the Fuc(Le) and Fuc(H) of this antigen simultaneously occupy the respective binding sites (Fig. 6A). However, a distortion in the Fuc(H) site appears to be present and to result in the low affinity of the wild-type P domain for the Le^b antigen (Fig. 6A), suggesting that the binding pocket is insufficient to comfortably accommodate the Le^b antigen (Fig. 6A). On the other hand, in the Q389N mutant P domain, the Le^b antigen is fitted to the binding pocket in a relaxed, stabilized conformation (Fig. 6B). Interestingly, Fuc(H) of the Le^b antigen in the mutant P domain is coordinated by water molecules in a similar manner to Fuc(H) of the H antigen in the wild-type protein, accompanied by flexible interactions at the Fuc(Le) site (Fig. 6C).

Concomitant with the Q389N mutation, slight movements of the Asn441 and Ser343/A residues (0.6 Å and 0.49 Å, respectively) were observed. Interestingly, the Gln389 in the wild-type protein directly interacts with Ser343/A in loop P (Fig. 1E), while the interaction between Asn389 and Ser343/A in the mutant protein is mediated via a water molecule (Fig. 6B). The presence of the water molecule consequently evokes the shift of Gly345 by 0.25 Å from the carbohydrate-binding pocket. It should be noted that Gly345 in loop P plays a critical role in the binding of both Fuc(H) and Fuc(Le). Moreover, in conjunction with the Q389N mutation, the loop region containing the residues from Ser391 to Thr396 moves away by up to 1.4 Å from the carbohydrate-binding pocket. Based on these results, it was concluded that the Q389N mutation expanded the space for Fuc(Le) and thus conferred an advantage in the binding of the Le^b antigen. All or some of these slight movements in several regions evoked by the Q389N mutation might have been disadvantageous for the binding of the Le^a antigen, resulting in a loose binding with a lowered affinity. By contrast, the Q389N mutant apparently lost its ability to bind to the A and H antigens (Fig. 5). Consistent with this result, we could not obtain models of the A and H complexes of the Q389N mutant P domain owing to the poor electron density map, strongly suggesting that the Q389N mutation perturbs a local structure around the carbohydrate-binding pocket.

The majority of GI NoVs interact with the A antigen but not the B antigen (34), despite their structural resemblance; the A antigen has a terminal GalNAc, whereas the B antigen has a Gal at the same position. As shown in Fig. 7, the carbonyl oxygen in the *N*-acetyl group of GalNAc of the A antigen interacts with the protein backbone via a water molecule. Similarly, a water molecule mediates the interaction between the protein and the O-5 atom of Fuc(H) of the H antigen. These features might help to explain why the B antigen is not recognized by the FUV258 VLPs (Fig. 5). Indeed, the P domain crystals soaked with the B antigen did not give sufficient electron density for the carbohydrates (data not shown). Here, we discuss two scenarios. (i) What would happen if the Fucα1-2Gal moiety of the B antigen is overlaid with Fuc(H)-Gal of the H antigen? Presumably the terminal α1-3-linked Gal of the B antigen interferes with the binding of the entire B antigen. (ii) What would happen if the terminal α1-3-linked Gal and Fuc(H) of the B antigen are overlaid with GalNAc and Fuc(H) of the A antigen, respectively? In this case, the only difference is whether the *N*-acetyl group or the hydroxyl group is linked to the second carbon of the Gal ring. As described above, the carbonyl oxygen in the *N*-acetyl group plays a crucial role in being recognized by the P domain. However, no atom is present at the equivalent position in the B antigen, resulting in a failure to form hydrogen bonds. The results of a previous study support our hypothesis (6). The authors suggested that the hydrophobic interaction with the *N*-acetyl group of GalNAc of the A antigen played a critical role in conferring NoV-HBGA binding specificity.

The evaluation of the hydrogen bonding interaction bridged by water molecules is complex because it is difficult to use computer programs to add and refine the hydrogen atoms around the sugar. This is a major future issue. (The calculations and evaluations are in progress.) On the other hand, the strong stacking interaction of the aromatic residues with the sugar chain that are typically found in the legume lectin was not found (44), although some hydrophobic contacts were seen (see Ligplot diagrams [43]) in Fig. S3 in the supplemental material.

Several X-ray crystallographic analyses were performed to investigate the structural basis for how HBGAs interact with the NoV capsid protein (3, 5, 6, 9, 33). The sites on HBGA for recognition by the prototype strain GI/1 NV/68 and the epidemic GII/4 variants have been analyzed in detail (3, 6, 33). They are situated at different locations on the protein surface. Consequently, GI and GII probably do not share a manner of Le^a recognition. Therefore, to avoid ambiguous speculation, we do not describe the differences between GI and GII in this study.

The carbohydrates used in this study were bound to the same spaces of the FUV258 (GI/2) and NV/68 (GI/1) P domains. The VLPs of GI/1 and GI/2 strains share the common feature of showing a high level of binding to carbohydrates in the saliva of type O, A, and AB secretors but not to those in the saliva of type B secretors (34). However, the levels of binding to carbohydrates from nonsecretors differed between GI/1 and GI/2 (34). In the present study, we showed that the difference in the length and hence the structure of loop P between the two strains affected the degree of recognition of the Lewis antigens. A unique characteristic of several GI strains, such as GI/2, GI/3, and GI/4, is the ability to bind to the Le^a antigen, which is not observed for any GII strains (34). The Le^a antigen, which has a type 1 core structure, is the dominant antigen found in the secretions of nonsecretors. NoV binds to the gastroduodenal junction, and this binding is correlated with the presence of a type 1 core structure but not of a type 2 core structure (23). Moreover, an *in vitro* study showed that the type 1 carbohydrate binds more tightly to NoV VLPs than the type 2 carbohydrate (34). Overall, the differences in the amino acid sequences of the loop P region are potentially one of the determinants of whether the NoV strains of interest bind to the Le^a antigen, and hence whether the strains infect nonsecretors.

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T.K. and H.S. conceived and designed the experiments. T.K., A.K., S.F., and H.S. performed the experiments. T.K. analyzed the data. H.I. provided synthetic carbohydrates. Y.S., N.T., and K.I. provided expert advice. T.W. and H.N. supervised the project. T.K., Y.S., N.T., and H.S. wrote the paper.

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Original Article

Possible widespread presence of hepatitis A virus subgenotype IIIA in Japan: Recent trend of hepatitis A causing acute liver failure

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Aim: Recently, the number of acute hepatitis A cases has decreased in Japan. However, six patients with acute liver failure caused by hepatitis A virus (HAV) have been admitted to Chiba University Hospital, Japan, in the last 18 months, between 2010 and June 2011. The aim of this study is to characterize the recent HAV genotypes from an urban hospital in Japan and to compare the clinical differences.

Methods: Hepatitis A virus RNA was detected by strand-specific reverse transcription. Then, HAV VP1/2A regions were amplified by nested polymerase chain reaction (PCR). Sequences were directly determined and phylogenetic trees were constructed for determining HAV subgenotypes.

Results: Analysis of these HAV genomes revealed that 4 and 2 belonged to subgenotypes IA and IIIA, respectively.

Conclusions: Fujiwara *et al.* reported a frequency of HAV subgenotype IIIA of only 2.1% in Japan. We conclude that HAV subgenotype IIIA might be widespread in our country.

Key words: acute liver failure, hepatitis A virus, Japan, subgenotype IIIA

INTRODUCTION

HEPATITIS A VIRUS (HAV) is a member of the genus *Hepatovirus* in the *Picornaviridae* family. HAV is a positive-stranded RNA virus with an approximately 7.5 kb genome, is usually spread via the fecal-oral route, causes acute hepatitis, and occasionally leads to acute liver failure with fatal outcome in unvaccinated individuals.[1,2] There is only one serotype of HAV, but based on sequences of the VP1/2A genomic region, at least six genotypes (I to VI) exist.[3] Three (I, II and III) of the genotypes are of human origin.

Several studies on HAV genotypes in Japan were reported.[3–6] In 1992, Robertson *et al.*[3] reported the existence of two predominant subgenotypes, IA and IIIB. In 2003, Fujiwara *et al.*[4] determined that 44 of 47 acute hepatitis A cases belonged to subgenotype IA, two to IB, and one to IIIA. In 2006, Takahashi *et al.*[5] also reported that 57 of 58 sequences belonged to IA and only one to IIIA. Toyoda *et al.*[6] reported that all 61 isolates they determined between 1992 and 2003 belonged to subgenotype IA. These reports revealed that the HAV subgenotype IA was endemic to Japan.[4–6]

Recent studies on HAV genotypes from South Korea have shown a distinct pattern change in circulating HAV genotypes over the past 10 years.[7] Until early 2000, almost all isolates tested had been identified as subgenotype IA.[8] A more recent study showed that subgenotype IIIA has been predominant since 2008.[7] In addition, a rise in the frequency of hepatitis A outbreaks has recently been observed in South Korea, our immediate neighbor, although the number of hepatitis A

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cases in Japan has been progressively decreasing during the last several years.[9] The two countries have some cultural similarities. There is no universal hepatitis A vaccination program in either country, whereas Korea, but not Japan, has such a program against hepatitis B. We also reported that HAV 5'NTR subgenotype IA from Korea had high homology to Japanese sequences.[9] These circumstances have raised concerns about a possible HAV epidemic in Japan. The aim of this study is to characterize the recent HAV genotypes from an urban hospital in Japan and to compare the clinical differences.

