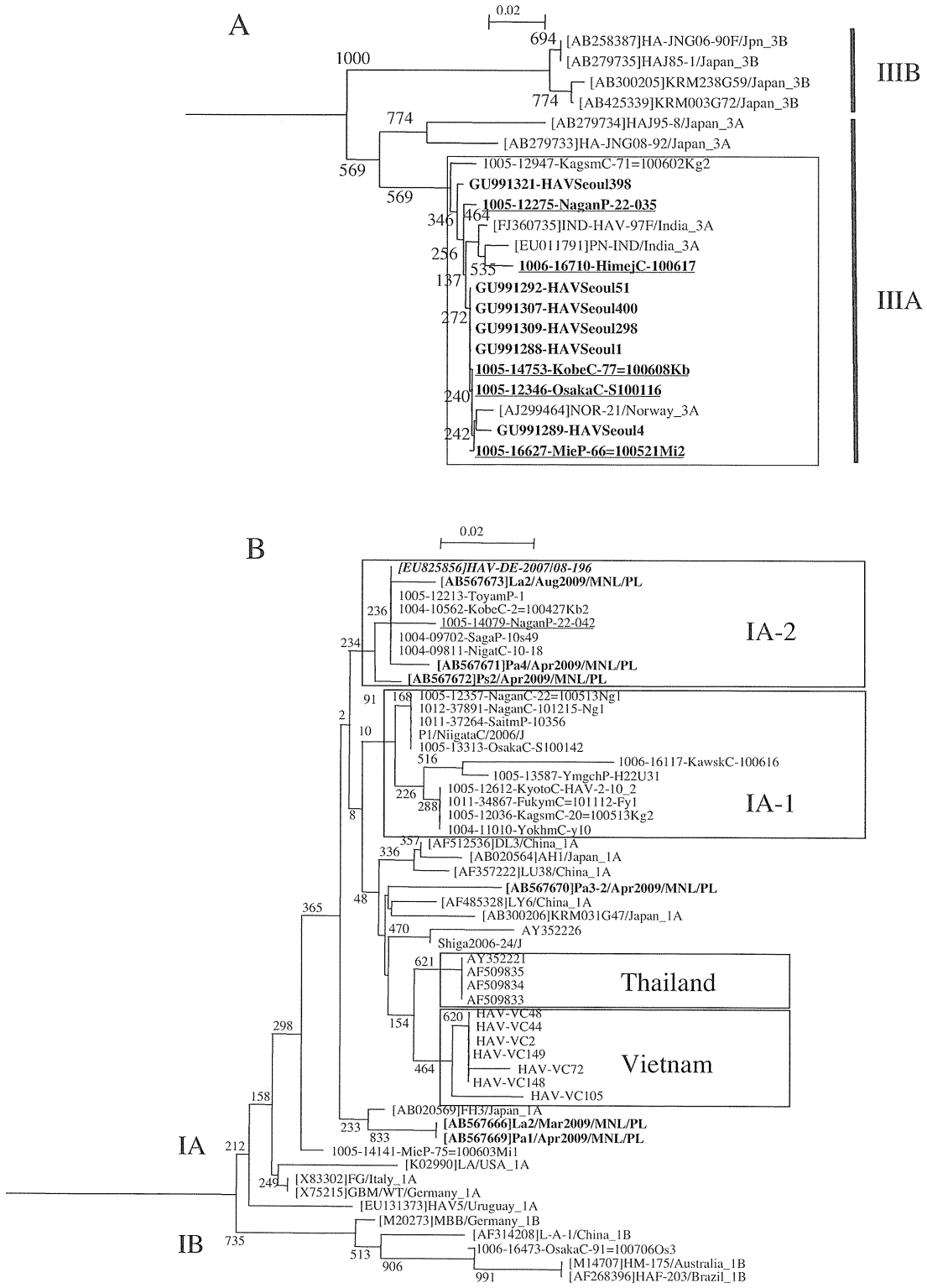

Fig. 4. (A) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from HAV strains (genotype IIIA) isolated from Japan (bold underline) and Korea (bold). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling. (B) Phylogenetic tree 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A slightly different region of VP1-2A (nt: 2975–3364) was used for phylogenetic analysis in South Korea (Yoo et al., unpublished, available on GenBank) compared to the region of VP1-2A (nt: 2930–3161) used in the present study.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

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

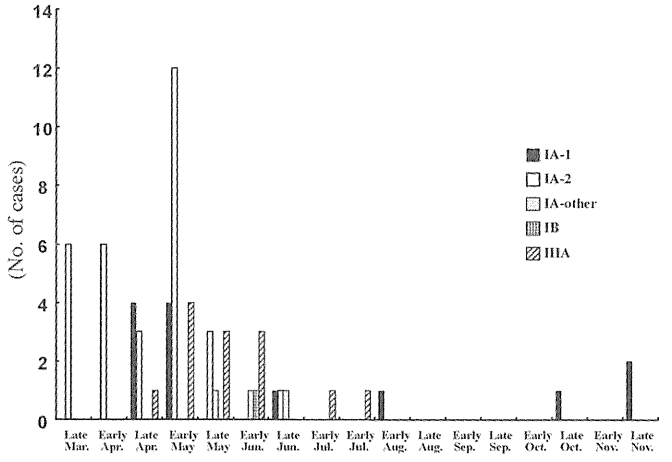

Fig. 5. Temporal distribution of HAV genotypes from late March to late November in 2010.

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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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.

