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Short Communication

Hepatitis A outbreak associated with a revolving sushi bar in Chiba, Japan: Application of molecular epidemiology

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Aim: The number of hepatitis A cases in Japan as well as in other developed countries has been progressively decreasing during the last several years. There is no universal hepatitis A vaccination program in Japan, and a hepatitis A virus (HAV) epidemic in Japan is not unlikely. In 2011, a hepatitis A outbreak associated with a revolving sushi bar occurred in Chiba, Japan. We aimed to analyze this outbreak.

Methods: Twenty-seven patients associated with this outbreak were admitted to the National Hospital Organization Chiba Medical Center. Molecular epidemiologic investigations were conducted.

Results: Twenty-six of the 27 patients had gone to the same revolving sushi bar, and then clinical symptoms appeared. HAV RNA was detected by reverse transcription polymerase chain reaction in 23 of the 27 (85.1%) patients whose sera had tested positive for anti-HAV immunoglobulin M. All isolates from this outbreak were clustered within subgenotype IA, displaying 100% sequence homology with each other in 232 bp from all 23 patients. All isolates belong to the IA-1 sublineage, which is endemic to Japan.

Conclusion: A revolving sushi bar was associated with a hepatitis A outbreak, and molecular epidemiological investigations proved useful.

Key words: hepatitis A virus, Japan, subgenotype IA, sushi bar

INTRODUCTION

HEPATITIS A VIRUS (HAV) is a positive-strand RNA virus that causes acute hepatitis in humans.[1] The spread of HAV is primarily by fecal–oral route, often contaminated food, drink or objects handled by infected persons, but rarely is transmitted sexually or parenterally.[2] Epidemiological studies have also shown that HAV exposure increases with low hygiene and increasing age.[3] With the availability of safe inactivated HAV vaccine, the epidemiological pattern of HAV in children and high-risk populations can be greatly influenced.[4]

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As hepatitis A is a reportable disease in Japan, statistics show that the number of hepatitis A cases in this country has been progressively decreasing during the last several years.[5] There is no universal hepatitis A vaccination program in Japan, and the possibility of an outbreak of a HAV epidemic cannot be ruled out.[6]

Between January and February 2011, 27 hepatitis cases were admitted to the National Hospital Organization Chiba Medical Center (Chiba, Japan). Twenty-six of them had gone to a revolving sushi bar, where customers select their food from a revolving conveyor belt, in the central ward of Chiba City. We isolated viral RNA and studied the molecular characteristics of HAV strains from the identified cases by a molecular epidemiological approach, which suggested the same transmission route. Furthermore, we compared the viral sequences with other reported sequences.

In the present study, we reported a hepatitis A outbreak associated with a revolving sushi bar in Chiba, Japan, in 2011, and we recognized that a molecular

Table 1 Profiles of 27 patients with hepatitis A in the present study

Patient no.	Age/sex	AST (IU/L)	ALT (IU/L)	Nadir PT (%)	Date of visiting sushi bar	Date of onset
1	43/M	2828	5743	84.1	Early December 2010	Early January 2011
2	37/F	300	805	95.3	Early October and December 2010	Early January 2011
3	61/M	219	479	99.2	Middle December 2010	Early January 2011
4	43/M	145	1134	102.5	Middle December 2010	Early January 2011
5	43/M	5764	4120	57.5	Middle December 2010	Middle January 2011
6	41/F	7918	5376	62.7	Early–middle December 2010	Middle January 2011
7	43/M	2504	5687	65.6	Twice every month	Middle January 2011
8	56/F	13 104	9690	51.4	Middle December 2010	Middle January 2011
9	35/F	3476	4859	69.6	Twice every month	Middle January 2011
10	76/F	790	2200	73.0	Early and late December 2010	Middle January 2011
11	56/F	2912	4386	48.5	Middle November 2010	Middle January 2011
12	35/M	1816	5297	66.8	Middle December 2010	Middle January 2011
13	61/M	821	1957	102.5	Early, middle and late December 2010 and early January 2011	Middle January 2011
14	28/F	1019	1950	76.8	Middle December 2010	Middle January 2011
15	53/F	5908	5495	47.6	Middle December 2010	Middle January 2011
16	60/F	100	186	113.8	Middle December 2010	Middle January 2011
17	31/F	8240	8033	35.0	Early and late December 2010 and early January 2011	Late January 2011
18	41/F	387	753	90.0	Middle December 2010	Late January 2011
19	56/M	2318	4637	108.0	Middle December 2010	Late January 2011
20	19/F	486	1608	63.7	Middle December 2010	Late January 2011
21	26/M	1651	2938	56.8	Middle December 2010	Late January 2011
22	53/F	3013	4807	47.0	Early January 2011	Late January 2011
23†	20/M	4490	3505	60.5	Middle November 2010 to late January 2011	Late January 2011
24	65/F	138	421	109.8	Middle December 2010	Late January 2011
25	18/F	1379	1624	60.0	Middle December 2010	Late January 2011
26	38/M	408	2011	69.1	No visiting	Early February 2011
27	43/F	1711	775	67.2	Middle December 2010	Early February 2011

†Sushi shop assistant.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; PT, prothrombin time.

epidemiological approach is useful for identifying the infection route of transmission.

METHODS

Study population

THIS OUTBREAK WAS based in a revolving sushi bar located in a central area of Chiba, Japan. During the course of this study, 27 patients aged between 18 and 76 years (mean, 43.7), 11 men and 16 women, were admitted to the National Hospital Organization Chiba Medical Center, Chiba, Japan (Table 1). The first case developed acute icteric hepatitis A in mid-January 2011, and the last case was discharged from this hospital in early March 2011. All patients' sera were collected and stored at −80°C until tested. This study was approved by the Ethics Committee, Chiba University Graduate School of Medicine, Chiba, and the National Institute of Infectious Diseases, Tokyo, and conformed to the Declaration of Helsinki.

