We noted a difference between the results obtained for children of rural and urban settings. Not only was there a lower seroprevalance for anti-HAV antibodies amongst rural children (8.95% *vs* 10.8% for urban schoolchildren) but also age-specific differences. In our study, the age-specific seroprevalence of anti-HAV antibodies in rural schoolchildren showed a linear increase with advancing age, in keeping with natural infection as the source. In contrast, there was an irregular pattern of age-specific seroprevalence observed in urban schoolchildren. Initially, there was a more rapid rise than that seen in rural children with 5% positive at age 4 years, 9.2% at 5-6 years, 13.0% at 7-8 years and 18.6% at 9-10 years. Following this, the prevalence declined at age 11-12 years (8.5%), increased again at age 13-14 years (15.8%) and declined again at age 15-16 years (9.2%). This phenomenon suggests the possibility of an outbreak of HAV infection among the group younger than 10 years in the urban school environment. Similar patterns of age-specific seroprevalence of anti-HAV antibodies among schoolchildren were demonstrated from Nakhon Si Thammarat, Southern region (Sinlaparatsamee *et al*, 1995) and Khon Kaen, northeastern region of Thailand (Kosuwan *et al*, 1996). Intermittent contamination of drinking water or food, not a particular behavior, was suspected to be responsible for such outbreaks (Poonawagul *et al*, 1995).

Over the past 30 years, HAV prevalence has shifted from hyperendemic to intermediate endemicity in Thailand, and could theoretically become a public health problem in the future. Recent studies still report the presence of HAV in canal water in urban environments such as Bangkok (Kittigul *et al*, 2000). The declining prevalence of naturally acquired immunity to HAV before adulthood may result in a greater number of cases of clinical infection and increased susceptibility to HAV outbreaks. The occurrence of HAV infection in an older population has practical implications, because the morbidity and mortality increases significantly with advancing age of the patient. HAV infection can now be prevented by active immunization with hepatitis A vaccine (Innis *et al*, 1994; Kuramoto *et al*, 1993; Loutan *et al*, 1994). Antibody levels remain detectable for 20-25 years (Van Herck and Van Damme, 2001). The availability of an effective hepatitis A vaccine, together with the changing epidemiology of HAV infection call for a re-evaluation of the costs and benefits, economic and social factors, associated with the various preventive strategies available for countries with endemic HAV, such as Thailand.

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Short Communication
Seroprevalence of hepatitis D virus infection among HBsAg carriers in northern Thailand

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Key words: HDV, Northern Thailand, HBsAg carrier

INTRODUCTION

The hepatitis delta virus (HDV) is a small, single stranded RNA hepatotropic virus that depends on hepatitis B virus (HBV) for its survival and replication (Polish *et al*., 1993; Purcell, 1994; Karayiannis, 1998). The hepatitis B virus (HBV) provides the envelope for the HDV, which consists of hepatitis B surface antigen (HBsAg). Because of this relationship, HDV infection occurs only in persons with hepatitis B, either as a co-infection or as a superinfection of a carrier of HBsAg (Rizzetto *et al*., 1980).

The mode of transmission of HDV infection appears to have two patterns: (i) endemic, associated with non-parenteral spread in Italy and (ii) sporadic, associated with parenteral transmission in almost all other areas of the world (Rizzetto *et al*., 1984). In regions where HDV infection is not endemic, the disease is mostly confined to groups at high risk of acquiring HBV infection and high-risk HBV carriers (Polish *et al*., 1993).

Human HBsAg carriers express delta antigen in the liver but do not circulate detectable delta antigen in the blood. Most patients develop antibody to HDV (anti-delta). Detection of anti-delta virus antibodies can indicate an ongoing or a past infection with HDV. The serological detection of this antibody by a sensitive enzyme immunoassay (EIA) provides a tool for recognizing HDV infection and for studying its epidemiology.

Because this viral infection can cause fulminant as well as chronic liver disease, spread of HDV into areas where HBV infection is endemic has serious clinical implications. Prevention depends on the widespread use of hepatitis B vaccine (Polish *et al*., 1993). But those who already have chronic HBV infection continue to be at risk of being infected with HDV (Purcell, 1994). Our study has indicated that northern Thailand belongs to an intermediate prevalence region of HBV infection with 8.7% (Jutavijittum *et al*., 1999) and it needs to be aware of the possibility of superinfection or co-infection of HBV with HDV. Hence, we plan to determine the prevalence of HDV infection among HBsAg carriers in northern Thailand.

MATERIALS AND METHODS

Serum samples from voluntary blood donors in Chiang Mai, Chiang Rai, Lampang, and Lamphun provinces in northern Thailand were screened for blood-transmitted pathogens at the 10th Regional Blood Center office in Chiang Mai. None of the blood donors complained the subjective symptoms of liver dysfunction. Testing for HBsAg was performed using a commercial ELISA kit, Enzygnost® HBsAg 5.0 (Dade-Behring, Marburg Germany). From 1998 to 2000, samples that were HBsAg-positive were collected and stored at −20℃. A total amount of 395 HBsAg-positive (287 males and 108 females) serum samples were obtained, 110 from Chiang Mai, 97 from Chiang Rai, 88 from Lampang, and 100 from Lamphun. The range of donor's age was from 17 to 52 years. The samples were tested for the presence of antibody to hepatitis delta antigen(anti-HD) using ETI-AB-DELTAK-2®, an ELISA kit from DiaSorin, Saluggia (Vercelli), Italy.

RESULTS

No anti-HDV was detected among 395 voluntary

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blood donors in 4provinces of northern Thailand.