METHODS

Patients

SERA WERE COLLECTED from immunoglobulin M (IgM) antibodies to HAV (IgM-HA) positive patients upon admission to Chiba University Medical School Hospital, Chiba, Japan. HAV infection was defined by positive reactions for IgM-HA and serum HAV RNA by polymerase chain reaction (PCR) with primers from the highly conserved 5' non-translated region (5'NTR).[9] These patients presented with acute liver failure without encephalopathy on admission between 2010 and June 2011 (Table 1). This study was approved by the ethics committee of Chiba University, Japan (permission number 1160), the ethics committee of the National Institute of Infectious Diseases Japan (permission number 305), and complied with the Helsinki Declaration.

RNA extraction and detection of HAV RNA by PCR

RNA was extracted from 100 μL of serum samples according to the guanidium thiocyanate method and subjected to RT-PCR for the VP1/2A region of the HAV genome.[3] Complementary DNA was synthesized with HAV-3273 (5'-CCA AGA AAC CTT CAT TAT TTC ATG-3'), then amplified with HAV-3273 and HAV-2799 (5'-ATT CAG ATT AGA CTG CCT TGG TA-3') for 40 cycles at 94°C, 50°C, and 72°C. Then, the first PCR product was further amplified with inner primer pairs HAV-2907 (5'-GCA AAT TAC AAT CAT TCT GAT GA-3') and HAV-3162 (5'-CTT CYT GAG CAT ACT TKA RTC TTT G-3') in the same manner. Amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Sequencing of the VP1/2A region

Sequences were directly determined as previously described.[9]

Phylogenetic analysis

A phylogenetic tree was constructed by using GENETYX, version 10 (Genetyx, Tokyo, Japan) based on the nucleotide sequences of the amplified VP1/2A region. The GenBank accession numbers for the nucleotide sequences of HAV isolates are AB643799 – AB643804. HAV complete genome sequences were retrieved from the DDBJ/EMBL/GenBank genetic database and used as references in this study.

RESULTS

SIX PATIENTS WITH acute liver failure caused by HAV were admitted during an 18-month period between 2010 and June 2011 (Table 1). All patients had >38.5°C fever on admission. All patients presented with acute liver failure with coagulopathy but without encephalopathy (non-fulminant cases) (Fig. 1). Patient no. 2 was a hepatitis B virus carrier. All patients recovered

Table 1 Profiles of six acute liver failure patients infected with hepatitis A virus in Japan

Patient no.	Age (years)/sex/ nationality	Month of onset	Nadir PT (%/INR)	Peak ALT (IU/L)	Peak total bilirubin (mg/dL)	Presumed route of transmission	Isolate name/ subgenotype
1	69/F/JPN	2010 Mar	23/2.88	7731	8.5	Raw scallop	Ch24/IIIA
2	46/M/JPN	2010 Apr	25/2.71	3388	12.6	Unknown	Ch23/IA
3	59/M/JPN	2010 Jun	35/2.01	5693	22.8	Raw oyster	Ch26/IA
4	30/F/KOR	2010 Jul	36/1.98	6958	5.0	Raw oyster	Ch25/IIIA
5	54/M/JPN	2011 Jan	20/3.20	2979	10.1	Sushi	Ch27/IA
6	37/M/JPN	2011 Jan	34/2.11	9826	3.9	Sushi	Ch29/IA

ALT, alanine transaminase; F, female; G, subgenotype; INR, international normalized ratio; JPN, Japan; KOR, South Korea; M, male; PT, prothrombin time.

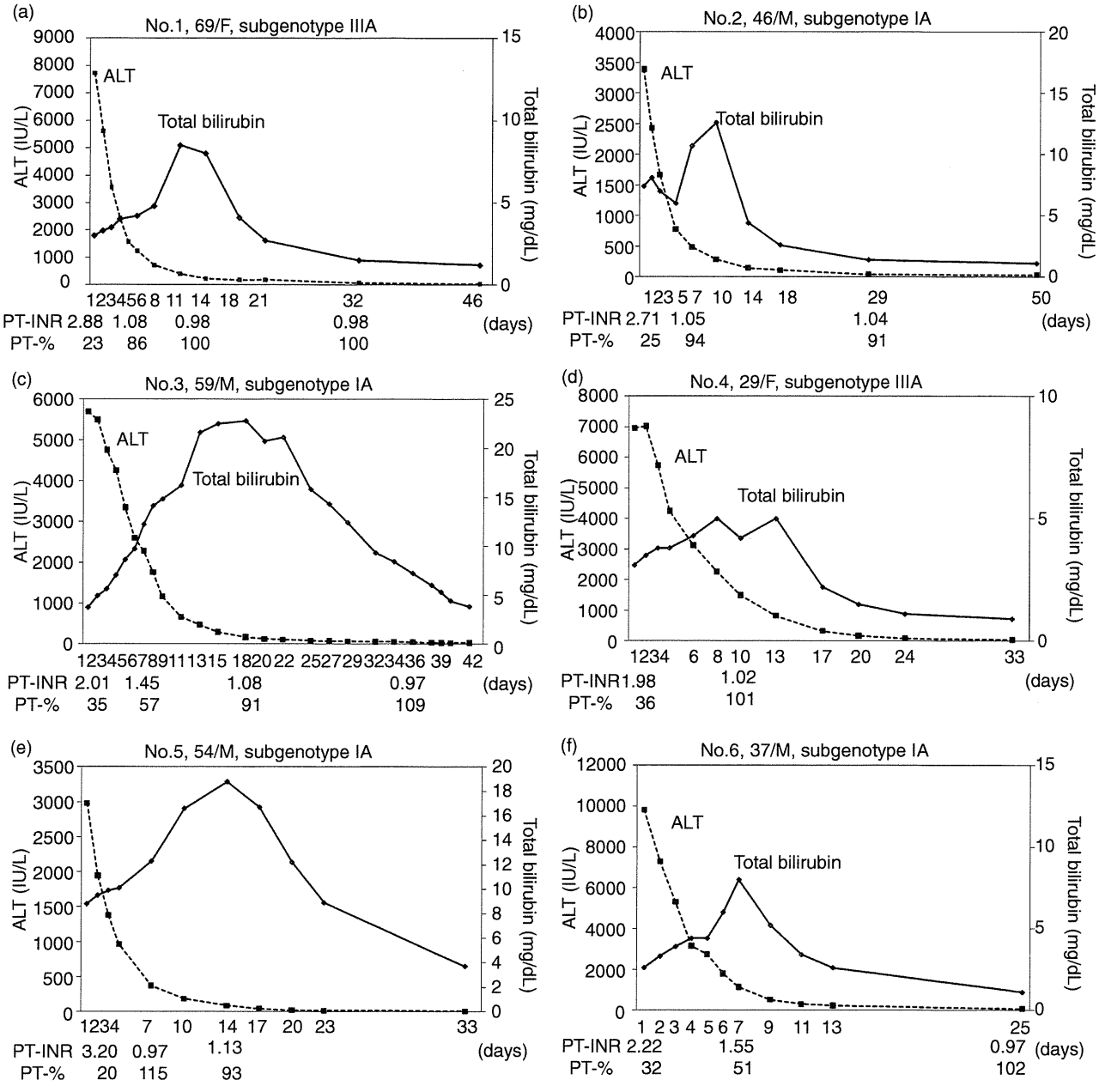

Figure 1 Clinical course of six acute liver failure patients infected with hepatitis A virus (HAV) in Japan. (a), (b), (c), (d), (e) and (f) indicates patient no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6 in Table 1, respectively. All patients presented with acute liver failure with coagulopathy but without encephalopathy (non-fulminant cases). PT, prothrombin time.

without liver transplantation, although patient no. 3 had interstitial pneumonia and was complicated by prolonged cholestasis while hospitalized and bone marrow suppression during the follow-up period, and patient no. 5 was complicated by mild acute kidney injury but recovered.

The nucleotide sequences of the six human HAV isolates in this study were compared with those of 24 published HAV sequences, and the genetic relatedness of the HAV isolates from different genotypes was investigated. Phylogenetic analysis of the nucleotide sequences from the VP1/2A region showed that four isolates (Ch23,

Ch26, Ch27 and Ch29) and two isolates (Ch24 and Ch25) belonged to subgenotype IA and IIIA, respectively (Fig. 2).

The sequences of the four isolates of subgenotype IA closely matched that of one well-characterized subgenotype IA virus: FH1 (GenBank accession no. AB020567) (96–97% nucleotide identity). Similarity of the nucleotide sequences of the VP1/2A region between the four isolates of subgenotype IA in this study ranged from 95% to 99%.