Biochemistry tests

Liver function tests for alanine aminotransferase (ALT), aspirate aminotransferase (AST) and prothrombin time (%) were performed by automated analyzer.

Serological study

Sera were tested for anti-HAV immunoglobulin (Ig)M antibodies by enzyme-linked immunosorbent assay using commercially available kits (Abbott Laboratories, North Chicago, IL, USA). The diagnosis of hepatitis A was only made when anti-HAV IgM was positive.[7,8]

HAV RNA detection

RNA was extracted from 100 μL of serum samples according to the guanidium thiocyanate method and subjected to reverse transcription polymerase chain reaction (RT–PCR) for the VP1/2A region of the HAV genome.[9] 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.[6]

Sequencing of the VP1/2A region and phylogenetic analysis

Sequences were directly determined as previously described.[5,6] A phylogenetic tree was constructed by using GENETYX ver. 10 (Genetyx, Tokyo, Japan) based on the nucleotide sequences of the amplified VP1/2A region. The GenBank accession numbers for the nucleotide sequences of the HAV isolates (termed KCH1–KCH22 and KCH30) will be AB690782–AB690804. The obtained sequences were compared with the corresponding GenBank references for subgenotypes IA (K02990, X75215, AB020564, AB020565, AB020566, AB020567, AB020568 and AB020569), IB (AF268396, M14707 and M20273.1), IIA (AY644676), IIB (AY644670), IIIA (AB258387, AB279735, AY644337, AJ299484, AB279732, AB279733 and AB279734) and IIIB (D00924), and two sequences (AB643803.1 and AB643804.1) from a different hospital involved with the same outbreak.[6]

RESULTS

SEROLOGICAL TESTING REVEALED anti-HAV IgM in the 27 patients. Twenty-six of the 27 patients had gone to the same revolving sushi bar in Chiba, where they ate sushi and other food between 20 November 2010 and 4 January 2011, and then clinical symptoms appeared between 8 January 2011 and 10 February 2011 (Table 1). Typical symptoms of acute hepatitis are jaundice, fever, appetite loss, fatigue, nausea, abdominal pain and headache, and they were seen in 100%, 92.5%, 55.5%, 37.0%, 14.8%, 11.1% and 3.7%, respectively. The peaks of ALT and AST levels were less than 500 IU/L in three and eight patients, 500–999 IU/L in three and two, 1000–1999 IU/L in five and five, 2000–2999 IU/L in three and four, 3000–4999 IU/L in six and four, 5000–9999 IU/L in seven and three, and 10 000 IU/L or higher in zero and one, respectively. Prothrombin time was 40% or less in one patient, 40–59% in six and 60% or higher in 20. No patient had hepatic encephalopathy and all were ambulatory when discharged from the hospital.

Hepatitis A virus RNA was detected by RT–PCR in 23 of 27 (85.1%) patients whose sera had tested positive for anti-HAV IgM. Four hundred and fifty-one and 232 bp of the HAV VP1–2A region were obtained from 19 and four patients, respectively. These sequences were aligned with those of the isolates of known genotype and subjected to phylogenetic analysis (Fig. 1). All isolates from this outbreak were clustered within subgenotype IA, displaying 100% sequence homology with each other in 451 bp from 19 patients and 100% sequence homology with each other in 232 bp from all 23 patients in the present study and two sequences of the same outbreak from a different hospital.[6] Recently, Ishii *et al.*[10] reported that HAV subgenotype IA consisted of two genomic sublineages, IA-1 and IA-2. All isolates in the present study belonged to the IA-1 sublineage, which is endemic to Japan.

DISCUSSION

IN THE PRESENT study, molecular phylogenetic analysis revealed that this outbreak was caused by a single HAV strain and 26 of 27 patients, including one shop assistant, had been at a revolving sushi bar in Chiba, with the dates of visiting the sushi bar being almost the same (Table 1), suggesting that this outbreak might have been related to this sushi shop. It has been reported that HAV-contaminated shellfish such as oysters, cockles, mussels and scallops can play a role as reservoirs and/or vehicles.[11–14] A shop assistant involved in this outbreak made sushi while wearing gloves, but he ate sushi himself during his rest time. We do not exactly know whether there is an association of the one patient who had not visited the revolving sushi bar with the outbreak. He was 38 years old, and medical interviews did not reveal that he had any relationship with the other patients or family members with hepatitis A (Table 1, patient no. 26). However, the fact that his onset was consecutive in this outbreak and no other HAV positive patients were seen in our hospital made us suspect an association with this outbreak. Further molecular epidemiologic studies might be needed.