DISCUSSION

The incidence of HDV varies around the world. Of patients with chronic liver disease in Africa, 73% are positive for HBsAg and 75% of these are also positive for anti-HDV antibodies (Cenac *et al*., 1995). Even in non-endemic areas, HDV antibodies are found in~20% of patients with chronic hepatitis B and acute hepatitis superimposed on chronic hepatitis B infection (Jacobson *et al*., 1985). In contrast, asymptomatic carriers of HBV are only rarely positive for anti-HDV antibodies (Jacobson *et al*., 1985; Louisirirotchanakul *et al*., 1988). In Asia, despite a rich reservoir of HBV carriers, the prevalence of HDV infection is considered to be low, found in~9% (Hao *et al*., 1992; Arakawa *et al*., 2000) although there are areas with high prevalence such as Fiji, Samoa and some areas of China (Vranckx *et al*., 1988).

HDV markers were more frequent in chronic liver disease with 18% than in asymptomatic HBV carriers with 2% (Jacobson *et al*., 1985). In Taiwan, the anti-HDV prevalence among HBsAg carriers was significantly high in STD patients (9.6%), prostitutes (33.1%), and drug abusers (68.1%) than in blood donors from the general population (2.2%) (Chen *et al*., 1992). In Thailand, HDV infection was generally found to be uncommon among cases of HBsAg-positive individuals, 0/27 of asymptomatic HBsAg carriers (Chainuvati *et al*., 1987). About 10% of patients with chronic liver disease and cirrhosis have anti-HDV antibodies, in contrast to~60% of intravenous drug users, and no anti-HDV demonstrated from 46 asymptomatic HBsAg carriers (Louisirirotchanakul *et al*., 1988). We demonstrated that all 395 voluntary blood donors in 4provinces of northern Thailand were negative for anti-HDV. This concurs with previous epidemiological surveys which indicate that in Thailand where HBV infection is endemic, delt. infection is rare among asymptomatic HBsAg carriers.

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Simian TT virus (s-TTV) infection in patients with liver diseases

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Abstract

Recently, we identified TTV isolates from nonhuman primates and named them simian TTV (s-TTV). To investigate the prevalence of s-TTV in humans, we examined sera from healthy individuals and patients with liver diseases in Japan for the presence of s-TTV DNA by PCR assay. s-TTV DNA was determined by nested PCR using s-TTV-specific primers designed from untranslated region of s-TTV genome. s-TTV DNA sequence was detected in three of 200 (1.5%) healthy adults but none of 48 infants without liver disease. On the other hand, s-TTV DNA was detected in 30 of 287 (10.5%) Japanese patients with liver disease. s-TTV coinfection with hepatitis B virus and hepatitis C virus were present in 16.7 and 30% of these patients, respectively, while 53.3% of patients were positive for s-TTV alone. Nucleotide sequence analyses in 20 patients confirmed that these PCR products were derived from s-TTV genome sequences and nearly 85% identical to those of s-TTV prototypes from chimpanzees. Phylogenetic analysis demonstrated that all s-TTV isolates from humans were distinguished clearly from the human TTV isolates. Furthermore, s-TTV in humans was classified into two different genotypes as well as simians. Our results indicate that generally 10.5% of Japanese patients with liver diseases were infected with s-TTV. The routes of s-TTV transmission from animal to human require clarification.

Keywords: TTV; Simian TTV (s-TTV); s-TTV infection in humans

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1. Introduction

The known viral agents of hepatitis do not account for all of the cases of hepatitis of purported viral etiology. Specifically, the screening of donated blood for serologic markers of HCV has not prevented all cases of non-B, non-C post-transfusion hepatitis, suggesting the existence of an additional unidentified agent(s) related to liver disease. Recently, the genome of a novel DNA virus, named TT virus (TTV), was discovered from a patient with acute post-transfusion hepatitis by representational difference analysis [1,2]. TTV is an unenveloped circular, single-stranded DNA virus (having 3852 nucleotides in the full-length sequence), with an isopycnic density of 1.31–1.34 g/ml in CsCl [3,4]. The TTV genome has three possible open reading frames, capable of encoding 770, 202 and 105 amino acids, respectively [3]. The genome structure and its banding in buoyant density gradient centrifugation suggest that TTV might be most related to *Circoviridae* virus among the known animal virus families [3,5]. Despite being a DNA virus, the TTV sequence has a wide range of sequence divergence, allowing classification into several genotypes [4–7]. TTV sequences can be detected in sera and liver tissues from liver disease patients, suggesting that TTV would be responsible for a part of acute and chronic liver disease of unknown etiology [2,8]. On the other hand, it has been reported that TTV infection does not induce significant liver damage [9]. We recently reported a very high prevalence of TTV in general populations worldwide, suggesting that this virus may be a common DNA virus with no clear disease association in humans [7]. However, the epidemiology, clinical significance and transmission patterns of TTV remain unclear. Very recently, we identified TTV isolates from nonhuman primates, including chimpanzees and crab-eating macaques, and tentatively named them simian TTV (s-TTV) [10,11]. Interestingly, our results revealed that the TTV isolates obtained from simians were distinct from the human TTV isolates. To investigate a possibility of s-TTV infection in humans, we examined sera from healthy individuals and patients with liver diseases for the presence of s-TTV DNA by PCR assay.

2. Materials and methods

2.1. Patients

We tested 287 Japanese patients' sera who had liver disease (195 men and 92 women, ranging in age from 18 to 85 years). They were patients at the International Medical Center of Japan, Tokyo, Japan. Diagnosis was based on liver function tests, hepatitis virus markers, autoantibodies, tumor markers and ultrasonography. Some patients, mainly in chronic hepatitis for interferon therapy, were diagnosed by histopathology of the liver. We also tested the sera of 200 healthy Japanese blood donors that were received from the World Health Organization and the National Serum Reference Bank, Tokyo, National Institute of Infectious Diseases, Tokyo, Japan, and 48 infants' sera without liver disease. These sera were collected between 1990 and 2000 and stored at −40 °C or below. Informed consent for participation in this study was obtained from each individual.