The sequences of the two isolates of subgenotype IIIA closely matched that of two well-characterized subgenotype IIIA viruses: A408 (GenBank accession no. AB046904) (99–100% nucleotide identity) and NOR-21 (GenBank accession no. AJ299464) (98% nucleotide identity). Similarity of the nucleotide sequences of the VP1/2A region between the two isolates of subgenotype IIIA in this study was 98%. Our two strains were clustered with A408 (Japan), NOR-21 (Norway), HA-JNG04-90F (Japan), HMH (Germany) and subgenotype IIIA strains reported from Japan in early 2010. Another subgenotype IIIA cluster was formed by two strains, HAJ95-8F (Philippines) and HA-JNG08-92F (Madagascar).

DISCUSSION

IN THE PRESENT study, of six recent patients with HAV-associated acute liver failure, two were caused by subgenotype IIIA. It was reported that almost all acute hepatitis A cases (93.6%) were caused by subgenotype IA and only 2.1% by subgenotype IIIA,[4] and that all acute liver failures were caused by subgenotype IA. Thus, the possibility of a changing pattern in circulating HAV genotypes such as that reported in Korea[7] might need to be entertained in Japan as well.

What about the transmission route? Many high-risk groups such as travelers visiting highly endemic areas, the military, healthcare workers, sewage workers,

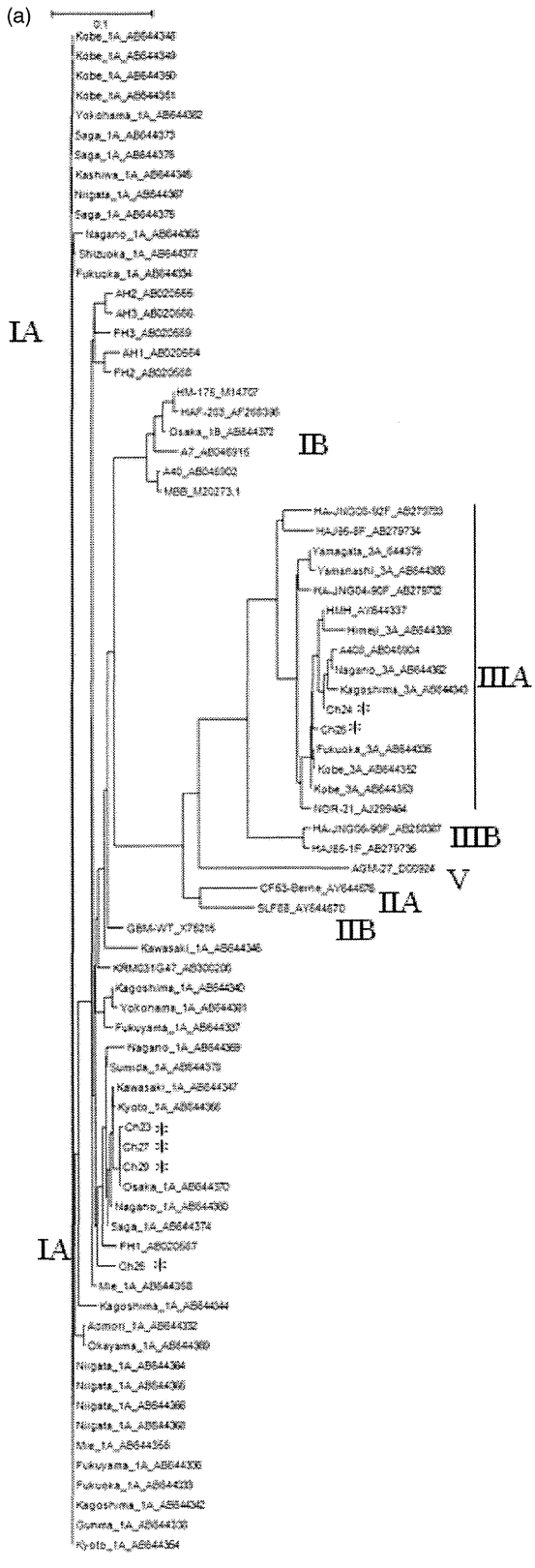

Figure 2 Phylogenetic analysis of hepatitis A virus (HAV) isolates from patients with acute liver failure from Japan. (a), (b) The neighbor joining tree was constructed based on a partial sequence of 451 nt in the VP1/2A region of HAV. Selected reference strains were also included in the phylogenetic analysis to represent the following subtypes: HAV-IA, IB, IIA, IIB, IIIA, IIIB, and V. *Strains sequenced in this study are indicated (Ch23, Ch24, Ch25, Ch26, Ch27 and Ch29), aligned with all the available reference sequences retrieved from data bases (DDBJ/EMBL/Gene Bank).

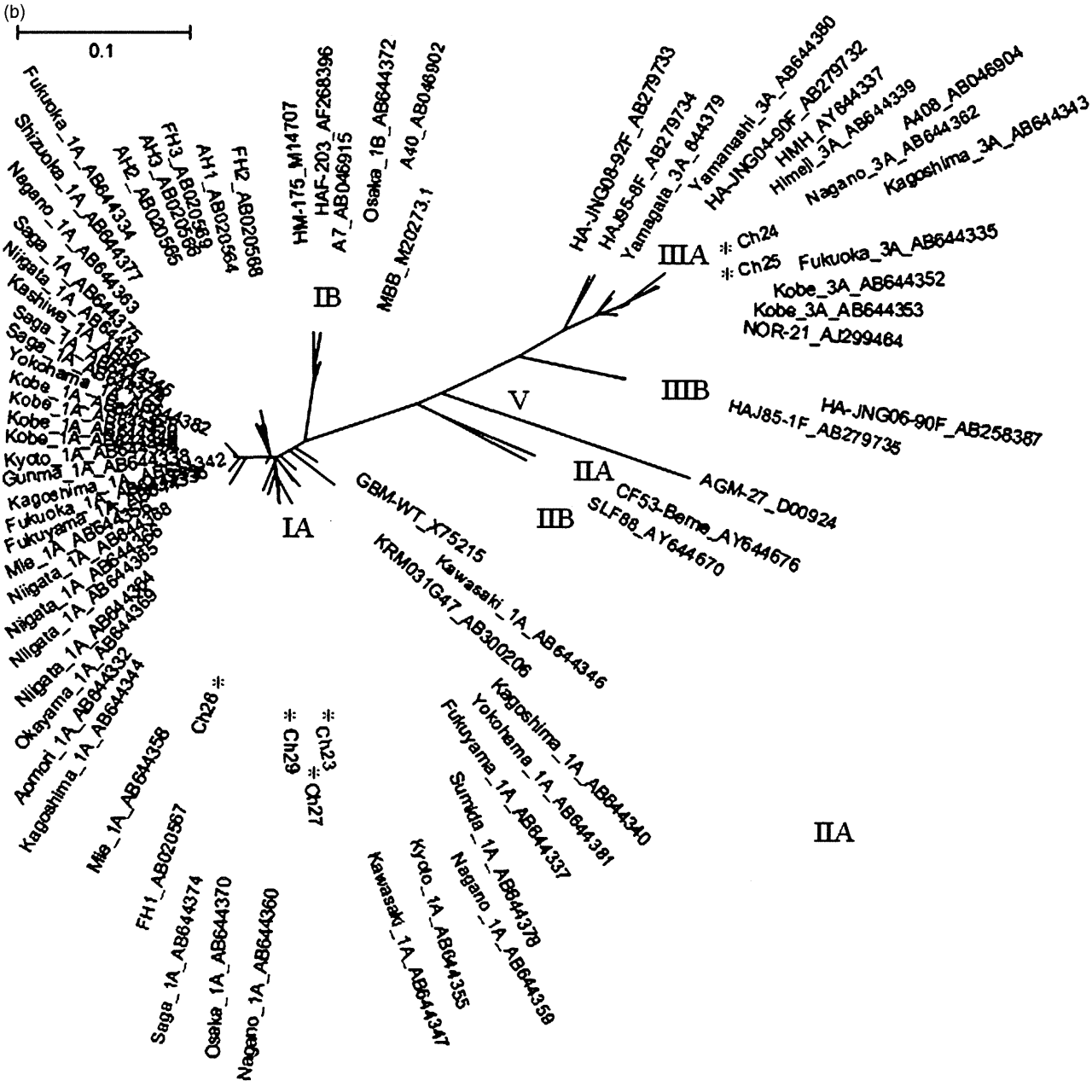

Figure 2 *Continued.*

day-care assistants, drug addicts, and homosexual people have been identified for potential HAV infection.[10] In the present study, four patients with subgenotype IA were male and two with subgenotype IIIA were female. We do not know why there were sex differences between the two subgenotypes. None in the present study was homosexual or HIV-positive. Patients no. 5 and no. 6 were associated with a recent HAV outbreak at a sushi shop in the Chiba area (Table 1).[11] None of the patients had traveled abroad, including to South Korea, during more than one year before admission. That is, all patients were infected with HAV in our country, suggesting that HAV subgenotype IIIA might be widespread in our country. Of interest is that these two patients (no. 1

and no. 4) had eaten raw scallops and raw oysters, respectively (Table 1).