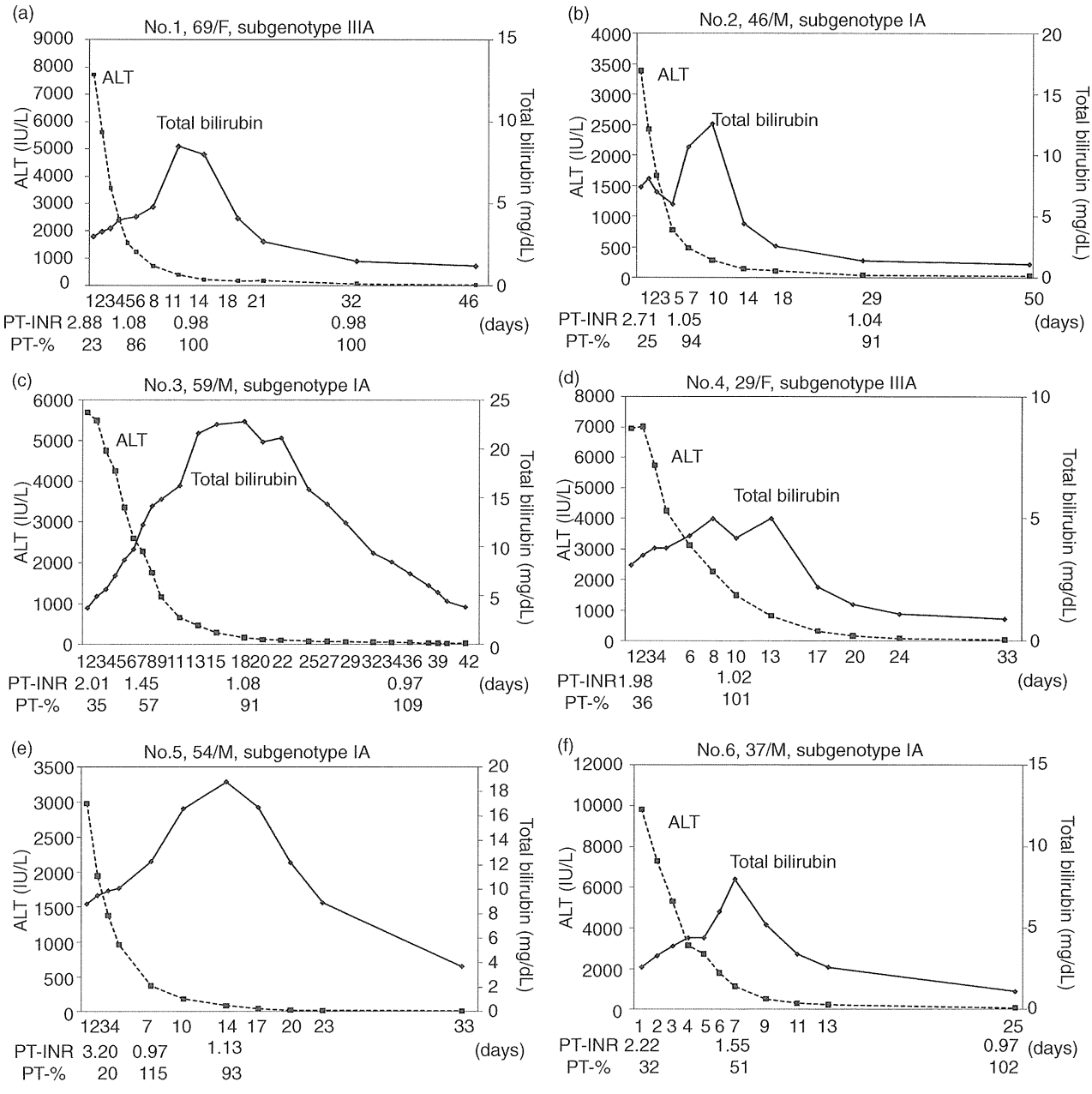

Figure 1 Clinical course of six acute liver failure patients infected with hepatitis A virus (HAV) in Japan. (a), (b), (c), (d), (e) and (f) indicates patient no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6 in Table 1, respectively. All patients presented with acute liver failure with coagulopathy but without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

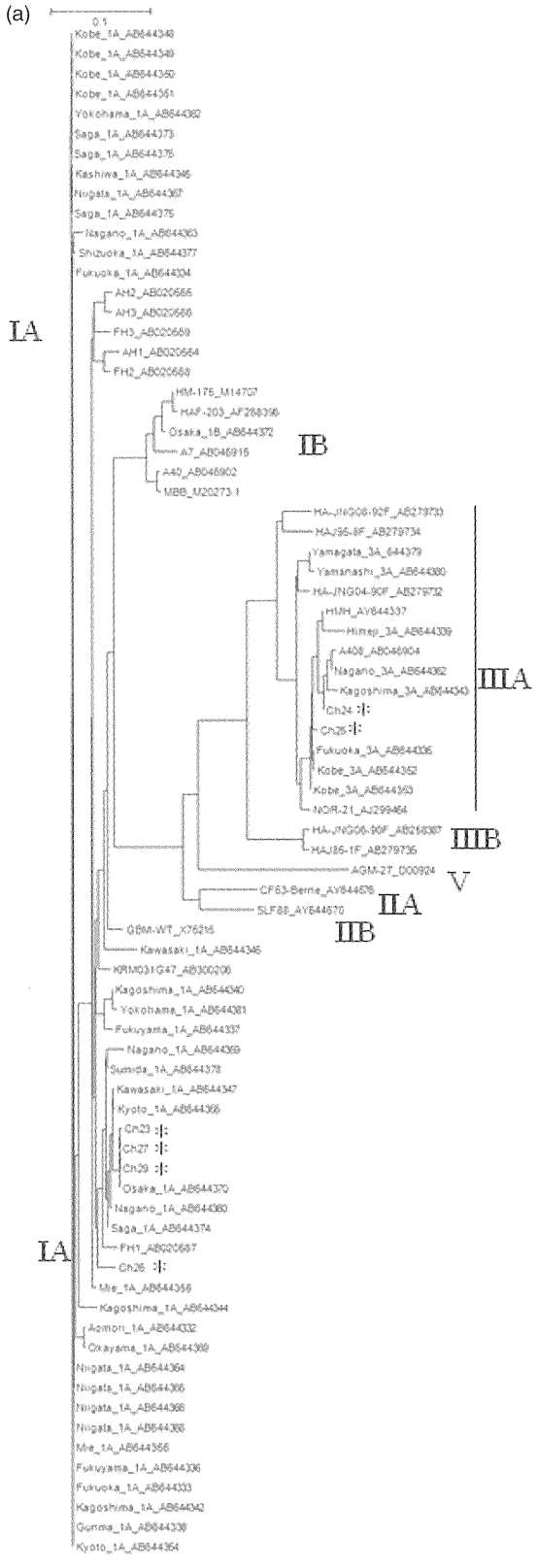

Figure 2 Phylogenetic analysis of hepatitis A virus (HAV) isolates from patients with acute liver failure from Japan. (a), (b) The neighbor joining tree was constructed based on a partial sequence of 451 nt in the VP1/2A region of HAV. Selected reference strains were also included in the phylogenetic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

