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Epidemiology of Hepatitis E Virus in Indoor-Captive Cynomolgus Monkey Colony

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Abstract

A serological survey of hepatitis E virus (HEV) antibody was conducted using 202 adult captive cynomolgus monkeys, who did not show any clinical signs of acute hepatitis. Out of these, 44 monkeys were sero-positive for anti-HEV IgG and all monkeys were negative for anti-HEV IgM. All positive monkeys came from either Vietnam or China, but none from the Philippines, Indonesia, or our facility. Selected 12 monkeys out of positive monkeys from Vietnum, including 9 positive and 3 negative, revealed mostly within the reference ranges for alanine aminotransferase (ALT) and asparatate aminotransferase (AST) by serum biochemistries. Their titers of anti-HEV IgG did not correlate with the concentrations of ALT and AST. Moreover, HEV-RNA could not be detected from any fecal specimens of the 12 monkeys. Thus, monkeys with anti-HEV IgG sero-pisitive did not seem to be source of the HEV-pollution, because 1) sero-positive monkeys did not excrete HEV-RNA from their feces, and 2) monkeys from the Philippines and Indonesia have remained to be sero-negative for anti-HEV IgG, even if the monkeys were kept in same animal room of our facility. From these results, it could be inferred that primary infection of HEV in our colony occurred in the exported countries, but not in our colony. The contamination of HEV in indoor-captive monkeys could be prevented by precise quarantine tests, including ELISA for detecting anti-HEV and RT-PCR for HEV RNA.

Key words: Epidemiology, Hepatitis E virus, Nonhuman primate

Introduction

Hepatitis E virus (HEV) infection causes acute hepatitis in humans living in tropical and semi-tropical areas, such as Asia, Africa, and southern areas of North America [22]. Typical symptoms of HEV, is a self-limited syndrome, include malaise, fatigue, nausea, fever, transaminase elevation, and hyperbilirubinemia [21]. Although most patients recover within a week, 7% are fatal; in particular, pregnant women are at a higher risk of developing these symptoms [2, 22]. After infection, anti-HEV IgM first appears in the patient's serum, which remains detectable for 2 to 3 months [4]; on the other hand, anti-HEV IgG elevates after acute phase and anti-HEV IgM response. Persistent increase of anti-HEV IgG for more than 14 years is found in 47% of patients [6]. HEV RNA can be detected in feces of most patients by RT-PCR during 2 weeks of acute phase [6]. The main route of transmission for HEV is oral infection via fecally contaminated water [11]. This occurs as a single-peaked outbreak, and the magnitude of the outbreak was associated with population density and hygienic environment, such as supply of clean water and maintenance of sewage [11].

Sporadic HEV infection has been recognized in the eating habits of raw or nearly raw meat and raw liver of wild boars in Japan [17]. However pigs do not show any symptoms under natural and experimental infections [15]. Because naturally acquired antibodies against HEV without symptoms have been detected in wild and domestic animals, including pigs, HEV infection is considered as a zoonotic disease [5, 9]. In the case of nonhuman primates, natural infection has also been reported in Old World monkeys, including macaque species, in India and Japan [3, 10]. On the other hand, nonhuman primates are often used for experimental infection of HEV, such as the development of a vaccine [12]. Monkeys used for biomedical science in Japan are mostly imported from tropical countries, e.g., Vietnam, the Philippines, and Indonesia. Because HEV is

also prevalent in these countries, a possibility of cross-infection of HEV between humans and monkeys or animals and monkeys is considered [18]. Although imported monkeys for medical science in Japan might have been infected with HEV, there are no reports describing the epidemiological aspect of HEV in indoor-kept nonhuman primates. Therefore, we confirmed 1) where cynomolgus monkeys infected with HEV and 2) whether the incidence of HEV infection differed among each imported country.

Materials and Methods

Animals and specimens: All 202 cynomolgus monkeys (*Macaca fascicularis*) were kept in the animal room maintained at 25 ± 2°C and 50 ± 5% at the Research Center for Animal Life Science (RCALS), Shiga University of Medical Science (SUMS). Most of them originated from southern east or east Asian countries, e.g., Vietnam (n = 114), China (n = 15), the Philippines (n = 23), Indonesia (n = 45), and from our facility (n = 5) (Table 1). They consisted of 164 females and 38 males (Table 1), whose ages ranged from 1 year & 2 months to 17 years & 5 months (average: 5.25-years ± 0.14 SE). All sera and fecal specimens used in the present study were collected at the regular health checks, and stored at −80°C until use. The regular heath checks were conducted based on Guide for the Care and Use of Laboratory Animals of SUMS, and Standard Operation Procedures of RCALS.