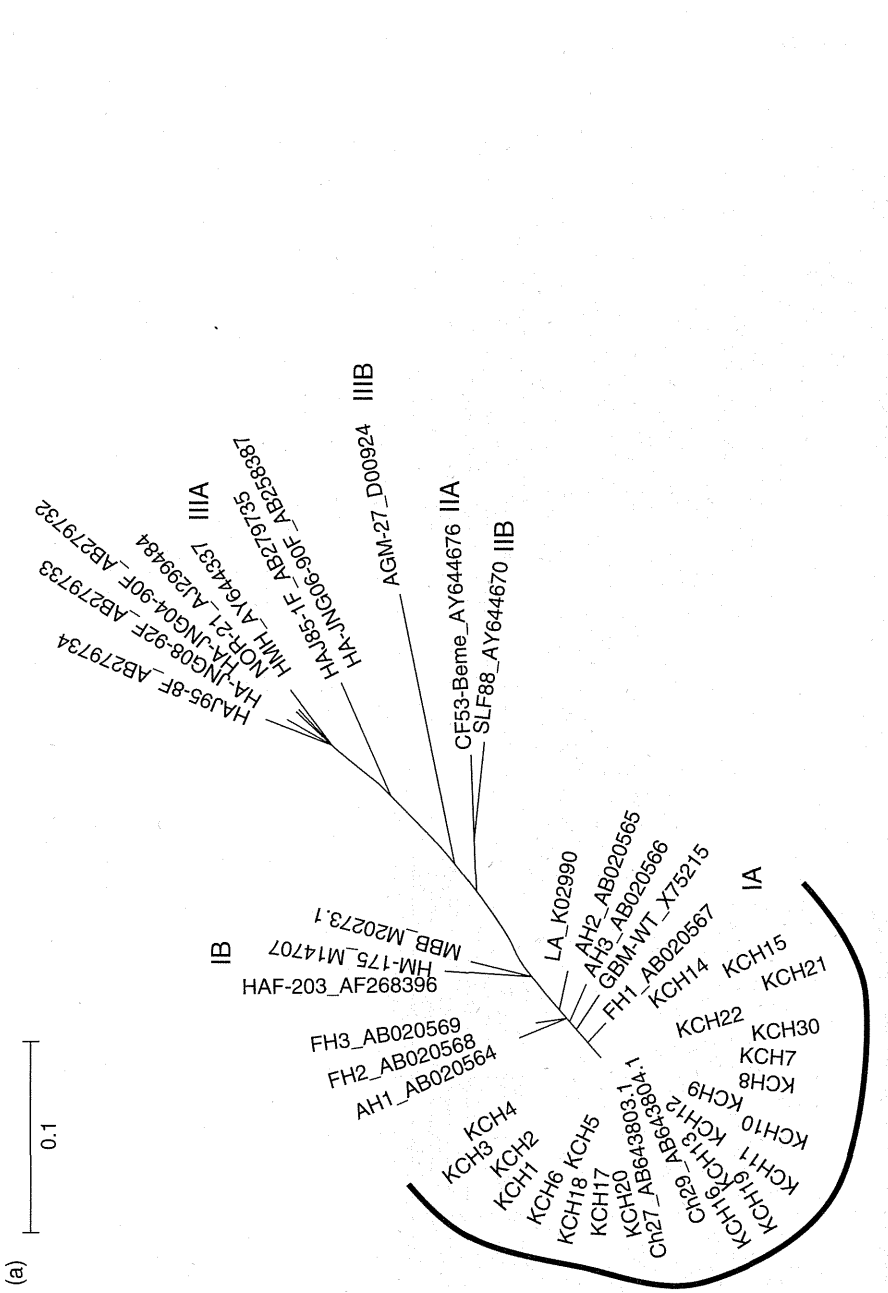

Figure 1 Phylogenetic tree analysis of hepatitis A virus (HAV) isolates in the present study and reported sequences. (a) Neighbor-Joining Tree. (b) Unweighted Pair Group Method With Arithmetic Averaging (UPGMA) tree. Black lines indicate HAV isolates derived from this outbreak. The GenBank accession numbers for the nucleotide sequences of HAV isolates (termed KCH1–KCH22 and KCH30) in the present study will be AB690782–AB690804. The obtained sequences were compared with the corresponding GenBank references for subgenotypes IA (K02990, X75215, AB020564, AB020565, AB020566, AB020567, AB020568 and AB020569), IB (AF268396, M14707 and M20273.1), IIA (AY644676), IIB (AY644670), IIIA (AB258387, AB279735, AY644337, AJ299484, AB279732, AB279733 and AB279734) and IIIB (D00924), and two sequences (AB643803.1 and AB643804.1) from a different hospital of the same outbreak.[6]