2.2. Detection of s-TTV DNA by PCR and nucleotide sequencing

DNA was extracted from 100 ul of serum samples using nucleic acid extraction kit (Sepa-Gene RV-R, Sanko Junyaku Co., Ltd., Tokyo, Japan) as directed by manufacturer. The resulting pellet was resuspended in RNase- and DNase-free water and then subjected to nested PCR. The sequence of the TTV specific primers were 5′-GCTACGTCACTAACCACGTG-3′ (T801, sense primer, nucleotides 6–25) and 5′-CTBCGGTGTGTAAACTCACC-3′ (T935, antisense primer, nucleotides 185–204, B = G, C or T) for the outer primer pairs (199 bases) as designed by Takahashi et al. [12] in untranslated region of TA278 isolate from human. The second PCR reaction was performed with the inner primer combination of TT17 and TT11R (144 bases), designed from 15 s-TTV isolates (database accession numbers AB035155–AB035168 and AB037926) that showed types 1 and 2. But, these primer sequences do not match the corresponding sequences of type 3. The sequence of the s-TTV specific primers were 5′-CCRCAGGC-

Table 1
Prevalence of s-TTV infection in humans in Japan

Category	Number of subjects	s-TTV total	s-TTV alone	Co-infection	
				HBV	HCV
Blood donors	200	3 (1.5)*	3/3 (100)	0	0
Infants without liver disease	48	0	0	0	0
Liver disease patients					
Acute hepatitis	15	2 (13.3)	2/2 (100)	0	0
Chronic hepatitis	221	12 (5.4)	7/12 (58.3)	1/12 (8.3)	4/12 (33.3)
Liver cirrhosis	31	6 (19.3)	2/6 (33.3)	4/6 (66.7)	0
Hepatocellular carcinoma	20	10 (50)	5/10 (50)	0	5/10 (50)
Total	287	30 (10.5)*	16/30 (53.3)	5/30 (16.7)	9/30 (30)

Number in parenthesis indicate percentages. *, $P < 0.001$.

CAAYCCGS-3′ (TT17, sense primer, nucleotide 30–45, R = A or G, Y = C or T, S = G or C) and 5′-GGACGCGGTCGGCCGTTSGG-3′ (TT11R, antisense primer, nucleotide 154–173). AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA) was used for the PCR. Amplification conditions included preincubation at 95 °C for 10 min to activate AmpliTaq Gold then followed by 40 cycles of the first-round PCR (94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s) using a Perkin–Elmer 2700 or 9700 Thermal Cycler. For the second-round PCR, the annealing temperature was set to 68.5 °C instead of 60 °C and followed by 45 cycles. The PCR products were detected by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed under UV light. Purified PCR products from gels were subjected to direct sequencing from both directions using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer). Sequences of amplified cDNA were determined using a sequencer (ABI model 373A; Applied Biosystems, Foster City, CA).

2.3. Sensitivity of the PCR assay

We used plasmid DNA containing UTR of s-TTV DNA for testing of PCR sensitivity. The sensitivity showed to be detected up to ten copies of s-TTV DNA by the method reported in the present study.

Table 2
Prevalence of s-TTV infection in patients with liver disease of unknown etiology in Japan

Disease	Number of subjects	s-TTV DNA positive (%)
Acute hepatitis	12	2 (16.7)
Chronic hepatitis	139	7 (5)
Liver cirrhosis	8	2 (25)
Hepatocellular carcinoma	9	5 (55.6)
Total	168	16 (9.5)

2.4. Phylogenetic analysis

Nucleotide sequences that are not including PCR primers sequences, were multiple aligned using CLUSTAL W version 1.4. The distance matrix of the nucleotide substitutions among each isolate was estimated by the eight-parameter method [13] and phylogenetic trees were constructed by the neighbor-joining method [14] from the matrix. These procedures were computed using PHYLO_WIN version 1.2 [15] on a DEC alpha 2000 server, and the trees were drawn by TREEVIEW version 1.5 [16]. The reliability and topology of each tree branch was tested by bootstrap analysis [17] of the data of 100 bootstrap resamplings of the columns in the untranslated region sequence alignment of s-TTV. Bootstrap values greater than 60% were considered supportive of the observed groupings. In addition to our isolates in this study, 28 previously reported TTV sequences isolated in

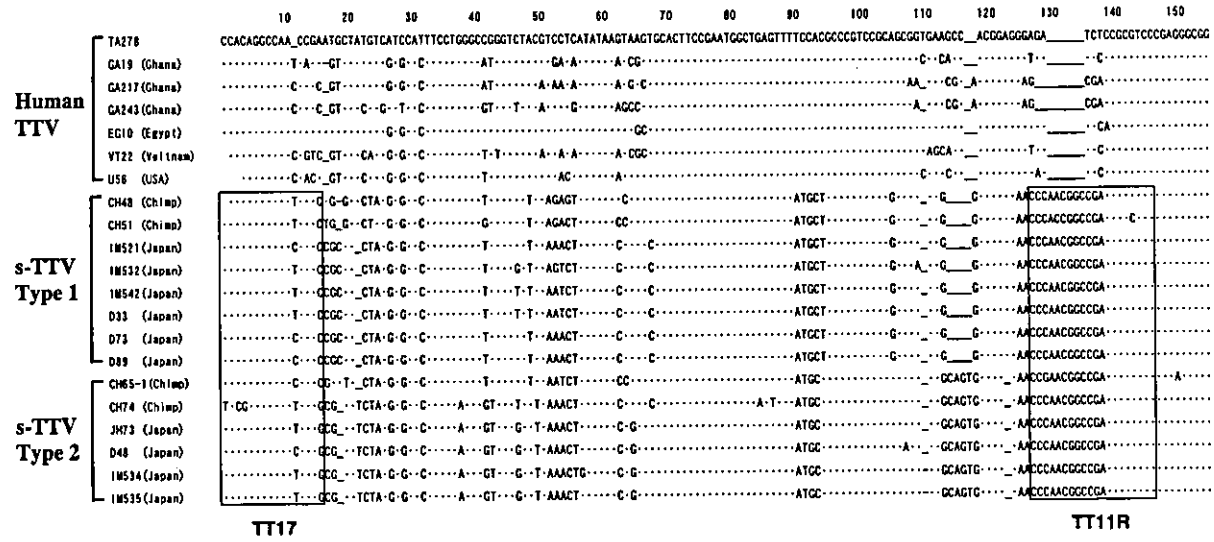

Fig. 1. Alignment of nucleotide sequences of TTV and s-TTV in the untranslated region recovered from humans and chimpanzees. Dots indicate identical to prototype of TA278, and lines indicate deletions. Primers used for s-TTV PCR are boxed.