ELISA: Anti-HEV IgG and IgM in sera were detected with an ELISA system using virus-like particles (VLPs) of HEV as described by Li TC et al [14]. Flat-bottom 96-well polystyrene microplates (Immulon 2, Dynex Technologies, Inc. Chantilly, VA) were coated with purified VLPs (1 μg/ml, 100 μl/well). The plates were incubated overnight at 4°C. Unbound VLPs were removed, and the wells were rinsed twice with 10 mM phosphate buffered saline (PBS) containing 0.05%

Tween 20 (PBS-T), and then blocked at 37°C for 1 h with 200 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in TBS-T. After the plates were washed 4 times with TBS-T, monkey sera (100 μl/well) were added in duplicate at a dilution of 1:200 in PBS-T containing 1% skim milk. The plates were then incubated at 37°C for 1h. The plates were rinsed 4 times as described above and were administrated with 100 μl of horseradish peroxidase-conjugated goat anti-human IgG and IgM (KPL, Guildford, UK) in TBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 h, and washed 4 times with TBS-T. Then, 100 μl of the substrate o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) was added to each well. The plates were incubated at room temperature for 30 min in a darkroom, and then, 50 μl of 4N H_2SO_4 was added to each well. After the plates were kept at room temperature for 10 min, the absorbance at 492 nm was measured. The OD_{492} cut off at 0.110, which was followed with results by Yamamoto et al [23].

Preparation of recombinant viral-like particles: A recombinant baculovirus, Ac5480/7126, harboring the G1 HEV capsid protein gene with a 111-amino-acid deletion at the N-terminal was constructed as described previously [13]. In brief, Tn5 cells (High FiveTM, Invitrogen, San Diego, CA) were infected with Ac5480/7126 at an infection at multiplicity of 10, and incubated at 26.5°C for 7 days. The intact cells and cell debris were removed from the culture medium, and the recombinant VLPs with 53 KDa were concentrated by centrifugation at 100,000 × g for 2 h in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLPs were further purified by isopycnic binding in CsCl gradient. A visible band containing VLPs was collected, and the VLPs were diluted five times with PBS. The VLPs were pelleted at 100,000 × g and resuspended in PBS. Purity of the VLPs was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy after negative staining, as described previously [13].

Serum biochemistries: Serum biochemistries were performed in the selected twelve female cases

from Vietnam, based on the OD value of anti-HEV IgG. They consisted of nine positive and 3 negative monkeys (-; cases #10-12) (Table 2). The nine positive monkeys were further divided into 3 groups with 3 monkeys each, revealing higher (+++; cases #1–3, OD > 0.9), moderate (++; cases #4–6, 0.9 > OD > 0.5), and lower (cases #7–9, 0.5 > OD > 0.11) titers for anti-HEV IgG, respectively (Table 2). Serum alanine aminotransferase (ALT) and asparatate aminotransferase (AST) of these 12 monkeys were measured as markers for hepatic disorders.

RT-PCR: Total RNAs from the above 12 monkeys were extracted with RNAzol from 10% fecal specimens. The RNA was purified with Oligotex-dT30 (Roche Diagnostic Systems, Tokyo) according to the manufacturer's protocol and converted into cDNA as described previously [19]. The cDNA was subjected to polymerase chain reaction with ExTaq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto) with an external sense primer HEV–D4 (5'–TGTAGAGAATGCTCAGCAGGATAA-3', nt 6391-6414) and an antisense primer HEV–U4 (5'–TAACTCCCGAGTTTTACCCACCTT-3', nt 7103-7126) using the GeneAmp PCR System 9700 (PE Biosystems, Poster City, CA) [13]. Each cycle consisted of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension reaction at 72°C for 60 sec followed by final extension at 72°C for 7 min. The nested PCR was performed using an internal sense primer HEV-D5 (5'-CTGCCGAGTATGACCAGTCCACTTA-3', nt 6576-6600) and an internal antisense primer HEV-U3 (5'-TTAAGGCGCTGAAGCTCAGCGA-3', nt 7077-7098) under the same conditions, resulting in a 523 nt fragment [13].