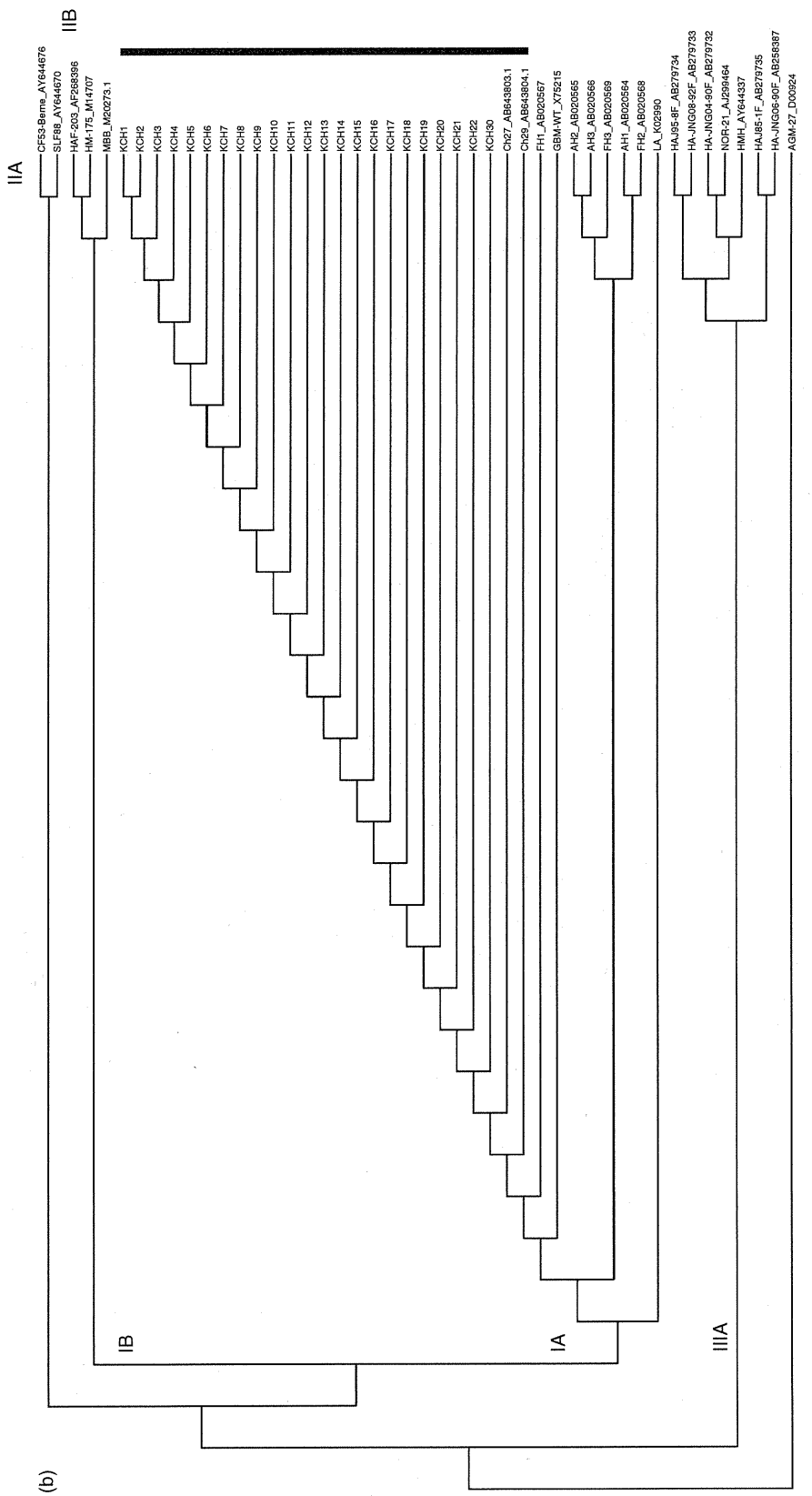

Figure 1 *Continued.*

It has also been suggested that accurate HAV levels in shellfish involved in outbreaks could be of use for the purpose of risk assessment.[15] Although there is no documented incidence rate of HAV infection at revolving sushi bars or sushi shops, HAV infection reportedly happens frequently from sushi bar visits.[16] There are several reports regarding HAV infections at revolving sushi bars and sushi shops.[17–19] In the present outbreak, some of the patients ate only cooked eel, not raw shellfish or oysters. Thus, we could not determine the exact food sources of the HAV. Food-borne outbreaks of HAV may represent an increasing problem in populations not immune to HAV, although it has recently been reported that genetic variants in ABCB1, TGFB1 and XRCC1 appear to be associated with a susceptibility to HAV infection among Mexican Americans.[20]

In Japan, HAV was added to the diseases of Infectious Agents Surveillance in 1987, and HAV infection was listed as one of the reportable diseases on 5 November 2003. At present, all HAV infections have to be reported to a prefectural governor by the physician in charge. In the present cases, the physicians reported the first HAV case to a prefectural governor, and a local public health center intervened in investigating the source of the HAV infection. Their investigation revealed that three shop assistants, one in the present study and two in two other hospitals, were positive for HAV RNA in their stools. Two of them prepared sushi in the revolving sushi bar, one presenting with fever and general fatigue on 19 December 2010, and later he was diagnosed with hepatitis A from another hospital, but he continued to work into January 2011.[21] We could not exclude the possibility that he was the source of the HAV infection because the onsets of the other patients followed his.

Sushi is a Japanese traditional food consisting of rice combined with varieties of raw/cooked fish and shellfish. A revolving sushi shop is a Japanese fast-food sushi restaurant with a revolving conveyor belt that carries plates of sushi. In the present study, 26 of 27 patients went to the same revolving sushi shop. It is unknown whether they ate HAV-contaminated food or not. However, molecular analysis of the HAV infection revealed that a single source might have caused this outbreak.

In Japan, universal vaccination programs against HAV as well as hepatitis B virus are not yet being performed. In recent years, the incidence of hepatitis A in Japan has dramatically decreased,[10] and therefore there might be a decrease in the proportion of persons who have immunity against HAV. Our previous study[5] suggested that hepatitis A cases could increase in the near future. The current outbreak was caused by the HAV subgenotype IA strain, different from HAV subgenotype IIIA that caused the recent Korean outbreak.[22]

In conclusion, we report a hepatitis A outbreak associated with a revolving sushi bar, and that the same HAV subgenotype I strain was detected in 23 of 27 patients. It was again recognized that molecular phylogenetic analysis is useful for detecting the source of HAV infection. In developed countries, because HAV may cause acute hepatitis, particular attention should be paid to hepatitis A.

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Hepatology Research

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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.

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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

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.