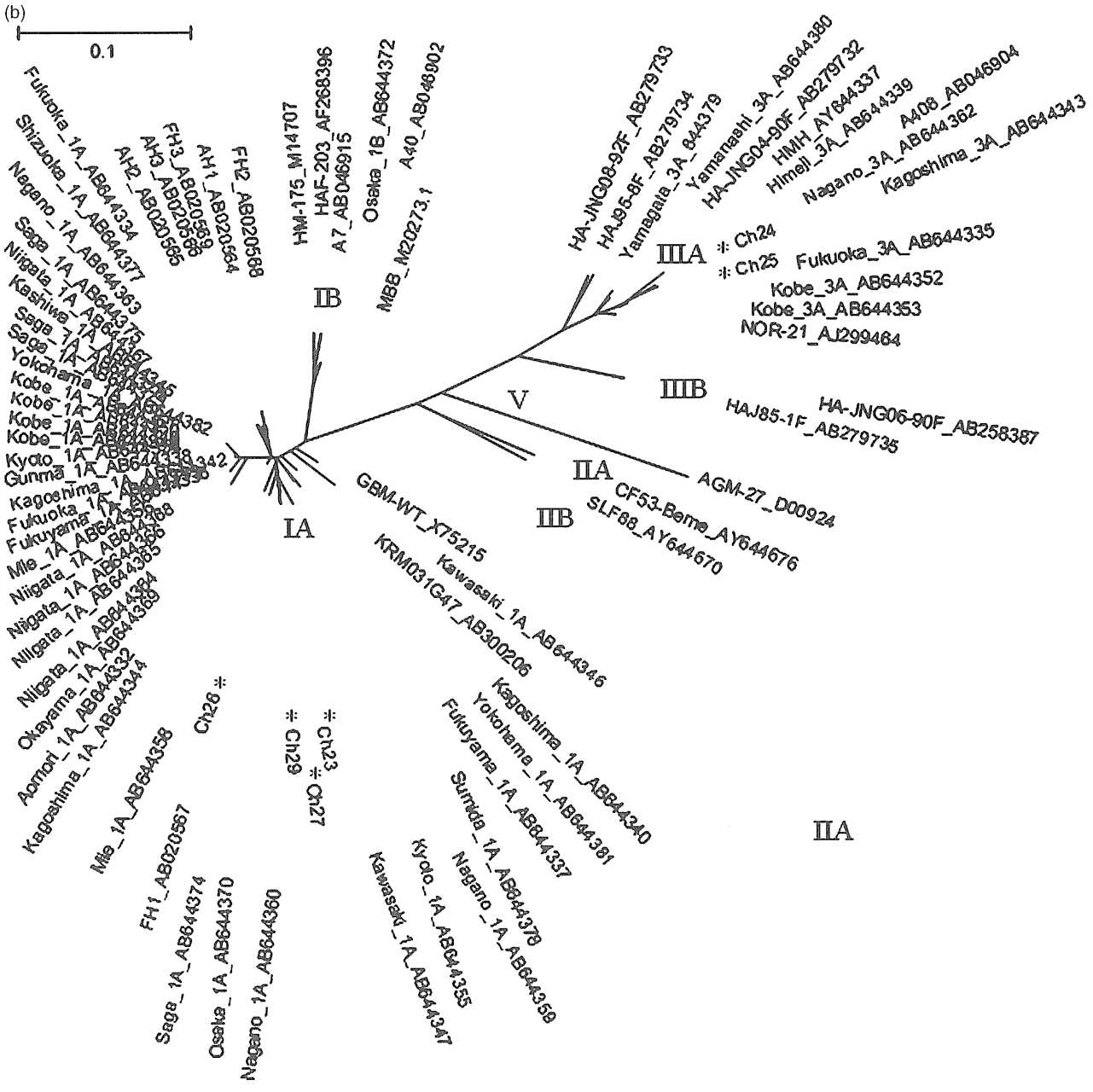

Figure 2 *Continued.*

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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Laboratory and Epidemiology Communications

Epidemiological Investigation and Analysis of Hepatitis A Virus Genomes in the Three Cases of Hepatitis A Infections That Occurred in April–May 2010

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Hepatitis A infection is classified as a category IV infectious disease. Under the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infectious Diseases in Japan, the doctor who diagnoses a patient with hepatitis A infection must notify the governor of the prefecture of any such case. For the past several years, the number of hepatitis A cases in Japan has been less than 200 per year. However, since the 10th week in 2010, the number of cases rapidly increased, mainly in western Japan, and was approximately 270 by the 28th week. In this period, three cases of hepatitis A infection occurred in central Japan. In this report, we describe the results of epidemiological investigation and genetic analysis of these cases.

The epidemiological investigation was performed by the staff at the health center that governs the medical facilities in which the cases were diagnosed. The investigation was conducted by meeting with and interviewing the patients. Genetic analysis of hepatitis A virus (HAV) was carried out according to the manual of the National Institute of Infectious Diseases (1). We extracted viral RNA from the patient's feces and then used the reverse transcription-polymerase chain reaction (RT-PCR) to detect a part of the HAV gene. Moreover, we determined the sequence of the PCR product of approximately 230 nt, a part of the VP1-2A region, by direct sequencing and performed analyses including phylogenetic tree analysis.

The three patients with hepatitis A infection included two females, Patient A (54 years old) and Patient C (20 years old), and one male, Patient B (52 years old), and they developed the infection between April 29 and May 19 in 2010 (Table 1). The patients showed typical symptoms of hepatitis A infection including jaundice, liver dysfunction, fever, and malaise. Of the three patients, two (Patients A and C) had traveled abroad. Patient A had traveled through Korea and Taiwan, and Patient C had traveled to the Philippines (Table 1). Considering the incubation period of hepatitis A infection, we strongly suspected that Patient C was exposed to HAV in the Philippines. On the basis of the interviews, we found that Patient B had eaten raw salted short-necked clams about 1 month before the onset of the disease.

We conducted an RT-PCR analysis of the RNA isolated from the feces of the three hepatitis A patients to detect the HAV gene; all specimens tested positive for the presence of the HAV gene. Nucleotide sequences of the PCR products were determined and phylogenetic

Table 1. Epidemiological information of hepatitis A patients and results of genotyping of detected viruses

Patient	Sex	Age (y)	Onset date	History of travel abroad		Consumption of raw bivalve	Virus strain name	Genotype
				Country	Period			
A	Female	54	Apr. 29, 2010	Korea Taiwan	Apr. 4–7, 2010 Apr. 26–28, 2010	No	1005-12275-NgnP-22-035	IIIA
B	Male	52	May 13, 2010		No	Raw salted short-necked clams (in mid-April)	1006-13956-NgnP-22-041	IIIA
C	Female	20	May 19, 2010	Philippine	Nov. ??, 2009–May 18, 2010	Unknown	1006-14079-NgnP-22-042	IA

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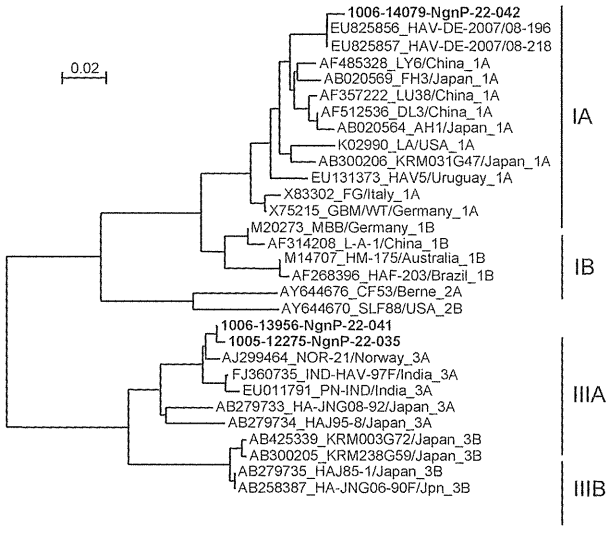

Fig. 1. Phylogenetic tree constructed on the basis of partial sequences (approximately 230 nt) of the hepatitis A virus VP1-2A region. The tree was plotted using the neighbor-joining method, and bootstrap was conducted 1000 times. Avian encephalomyelitis virus (accession no. AY275539) was used for outgroup but it was not shown.

analysis was performed. HAV strains isolated from Patients A and B were genetically similar and were classified into genotype IIIA (Fig. 1). In Korea, hepatitis A infection has recently been spreading mainly in the younger age group, and studies have reported that most of the hepatitis A strains isolated from these patients belonged to genotype IIIA (2). Thus, we cannot rule out the possibility that Patient A was infected with the HAV strain that had spread in Korea during the patient's stay in that country. We thought that Patient B was infected by eating raw salted short-necked clams about 1 month before the onset of the illness. We could not trace the place of origin of the infection because the patient could not clearly remember where he had consumed the raw short-necked clams.