The clinical spectrum of HAV infection ranges from asymptomatic infection to fulminant hepatitis.[12] Clinical presentation of hepatitis A depends on the age of the patient, being more severe in adults than in children.[13] In the present study, the mean age of subgenotype IA and IIIA patients was 49 ± 9.6 and 49.5 ± 27.5 years, respectively. A recent study from Korea reported that HAV genotype influences the severity of liver disease and that a higher ALT level (>1000 IU/L) and longer hospitalization were significantly associated with subgenotype IIIA.[7] All HAV-associated acute liver failure patients in the study of Fujiwara *et al.*[4] belonged to subgenotype IA. In this regard, we also examined whether HAV genotype is directly related to the disease severity of hepatitis A. Two of the six acute liver failure patients in the present study were subgenotype IIIA. It is well-known that viral genotypes occasionally affect disease progression, severity and treatment response in hepatitis B and C.[14,15] Mean ALT levels of subgenotype IA and IIIA patients were 5470 ± 3130 and 7340 ± 546 IU/L, respectively. Further studies will be needed to examine whether there are associations between HAV genotypes and disease severities, as the number of patients was limited and most of the patients in Chiba University Hospital were cases with acute liver failure.

In conclusion, the current study suggested that HAV subgenotype IIIA is also associated with acute liver failure in Japan. We need to make a cautious interpretation of the relation between HAV genotypes and their disease severities.

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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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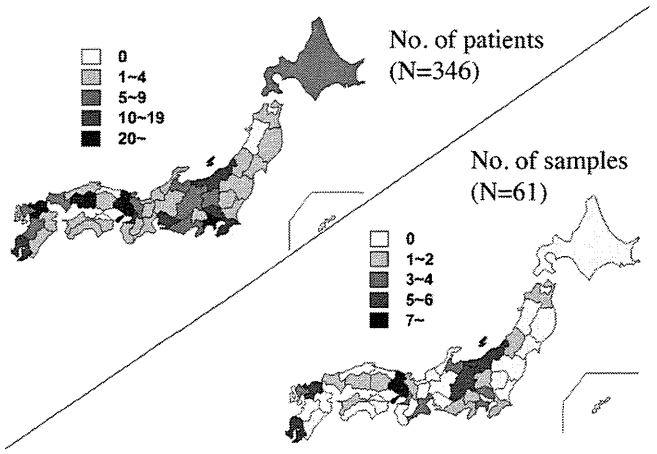

Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

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.

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.

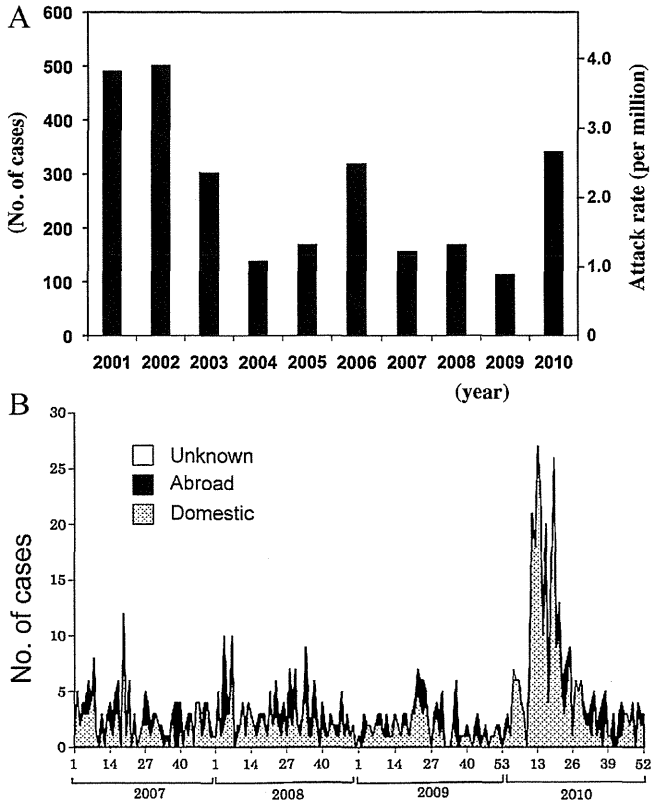

Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t = 5.4 \times 10^{-}$), 2008 ($t = 5.6 \times 10^{-5}$) and 2009 ($t = 1.8 \times 10^{-5}$). (B) Weekly acute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

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,

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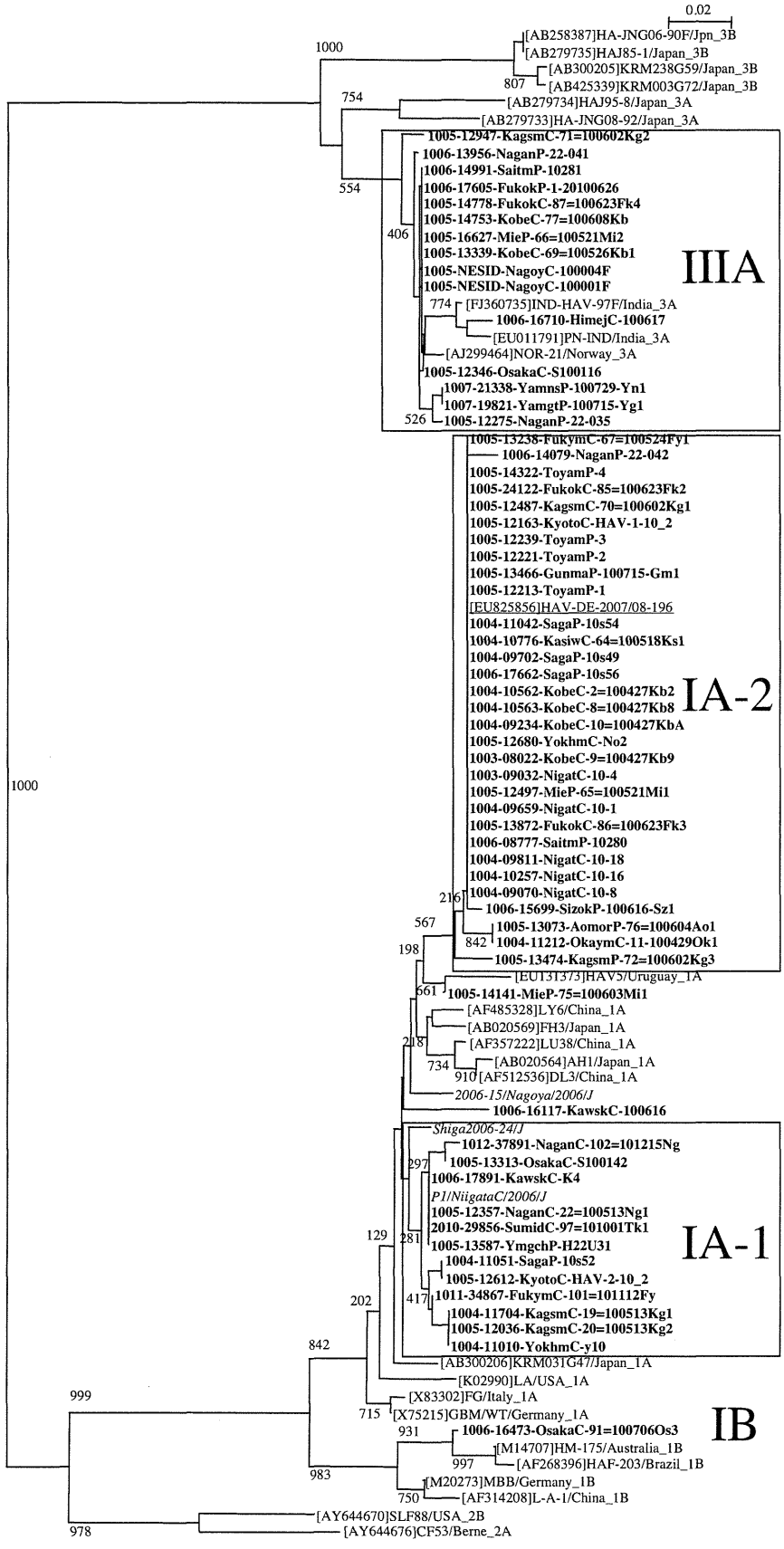

Fig. 3. Neighbor-joining phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus isolates. Reference strains are used in this study and indicated as GenBank accession numbers. Sequences of 61 isolates from this study are shown as YYMM-NESID-KKKKKKKKK (YYMM represents 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.