Statistic analyses: The statistic differences of positive percentages for anti-HEV IgG between two positive countries and between female and male were compared using Chi-square test. The statistic differences of ALT and AST concentrations among 4 groups were compared using two-way ANOVA. All statistical significance was attained at P less than 0.05.

Results

None of the examined animals showed any symptoms related to acute hepatitis, e.g., jaundice, hepatic coma. General health conditions, such as appetite, physical activity, and fecal and urinary features, were normal.

ELISA: Positive cases for anti-HEV IgG were found in 44 (34 females, 10 males) out of 202 animals (Table 1). The 34 (32 females, 2 males) of all positive cases were originated from Vietnam and the other 10 cases (2 males, 8 females) were originated from China (Table 1). Positive percentage for anti-HEV IgG in monkeys from Vietnam was significantly higher than that from China (Table 1). No significant difference of positive percentages for anti-HEV IgG was found between females and males (Table 1). No animals positive for anti-HEV IgM were found from our colony (data not shown).

Blood biochemistries: Averages of ALT in the positive (+++, ++, and +) groups for anti-HEV IgG and negative (-) group for anti-HEV IgG were 35, 57, 45, and 33 IU/L, respectively (Fig. 1 and Table 2). Averages of AST in same groups as above were 16.7, 18.3, 15.3, and 39.7 IU/L (Fig. 1 and Table 2). No differences were found in the averages for the ALT, whereas significant differences were found in the averages for AST between negative and all other three positive groups ($p=0.017$ on +++ vs. -, $p=0.023$ on ++ vs. -, $p=0.013$ on + vs. -) (Fig. 1).

RT-PCR: Viral RNA could not be detected from any fecal specimens from the 12 monkeys (Fig. 2 and Table 1).

Discussions

HEV infection in nonhuman primates has been investigated by experimental studies [1, 12].

Cynomolgus monkeys infected by intravenous inoculation of HEV provoked an increase in ALT, elevation of anti-HEV IgG, and slight lymphatic infiltration or necrotic change in the liver, although obvious clinical symptoms were not observed [1, 20]. Furthermore, cynomolgus monkeys infected with HEV by the oral route did not show any clinical and hematological changes associated with acute hepatitis or HEV infection [12]. These events in nonhuman primates are very similar to those in pigs, which is a typical and well-known asymptomatic reservoir [5–7]. Although carrier animals do not have any clinical problems, they sometimes provoke HEV sporadic infection in humans [17]. Therefore, HEV infections in nonhuman primates kept in indoor facilities, imported from southern east or eastern Asian countries, should be controlled under precise management of public hygiene.

Monkeys examined in the present study did not show any symptoms or disorders in their blood biochemistry associated with acute hepatitis. Although statistical differences for AST concentration were found between negative and all positive groups, those were not affect to health conditions and any infectious hepatitis in the monkey examined. Namely, AST concentrations of all groups were held less than the reference value (42 ± 12) [16], and this difference was due to the higher concentration in a negative group, but not in positive groups. All anti-HEV IgG-positive monkeys were from either China or Vietnam, but none were from the Philippines or Indonesia. Although they were fed in the same room, regardless of their origins and anti-HEV IgG status, monkeys from the Philippines and Indonesia remained negative for anti-HEV IgG. These events suggest the possibility that HEV does not spread in our facility. This was also supported by the results that anti-HEV IgM was negative for all monkeys examined, and that HEV RNA could not be detected from any fecal specimens who were positive for anti-HEV IgG. Anti-HEV IgM precedes anti-HEV IgG and appears a few days after infection, and then decreases after 4 or 5 months [2, 8], whereas anti-HEV IgG appears rapidly after IgM response and remains persistent for several years

[6]. On the other hand, HEV RNA from fecal samples appears a week before the first increase in IgM, and the RNA appears for about a month and then disappears [2]. According to these results, our monkeys positive for anti-HEV IgG have tolerated and recovered completely from HEV infection.