The HAV strain from Patient C was classified into genotype IA, and it belonged to the same cluster as that of HAV-DE-2007/08-196 (accession no. EU825856) and HAV-DE-2007/08-218 (accession no. EU825857) detected in Philippine tourists visiting Germany (Dr. Mirko Faber, personal communication) (Fig. 1). Although the HAV strain isolated from Patient C is not shown in the figure, it was similar to the strains isolated form river water samples in the Philippines. The results of these genetic analyses strongly suggested that Patient C was infected with HAV in the Philippines.

Most of the HAV strains that spread in Japan in 2010 were classified into genotype IA, and these strains are closely related to the HAV strain from Patient C. More detailed and extensive genetic and epidemiological analyses are needed to determine the cause of the rapid increase in the number of hepatitis A patients.

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Conflict of interest None to declare.

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CLINICAL STUDIES

New findings regarding the epidemic history and population dynamics of Japan-indigenous genotype 3 hepatitis E virus inferred by molecular evolution

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Keywords
Bayesian skyline plot – coalescent analysis – epidemic history – genotype 3 – hepatitis E virus – Japan – large-race pig – large-scale pig breeding – phylogenetic tree analysis – zoonosis

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Abstract

Background: Since previous studies have investigated the population dynamics of Japan-indigenous genotype 3 hepatitis E virus (HEV) using virus sequences, more nucleotide sequences have been determined, and new techniques have been developed for such analysis. *Aims:* To prevent future hepatitis E epidemic in Japan, this study aimed to elucidate the cause of past HEV expansion. *Methods:* The epidemic history of Japan-indigenous genotype 3 HEV was determined using the coalescent analysis framework. Bayesian skyline plot (BSP) and Bayesian estimate of phylogeny with relaxed molecular clock models were calculated using Markov chain Monte Carlo sampling. *Results:* Japan-indigenous strains consist of New World strains (subtype 3a), Japanese strains (3b) and European strains (3e). The oldest lineage, 3b, appeared around 1929. Lineages 3a and 3e appeared around 1960. BSPs indicated similar radical population growth of the 3a and 3b lineages from 1960 to 1980. *Conclusions:* Population dynamics of the three lineages shared some common characteristics, but had distinguishing features. The appearance of 3a and 3e lineages coincides with the increase of large-race pig importation from Europe and the USA after 1960. The epidemic phase of 3a and 3b strains from 1960 to 1980 could be related to increased opportunity for HEV infection arising from large-scale pig breeding since 1960. Our observations revealed new findings concerning the close relationship between the epidemic history of Japan-indigenous genotype 3 HEV and the improvement of the Japanese pig industry. Infection control in pig farms should be an effective method of preventing HEV infection in humans.

Hepatitis E virus (HEV) is a member of the genus *Hepevirus* in the family *Hepeviridae* (1). HEV is a non-enveloped virus, and its genome comprises positive-sense single-stranded RNA of approximately 7200 nt (2, 3). The genome contains three open reading frames (ORFs). ORF1 codes for non-structural genes, ORF2 encodes the structural protein comprising the virion capsid and ORF3, which mostly overlaps ORF2, encodes a short protein of 113–114 amino acids that is required for virus egress from cells, and is proposed to perturb numerous cellular pathways (4–6).

The GenBank/EMBL/DDBJ accession numbers of the new HEV sequences in this study are AB578953-AB578957, AB578959-578963 and AB581581- AB581598.

Hepatitis E virus is the causative agent of hepatitis E. The first outbreaks of hepatitis E described in the literature were waterborne and associated with faecal contamination of water sources (7, 8). Until its discovery in swine, hepatitis E was assumed to be limited to developing countries and was seen in industrialized countries only as imported cases (7, 9). However, sufficient evidence from Japan and Europe, in the form of sporadic cases who had never travelled to developing countries, suggests that HEV can be transmitted zoonotically to humans from consumption of meat or offal of swine, wild boar and wild deer (10–14).

Hepatitis E virus had been segregated into four genotypes, i.e. 1–4. Lu *et al.* proposed genotyping and subtyping of global HEV strains using phylogenetic analyses (15). Recently, new genotype candidates have been reported in rats (16), rabbits in China (17) and wild boars in Japan (18, 19). The contribution of the new genotype candidates to human disease remains unknown.

Genotypes 1 and 2 do not infect swine (20), infect only humans and are associated with hepatitis E outbreaks by faecally contaminated water sources in developing countries (7–9). Genotypes 3 and 4 have been isolated from sporadic cases in humans who had never travelled to developing countries (21) and from some mammals (13). Genotype 3 strains have been isolated all over the world – Europe, Asia, New World, Oceania and Africa (15, 22). Genotype 4 cases have been reported in Asia, mainly Japan and China (15). Although some cases of genotype 3 and 4 hepatitis have been suggested to occur via zoonotic transmission by exposure to the body fluids of infected swine (23) and ingestion of food products from pigs, boars and deer, the aetiology of other cases with these genotypes still remains to be determined.

In Japan, autochthonous acute hepatitis E accounted for 10–15% of non-ABC hepatitis after 2002 (24). Our previous study revealed a high prevalence of HEV in Japanese swine and suggested that swine serves as reservoirs for HEV infection (25). The food-borne transmission of HEV through ingestion of raw or undercooked meat including liver and intestine from infected swine is one of the most plausible transmission routes (10). A nationwide survey revealed that genotype 3 is the most prevalent genotype in humans (26, 27) and swine in Japan (25). The existence of multiple lineages of genotype 3 HEV has been reported in swine and humans in Japan (25, 28).

The epidemic history of Japan-indigenous HEV strains has already been reported using a coalescent-based method to analyse viral sequences (29). However, only a small number of sequences were previously available for the analyses. Furthermore, the epidemic history was inferred from a single estimated genealogy, and thus the error associated with phylogenetic reconstruction, which may be large, was ignored. Herein, we utilized a more powerful method called the Bayesian skyline plot (BSP) (30, 31), which is not influenced by phylogenetic error. Moreover, a more realistic Bayesian inference method with relaxed clock models that incorporate variation in evolutionary rates among lineages has been developed (32). Purdy and Khudyakov reported an epidemic history of all genotypes of HEV strains using this method (33).

In this study, we focused on the epidemic history of Japan-indigenous genotype 3 HEV and analysed the sequence of Japanese strains on a large scale using the sophisticated Bayesian inference method. Comprehensive phylogenetic analyses comparing Japanese genotype 3 strains and genotype 3 strains from other countries were also performed, and reconfirmed the existence of multiple lineages in Japan. New interesting findings on the epidemic history and past population dynamics of Japan-indigenous genotype 3 HEV were revealed using molecular evolutionary analyses considering multiple lineages. We also speculate on a possible dissemination scenario for Japan-indigenous genotype 3 HEV, which could be applied to the prevention of future endemic or epidemic HEV infection in Japan and other developed countries.