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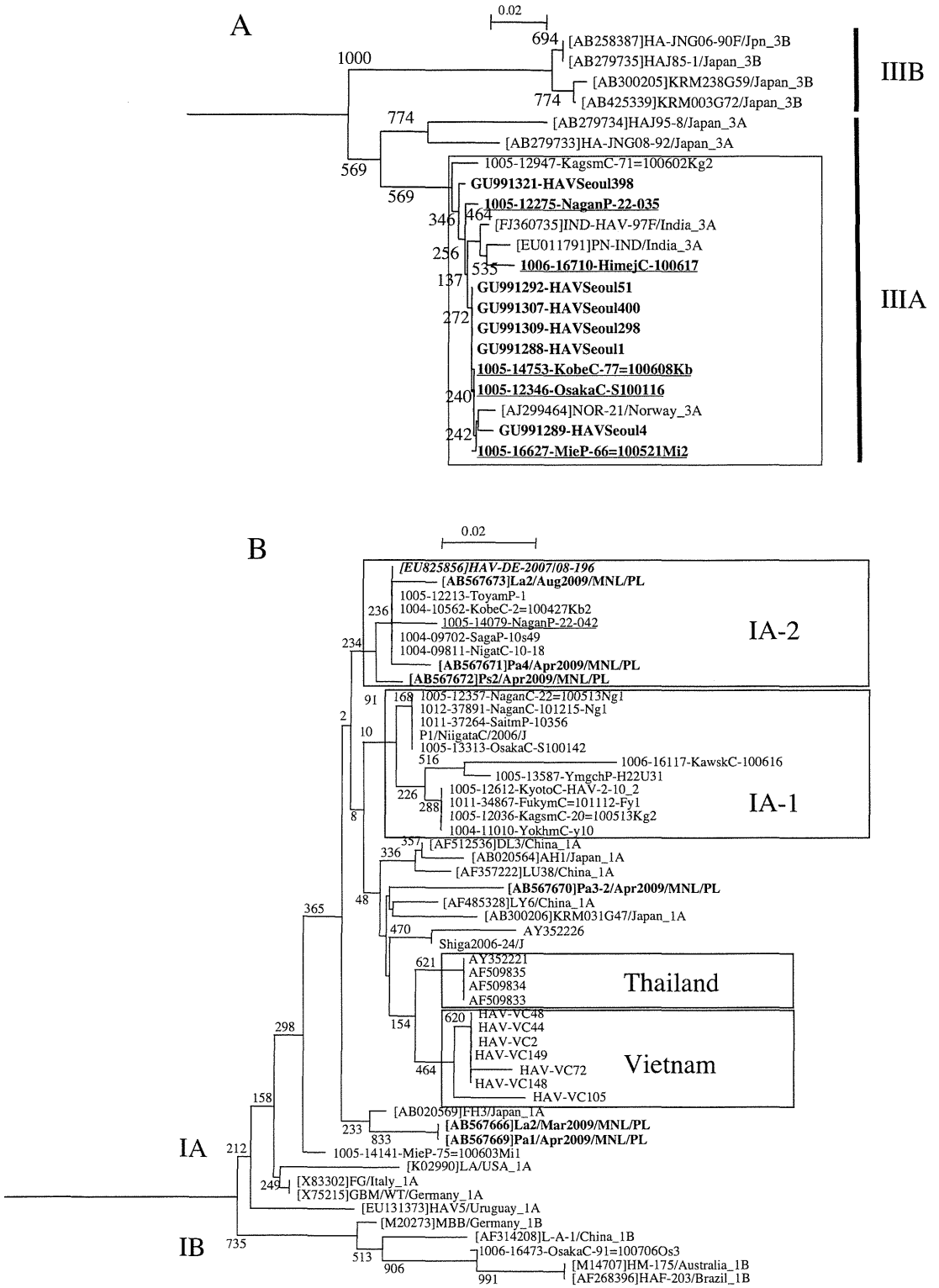

Fig. 4. (A) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from HAV strains (genotype IIIA) isolated from Japan (bold underline) and Korea (bold). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling. (B) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus strains (genotype IA) isolated from Japan, Thailand, Vietnam and river and sewage from Philippines (shown in bold). HAV sequences of Japanese patients who developed acute hepatitis shortly after travel to Philippines are underlined. HAV-DE-2007/08-196 is shown in italics. In IA-2 sub-lineage, 26 identical sequences are represented by four sequences (1005-12213, 1004-10562, 1004-09702, 1004-09811). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling.

suggesting the relationship of this lineage with HAV viruses from that geographical source.

A slightly different region of VP1-2A (nt: 2975–3364) was used for phylogenetic analysis in South Korea (Yoo et al., unpublished, available on GenBank) compared to the region of VP1-2A (nt: 2930–3161) used in the present study. Unfortunately, the overlap between these sequences was not long enough for comparison between the two studies. To permit such a comparison, we

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Table 1
Clinical descriptions of hepatitis A cases during diffuse outbreak period (from 10th to 28th week of 2010, 236 cases).

Items	Data
Age (median)	5–88 yr (48 yr)
Sex	Male 138 (58%), female 98 (42%)
Suspected infection route	Fecal–oral 199 (84%), others/unknown 37 (16%)
Suspected food vehicle	Oyster 58 (29%), fishery product 27 (14%), well water/tap water in foreign country 4 (2%), others/unknown 46 (23%), unnoted 64 (32%)
Icteric	171 (72%)
Fulminant (severe) hepatitis	6 (3%)
Diagnosed by	IgM 223 (94%), PCR 2 (1%), IgM and PCR 11 (5%)

sequenced VP1-2A fragments (nt: 2822–3272) generated by the first PCR reaction on some of the Japanese genotype IIIA strains. These sequences were compared with Korean genotype IIIA strains. Phylogenetic analysis revealed that the Japanese and Korean genotype IIIA isolates could be classified into a single cluster (Fig. 4A). This observation suggests a close relationship between the Japanese genotype IIIA strains and those derived from the recent Korean outbreak.

5. Discussion

In recent years, the incidence of hepatitis A in developed countries has decreased dramatically. Changes in the genotypes or subtypes of HAV strains, including the emergence of HAV strains that are new to the area, have been observed in patients with acute hepatitis A in developed countries,[26] probably due to the transport of HAV strains via international transport of foods and agricultural products. HAV strains also could be imported by unvaccinated human carriers who have traveled to endemic countries. National surveillance of HAV in Japan has shown that more than 90% of people over 65 years of age, but fewer than 10% of people under 34 years of age, are seropositive for HAV.[27] Most of the infections that have occurred in Japan represent sporadic events, with exceptional occurrences of small-scale outbreaks. In 2010, however, there was a spike of hepatitis A infections in Japan, with 346 cases reported by the Infectious Disease Surveillance Center, NIID.

One of the genotype IA sub-lineages (referred to as IA-1 in this paper) was related to an isolate found in small outbreaks in Shiga and Niigata prefectures in 2006.[23,24] The isolates belonging to this sub-lineage have been detected in Japan since at least 2001 (Tamada and Yano, personal communication), suggesting that the isolates of this sub-lineage were locally endemic strains of Japan. On the other hand, more than half of genotype IA isolates displayed identical or virtually identical sequences across a 230-nt interval of the VP1-2A segment of the genome. Among the isolates in this sub-lineage (IA-2 in this paper), two (Fig. 4B, underlined) were from patients who had recently visited the Philippines, suggesting a relationship between IA-2 sub-lineage and this geographical site. This sequence also was found to be identical to HAV-DE-2007/08-196 (Fig. 4B, italics), which was identified in Germany in 2007.[28] The patient of HAV-DE-2007/08-196 was an 11-year old female who developed acute hepatitis shortly after traveling to the Philippines (Faber et al., personal communication). To assess this proposal, we also obtained sequence data for HAV derived from river and sewage of Manila and included these sequences in our phylogenetic analysis (Fig. 4B; HAV from river and sewage of Manila are shown in bold). Some sequences classified with the IA-2 sub-lineage, supporting the hypothesized Philippine connection. Genotype IA isolates of HAV from other Southeast Asian countries, such as Vietnam[29] and Thailand,[30] formed distinct clusters (Fig. 4B). However, caution is necessary with this result, because

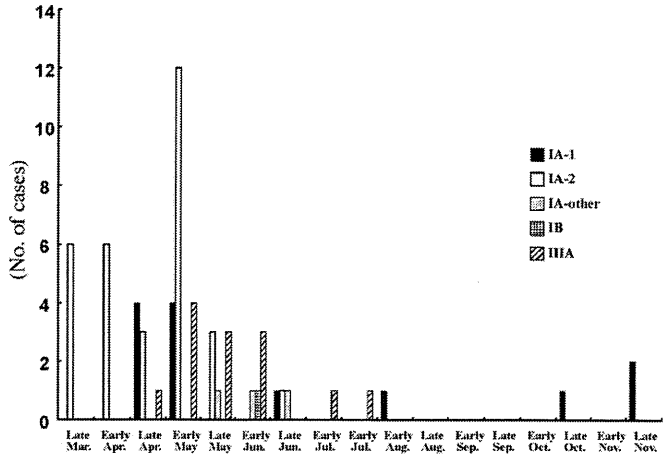

Fig. 5. Temporal distribution of HAV genotypes from late March to late November in 2010.

the sequences of HAV from these countries were determined 4–5 years before the Japanese diffuse outbreak in 2010, and a shorter 168-bp fragment (nt: 3024–3191, corresponding to the sequence data of Thai isolates) was used for the analysis. The isolates belonging to the IA-2 sub-lineage were detected mainly from late March through May, and could not be detected after June (Fig. 5). On the other hand, a regional imbalance of hepatitis A cases associated with this strain was not observed. Together with the uniformity of this cluster, we propose that this strain expanded from a single infection source (possibly an imported food product) that caused diffuse outbreak without a secondary expansion. Unfortunately the source(s) of HAV isolates belonging to the IA-2 remain unidentified.