Monkeys used for biomedical science in Japan are mostly imported from southern east or eastern Asian countries, such as the Philippines, Indonesia, Vietnam, and China, epidemic areas of HEV [2]. The incidence of anti-HEV IgG differed among each country examined in the present study. Although we do not clarify the detail reason why this difference of the incidence could be seen, procedures of breeding and hygienic managements also might differ at the reproductive facilities in each exported country. The imported monkeys in Japan are required for 60 days quarantine, 30days at the exported and 30 days at the imported countries. Monkeys are tightly isolated each other during quarantine. Moreover, HEV RNA disappears for about a month [2], even if a monkey have infected at the reproductive facility of exported country. Therefore, the opportunities of HEV infection during and after quarantine are hard to be considered. That is, onset of HEV infection in the present positive monkeys might occur in the exported countries. Especially, monkeys from Vietnam and China should be carefully treated for the possibility of HEV. Autonomous-examinations (not legal examination) for HEV using ELISA and RT-PCR during quarantine would help to notice the fact of infection at the exported country. Indoor-captive cynomolgus monkeys in Japan, stated anti-HEV IgG-positive and anti-HEV IgM-negative, can be managed under general feeding and hygienic conditions.

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Figure legends

Figure 1: Blood biochemical examinations associated with hepatic disorders are indicated in the graphs. Average of ALT (a) was the highest in an intermediate positive group (++) for anti-HEV IgG, followed by the lower positive (+), higher positive (+++), and negative (-) groups. Average of AST (b) was the highest in the negative group (-), and those in the other groups were almost equal (+++, ++, and +). No differences on ALT were statistically observed among each group, whereas obvious differences on AST were statistically observed between positive and negative groups.

Figure 2: RT-PCR did not detect any viral RNAs from monkey specimens. Numbers of specimens were indicated on the upper side of this figure with severity of anti-HEV IgG (+++; higher positive, ++; intermediate positive, +; lower positive, -; negative,). M; molecular marker, N; negative control, P; positive control

Table 1. Positive or negative number for anti-HEV IgG in indoor cynomolgus monkeys

Birth place	Sex	Total number of each origin		Negative		Positive (OD) 0.11~0.5		0.5~0.9		0.9<		Total positive number		Positive percentage of individual countries	
Vietnum	Female	114	107	80	75	22	20	4	4	8	8	34	32	29.8[a]	29.9
	Male		7		5		2		0		0		2		28.6
China	Female	15	3	5	1	8	2	1	0	1	0	10	2	66.7[a]	66.7
	Male		12		4		6		1		1		8		66.7
Phillipine	Female	23	7	23	7	0	0	0	0	0	0	0	0	0.0	0.0
	Male		16		16		0		0		0		0		0.0
Indonesia	Female	45	45	45	45	0	0	0	0	0	0	0	0	0.0	0.0
	Male		0		0		0		0		0		0		0.0
RCALS*	Female	5	2	5	2	0	0	0	0	0	0	0	0	0.0	0.0
	Male		3		3		0		0		0		0		0.0
Total	Female	202	164	158	130	30	22	5	4	9	8	44	34	21.8	20.7[b]
	Male		38		28		8		1		1		10		26.3[b]

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[a]; Significant difference between Vietnum and China origin by Chi-square test

[b]; No significant difference between female and male by Chi-square test

Table 2 Profiles of selected 12 animals examined for blood chemistries and RT-PCR

No	Birthplace	Sex	Age	Anti-HEV IgG (OD)	Criteria	Blood biochemistries		RT-PCR
						ALT (IU/L)	AST (IU/L)	
1	Vietnam	F	6y3m	2.476		17	13	-
2	Vietnam	F	6y	1.474	+++	46	13	-
3	Vietnam	F	4y10m	0.902		39	24	-
4	Vietnam	F	4y5m	0.807		42	10	-
5	Vietnam	F	5y11m	0.746	++	30	16	-
6	Vietnam	F	5y1m	0.739		99	29	-
7	Vietnam	F	4y4m	0.486		11	12	-
8	Vietnam	F	4y5m	0.321	+	94	<10	-
9	Vietnam	F	5y1m	0.184		30	24	-
10	Vietnam	F	4y7m	-		33	31	-
11	Vietnam	F	5y1m	-	-	<10	34	-
12	Vietnam	F	4y4m	-		56	54	-