humans were obtained from the GenBank database and used for comparison with the sequence of the isolates in this study.

2.5. Statistical analyses

Statistical analyses were performed using χ^2-test or Fisher's exact test. A difference with a P value of < 0.05 was considered significant.

3. Results

s-TTV DNA was detected in 30 of 287 (10.5%) liver disease patients, while three of 200 (1.5%) healthy adults and none of 48 infants without liver disease in Japan. Among liver disease patients, s-TTV DNA was present in liver disease 12 of 221 (5.4%) with chronic hepatitis, six of 31 (19.3%) with liver cirrhosis, and ten of 20 (50%) with hepatocellular carcinoma (Table 1). The prevalence of s-TTV infection in chronic hepatitis patients who were diagnosed as non-A–E hepatitis was 9.5% (16/168) (Table 2). Furthermore, s-TTV DNA was detectable in two of eight (25%) liver cirrhosis and five of nine (55.6%) hepatocellular carcinoma patients who were diagnosed as unknown etiology. Among 30 s-TTV-seropositive patients, s-TTV coinfection with HBV and/or HCV were observed in five (16.7%) and nine (30%), respectively. About 53.3% (16/30) of liver disease patients examined were infected with s-TTV alone. Neither age nor sex of the patients as a background factor was correlated to the prevalence of s-TTV infection in this study. To verify that the amplified products by PCR were of s-TTV origin, sequence analyses in 20 cases were performed. The results revealed that specificity of the PCR products of s-TTV DNA was confirmed by nucleotide sequence analysis (Fig. 1). The nucleotide sequences from 20 individuals were 84–85% identical to those of s-TTV isolated from chimpanzees. Phylogenetic analysis demonstrated that all s-TTV isolates obtained from both of humans and simians were distinguished clearly from TTV and this difference was strongly supported by bootstrap analysis (Fig. 2). Furthermore, s-TTV in humans was also further divided into two genotypes and showed major genotype was type 1 as well as the simians.

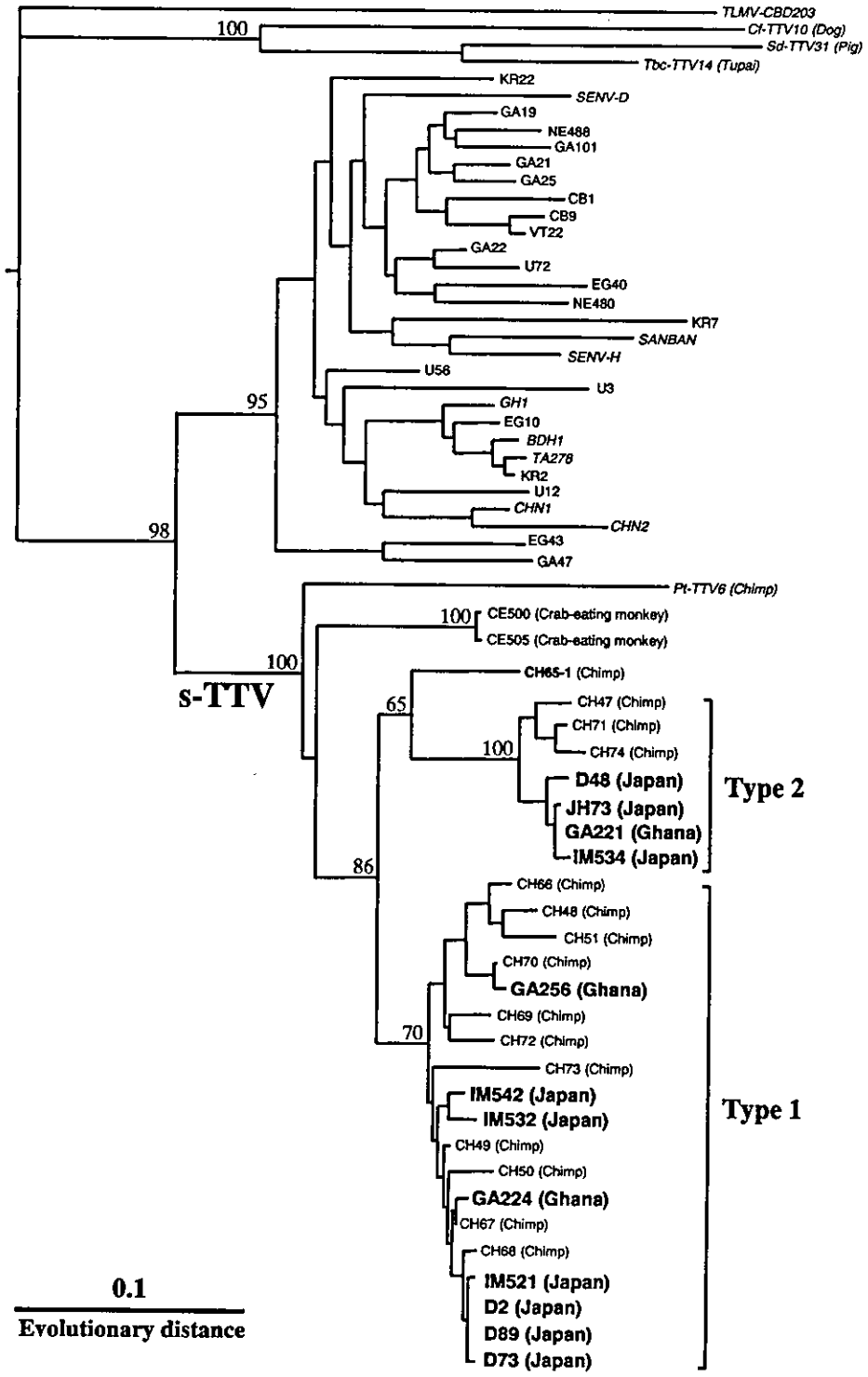
TLMV-CBD203
Cf-TTV10 (Dog)
Sd-TTV31 (Pig)
Tbc-TTV14 (Tupai)
100
KR22
SENV-D
GA19
NE488
GA101
GA21
GA25
CB1
CB9
VT22
GA22
U72
EG40
NE480
KR7
SANBAN
SENV-H
U56
U3
95
GH1
EG10
BDH1
TA278
KR2
U12
CHN1
CHN2
98
EG43
GA47
Pt-TTV6 (Chimp)
100
CE500 (Crab-eating monkey)
CE505 (Crab-eating monkey)
100
CH65-1 (Chimp)
s-TTV
65
CH47 (Chimp)
CH71 (Chimp)
100
CH74 (Chimp)
D48 (Japan)
JH73 (Japan)
GA221 (Ghana)
IM534 (Japan)
Type 2
86
CH66 (Chimp)
CH48 (Chimp)
CH51 (Chimp)
CH70 (Chimp)
GA256 (Ghana)
CH69 (Chimp)
70
CH72 (Chimp)
CH73 (Chimp)
IM542 (Japan)
IM532 (Japan)
CH49 (Chimp)
CH50 (Chimp)
GA224 (Ghana)
CH67 (Chimp)
CH68 (Chimp)
IM521 (Japan)
D2 (Japan)
D89 (Japan)
D73 (Japan)
Type 1
0.1
Evolutionary distance