Materials and methods

Sampling, isolation, and sequencing of HEV isolated from 2009–2010 HEV infections in Japan

Nine genotype 3 HEV strains isolated from patients admitted to Toshiba General Hospital and Tokyo Teishin Hospital, Tokyo, Japan in 2009 and 2010 were included. The patients had undergone an interview for risk factors pertaining to HEV infection and represented sporadic cases that were not correlated to each other. Informed consent was obtained from each patient. This study was approved by the institutional review committees of Toshiba General Hospital and Tokyo Teishin Hospital.

A total of 313 packages of raw pig liver and colon sold as food were purchased from 22 grocery stores in Tokyo in 2009. Tissue specimens were obtained from each package. Eight pig liver or colon specimens had detectable HEV RNA (34). The nucleotide sequences from the patients and pig livers or colon were determined using a previously reported method (35), with some modifications. In brief, nucleic acids were extracted from serum with the QIAamp MinElute Virus Spin Kit (QIAGEN GmbH, Hilden, Germany). HEV RNA genomes were reverse transcribed, and cDNA was amplified using PCR with primers specific for targeted partial ORF1 and ORF2 regions of the HEV genome (28, 36). Reverse transcription and first-round PCR were conducted using the SuperScript III One-Step RT-PCR System (Invitrogen Corporation, Carlsbad, CA, USA); second-round PCR was conducted using Platinum Taq DNA polymerase (Invitrogen). The final products were sequenced in the 377 DNA Sequencer using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For five

swine and nine human strains, both the ORF2 and ORF1 region sequences were determined in this study, and these strains were included in the subsequent phylogenetic and coalescent analyses. Only one region of the ORF2 or ORF1 sequence was successfully sequenced for the remaining three swine strains; therefore, those three strains were not included.

Phylogenetic analysis of the ORF2 and ORF1 datasets

All available nucleotide sequences of 301 nt of ORF2 (nucleotide number 5994–6294: M73218) and 287 nt of ORF1 (nucleotide number 104–390) were downloaded from the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/index.html) (37). These two regions have been sequenced and frequently used for genotyping and subtyping since Mizuo *et al.* compared Japanese HEV strains with global HEV sequences (28). Lu *et al.* reviewed genotyping and subtyping of global HEV strains by phylogenetic analyses using these regions (15). Database sequences were divided into each genotype using phylogenetic analyses after comparison with reference sequences for each genotype: genotype 1, M73218; genotype 2, M74506; genotype 3, AB073912; genotype 4, AB074915; Chinese rabbit HEV, FJ906895 (17); genotype 5, AB576193 (18); and newly reported novel genotype, AB602441 (19).

Using SeaView version 4 (38), large trees were estimated using neighbour-joining (Kimura 2-parameter substitution model). Only genotype 3 sequences and the above reference sequences were retained for the analyses. Next, information from the database, from original publications and from our own knowledge was used to classify the sequences. Only one sequence from each infected individual was retained. Closely related sequences obtained from single outbreaks of hepatitis E from the same origin were excluded except for one sequence that was considered as the representative. Similar sequences isolated from the same swine herd were also excluded except for one sequence. Experimental clones were excluded. Sequences whose nationality remained unknown were also excluded. However, identical or very similar sequences whose epidemiological linkage was not certified by any means could not be excluded because of the absence of a rational exclusion criterion for random sampling.

Database sequences were then collated and aligned with our 14 newly generated Japanese genotype 3 sequences, and were subsequently adjusted manually. The resulting ORF2 and ORF1 alignment contained 473 and 131 global genotype 3 sequences respectively (Tables 1 and 2). Neighbour-joining trees and maximum likelihood (ML) phylogenies of the two datasets were constructed to determine the phylogenetic distribution of the included Japanese strains within the global epidemic using SeaView version 4. The program jModelTest was used to select the most appropriate nucleotide substitution model that adequately fit the sequence datasets (39). Maximum likelihood phylogenies were heuristically searched using the SPR (subtree pruning and regrafting) and NNI (nearest neighbour interchange) perturbation algorithms. The statistical robustness levels of phylogenetic groupings were subsequently assessed using bootstrap analyses (1000 replicates for neighbour-joining trees and 100 replicates for ML phylogenies). Phylogeographic structure was then identified using FigTree (available from http://tree.bio.ed.ac.uk), and ancestral lineage states of clades and lineages of Japanese strains were coloured using a parsimony approach (40). Colours were used according to the subtyping, tentatively determined by Lu *et al.* (15).

Table 1. Origin of and number of isolates included in each phylogeny cluster of the 301-nt ORF2 region

3a		3e	
Japan	59*	Japan	17
Korea	24	Mongolia	1
Thailand	5	UK	16
China	1	France	5
Taiwan	1	Hungary	2
USA	28	Germany	1
Canada	20	Russia	1
Mexico	5	total	43
Hungary	6		
Netherland	2	3f	
Total	151	Japan	2
		Thailand	5
3b		France	63
Japan	137†	Spain	14
China	4	Netherland	7
Canada	1	Germany	2
France	1	UK	2
Total	143	Sweden	1
		Total	96
3c			
Mongolia	1	3g	
France	10	Kyrgyzstan	1
Netherland	4	Total	1
Germany	3		
Brazil	4	3j	
Congo	1	Canada	5
Total	23	Total	5
		Magenta (undetermined)	
3d		Japan	6
Taiwan	3		
Total	3	Cyan (undetermined)	
		Japan	2

*Two newly determined isolates in this study are included.
†Twelve newly determined isolates in this study are included.

Coalescent analyses of Japan-indigenous genotype 3 HEV strains

From the genotype 3 Japanese ORF2-301 nt dataset and ORF1-287 nt dataset, sequences of imported cases were

Table 2. Origin of and number of isolates included in each phylogeny cluster of the 287-nt ORF1 region

3a		3f	
Japan	22*	Japan	2
Korea	2	Thailand	2
USA	3	Mongolia	1
Hungary	4	Netherland	10
Germany	2	Spain	6
Netherland	2	France	1
Total	35	Germany	1
		Greece	1
3b		Sweden	1
Japan	68†	Total	25
China	1		
Total	69‡	3g	
		Kyrgyzstan	1
3c		Total	1
Netherland	5		
Germany	1	3h	
total	6	Mongolia	1
		Itay	1
3e		NewZealand	1
Japan	6	Total	3
UK	1		
Hungary	1	3i	
Germany	1	Argentina	3
Greece	1	Germany	1
Total	10	Austria	1
		Total	5
		3j	
		Canada	1
		Mexico	1
		Australia	1
		Total	3

*Two newly determined isolates in this study are included.
†Twelve newly determined isolates in this study are included.
‡One undetermined isolate in ORF2 phylogeny is included.

excluded, and human, swine and wild animal sequences that strongly suggested domestic infection within Japan were retained. Many of the sequences with known sampling dates were longer sequences including the 301-nt ORF2 region or 287-nt ORF1 region. Sampling dates of each sequence were accurate because most dated sequences were obtained by this study's authors. Including 14 newly generated sequences in this study, 174 Japan-indigenous ORF2 sequences (412 nt: nucleotide number 5944–6355) and 76 ORF1 sequences (326 nt: nucleotide number 125–450) were available as sequences with known sampling dates. The sampling dates of the longer ORF2 dataset and the longer ORF1 dataset ranged from 9 October 1979 to 23 June 2010 and from 21 September 1993 to 23 June 2010 respectively. Furthermore, a concatenated dataset was also analysed to increase statistical power, but at the expense of a reduction in the number of available strains for analyses. The concatenated dataset contained 59 Japan-indigenous sequences.