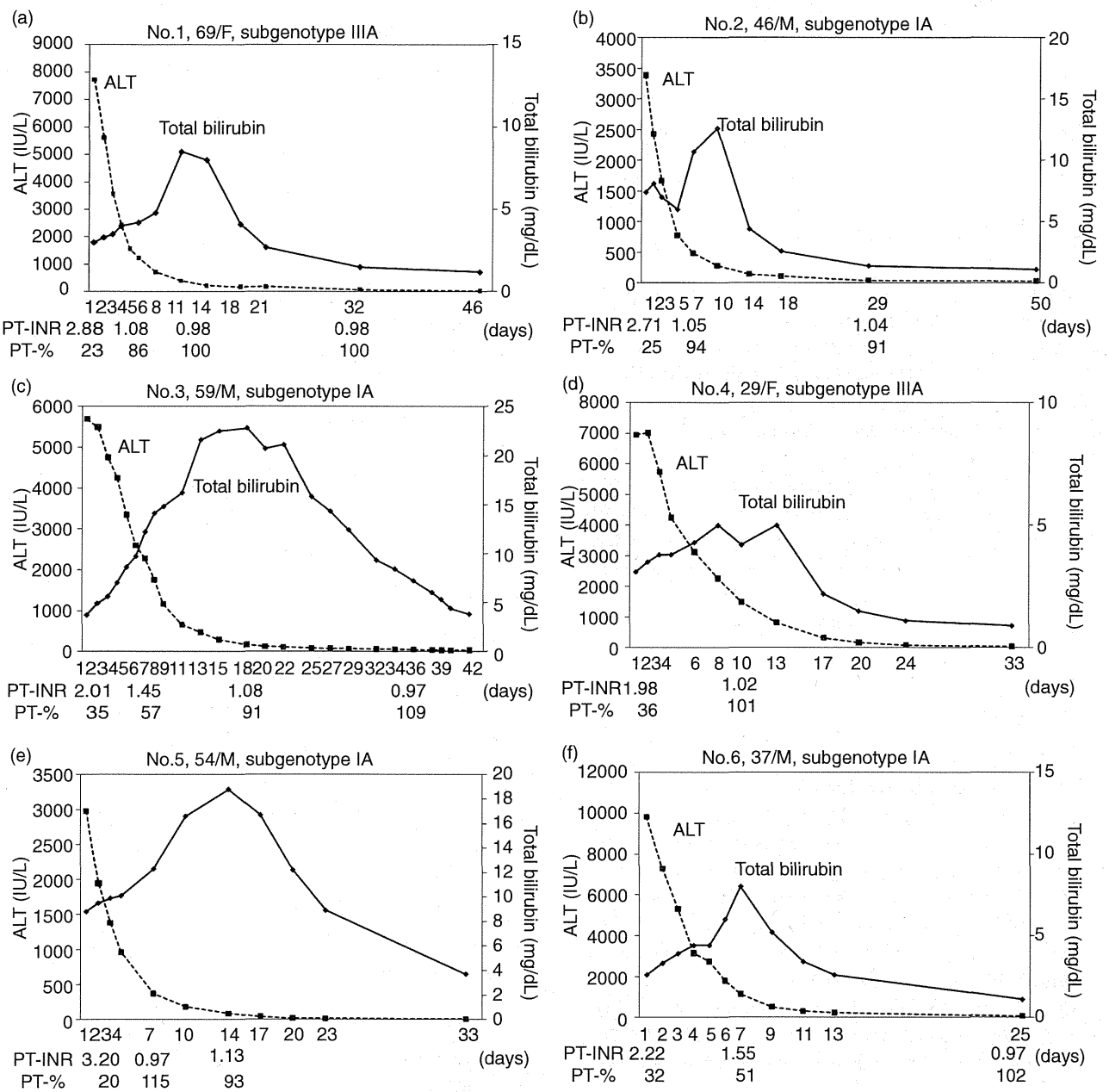

Figure 1 Clinical course of six acute liver failure patients infected with hepatitis A virus (HAV) in Japan. (a), (b), (c), (d), (e) and (f) indicates patient no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6 in Table 1, respectively. All patients presented with acute liver failure with coagulopathy but without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