Fig. 2

4. Discussion

Despite the molecular biologic discovery of TTV, the clinical implications of the identification of this new agent remain largely unresolved. It has been reported that TTV DNA sequences have been found in nonhuman primates and farm animals [18–24]. Very recently, we identified simian-specific TTV isolates from nonhuman primates and tentatively named them s-TTV [10]. Furthermore, we cloned the entire nucleotide sequence of s-TTV isolate recovered from a chimpanzee [11]. Based on an analysis of full-length sequence data, s-TTV represent a new TTV-like viral species or genus, although it is closely related to human TTV. In the present study, we found s-TTV infection in humans. Furthermore, we were able to confirm that PCR products were of s-TTV origin by sequencing and phylogenetic analysis. The reliability of PCR results is always in question because of the risk of false-positive results caused by cross-contamination. In our laboratory, strict precautions against such events have been undertaken to overcome this problem. It is noteworthy that the prevalence of s-TTV infection was 10.5% in patients with liver diseases, and this frequency was greater than those of positive rate of 1.5% in healthy individuals. Interestingly, single infection of s-TTV was found in 50% of chronic liver disease patients including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. This suggests the need for prospective studies to document histopathologicaly the progression from acute s-TTV infection through various stages of chronic hepatitis to the development of cirrhosis, hepatocellular carcinoma, or end-stage liver disease, as was documented for hepatitis B and C. In general, patients with TTV infection usually appear to have normal serum ALT [9]. It is also known that the TTV viremia is widespread in the general population worldwide, since the prevalence among patients including healthy populations was found to be over 70% [7]. Such an extremely high prevalence of TTV infection in the general population suggests that TTV may be transmissible not only the blood, but also by a nonparental route. The presence of TTV in body fluids other than serum, such as saliva, semen and bile juice, may affect the routes of viral transmission [25–27]. Our preliminary results showed that high prevalence of s-TTV infection among drug addicts and we found that s-TTV DNA was detectable in saliva and semen in these patients examined (data not shown). Furthermore, Okamoto et al. reported that TTV was excreted into the feces, thereby suggesting that TTV would be transmitted not only parenteraly, but also non-parenteraly by a fecal–oral route [28,29]. It remains to be investigated whether or not s-TTV is pathogenic in humans. Investigation of prevalence of s-TTV in different countries is underway and we shall report as a further study. As a preliminary data, s-TTV DNA was detected in 11 of 88 (12.5%) blood donors in Ghana (data not shown) and suggested that s-TTV is prevailing in West African countries. The routes of s-TTV transmission from animal to human in Japan require clarification. Further studies to evaluate the clinical and pathological significance of s-TTV infection in humans will be needed in order to draw valid conclusions. Even if s-TTV is not pathogenic to humans, the fact that humans are infected with s-TTV of the animal origin is important to consider the zoonosis.

Fig. 2. Phylogram generated by neighbor-joining analysis of genetic distances in the untranslated region of TTV and s-TTV. The percentage of bootstrap replicates supporting these branches are shown in number. The database-derived isolates are presented in italic and their accession numbers are as follows: TA278 (AB008394), GH1 (AF122913), CHN1 (AF079173), CHN2 (AF129887), BDH1 (AF116842), SANBAN (AB025946), SENV-D (AX025730), SENV-H (AX025838), TLMV-CBD203 (AB026929), Pt-TTV6 (AB041957), Cf-TTV10 (AB076002), Sd-TTV31 (AB076001) and Tbc-TTV14 (AB057358). The remaining human TTV and s-TTV sequences in chimpanzees and crab-eating monkeys are isolates that we submitted previously to database with accession numbers of AB035154 through AB035171.