From the dated sequence datasets, the evolutionary rate and epidemic history of Japan-indigenous genotype 3 HEV were inferred using the framework of coalescent analysis implemented in BEAST (41). Markov chain Monte Carlo (MCMC) sampling was performed for at least 1×10^8 generations, sampling a tree for every 10 000 generations. The general time-reversible model with rate heterogeneity among sites and invariable sites (GTR + Γ + I) model was selected for the dated sequence sets using jModelTest from among 24 models. To select the best-fitting molecular clock from among a strict molecular clock and relaxed molecular clocks (uncorrelated lognormal and uncorrelated exponential), Bayes factors (BFs) were estimated for each dataset in a Bayesian framework using the Tracer program (http://tree.bio.ed.ac.uk). The Tracer was used to check for convergence and to determine whether appropriate mixing of the posterior target distribution had been achieved (effective sample size > 200). Tracer was also used to reconstruct BSPs to show the epidemic history of Japan-indigenous genotype 3 HEV. The BSP is a combined plot of variants of the generalized skyline plots determined by generating a posterior distribution of effective population size through time using MCMC sampling procedure, given the sequence data (31).

A Bayesian estimate of phylogeny was obtained from the posterior distribution of trees arising from the best-fitting BEAST analysis. Firstly, the program, TreeAnnotator (41), was used to construct a phylogeny that best summarizes the set of credible trees and is called the maximum clade support phylogeny. As a relaxed clock was used in the Bayesian MCMC analysis, the branch lengths and node heights of the maximum clade support phylogeny are in units of years (32). Phylogeographic structure was then identified using FigTree, and clades and lineages were coloured in the same manner as the trees of shorter sequences.

Results

Phylogenetic analyses of the global ORF2 and ORF1 datasets

In the neighbour-joining tree of a 301-nt sequence within the ORF2 region, subtypes of clusters were guided by subtyping tentatively determined by Lu *et al.* (15) (Fig. 1). According to the increasing number of sequences, the subtyping of the clusters became ambiguous, and no significant bootstrap support was obtained for each subtype. In the ML tree, no significant bootstrap support was obtained for each subtype (data not shown). In spite of the ambiguity of the subtyping in the large phylogeny of the short sequences, the structure was generally consistent with that of the small phylogeny based on corresponding full genome sequences (data not shown).

Then, 473 genotype 3 strains were tentatively divided into 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3j and undetermined subtypes respectively (Fig. 1). Each cluster contained different ratios of Asian, European, New World and African

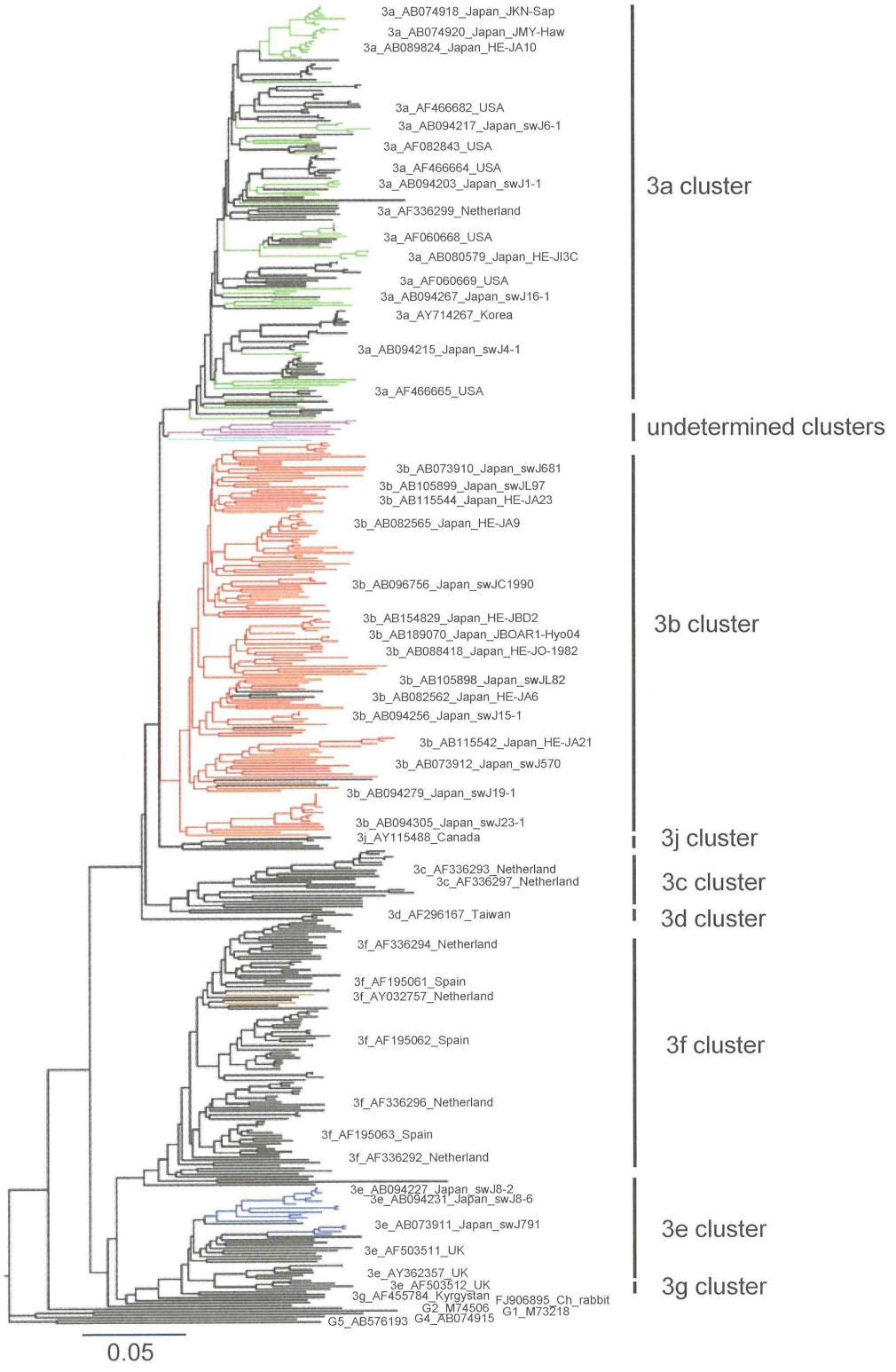

Fig. 1. Neighbour-joining tree of 301-nt sequence alignment within the ORF2 region containing 473 genotype 3 strains and outgroup strains. As the large size of the tree arising from the high number of sequences makes indicating all strain names difficult, only sequences reported by Lu *et al.* are indicated as guides for subtyping (15). The identical tree showing all strain names is available upon request. The ancestral lineage states of clades are indicated using a parsimony approach. The lineages of Japanese strains are coloured: 3a, green; 3b, red; 3e, blue; 3f, brown; and undetermined lineages, magenta and cyan. The lineages from other countries remain black.

strains, as shown in detail in Table 1. Overall, the 3a cluster consisted of many groups of Asian strains including Japanese strains and many groups of New World strains. The 3b cluster consisted mostly of Japanese strains. The 3c cluster consisted chiefly of European and some other strains. The 3e cluster consisted of two groups of 17 Japanese strains and many European strains. The 3f cluster consisted of mostly European strains. Two Japanese strains were included in this cluster, but these strains were strongly suggested as imported cases (42) (published only online). European strains were included in most of the subtypes. As a result, Japan-indigenous strains were divided into many groups: 3a strains that related to New World strains, 3b strains that consisted mostly of Japanese strains, 3e strains that were related to European (mainly UK) strains and unassigned strains.