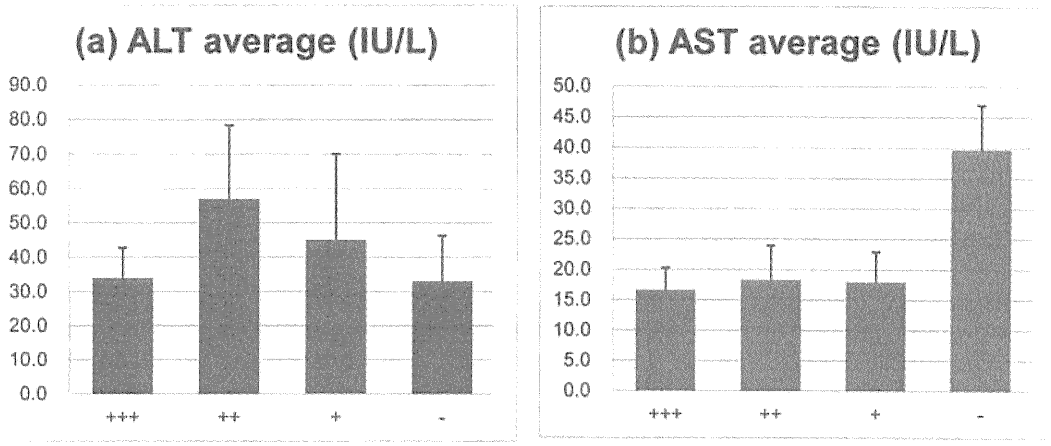

Figure 1

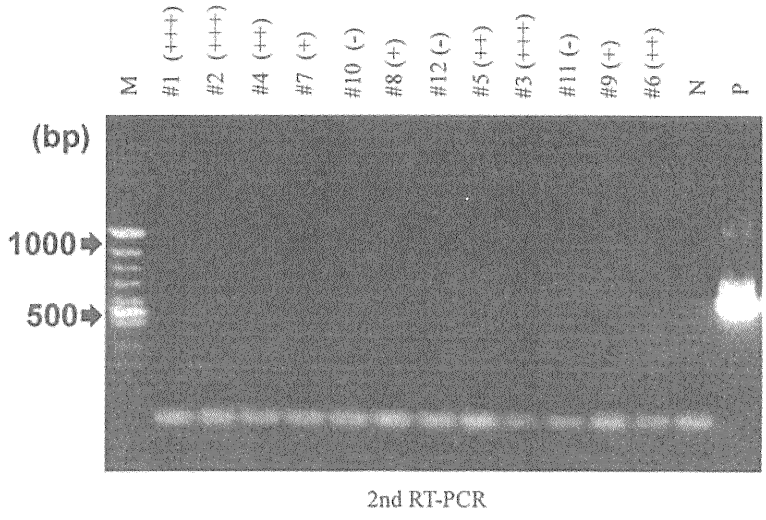

Figure 2

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Epidemiological and genetic analyses of a diffuse outbreak of hepatitis A in Japan, 2010

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ABSTRACT

Background: Hepatitis A virus (HAV) is still one of the most common causative agents of acute hepatitis in Japan. Although a relatively small number of annual acute hepatitis A cases (approximately 100–150, 0.78–1.17 per million) were recently reported, a larger number of cases (346, 2.71 per million) were reported in 2010.
Objectives: To investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses.
Study design: HAV specimens were obtained from 61 cases from 22 different prefectures. These viral specimens were genotyped by PCR amplification and sequencing of the VP1/2A region of HAV genome.
Results: Phylogenetic analysis revealed that 61 HAV strains could be divided into three genotypes: IA (44 cases), IB (1 case) and IIIA (16 cases). The IA genotype consisted of two genomic sub-lineages. The sequences of one of the two IA sub-lineages (corresponding to 31 cases) were very similar, 26 of these 31 isolates had 100% identity. The other IA sub-lineage corresponded to strains endemic to Japan. The sequences of Japanese IIIA strains were similar to those of strains that caused a large epidemic in the Republic of Korea from 2007 to 2009.
Conclusions: The resurgence of HAV in 2010 can be attributed to importation of two newly emerged HAV genotypes.