Until recently, Japanese isolates of genotype IIIA were detected only on rare occasion, with the exception of some imported cases.[31–33] However, in 2010, approximately 26% of HAV isolates were classified as genotype IIIA. In South Korea, the incidence of reported HAV cases were increased dramatically since 2005, and most of the HAV isolates from this period clustered within genotype IIIA lineage. These results suggest genotype IIIA as the major epidemic strain for this outbreak, despite the fact that the predominant genotype in Korea, until 2005, was genotype IA.[12,34] Since the VP1-2A region of HAV genome amplified by nested RT-PCR for phylogenetic analysis in Korea differed from that in our study, we could compare only those Japanese IIIA isolates for which we obtained sequences of the region amplified by the first PCR reaction. Phylogenetic analysis revealed that the Japanese and Korean IIIA isolates clustered together (Fig. 4A), suggesting a correlation between the Japanese IIIA strain in 2010 and the recent Korean outbreak.

In conclusion, our data revealed that the diffuse outbreak of hepatitis A in Japan in the spring of 2010 was derived not only from locally circulating strains, but also from two other newly emerged HAV strains, possibly imported from the Philippines (IA-2) and Korea (IIIA). More detailed and extensive epidemiological analyses, ideally in collaboration with these countries, are needed to determine the source of the imported strains. However, in order to provide a better phylogeny, the use of a longer fragment, such as the entire VP1 gene and/or VP3 gene, is highly desirable. Together with the changing epidemiology of HAV infection, our findings may help the authorities in formulating public guidelines, including HAV vaccination policies targeted at susceptible populations.

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Competing interests

None.

Ethical approval

Not required.

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Hepatitis E Virus Outbreak in Monkey Facility, Japan

Hiroshi Yamamoto, Juri Suzuki, Atsushi Matsuda, Takafumi Ishida, Yasushi Ami, Yuriko Suzaki, Isao Adachi, Takaji Wakita, Naokazu Takeda, and Tian-Cheng Li

An outbreak of hepatitis E virus occurred in an outdoor monkey breeding facility in Japan during 2004–2006. Phylogenetic analysis indicated that this virus was genotype 3. This virus was experimentally transmitted to a cynomolgus monkey. Precautions should be taken by facility personnel who work with monkeys to prevent infection.

Wild or reared monkeys have been used as disease models in animal facilities worldwide. Because disease caused by hepatitis E virus (HEV) is a zoonosis (*1–4*), monkeys might be infected. We examined the prevalence of antibodies against HEV in serum and fecal samples collected from monkeys in animal facilities at the Primate Research Institute of Kyoto University in Japan for 6 years (2004–2009). We found that spontaneous infection and transmission of HEV occurred in a monkey facility.

The Study

There are 9 monkey colonies (A–I) at the Primate Research Institute of Kyoto University. Colonies A–G contained Japanese monkeys (*Macaca fuscata*), and colonies H and I contained rhesus monkeys (*Macaca mulatta*). Each colony was bred in a separate outdoor breeding facility. A total of 588 monkey serum samples were collected during September–November 2004–2009 and tested for IgG and IgM against HEV and for HEV RNA by ELISA or reverse transcription PCR (RT-PCR) as described (*5–7*). Samples from colonies G and F were collected during 2004–2006, whereas in 2009 samples were collected from colonies A, C, D, and I.

The prevalence of IgG against HEV was 0% in 2004, 20.0% in 2005, and 78.5% in 2006, followed by a gradual decrease to 35.9% in 2009 (Table 1). The prevalence of IgM against HEV increased from 0% in 2004 to 2.5% in 2005 and to 6.6% in 2006, and then decreased to 1.1% in 2007 and 0% in 2008 and 2009.

Table 1. Prevalence of IgG and IgM against hepatitis E virus in monkeys at monkey facility, Japan, 2004–2009

Year	No. positive/no. tested (%)	
	IgG	IgM
2004	0/110	0/110
2005	24/120 (20.0)	3/120 (2.5)
2006	96/121 (78.5)	8/121 (6.6)
2007	73/96 (76.0)	1/96 (1.1)
2008	47/90 (52.2)	0/90
2009	18/51 (35.3)	0/51

IgG against HEV was not detected in any of the 9 colonies in 2004, indicating that HEV infection did not occur before October 2004. However, in 2005, the prevalence of IgG reached 100% in colony D and 20% in colony G (Figure 1). ELISA titers were high, ranging from 0.293 to 1.641 in colony D and from 0.230 to 0.845 in colony G. These results suggested that HEV infection occurred after October 2004 in the monkey facility. The prevalence of IgG was higher in colony D than in colony G, and IgM was not detected in colony D, suggesting that HEV infection occurred earlier in colony D than in colony G. These colonies adjoined each other, indicating that the first HEV infection occurred in colony D and was then transmitted to colony G. Colonies A, C, D, E, and H each had an IgG prevalence of 90%–100%, and colonies B and G had an IgG prevalence >80% in 2006 (Figure 1). These results demonstrated that infection spread over a large area, except for colony F, during 2005 and 2006.

To compare the kinetics of IgG formation during 2004–2009, serum samples from 25 monkeys awhose peak ELISA optical density (OD) values for IgG against HEV were each higher than 1.0 were selected. In most monkeys, OD values for IgG increased rapidly and then decreased gradually year by year. The kinetic pattern of monkey M1543 was different from those of other monkeys that had high OD values (2.568–2.738). IgM was detected exclusively in this monkey in 2006 (OD value 0.620).

Serum samples from the 25 monkeys were used to detect HEV RNA by RT-PCR. Four serum samples were positive for HEV RNA; all were from the same monkey (M1543) from which samples were collected in 2006, 2007, 2008, and 2009. Nucleotide sequences of 348 bp coding the partial open reading frame 2 showed 100% identity. This result indicated that monkey M1543 was infected persistently with HEV and produced virus continuously.

To examine whether HEV was present in feces, 2 fecal samples were collected from monkey M1543 in September and November 2009 for detection of HEV RNA. Both samples were positive for HEV RNA. Nucleotide sequences of these samples were identical to those detected from serum samples.

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Primers were designed on the basis of sequences of swine HEV (GenBank accession no. AB248522), and RT-PCR was performed to amplify the viral genome except for the N terminus noncoding region. This strain was designated the monkey HEV Inuyama strain (JQ026407). Phylogenetic analysis of its genome indicated that this strain belongs to HEV genotype 3 (Figure 2). Infectivity of the monkey HEV strain was examined ex vivo with a human hepatocarcinoma cell line (PLC/PRF/5), and in vivo with 2 HEV-negative cynomolgus monkeys. Both experiments showed that the virus was infectious (online Technical Appendix Figures 1 and 2, wwwnc.cdc.gov/EID/pdfs/12-0884-Techapp.pdf).

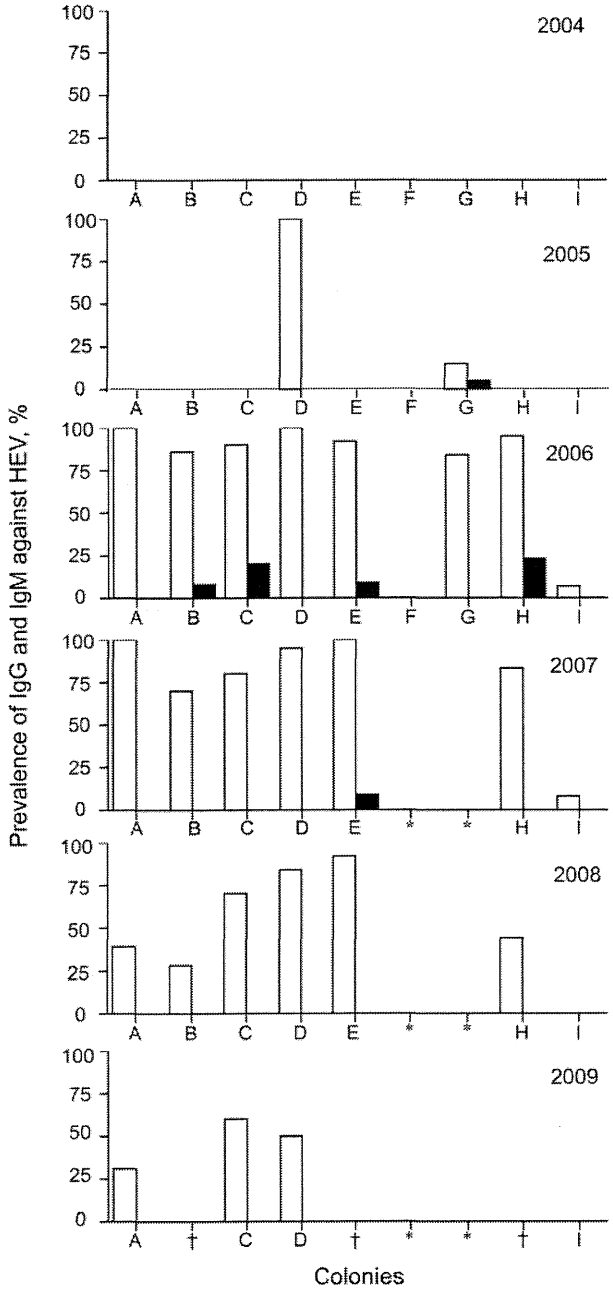

Figure 1. Prevalence of IgG (white bars) and IgM (black bars) against hepatitis E virus (HEV) in monkey facility, Japan, 2004–2009. *Monkeys were moved to another animal facility; †specimen not available.