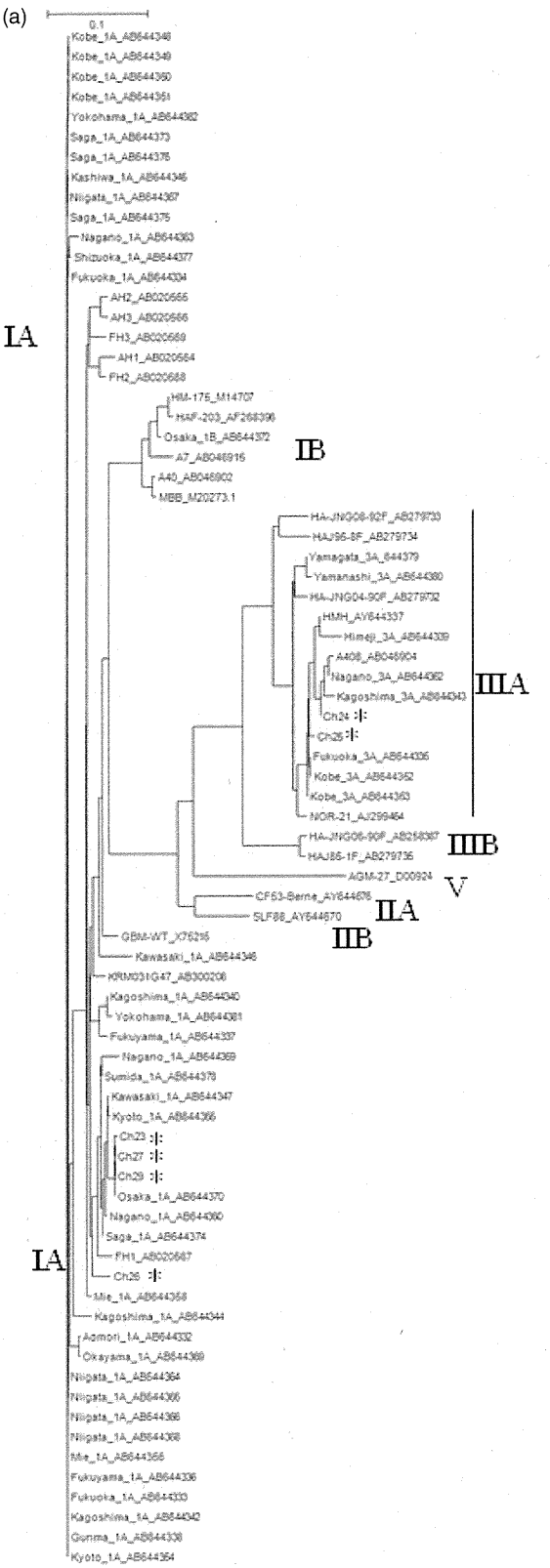

Figure 2 Phylogenetic analysis of hepatitis A virus (HAV) isolates from patients with acute liver failure from Japan. (a), (b) The neighbor joining tree was constructed based on a partial sequence of 451 nt in the VP1/2A region of HAV. Selected reference strains were also included in the phylogenetic 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).

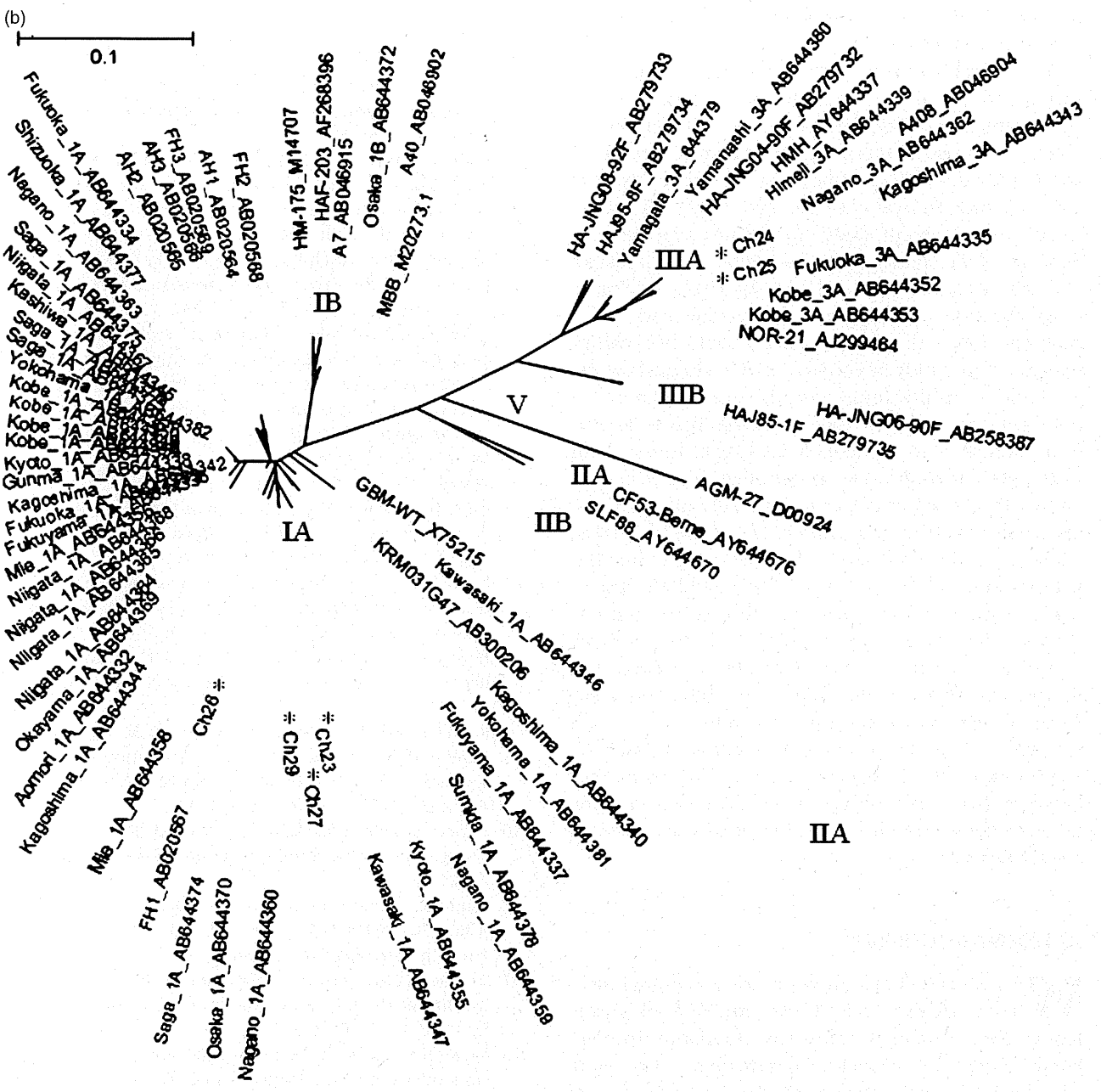

Figure 2 *Continued.*

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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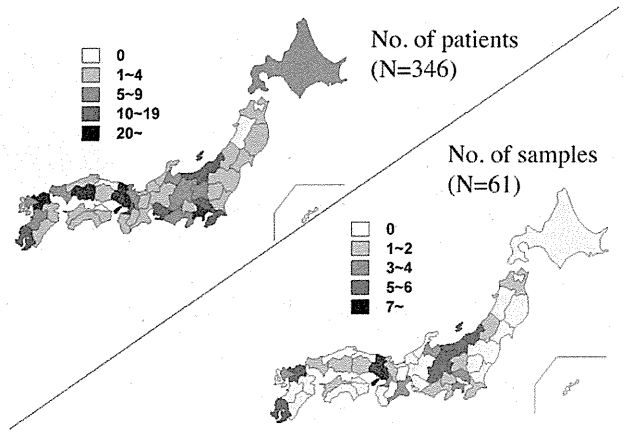

Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

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.