In the neighbour-joining tree of the 287-nt sequence in the ORF1 region, subtyping by Lu *et al.* also served as a provisional standard (Fig. 2). In this tree, the structure of the subtyping also became ambiguous, and no significant bootstrap support was obtained for each subtype. In the ML tree, no significant bootstrap support was obtained for each subtype (data not shown). However, the structure was generally consistent with that of the above-mentioned ORF2 phylogeny. In the ORF1 tree, strains were divided into 3a, 3b, 3c, 3e, 3f, 3g, 3h, 3i and 3j clusters. The detailed composition of the countries of origin of each strain in each subtype is shown in Table 2. Overall distribution of Asian, European and New World strains in each cluster were the same as the ORF2 tree. The 3d cluster did not exist, and instead, 3h and 3i clusters were observed. Two Japanese 3f strains were the same strains as those in the ORF2 tree, that is, imported cases. As a result, Japan-indigenous strains were divided among 3a strains, 3b strains and 3e strains as in the ORF2 tree. One Japanese strain (Magenta) that was unassigned in the ORF2 tree was included in cluster 3b, probably because of the weak phylogenetic signal of the short region.

Two newly determined Japanese sequences in this study were included in cluster 3a, and 12 were included in cluster 3b in both ORF2 and ORF1 phylogenies. From the results of the ORF2 and ORF1 region trees, the Japanese strains in the 3a, 3b, 3e and undetermined clusters were analysed as Japan-indigenous strains in the following analyses.

Estimation of epidemic history and population dynamics of Japan-indigenous genotype 3 HEV using dated ORF2 dataset

Evolutionary analysis of the dated ORF2 dataset was performed with a strict molecular clock and a relaxed molecular clocks (uncorrelated lognormal and uncorrelated exponential) in BEAST. The combination of the three clock models and BSP gave similar median estimates of the evolutionary rate and time of the most recent common ancestors (TMRCAs) of the total tree and each clade (Table 3). Although the strict clock model gave a narrow range of evolutionary rate, the uncorrelated exponential clock model gave the best BF (vs. strict clock: 11, vs. uncorrelated lognormal clock: 10) and also gave the widest range of evolutionary rate and TMRCAs. The evolutionary rate was 1.44×10^{-3} (95% credible interval (CI), 0.95×10^{-3} to 1.99×10^{-3}) substitutions/site/year. The TMRCAs dated 1847 (1675–1940) for the total tree, 1959 (1940–1974) for cluster 3a, 1929 (1873–1961) for cluster 3b and 1960 (1937–1974) for cluster 3e respectively.

Figure 3 shows the maximum clade support phylogeny for the dated ORF2 sequence set, reconstructed from the phylogenies sampled under the best-supported model combination: the uncorrelated exponential clock model and BSP. Japan-indigenous 3a, 3b, 3e and undetermined cluster strains each clustered together. The tree demonstrated a similar structure to the global ORF2 region tree. The 3e cluster branched off from other clusters at the earliest time (around 1850), without more branching until after 1950. The 3b cluster, which consisted mostly of Japanese strains, began to branch between 1900 and 1950 followed by more frequent branching after 1950. The 3a cluster and the undetermined clusters also started to branch after 1950.

Figure 4a shows the BSPs estimated from the Japan-indigenous 3a and 3b clusters of the ORF2 dataset. The BSP is a flexible, non-parametric estimate of past changes in effective population size (number of infections). The most notable difference in BSPs between clusters 3a and 3b was the different time of onset. The BSP of cluster 3a began around 1960, but the BSP of cluster 3b began around 1930. The same difference between the two clusters was seen in TMRCAs (Table 3) and the beginning of branching (Fig. 3). The effective population size of 3a rapidly increased from around 1960. The population size of 3b demonstrated a relatively constant phase from 1930 to 1960, and then indicated rapid epidemic growth. This rapid growth of the two BSPs coincided with the onset of rapid diversification in the lineages of the two clusters after 1960, as shown in Fig. 3. The rate of growth appeared to slow around 1980, and the population size entered a constant phase until around 2000 in both BSPs. From 2000 to the present, the population size decreased in both BSPs. The number of 3e strain sequences was too small to be analysed by BSP.

Estimation of epidemic history and population dynamics of Japan-indigenous genotype 3 HEV using a dated ORF1 dataset and concatenated dataset

To support the results of the ORF2 dataset, evolutionary analyses of the dated ORF1 dataset and the dated concatenated dataset were performed in BEAST by the same methods used for the dated ORF2 dataset. The uncorrelated exponential model gave the best BF among the