The nucleotide sequence data of s-TTV isolated from humans reported in this paper have been submitted to the DDBJ, EMBL, and GenBank databases under accession no. AB073473 through AB073489.

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Recombination of Genotypes B and C in Hepatitis B Virus Isolated from a Vietnamese Patient with Fulminant Hepatitis

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Hepatitis B virus (HBV) can now be classified into seven major genotypes designated from A to G (1). Accumulated evidence suggests that recombination among different genotypes is relatively frequent (2,3). Within the HBV genome, three recombination hot spots have been identified: in the vicinity of DR1 (direct repeat 1; nucleotide [nt] 1800); at the 3′ end of the core region (nt 2359); and within the 3′ end of the S gene (2,3).

The distribution of HBV genotypes varies geographically, and genotypes B and C are known to be prevalent in Asian countries, including Vietnam (4). Recently, phylogenetic analysis has indicated that B/C recombinants have spread throughout East Asia and A/D recombinants in Italy and South Africa (5,6).

We report here a complete genomic sequence of HBV isolated in Vietnam. The patient was a Vietnamese (male, 30 years old) who was admitted to the Bach Mai Hospital, Hanoi, Vietnam with diagnosis of fulminant hepatitis. At that time, the patient was positive for HBsAg and the IgM class of anti-HBc antibody, but negative for HCV RNA, HDV RNA, anti-HAV IgM, and anti-HEV IgM.

Nucleic acids were extracted from a 100-μl serum sample using a SepaGene RV-R Kit (Sanko-Junyaku, Tokyo). Five microliters of nucleic acids were used for amplification of HBV DNA by PCR. PCR was carried out using a set of primers to amplify five overlapping fragments that cover the full genome of HBV. PCR was performed as reported previously (7). Genotyping of HBV was done by PCR using type-specific primers reported by Naito et al. (8). Purified amplicons were subjected to direct sequencing from both directions using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, Conn., USA). Sequences of amplified DNA were determined using an automated DNA sequencer ABI 377 (Applied Biosystems, Foster City, Calif., USA). A phylogenetic tree was constructed with the MEGA program version 2.1, using the Kimura two-parameter matrix and the neighbor-joining method. To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 500 times. Recombination of the HBV genome was determined using the SimPlot program (distributed by Stuart Ray, http://www.welch.jhu.edu/~sray) and bootscanning analysis.

We obtained a full-length HBV genome 3215 nt in length that was designated as HBV-VH133. PCR using type-specific primers designed from pre-S1 through S gene and phylogenetic analysis indicated that the sample belonged to genotype B. However, the genome was clustered with a genotype C-derived sequence in the pre-Core/Core gene (Fig. 1a). Phylogenetic analysis of the HBV full genome indicated that VH133 belongs to subgroup Ba, according to Sugauchi et al. (6) (Fig. 1b). Analysis using SimPlot and bootscanning programs based on six different genotypes revealed that VH133 has a resolution of the breakpoint of genotypes B/C recombination at the position of nt 1880 to 2260 (pre-Core/Core region) (Fig. 2). Thus, VH133 is a recombinant of genotype B with genotype C, which is prevalent in Vietnam.

The nucleotide sequence reported in this paper has been submitted to DDBJ/GenBank/EMBL databases under accession no. AB100695 for HBV-VH133.

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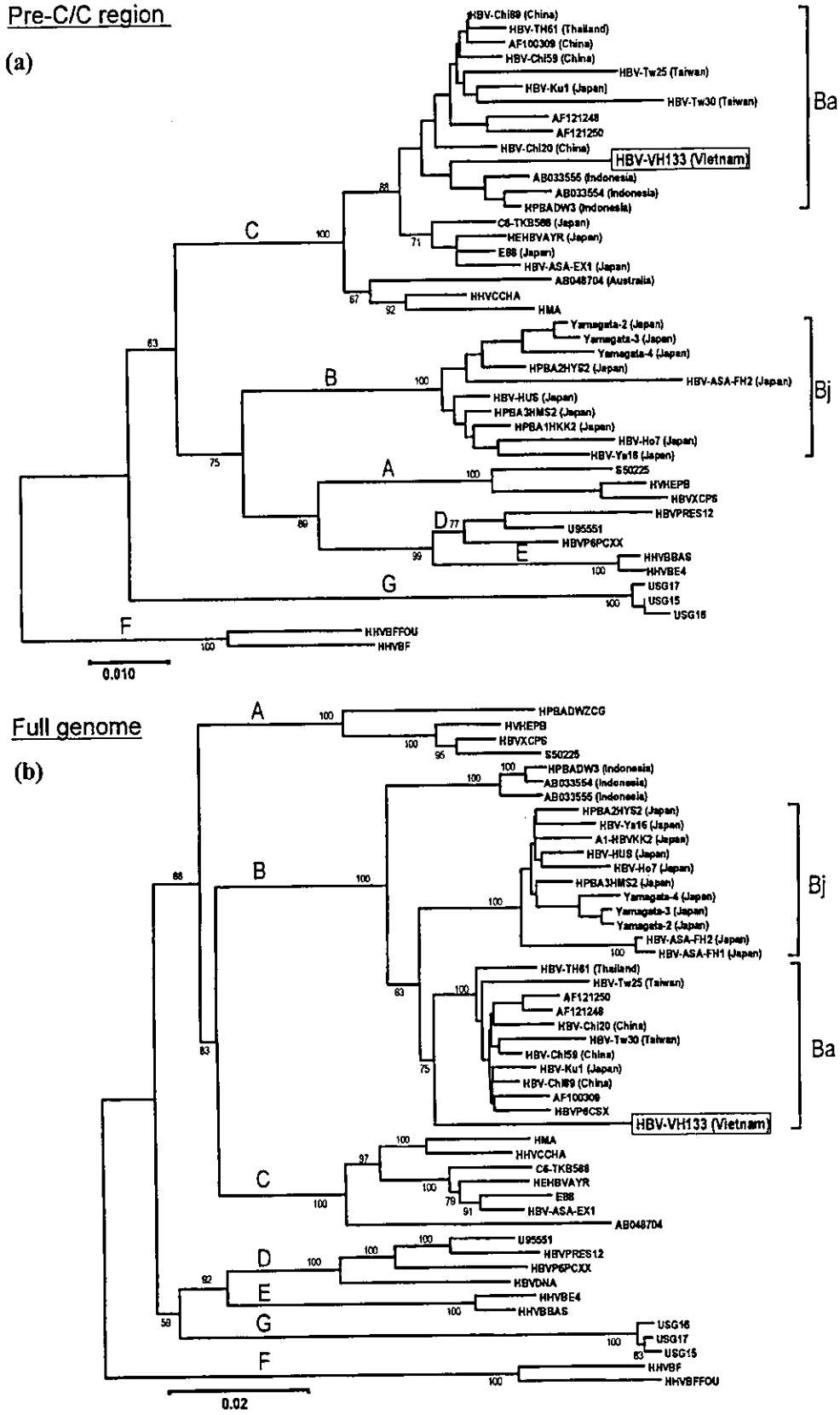