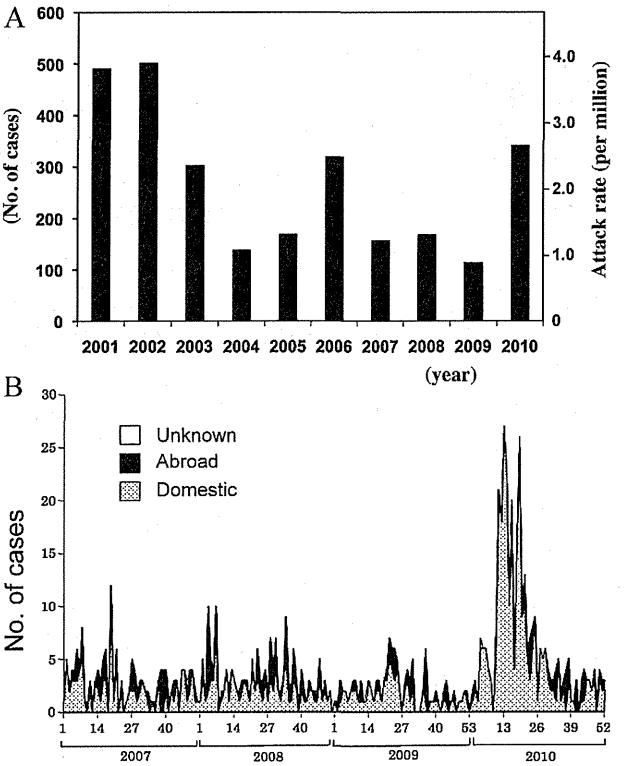

Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t = 5.4 \times 10^{-}$), 2008 ($t = 5.6 \times 10^{-5}$) and 2009 ($t = 1.8 \times 10^{-5}$). (B) Weekly acute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

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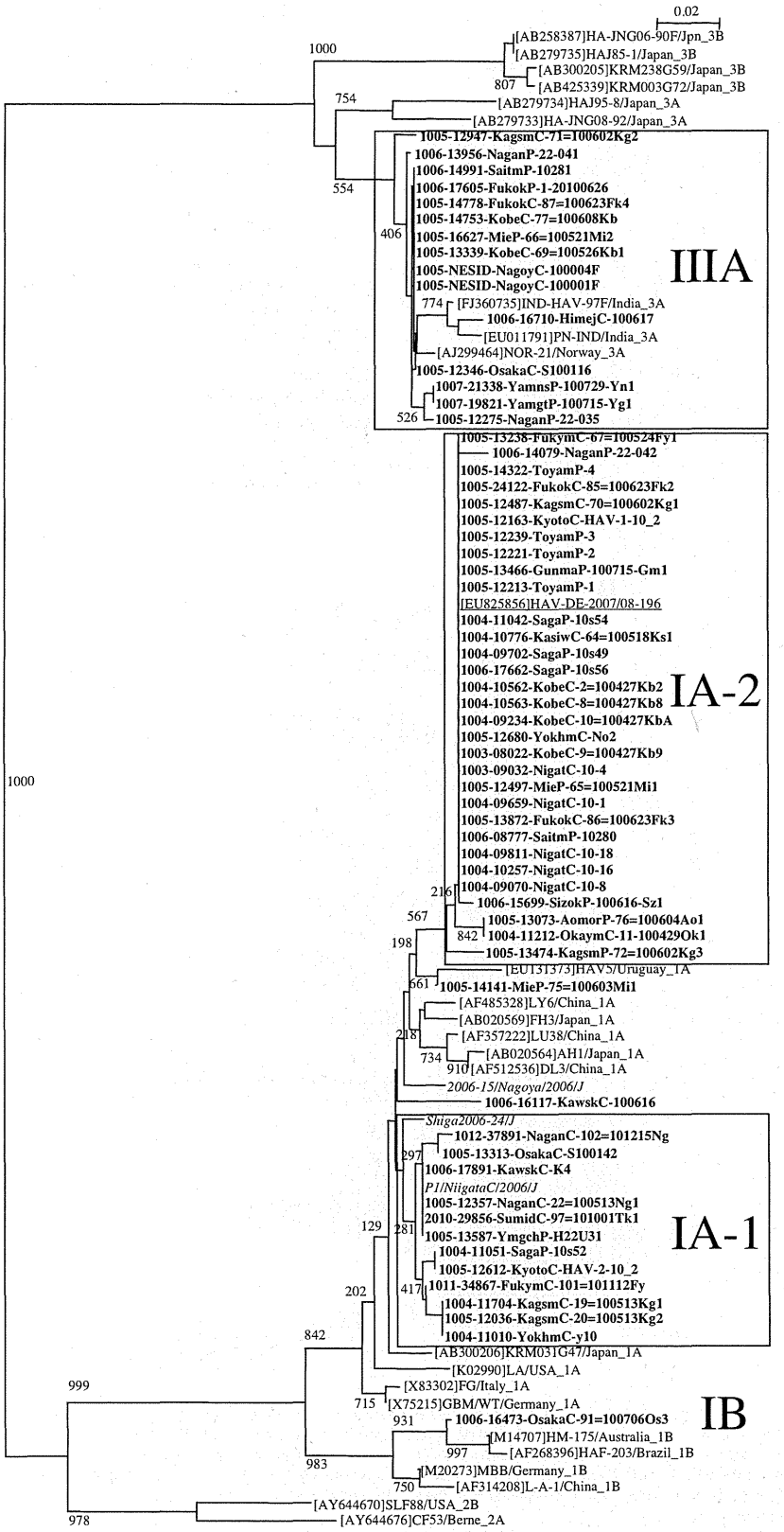