1. Background

HAV is a member of the genus *Hepatovirus* within the family *Picornaviridae*, and contains a positive-sense, single-stranded RNA genome of approximately 7.5 kb in length. HAV strains isolated from different parts of the world have been classified into six genotypes (I–VI); genotypes I–III are found in humans, and each of these is further divided into subgenotypes A and B. Most of the human HAV strains belong to genotypes I and III.[1–3] Subgenotypes IA and IB are most often found in North and South America, Europe, China and Japan.[1,4,5] Subgenotype IA appears to be the predominant virus of hepatitis A cases worldwide, whereas subgenotype IB has been prevalent in the European and Mediterranean regions.[3,6–8] Subgenotype IIIA was recovered from various countries in Asia, Europe (especially in Roman ethnic population), Madagascar and the USA,[1,5,9–12] and subgenotype IIIB was responsible for some cases of HAV infection in Denmark and Japan.[1,10,11,13]

HAV infection has been a major public health problem in many countries worldwide. The annual incidence of hepatitis A is 1.5 million cases of clinical disease.[14] HAV is transmitted primarily via the fecal–oral route by contaminated food or water,[15–17] but also has been associated with outbreaks in injecting drug users and men who have sex with men (MSM).[18]

The number of acute hepatitis A patients in Japan has been steadily decreasing since the 1990s. Most of the infections that occurred in Japan were sporadic, with the exceptional occurrence of small-scale outbreaks. In 2007–2009, a relatively low number of annual cases (approximately 100–150, 0.78–1.17 per million) of acute hepatitis A were reported. In 2010, however, 346 cases (2.71 per million) were reported. To investigate the epidemiology of this 2010 HAV resurgence, we collaborated with 28 local institutes of health in Japan to obtain stool and plasma specimens from 98 acute hepatitis A patients. The DNA of these viral isolates was PCR-amplified and sequenced, and the sequences were used to perform phylogenetic analyses.

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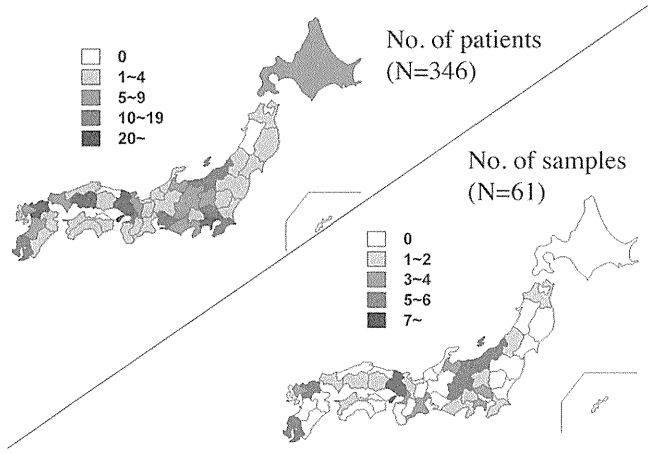

Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

2. Objectives

The primary objective of this study was to investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses. This study, performed in collaboration with local institutes of public health, is expected to provide insights useful for setting appropriate public health guidelines for HAV control.

3. Study design

3.1. Data collection

We collected stool and plasma specimens from 98 acute hepatitis A patients in collaboration with 28 local institutes of health in Japan. The collection sites were located at 22 different prefectures (regions in Japan) (Fig. 1).

3.2. RNA extraction, RT-PCR and phylogenetic analysis

A 10% fecal suspension (wt/vol) was prepared with phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 10,000 × *g* for 10 min. Viral RNA was extracted from the fecal suspension or sera by using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT) was performed with the SuperScript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Seven microliters of the purified RNA was added to a reaction mixture (final volume, 20 μl) containing 50 pmol of random hexamer, 25 mM $MgCl_2$ buffer, 10 mM deoxynucleotide triphosphates, 10× RT buffer, 0.1 M dithiothreitol, and 200 U SuperScript III RT. The mixture was incubated at 42 °C for 1 h, after which 10 U of RNase H was added at 37 °C for 20 min.