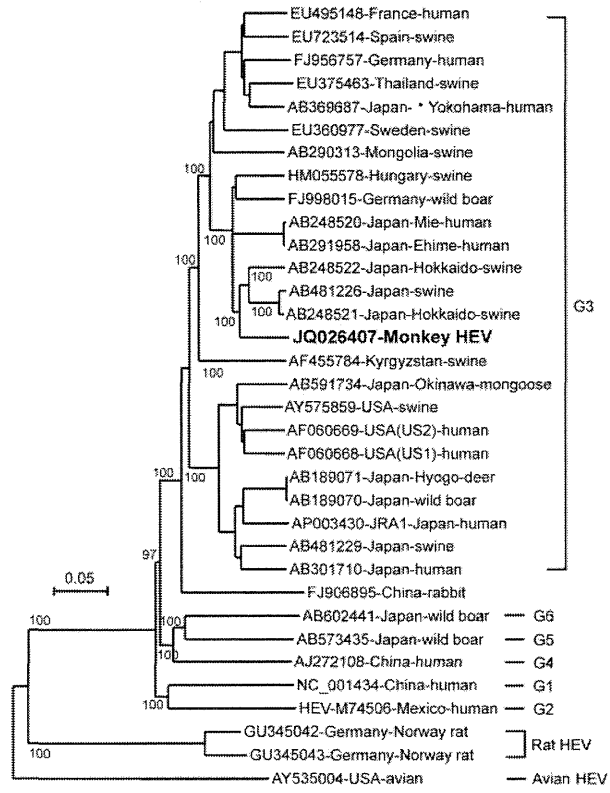

Figure 2. Phylogenetic analysis of monkey hepatitis E virus (HEV) Inuyama strain on the basis of nucleotide sequence of the HEV genome except for a 5′ noncoding region (7,206 nt) by using avian HEV as an outgroup. Values along the branches are bootstrap values determined on the basis of 1,000 resamplings of datasets. **Boldface** indicates strain isolated in this study. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

A total of 94 human serum samples were collected from staff of the Primate Research Institute and subjected to ELISA for detection of IgG and IgM against HEV. All serum samples were negative for IgM against HEV, but the prevalence of IgG was 6.9% in 2007, 9.7% in 2008, and 11.8% in 2009, although differences among these years were not significant ($p>0.05$) (Table 2). No HEV RNA was detected in serum samples, and none of the staff had symptomatic hepatitis E during the 6-year study.

Conclusions

We conducted long-term monitoring of HEV infection in monkeys and report natural infection and transmission of HEV in a monkey facility. We sought to determine the source of the HEV outbreak and where HEV was intro-

Table 2. Prevalence of IgM against hepatitis E virus in serum samples from animal handlers at monkey facility, Japan, 2007–2009*

Year	No. positive/no. tested (%)
2007	2/29 (6.9)
2008	3/31 (9.7)
2009	4/34 (11.8)

*All samples were negative for IgG against hepatitis E virus and for virus RNA.

duced to colony D. At our research institute, each monkey colony is bred in a separate outdoor breeding facility built on a mountain, and the monkeys live in an environment similar to their natural habitat. Because each outdoor feeding facility is isolated by a double fence, natural reservoirs of HEV (wild boars and deer) cannot enter it. Phylogenetic analysis of monkey HEV strains indicated that this virus was genotype 3, and BLAST analysis showed that the monkey isolate is closest to HEV strains isolated from pigs in Japan. Nucleotide identities were 92%–93% (AB248521, AB248522, and AB481226). However, no evidence indicates that HEV is transmitted from pigs or wild boars to monkeys.

A notable finding in this study was the persistence of HEV infection. Generally, HEV infection is self-limiting and symptoms are transient. Persistent HEV infection occurs in solid-organ transplant recipients who have received immunosuppressive drugs (*8*) or in patients with other conditions associated with immunosuppression, such as HIV infection (*9*) and hematologic malignancies (*10*,*11*). However, there is no evidence of immunosuppression in monkey M1543, and the cause of the persistent HEV infection in this monkey is unknown.

The fact that the infectious HEV strain was detected in a monkey facility and caused an HEV outbreak cast doubt and apprehension on the safety of handling monkeys. Although no staff member showed development of symptomatic hepatitis E, precautions should be taken by facility workers who work with monkeys to prevent infection with HEV.

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Characterization of Full Genome of Rat Hepatitis E Virus Strain from Vietnam

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We amplified the complete genome of the rat hepatitis E virus (HEV) Vietnam strain (V-105) and analyzed the nucleotide and amino acid sequences. The entire genome of V-105 shared only 76.8%–76.9% nucleotide sequence identities with rat HEV strains from Germany, which suggests that V-105 is a new genotype of rat HEV.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (*1*), classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (*2*). Hepatitis E, caused by HEV infection, is a serious public health concern in developing countries and is recognized as sporadic and endemic acute hepatitis (*3*). To date, at least 4 genotypes of HEV have been isolated from humans (*4*). In addition, HEV has been isolated from other mammals, including pigs, wild boars, wild deer, rabbits, ferrets, bats, chickens, and wild rats (*5–9*). Much direct evidence indicates that HEV is transmitted from pigs or wild boars to humans, and therefore hepatitis E caused by genotypes 3 and 4 is recognized as a zoonotic disease (*6,8,10*).

Rat HEV was first isolated from Norway rats in Germany (*7,11*). Since then, rat HEV strains have been isolated from wild rats in other areas of Germany and detected in wild rats in the United States and Vietnam (*12–14*). Those results suggest that rat HEV infection is not restricted to Germany but is broadly distributed in wild rats throughout the world. The nucleotide sequences of the rat HEV isolated in Germany and the United States are similar; however, the partial sequences of the Vietnam rat HEV strain (V-105, JN040433) have been found to have 78.18%–79.43% identities with isolates from Germany, R63 and R68 (*14*). To confirm whether new genotypes of rat HEV exist, we amplified the entire genome of the rat HEV V-105 strain and analyzed the sequences. We confirmed that the rat HEV strain isolated in Vietnam belongs to a new genotype of rat HEV.

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The Study

The rat HEV used in this study was isolated from a 10% lung homogenate of a wild rat from Vietnam, which was positive for rat HEV RNA by reverse transcription PCR (RT-PCR) (*14*). Because of the limited availability of rat specimens that are positive for HEV RNA, we first transmitted the rat HEV to a laboratory rat (Wistar) to produce a large amount of virus for RNA extraction and genome amplification. After intravenous inoculation of the rat, fecal specimens positive for HEV RNA were collected, and a 10% suspension was prepared as described (*15*). RT-PCR was performed by using Superscript II RNase H^- (Invitrogen, Carlsbad, CA, USA) and primer TX30SXN (*14*). The full-length genome of the V-105 strain was amplified by RT-PCR with primers based on the nucleotide sequences of GU345042 and JN040433 (Table 1). All PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequencing was carried out by using an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA).

Because 901 nt of V-105, corresponding to nt 4108–5008 of the R63 genome, were already known (*14*), primers F13 and open reading frame (ORF) 1–R12 were designed. An ≈2,100-nt fragment of the C-terminus of the rat HEV V-105, nt 4923-poly (A) tail, was amplified with a pair of primers, F13 and TX30SXN, by the first RT-PCR. The ORF1 region was amplified with primers ORF1-F1 and ORF1-R12. Two fragments, 440 nt (nt 11–450) and 1,182 nt (nt 2990–4171), were amplified by nested PCR with 2 sets of primers, ORF1-F2/ORF1-R1 and ORF1-F7/ORF1-R12, respectively. On the basis of the nucleotide sequences of those amplified fragments, ORF1-F9, ORF1-F16, ORF1-R16, ORF1-F18, and ORF2-R21 were designed, and 3 fragments, 1,830 nt (nt 388–2217), 996 nt (nt 2080–3075), and 1,110 nt (nt 3991–5100), were amplified with 3 sets of primers, ORF1-F9/ORF1-R10, ORF1-F16/ORF1-R16, and ORF1-F18/ORF2-R21, respectively.