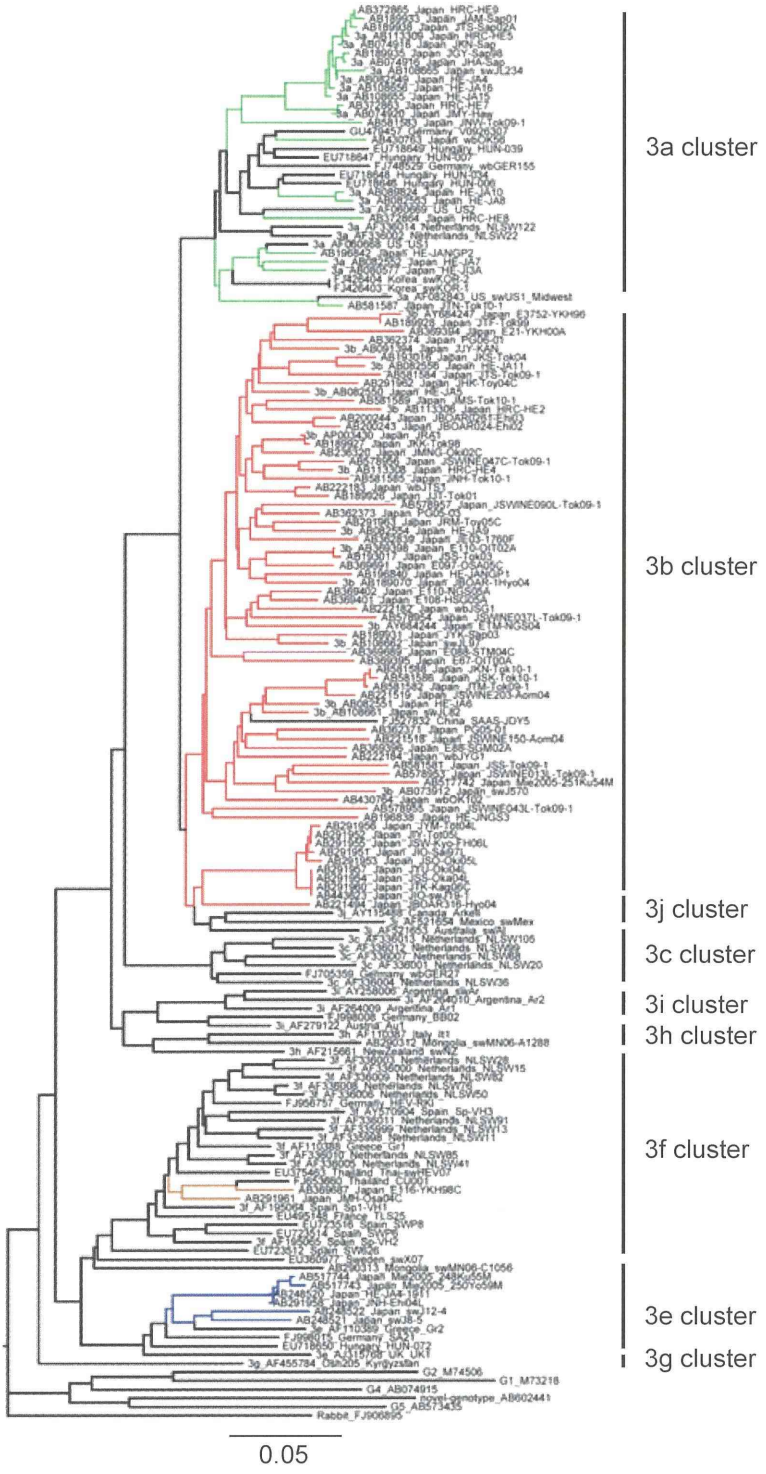

Fig. 2. Neighbour-joining tree of 287-nt sequence alignment within the ORF1 region containing 131 genotype 3 strains and outgroup strains. The ancestral lineage states of clades are indicated using a parsimony approach, and colours have the same indications as in Fig. 1. Strain subtypes reported by Lu *et al.* are indicated as guides for subtyping at the beginning of strain names. The origin of each strain is also indicated in strain names.

Table 3. Clock rates and the time of the most recent common ancestors (TMRCAs) (median and 95% credible intervals)

		Relaxed uncorrrelated clock	
Clock	Strict clock	Lognormal	Exponential
ORF2			
Clock rate*	1.35 (1.01–1.69) × 10^{-3}	1.35 (1.01–1.70) × 10^{-3}	1.44 (0.95–1.99) × 10^{-3}
TMRCAs			
Total	1804 (1719–1871)	1805 (1714–1875)	1847 (1675–1940)
3a+3b	1925 (1904–1942)	1924 (1903–1942)	1914 (1846–1953)
3a	1961 (1950–1969)	1961 (1949–1969)	1959 (1940–1974)
3b	1943 (1927–1955)	1943 (1926–1956)	1929 (1873–1961)
3e	1958 (1942–1968)	1959 (1943–1969)	1960 (1937–1974)
ORF1			
Clock rate*	1.10 (0.35–1.93) × 10^{-3}	1.09 (0.32–1.95) × 10^{-3}	1.47 (0.04–3.42) × 10^{-3}
TMRCAs			
Total	1661 (1181–1871)	1655 (1114–1876)	1762 (893–1968)
3a+3b	1891 (1747–1954)	1888 (1722–1954)	1882 (1468–1979)
3a	1936 (1849–1973)	1935 (1836–1974)	1943 (1760–1989)
3b	1918 (1808–1965)	1916 (1791–1966)	1923 (1648–1987)
3e	1940 (1861–1974)	1939 (1850–1974)	1953 (1797–1991)
Concatenated set			
Clock rate*	1.19 (0.46–1.90) × 10^{-3}	1.07 (0.32–1.82) × 10^{-3}	1.32 (0.09–3.32) × 10^{-3}
TMRCAs			
Total	1698 (1367–1855)	1664 (1191–1864)	1749 (892–1969)
3a+3b	1896 (1783–1946)	1882 (1721–1947)	1870 (1462–1978)
3a	1947 (1888–1974)	1940 (1855–1974)	1941 (1760–1989)
3b	1925 (1840–1961)	1915 (1797–1962)	1920 (1681–1987)
3e	1942 (1883–1971)	1937 (1852–1970)	1951 (1798–1990)

*Substitutions/site/year.

three clock models, but it was not significant, possibly because of insufficient information from the small number of sequences. As a result, the estimates of evolutionary rate and TMRCAs under the uncorrelated exponential model had considerably wide 95% CI (Table 3). However, the median estimates were not very different from those in the ORF2 region analysis.

Figure 5a, b show the maximum clade support phylogenies for the dated ORF1 sequence set and the dated concatenated dataset. Japan-indigenous 3a, 3b, 3e and undetermined strains clustered together. The trees showed structure similar to the ORF2 region maximum clade support phylogeny. The 3e strains branched at the oldest age without more branching until 1950. The 3b cluster began to branch between 1900 and 1950, with branching frequently after 1950. The 3a cluster started to branch after 1950.

Figure 4b, c show the BSPs estimated from the Japan-indigenous 3a and 3b clusters of the dated ORF1 dataset and the dated concatenated dataset. The BSPs of the two clusters had a wide range of 95% highest posterior density confidence intervals, corresponding to ambiguous estimates of the evolutionary rate and wide range of TMRCAs. However, the median estimates of the four BSPs represented an increase around 1960, constant phase from 1980 to 2000 and decrease in effective population size after 2000, which were all seen in the ORF2 BSPs.

Discussion

In a report on the epidemic history of Japan-indigenous genotype 3 HEV strains by Tanaka *et al.* (29), the identity of the Japan-indigenous strains could not be investigated sufficiently, and differences in epidemic histories by lineage could not be considered adequately because of the small number of analysed sequences. In this study, we first tried to define the Japan-indigenous genotype 3 HEV strains compared with the global strains. Most sequences reported from various countries are relatively short in the ORF2 and ORF1 regions, although analysis of longer or even whole-genome sequences should be more reliable for accurate identification of genetic relatedness among HEV strains. Especially for European strains, longer or full genome sequences were so limited that analysing two short regions was unavoidable for the identification of Japan-indigenous strains compared with the global strains. The more the sequences are added, the more ambiguous the subtyping becomes, and clusters for each subtype are not supported using bootstrap analyses in neighbour-joining trees and ML trees. However, the structure of the trees of short sequences is almost the same as that of the full genome sequence tree (data not shown).

In this study, three major lineages of Japan-indigenous strains were discerned, clusters 3a, 3b and 3e, according to the tentative subtyping by Lu *et al.* (15).

Fig. 3. Maximum clade support phylogeny for the dated Japanese sequence set of the ORF2 region (412 nt). Japan-indigenous 3a (green), 3b (red), 3e (blue) and undetermined (magenta and cyan) clusters indicate a structure similar to the global ORF2 region tree. The branch lengths and node heights are in units of years.