Four degenerate primers (P1 to P4) were used in PCR to amplify the VP1/2A region of the HAV genome.[1] The sequences of these primers were:

HAV-2799 (5′-ATTCAGATTAGACTGCCTTGGTA-3′)
HAV-2907 (5′-GCAAATTACAATCATTCTGATGA-3′)
HAV-3162 (5′-CTTCYTGAGCATACTTKARTCTTTG-3′)
HAV-3273 (5′-CCAAGAAACCTTCATTATTTCATG-3′)

PCR was carried out using the HAV-2799 and HAV-3273 primer pair, followed by nested PCR with the HAV-2907 and HAV-3162 primer pair. PCR was performed with EX-taq (Takara, Shiga, Japan) according to the manufacturer's instructions. Amplification was performed for 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 15 min. Three microliters of the PCR product was used as the template for a second round of PCR amplification under the same conditions. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) and used as a template for direct sequencing.

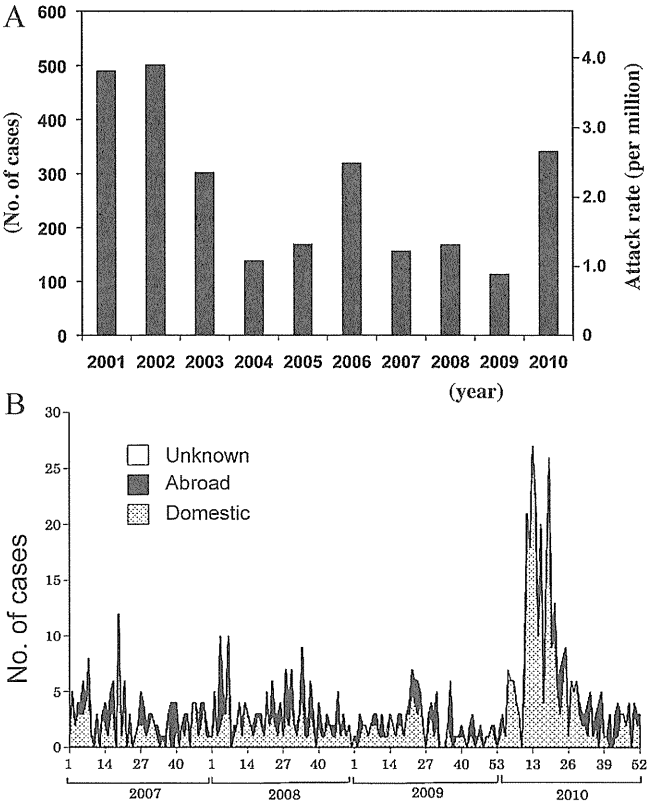

Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t = 5.4 \times 10^{-}$), 2008 ($t = 5.6 \times 10^{-5}$) and 2009 ($t = 1.8 \times 10^{-5}$). (B) Weekly acute hepatitis A cases from week 1 of 2007 to week 52 of 2010.

Phylogenetic trees were constructed with the MEGA software (DNA DATA Bank of Japan) by the neighbor-joining method from a Kimura two-parameter distance matrix, and bootstrap values were determined from 1000 bootstrap re-samplings of the original data.[19–22] All reference sequences used in this study were obtained from GenBank.

4. Results

In 2010, the number of acute hepatitis A cases increased to 346 (2.71 per million) (Fig. 2A) because of a diffuse outbreak that occurred from March through May (Fig. 2B). Most of the patients in this outbreak reflected domestic infection events (Fig. 2B). Clinical descriptions of these patients are summarized in Table 1.

Sera and fecal samples from 98 patients were available for PCR. Of these, 61 yielded a PCR product that could be used for sequencing. Among these 61 isolates, 44 were of genotype IA, one was of genotype IB and 16 were of genotype IIIA by phylogenetic analysis (Fig. 3). The genotype IA isolates could be sorted into two sub-lineages. One sub-lineage (referred to as IA-1 in this paper) grouped with several isolates found in 2006,[23–25] suggesting that the isolates in this lineage were endemic to Japan. In contrast, the sequences of most of the genotype IA isolates belonged to a second sub-lineage (referred to as IA-2 in this paper) with sequences almost identical to one another. Among the IA-2-infected patients, two had developed acute hepatitis shortly after returning from Philippines,