Fig. 1. Phylogram generated by neighbor-joining analysis of genetic distances in the pre-C/C gene (a) and the full-length gene (b) of HBV.

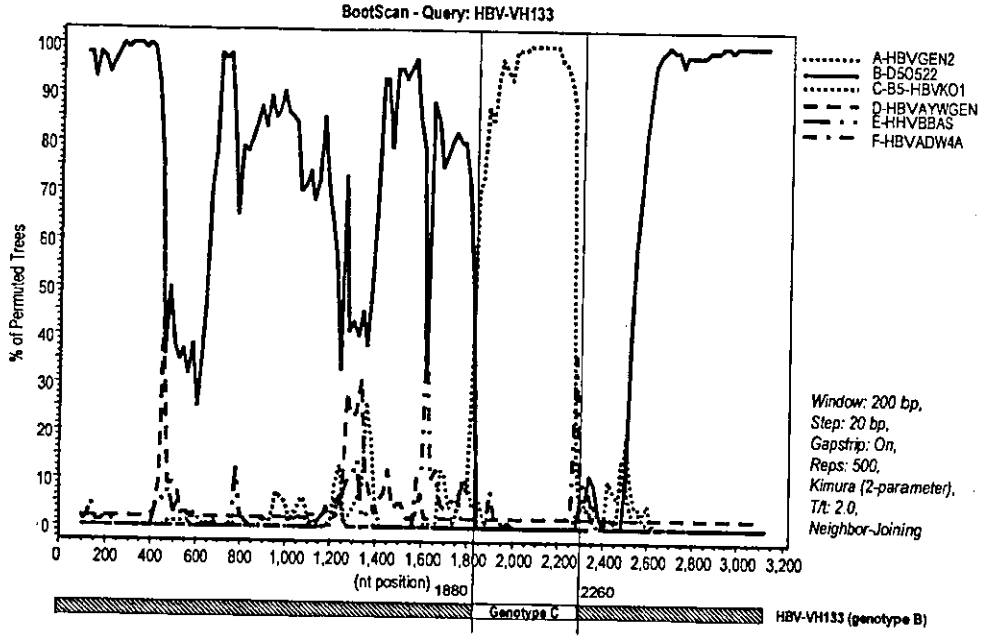

Fig. 2. Resolution of the recombinant event in HBV genomes of distinct genotypes was determined using the SimPlot program and bootscanning analysis.

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Present state of hepatitis E virus epidemiology in Tokyo, Japan

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Abstract

Recently studies have reported the possibility that an indigenous hepatitis E virus (HEV) exists in Japan, but the epidemiological features of HEV in Japan are inadequate to make a judgment. In order to search the present state of HEV infection in Japan, we used ELISA to test 1033 sera from residents living in Tokyo and the Tokyo suburbs, for the presence of the antibody against HEV. The positive rate of anti-HEV IgG was 15.4% in all liver disease patients (68 of 440), 3% (6/200) in healthy individuals and 0.4% in infants (1/246), respectively ($P < 0.01$). Anti-HEV IgG was seen in 17.6% (35/199) of liver disease patients of unknown etiology; 29.4% (5/17) of fulminant hepatitis, 17% of acute hepatitis (15/88) and 16% of chronic hepatitis (15/94). Anti-HEV IgG co-existed with hepatitis B virus and hepatitis C virus in 23.6% (21/89) and 7.9% (12/152), respectively. Furthermore, the prevalence of anti-HEV IgG was significantly higher in hemodialysis patients (18/60: 30%) and hospital workers (8/87: 9.2%) than in the healthy population ($P < 0.01$). Anti-HEV IgM was detected in 0.1% of all samples tested (1/1033). The prevalence of anti-HEV IgG increased with age. No individuals with HEV antibody had a recent history of visiting countries where hepatitis E is endemic. These results indicate that generally 15.4% of Japanese patients with liver diseases had a history of HEV infection in the past. The routes of transmission of HEV require clarification in Japan.