Fig. 3. Neighbor-joining phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus isolates. Reference strains are used in this study and indicated as GenBank accession numbers. Sequences of 61 isolates from this study are shown as YYMM-NESID-KKKKKKKKK (YYMM represents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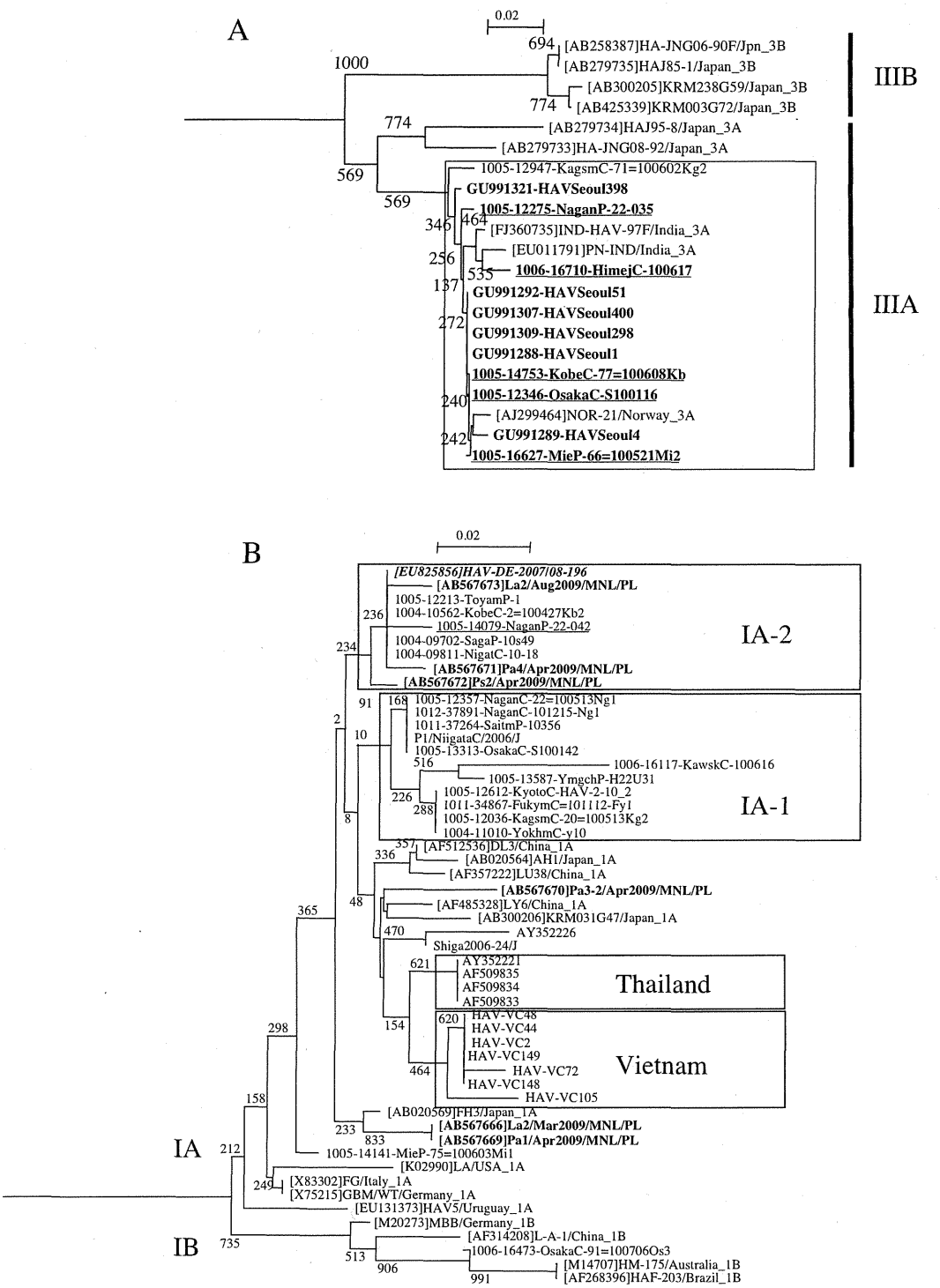

Fig. 4. (A) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from HAV strains (genotype IIIA) isolated from Japan (bold underline) and Korea (bold). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling. (B) Phylogenetic tree 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

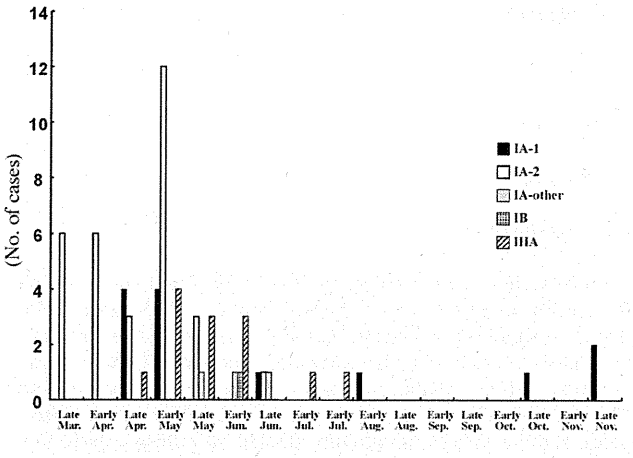

Fig. 5. Temporal distribution of HAV genotypes from late March to late November in 2010.

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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