To amplify the N-terminus nonstructural region of V-105, we synthesized cDNA with primer ORF1-R14, and a DNA anchor (P-CACGAATTCACTATCGATTCTGG AACCTTCAGAGG-NH_3) was linked to the N-terminus of the cDNA by T4 RNA Ligase I (BioLabs, Tokyo, Japan). By using this anchor-cDNA as the template, the first and the nested PCRs were carried out with 2 sets of primers, anchor-1/ORF1-R14 and anchor-2/ORF1-R13, respectively.

Table 1. Oligonucleotides used in amplifying the complete genome of the rat HEV Vietnam strain, V-105*

Primers	Product length,
Forward ORF1-F1 (1-21)† 5'-GCAACCCCCGATGGAGACCCA-3'‡ Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	§
Forward ORF1-F2 (11-30) 5'-ATGGAGACCCATCAGTATGT-3'† Reverse ORF1-R1 (431-450) 5'-GTGCAAAAGGAAAGATCAGT-3'	440
Forward ORF1-F9 (388-408) 5'-AGCTAACAACATCCGCCGTTG-3' Reverse ORF1-R10 (2197-2217) 5'-TGGGTTCGGTCGAAGGCCTCT-3'†	1,830
Forward ORF1-F16 (2080-2100) 5'-TGCAGCCGTTTATGAGGGAGA-3' Reverse ORF1-R16 (3055-3075) 5'-CGCCATTCTGTGGGTTCTAGA-3'	996
Forward ORF1-F7 (2990-3009) 5'-GACCCAAGGCAGATCCCTGC-3'† Reverse ORF1-R12 (4149-4171) 5'-GGCGGCCTCGAACTTCTCCTGAA-3'	1,182
Forward ORF1-F18 (3991-4011) 5'-ATTCACCACAGACGAGCCAGT-3' Reverse ORF2-R21 (5079-5100) 5'-GGTGATAGCCAATTGGTAAGCT-3'	1,110
Forward F13 (4896-4915) 5'-AATAACACTCTGGGCTGTAG-3' ReverseTX30SXN 5'-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	2,092
Forward primer Anchor-1: 5'-CCTCTGAAGGTTCCAGAATCGATAG-3' Reverse primer ORF1-R14 (276-296) 5'-TAGACCTAGGGTGCGCACCGA-3'	§
Forward primer Anchor-2: 5'-GAATCGATAGTGAATTCGTG-3' Reverse primer ORF1-R13 (200-220) 5'-AACACGCTGTACCGGATGCGA-3'	240

*HEV, hepatitis E virus; ORF, open reading frame.
†Numbers in a parentheses show the positions of primers corresponding to the entire genome of rat HEV V-105.
‡Primer designed based on rat HEV (GU345042).
§The PCR product was not detected.

The V-105 genome consisted of 6,927 nt plus a poly (A) tail of a still-undetermined length (GenBank accession no. JX120573). The genomic structure of V-105 was, from the N-terminus toward the C-terminus, the N 5′–untranslated region (UTR) at nt 1–10, ORF1at nt 11–4900, ORF3 at nt 4917–5225, ORF2 at nt 4928–6862, the 3′-UTR at nt 6863–6927, and the poly (A) tail starting at nt 6928. ORF2 and ORF3 encode 644 aa and 102 aa, respectively, as do R63 and R68. However, ORF1 of V-105 encodes 1,629 aa, which is 7 aa shorter than either R63 or R68. The V-105 genome possessed 2 aa insertions (Ser-Pro) between the aa residues 591 and 592 and 9 aa deletions (Ser-Pro-Pro-Gly-Pro-Pro-Pro-Ala-Gly) between aa residues 852 and 853, corresponding to those of R63. The 3′-UTR was 65 nt as were R63 and R68. Unlike R63 and R68, only 1 additional putative ORF, corresponding to ORF4 (nt residues 27–578), was found in V-105, suggesting that other putative ORFs, ORF5 and ORF6 found in R63 and R68, are not common in rat HEV.

When the V-105 genome was compared with reported HEV genomes, the V-105 genome shared identities of only 50.5% with avian HEV, 53.6% with rabbit HEV, 53.7%–54.0% with wild boar HEV, and 53.1%–53.5% with HEV genotypes 1–4. In contrast, V-105 shared relatively high nucleotide sequence identities (76.8%–76.9%) with rat HEV strains (R63 and R68) (Table 2). The nucleotide and amino acid sequences of ORF1, ORF2, and ORF3 of V-105 were compared with those of other HEV genotypes, and the identities among them are shown in Table 2. Together, these results suggest that V-105 is more similar to rat HEV than to other HEV genotypes.

Phylogenetic trees were generated on the basis of the nucleotide sequences derived from the entire genome and ORF3 of the genotypes 1–4, wild boar, rabbit, chicken, and rat HEV isolates. These trees demonstrated that V-105 does not belong to any known genotype and should probably be classified into a new genotype (Figure).

Table 2. Nucleotide and deduced amino acid sequence identities between human, wild boar, rabbit, rat, and avian HEV strains, compared with Vietnam rat HEV V-105 strain*

HEV strain (GenBank accession no.)	Vietnam rat HEV strain						
	Entire genome	Nucleotides, %			Amino acids, %		
		ORF1	ORF2	ORF3	ORF1	ORF2	ORF3
Genotype 1 (NC_001434)	53.5	50.7	60.8	51.0	54.1	55.5	33.3
Genotype 2 (M74506)	53.3	51.2	59.1	51.4	53.1	54.6	30.6
Genotype 3 (AF060668)	53.3	50.8	59.2	53.8	51.6	57.1	26.5
Genotype 4 (AJ272108)	53.1	50.9	58.9	52.6	50.7	55.4	24.5
Wild boar HEV (AB573435)	53.7	51.5	59.6	53.4	50.3	56.3	28.2
Wild boar HEV (AB602441)	54.0	51.5	59.6	53.4	50.3	56.3	28.2
Rabbit HEV (FJ906895)	53.6	51.3	59.3	51.5	52.1	56.2	26.2
Rat HEV (GU345042)/R63	76.9	75.7	79.6	80.6	87.0	91.6	66.7
Rat HEV (GU345043)/R68	76.8	75.5	79.8	80.9	86.4	92.1	66.7
Avian (chicken) HEV (AY535004)	50.5	49.7	54.2	47.0	44.7	47.4	33.9

*HEV, hepatitis E virus; ORF, open reading frame.

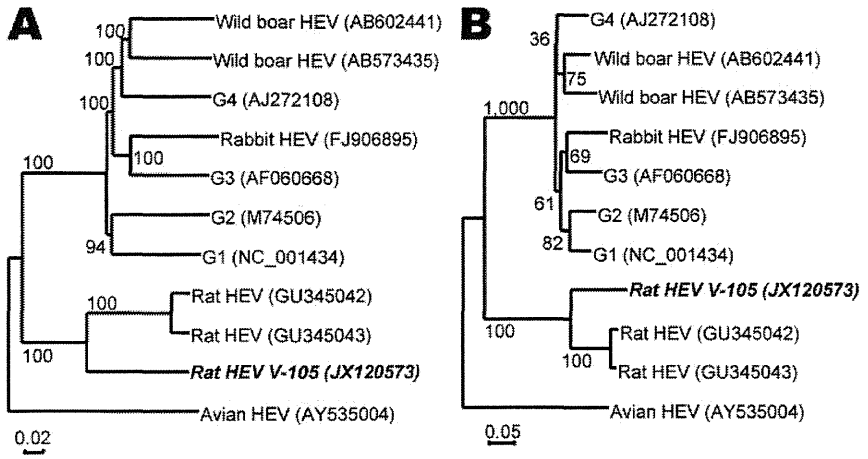

Figure. Phylogenetic relationships among genotypes 1–4, wild boar, rabbit, avian (chicken), and rat hepatitis E virus (HEV) isolates. The nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by the Kimura 2-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method, based on the entire genome (A) and open reading frame 3 (B) of the genotypes 1–4, wild boar, rabbit, avian (chicken), and rat HEV isolates. Scale bar indicates nucleotide substitutions per site. **Boldface** indicates isolate used in this study.

Conclusions

In this study we successfully amplified the entire genome of an HEV strain isolated from a wild rat in Vietnam. Phylogenetic analyses and nucleotide and amino acid sequence comparisons demonstrated that the complete rat HEV genome sequences were consistently well separated from those of mammalian genotypes 1–4, wild boar, rabbit, and chicken HEV and close to those of the rat HEV strains. Although the entire genome of V-105 shared nucleotide sequence identities of only 76.8%–76.9% with the isolates from Germany (R63 and R68), the ORF1 and ORF3 amino acid identities between V-105 and these isolates were 86.4%–87.0% and 66.7%, respectively, which suggests that V-105 can be classified into a new genotype of rat HEV. However, ORF2 has relatively high amino acid identities with R63 and R68 (91.6%–92.1%), indicating that the V-105 and rat HEV isolates from Germany share similar antigenicity. In fact, rat HEV–like particles derived from R63 are cross-reactive to serum from V-105–infected wild rats (*14*).

In conclusion, we isolated and identified rat HEV strain V-105 from a wild rat in Vietnam, and this strain was highly divergent from known rat HEV isolates. We propose that the strain from Vietnam, V-105, is a new member of the rat HEV genotype.

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