Keywords: HEV; HEV epidemiology in Tokyo, Japan; Anti-HEV prevalence in hemodialysis patients; Anti-HEV prevalence in medical staffs

1. Introduction

Hepatitis E virus (HEV) is the etiologic agent of hepatitis E, previously called enterically transmitted non-A, non-B hepatitis. HEV is thought to spread via the fecal-oral route, and outbreaks of hepatitis E are attributed to water contaminated with HEV. The endemic areas of hepatitis E are confined to developing nations where most people live under conditions of an inadequate water supply [1–3]. In industrialized nations, hepatitis E is not endemic, but the prevalence of anti-HEV antibody is 1–3% among healthy adults in the United States [4] and 4.6–6.7% in Japan [5]. In Japan, hepatitis E is rarely reported and most cases have usually been associated with travel to endemic areas, which explains why hepatitis E has been regarded as an imported hepatitis to the present time. However, several recent studies have suggested the existence of indigenous HEV strains in Japan as several HEV strains were recovered from Japanese patients with acute hepatic failure of unknown etiology, but who had not traveled abroad [6–9]. The route of HEV infection in those patients is still unknown. Recently, a highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) for anti-HEV antibody was developed by Li et al. [10,11]. Using this method to investigate the present state of HEV infection, we carried out a seroepidemiological survey among healthy individuals and liver disease patients in Tokyo, Japan for the presence of antibody against HEV.

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2. Materials and methods

2.1. Study population

We tested 1033 Japanese patients' sera from 200 healthy individuals (100 men and 100 women, ranging in age from 30 to 69 years; mean = 49.5 ± 11.1 years), 246 infants (119 men and 127 women, ranging in age from 0 to 14 years; mean = 3.2 ± 3.6 years), 440 liver disease patients (258 men and 182 women, ranging in age from 17 to 86 years; mean = 48.8 ± 15.5 years), 60 hemodialysis patients (30 men and 30 women, ranging in age from 30 to 60 years, mean = 46.2 ± 7.9 years) and 87 hospital workers (29 men and 58 women, ranging in age from 21 to 58 years; mean = 36.6 ± 10.5 years). The mean age in both groups was 46.7 ± 13.8 years in non-liver disease group and 48.8 ± 13.9 years in liver disease group, respectively ($P = 0.53$; statistically not significant). Clinical diagnosis was based on liver function tests, hepatitis virus markers, autoantibodies, tumor markers and ultrasonography. Some patients, mainly in chronic hepatitis for interferon therapy, were diagnosed by histopathology of the liver. Two hundreds serum samples from healthy individuals received from the World Health Organization and the National Serum Reference Bank/Tokyo, National Institute of Infectious Diseases, Tokyo, Japan. In healthy individuals, we collected 50 serum samples in each age group consisting of the 31–40 years old group, the 41–50 years old, the 51–60 years old and the 61–70 years old, respectively. All individuals were residents in Tokyo or its suburbs. Informed consent for participation in this study was obtained from each individual. These serum samples were collected between 2000 and 2002 and stored at −40 °C or below until analysis.

2.2. Detection of anti-HEV antibodies by ELISA

A recombinant open reading frame (ORF) 2 protein of HEV expressed by a recombinant baculovirus was used as the antigen in ELISA as previously described [6]. Briefly, sera were diluted at 1:200 and added to the assay plates coated with the recombinant HEV ORF2 protein. Horseradish peroxidase (HRP)-conjugated goat anti-human IgM with 1:1000 dilution and anti-human IgG with 1:5000 dilution (Cappel, Durham, NC, USA) was used to detect antigen-bound human IgM and IgG, respectively. Human serum known positive for both anti-HEV IgG and IgM was included in every assay plate as a positive control. The cutoff value was set at 0.2 of OD492, because the mean + 3S.D. values of human sera known negative for both anti-HEV IgG and IgM never exceeded 0.2 in the above-mentioned assays.

2.3. HEV RNA detection

HEV RNA was detected by the nested RT-PCR. We targeted the ORF3 of HEV gene for PCR and designed the primer sequences to be covered all genotypes of HEV for screening. Total RNAs were extracted from 100 μl of serum using SepaGene RV-R Kit (Sanko-Junyaku, Tokyo, Japan). The resulting pellet was resuspended in 50 μl RNase-free water, following the manufacturer's instruction. Extracted nucleic acids were stored at −20 °C until use. Five μl of nucleic acid was used for amplification of HEV RNA by the nested RT-PCR. PCR was carried out using a set of primers with 5′-GTW CAT AAC CTK ATT GGB ATG C-3′ (E3; sense, nucleotide [nt] 4996-5017) and 5′-RAA GGG GTT GGT TGG ATG-3′ (E3R; antisense, nt 5315-5332) for the outer primer pairs (337 bases), and 5′-CGG GHD GAA TGA ATA ACA TG-3′ (E4; sense, nt 5098-5117) and 5′-AAG GGC TGA GAA TCA RCC C-3′ (E4R; antisense, nt 5280-5298) for the inner primer pairs (201 bases). Nucleotide positions of each primer correspond to those of Nepali HEV isolate of genotype 1 (DDBJ/GenBank/EMBL accession no. AF051830).

2.4. Statistical analyses

Statistical analyses were performed by the Student's *t*-test or Fisher's exact test. A difference with a P value of < 0.05 was considered significant.

3. Results

3.1. Anti-HEV IgG prevalence

As shown in Table 1, anti-HEV IgG was detected in 3% (6/200) of healthy populations and none of the infants tested. On the other hand, 15.4% (68/440) were positive for anti-HEV IgG among patients with liver diseases. The

Table 1
Prevalence of antibody to hepatitis E virus among residents living in Tokyo (2000–2002)

Category	*n*	Anti-HEV	
		IgG class	IgM class
Liver diseases			
Non-B, non-C			
Fulminant hepatitis	17	5 (29.4%)	0
Acute hepatitis	88	15 (17%)	0
Chronic hepatitis	94	15 (16%)	0
Hepatitis B	89	21 (23.6%)	0
Hepatitis C	152	12 (7.9%)	0
Subtotal	440	68 (15.4%)	0
Healthy individuals	200	6 (3%)	0
Infants	246	1 (0.4%)	1 (0.4%)
Hemodialysis	60	18 (30%)	0
Hospital workers	87	8 (9.2%)	0
Total	1033	101 (9.8%)	1 (0.1%)