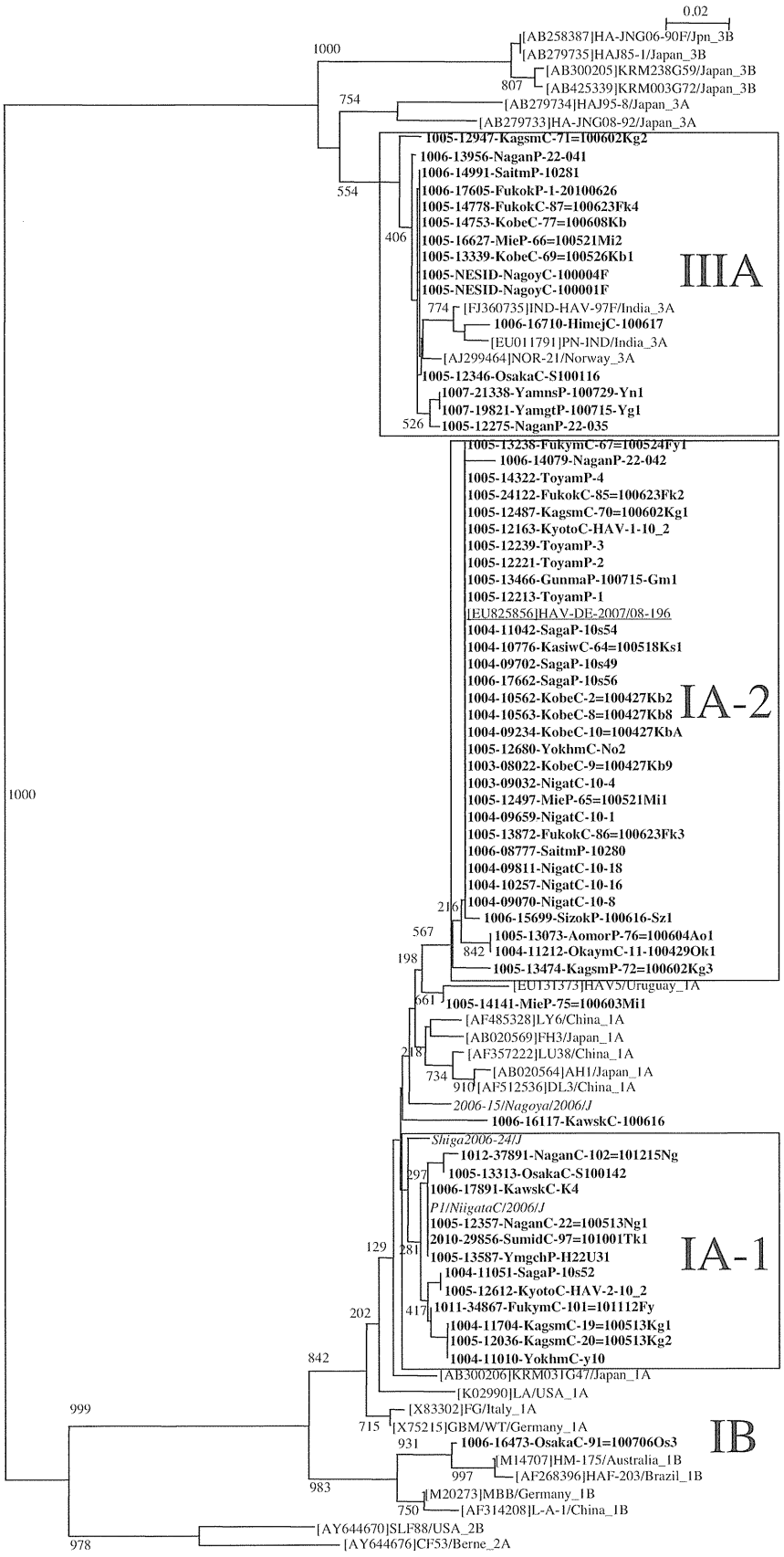

Fig. 3. Neighbor-joining phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus isolates. Reference strains are used in this study and indicated as GenBank accession numbers. Sequences of 61 isolates from this study are shown as YYMM-NESID-KKKKKKKKK (YYMM represents the reported year (YY) and month (MM); NESID (National Epidemiological Surveillance of Infectious Diseases) is the ID number of the patient; KKKKKKKKK is the name of the isolate given by local institute). The scale bar at the bottom indicates nucleotide distance. Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling.