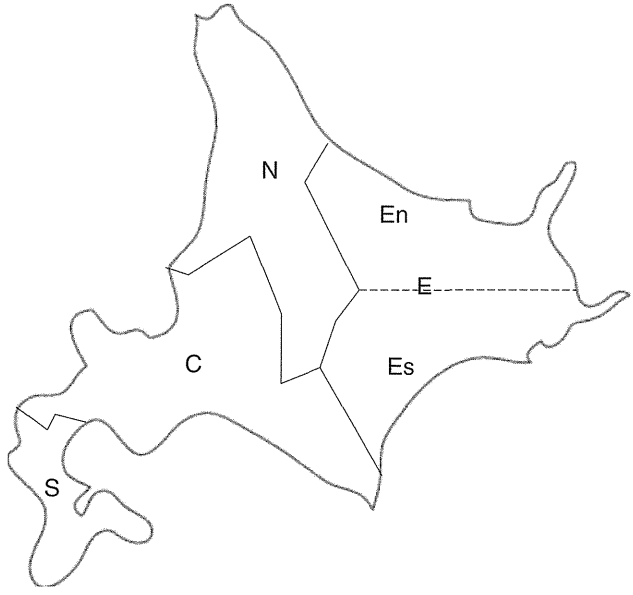

Fig. 1 Geographic map of the regions from which the samples were collected and the strains were isolated in Hokkaido

were identified by nucleic acid amplification screening for HEV in blood donors in Hokkaido at the Japanese Red Cross Blood Center. Viral RNA was extracted from serum samples using a QIAamp Viral RNA Mini Kit or a QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany). For the clinical samples, nested RT-PCR was performed using HE7-1/HE7-2/HE7-3/HE7-4 and HE7-5/HE7-6/HE7-7/HE7-8/HE7-9 primers for the ORF1 region [26], and HE040/HE044 and HE041/HE110-2 primers for the ORF2 region [16]. A phylogenetic tree was constructed by the neighbor-joining method on the basis of sequences of the central region of the ORF2 gene. For further sequence analysis of the ORF1 gene, the following additional primers were used: HE5-1/HE5-2/HE5-3/HE5-4/HE5-5/HE5-6 [25].

HEV analysis with putative animal reservoirs and environmental samples is shown in the Table 1. RNA in the liver of putative animal reservoirs was extracted from a piece of tissue specimen (3 mm × 3 mm) using an RNeasy Mini Kit (QIAGEN). A total of 954 environmental samples, samples of raw sewage (50 ml), treated sewage (200-500 ml), river water (200-500 ml), and seawater (20 liters), and oysters, which are known to naturally concentrate human pathogens, were periodically collected from part of region E: the En and Es areas. Seawater sample was concentrated using the adsorption-elution method by an electronegative filter (catalog no. HAWP-142-50; Millipore, Tokyo, Japan) [8] and other water samples were concentrated using a cation-coated filter (catalog no. HAWP-047-00; Millipore) [4]. A Centriprep YM-50 device (Millipore) was used to obtain a final volume of 700 µl as described previously [4, 8]. The stomach and digestive diverticula of oysters were homogenized, and viruses were precipitated with polyethylene glycol 6000 [3]. Viral RNA in the environmental samples was extracted using a QIAamp Viral RNA Mini Kit. The RNA was first examined by nested RT-PCR using a set of ORF1 primers (HE7 series) because of their high sensitivity, and the positive samples were subjected to nested RT-PCR using the ORF2 primers.

Forty-two strains from the serum samples of 31 patients and 11 carriers collected during 2004-2011 tested positive for HEV. Sequence analysis of the central region of the ORF2 gene revealed that 25 and 17 strains were genotype 4 and genotype 3, respectively. All of strains from blood donors except one were genotype 3. Of 390 swine liver samples from meat inspection centers and health centers in

Table 1 Summary of the HEV strains that were detected in putative animal reservoirs and environmental samples

Sample type	No. of samples	Sampling period	Region	Isolated HEV strain (genotype)
Swine liver	61	Jul 2005	S	swine-S-050705(G3)
	130	May-Sep 2005	C	swine-C-050624(G4)
	48	Aug-Sep 2005	N	
	151	May-Aug 2005	E	swine-E-050801(G3)
				swine-E-050704(G4)
Deer liver	10	Jan 2006	C	
	17	Dec 2005-Feb 2006	N	
	51	Jan-Aug 2006	E	
Raw sewage	62	Aug 2003-Jan 2005	En	
Treated sewage	53	Aug 2003-Jan 2005	En	
Seawater	37	Nov 2003-Mar 2005	En	seawater-En-050301(G3)
Oyster	114	Nov 2003-Feb 2005	En	
Raw sewage	37	Oct 2007-Mar 2009	Es	sewage-Es-080520(G3)
River and seawater	248	Sep 2006-Mar 2009	Es	
Oyster	403	Oct 2007-Feb 2011	Es	

Hokkaido, four swine livers (1 %) were positive for HEV RNA; two strains were classified as genotype 3 and two as genotype 4. Seventy-eight liver samples from Yezo deer from slaughterhouses were negative. HEV RNA was detected in one sewage sample from the Es area and one seawater sample from the En area. These two strains were classified as genotype 3. Noroviruses (NoVs) were also detected in the two environmental samples.

The results of phylogenetic analysis of the HEV strains are shown in Figure 2 and reveal that some strains from clinical samples that were collected during similar periods and in the same regions showed clustering with almost 100 % sequence identity (e.g., strains patient-C-101110, donor-C-100810 and donor-C-100816). This finding indicates that an HEV strain that is prevalent in humans during the same period may have had a common infection source in this area. Geographical differences have been noted among HEV strains in Japan. Genotype 3 is distributed in Honshu Island and Hokkaido, and genotype 4 is predominantly distributed in Hokkaido [20]. Although genotype 4 strains were predominant in this study, most of the strains from asymptomatic blood donors were classified as genotype 3. A previous report showed that the incidence of genotype 3 is higher than that of genotype 4 among blood donors in Hokkaido [21]. Patients with genotype 4 HEV infections are more likely to display severe clinical manifestations than those with genotype 3 HEV infections [18]. Our results do not contradict the results of these two reports and suggest that more cases of genotype 3 HEV infections are likely to be latent compared to genotype 4 infections.

Swine-S-050705 was genotype 3 and had 99 % similarity to the strain (accession no. AB434141) that originated from a blood donor in October 2003 in region N. Swine-E-050704 was genotype 4 and exhibited almost 100 % sequence similarity to a clinical strain (accession no. AB602890) that was identified in a blood donor in September 2004 in region C. The other strains from swine samples, swine-E-050801 and swine-C-050624, were also located on the phylogenetic branches that included the clinical isolates (swine-E-050801 vs. donor-N-100722, swine-C-050624 vs. HE-JI4/G4). Most of the genotype 4 strains including swine-E-050704 formed one main cluster and shared more than 96 % sequence similarity.

The nucleotide sequence of the strain sewage-Es-080520 was classified as genotype 3 and was 100 % identical to those of three clinical strains from different geographic regions (patient-C-101210, donor-C-101122 and patient-N-090615). Further analysis showed that the nucleotide sequence of ORF1 gene (326 bp) of sewage-Es-080520 was 100 % identical to that of patient-N-090615. The nucleotide sequences of the ORF1 genes of the other two clinical strains (patient-C-101210 and donor-C-101122) were 100 % identical to each other and shared 99.7 % sequence similarity with that of sewage-Es-080520. The strain seawater-En-050301 was also genotype 3 and exhibited 93-94 % sequence similarity to donor-S-091124.

There was not enough geographical evidence to show a direct link between the strains from clinical samples and those from swine and the environmental samples. Only patient-E-060308 and swine-E-050704 were geographically related, and they exhibited 99 % similarity. Although the swine samples in this study were collected in 2005, a continuous survey of genetic diversity among swine HEVs together with a study of marketing of swine liver is required to investigate whether a correlation between consumption of swine liver and human infection exists.

Genotype 3 HEVs were detected from two environmental samples that also contained NoVs. In addition, sewage-Es-080520 shared 100 % similarity with three strains from clinical samples that were collected during similar periods. Additionally, HEV strains derived from swine liver, seawater and raw sewage samples shared 93-100 % sequence similarity with human HEV strains. It is highly possible that these environmental strains originated from infected humans or animal reservoirs. Detection of HEV RNA from environmental samples, especially from seawater, suggests that there are more cases of latent HEV infection than have been reported. This study describes novel findings on the prevalence and genotype distribution of HEVs in various sources, suggesting that genetically diverse HEVs are circulating among humans, animal reservoirs and environments in Hokkaido. It should be noted that there is a potential risk of HEV infection by consumption of raw or insufficiently cooked shellfish and recreational contact. HEV detection in a bivalve called the Yamato-Shijimi (*Corbicula japonica*) and in oysters has been reported previously [13, 23]. However, all of the oysters and most of water samples were negative for HEV in our analysis. The method for virus concentration from water samples used here could recover viruses efficiently [4] and was applicable for detecting human enteric viruses [9, 10]. In this study, the detection of NoVs in the environmental samples is consistent with the increase in the number of gastroenteritis patients with NoVs infection. But the levels of HEV contamination in environmental samples may not be sufficient for detection [1, 7]. Because of the host specificity of viruses, molecular detection and characterization would allow the determination of sources of contaminants in environmental samples and improve surveillance for public health. Therefore, improved methods for concentration and purification of viruses as well as sensitive and specific detection methods are required to determine the route of HEV transmission among putative animal reservoirs, humans and the environment.

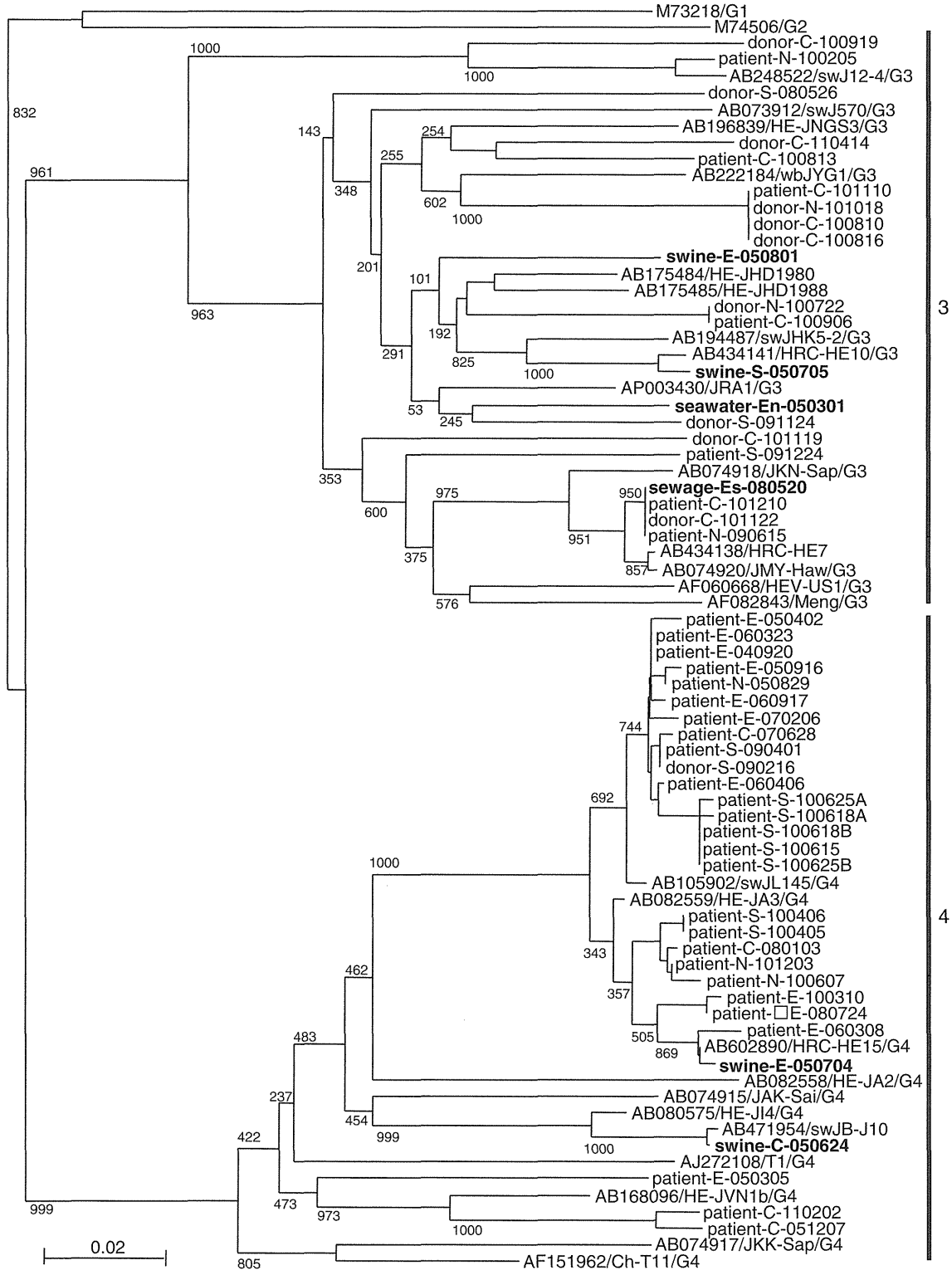

Fig. 2 Phylogenetic tree of the HEV sequences detected in clinical, swine and environmental samples. This tree was constructed by the neighbour-joining method based on the central region of the ORF2 gene (397 bp). Genotypes are indicated by numbers on the right. The sequences are designated by the sample category, sampling region and sampling date (YYMMDD). For example, donor-C-100919 is the sequence obtained from the donor sample from region C collected in September 19, 2010. The swine and environmental strains are indicated in bold type. Bootstrap values based on 1000 replicates are indicated for the major nodes

Nucleotide sequence accession numbers. The 40 nucleotide sequences reported to DDBJ were given the following accession numbers: swine-E-050704 (AB679634), swine-S-050705 (AB679635), swine-C-050624 (AB679636), swine-E-050801 (AB682756), seawater-En-050301(AB679637), sewage-Es-080520 (AB679638) and clinical samples (AB683157-AB683186, AB721083-AB721086).

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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.

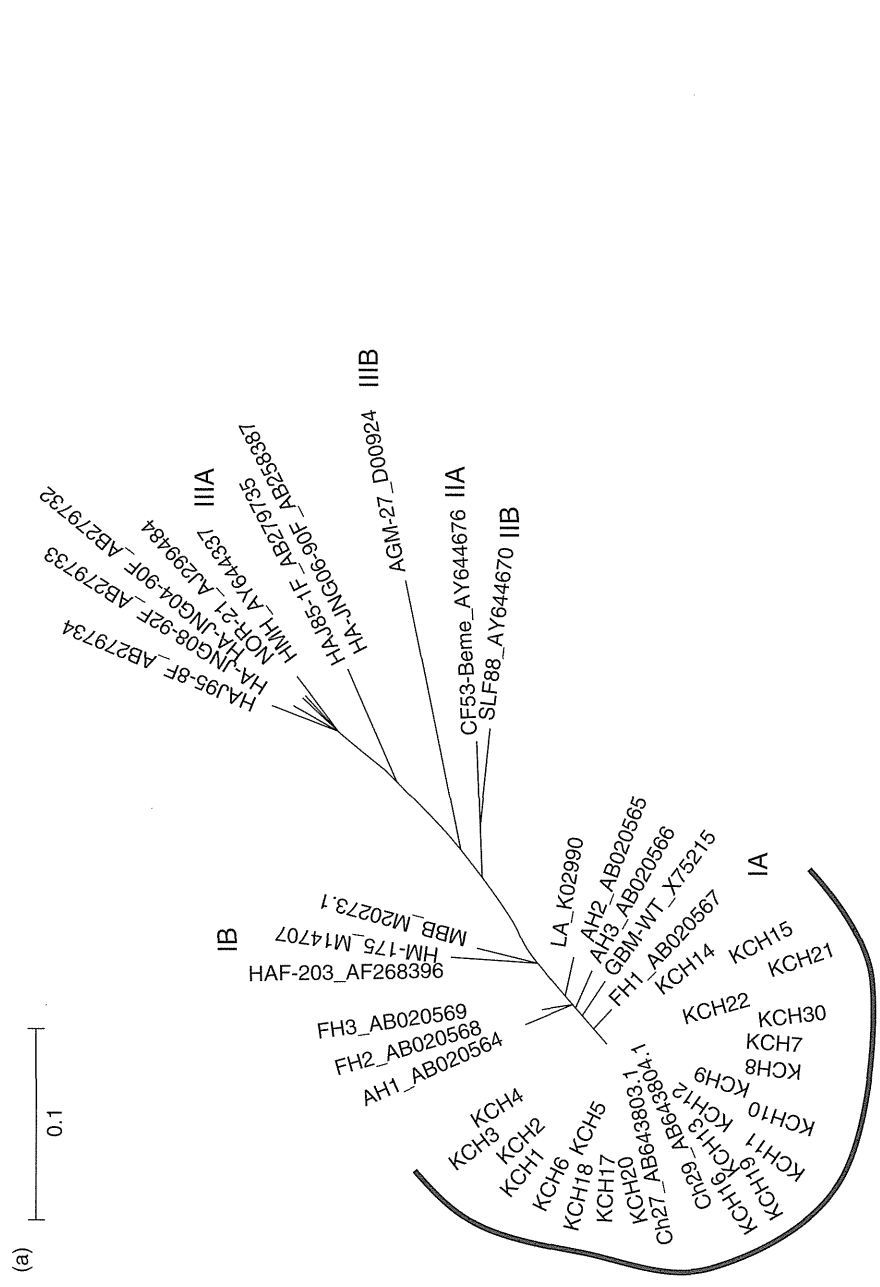

Figure 1 Phylogenetic tree analysis of hepatitis A virus (HAV) isolates in the present study and reported sequences. (a) Neighbor-Joining Tree. (b) Unweighted Pair Group Method With Arithmetic Averaging (UPGMA) tree. Black lines indicate HAV isolates derived from this outbreak. The GenBank accession 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]

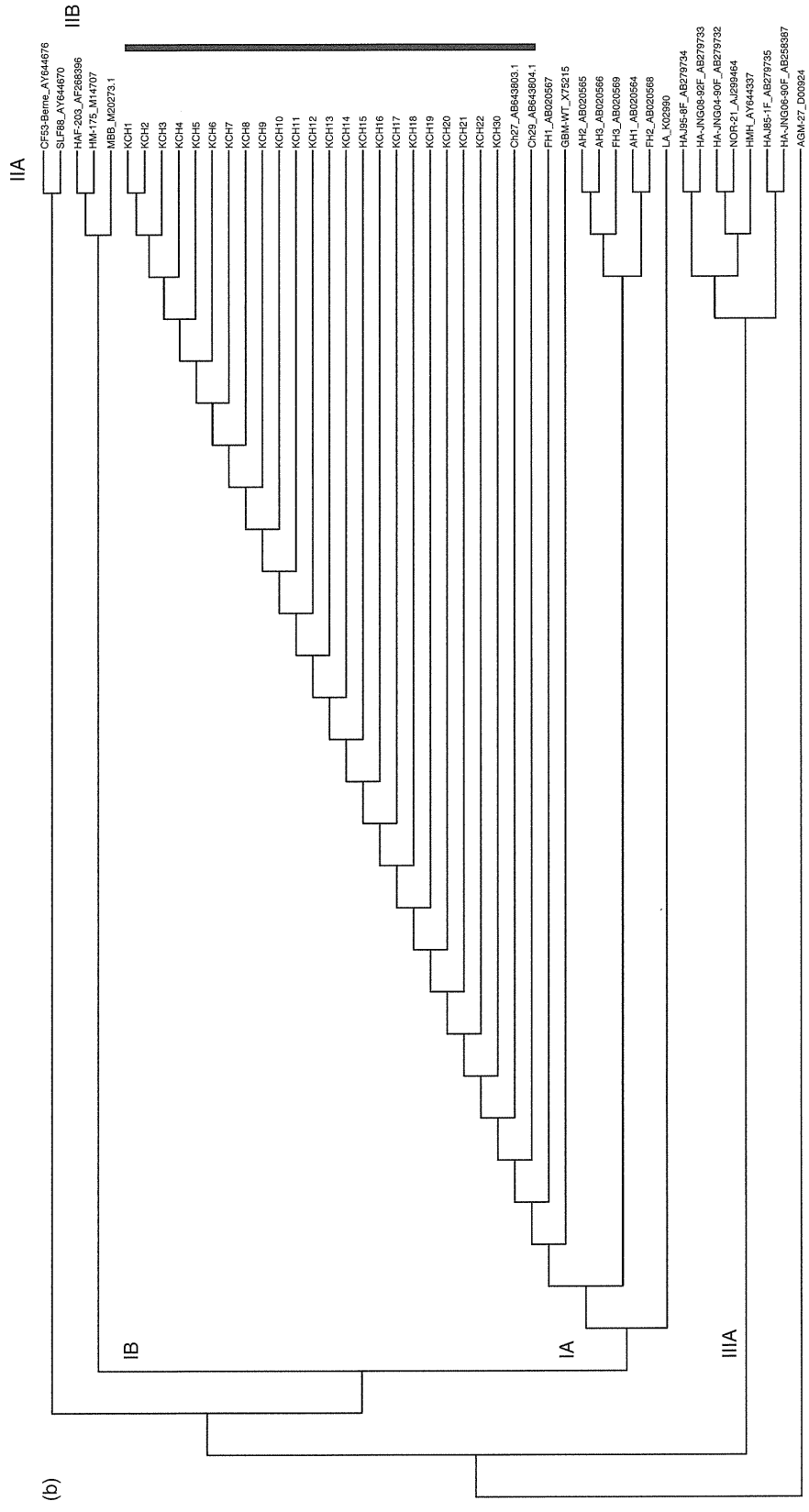

Figure 1 *Continued.*

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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Original Article

Hepatitis A, B, C and E virus markers in Chinese residing in Tokyo, Japan

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Aim: Recently, the number of foreigners living in Japan has been increasing, with the majority originating from China. It is important for us to know the prevalence of hepatitis virus markers among them, as proper medical practices and vaccinations should be prepared when seeing them and their offspring.

Methods: We examined the relationship between the prevalence of hepatitis virus markers: hepatitis B surface antigen (HBsAg), anti-HBs, anti-hepatitis C virus (HCV), anti-hepatitis A virus (HAV) and anti-hepatitis E virus immunoglobulin (Ig)G, and background such as age, birthplace and length of stay in Japan, of 568 Chinese residing in Tokyo, and also of 55 indigenous Japanese.

Results: The prevalence of HBV and HAV markers in Chinese staying in Tokyo is higher than in indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%). There were no differences in anti-HCV and anti-HEV IgG between the two groups.

Conclusion: Indigenous Japanese subjects have less immunity against HAV and HBV. The HBV carrier rate is higher in Chinese subjects, and attention should be paid to this issue in clinical practice. It might be important to control hepatitis viruses in Chinese subjects when doctors see them in Japan.

Key words: Chinese, HAV, HBV, HCV, HEV, Tokyo

INTRODUCTION

HEPATITIS A, B, C and E virus (HAV, HBV, HCV and HEV, respectively) cause acute hepatitis, and occasionally fulminant hepatitis, and HBV and HCV also lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma in Japan as well as throughout the world.[1–6] In general, the prevalence of hepatitis viruses follows a wide range of diverse patterns, being dependent on different areas and countries.[7–10]

In Japan, hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV), respectively, were detected in 0.63% and 0.49% in sera from first-time blood donors aged 16–64 years.[5] It was also reported that only fewer than 50% of people have immunity against HAV, estimated from anti-HAV prevalence.[11] Of qualified blood donors, 3.4% were regarded as positive for anti-HEV immunoglobulin (Ig)G.[12] On the other hand, as an example, in China, the prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was reported to be 5.84%, 41.3%, 0.58%, 72.8% and 17.66%, respectively, although this prevalence pattern is well known to differ among different areas in China.[13]

By the end of 2009, 2 186 121 foreigners were living in Japan, and the largest proportion, 31.6%, was born in China, Taiwan and Hong Kong.[14] With increasing numbers of foreigners living in Japan, we will have more opportunities to see them as patients in clinical practice. It is important for us to know, among other things, their prevalence of hepatitis virus markers, as vaccinations and appropriate medical practices should be provided when seeing them and their offspring.

Therefore, in the present study, we examined the relationship between the prevalence of hepatitis virus markers and background such as age, birthplace and their duration of domicile in Japan among Chinese living in Tokyo, Japan.

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METHODS

Study subjects and serum collection

THE SUBJECTS IN this study were 623 consecutive outpatients attending the Kyowa Clinic in Tokyo. Of these patients, 568 (80%) were Chinese who were staying in Japan. The others were 55 indigenous Japanese, and all patients were seen between August 2010 and January 2011 (Table 1). The duration of the Chinese subjects' stay in Japan was 103 ± 76 days. There were no differences in age, sex or alanine aminotransferase (ALT) levels between the two groups, but the platelet counts of the Chinese were lower than those of the Japanese subjects (Table 1). Chinese patients were divided into eight groups according to their birthplace in China, as follows: 32, nine, one, 180, 331, 10, zero and five were from North China (Beijing, Tianjing, Hebei, Shanxi and Inner Mongolia), Central China (Henan, Hunan and Hubei), South China (Guangdong, Guangxi and Hainan), East China (Shanghai, Jiangsu, Zhejiang, Fujian, Shandong, Jiangxi and Anhui), North-East China (Heilongjiang, Liaoning and Jilin), South-West China (Sichuan, Chongqing, Yunnan, Guizhou and Tibet), North-West China (Xinjiang, Shanxi, Gansu, Ningxia and Qinghai) and Hong Kong, Macao and Taiwan, respectively. All patients were adults and the most common symptoms were other than liver diseases. Family history of liver diseases, history of surgeries, blood transfusion, drug abuse and tattoo were investigated from patients' interviews and medical records.

Serological diagnosis

All patients were screened by serological tools for hepatitis A, B, C and E virus infections. HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were tested in each sample by magnetizing particle aggregation (MAT; Shino-Test Tokyo, Japan), particle agglutination (PA; Fujirebio, Tokyo, Japan), chemiluminescent enzyme immunoassay (CLEIA; Fujirebio), chemiluminescent immunoassay (CLIA; Abbott Laboratories, North Chicago, IL, USA) and enzyme immune assay (EIA; Institute of Immunology, Tokyo, Japan), respectively. A positive reaction was indicated when the cut-off index (COI) exceeded 1.0 in anti-HCV, anti-HAV and anti-HEV IgG. The lower detection limit for HBsAg tested by MAT was 8 IU/mL, corresponding to approximately 10 COI measured by CLEIA method. The lower detection limit for anti-HBs examined by PA corresponded to 30 mIU/mL measured by CLEIA.

Hepatitis B virus genotype of patient sera was determined by ELISA (Institute of Immunology) based on the methodology described by Usuda *et al.*[7,15] Informed consent was obtained at the time of blood sampling from each patient included in the study. This study was approved by the ethics committee of Chiba University, Japan, and that of Kyowa Clinic, and conformed to the Declaration of Helsinki. Sera were collected as part of clinical practice and stored at −20°C until laboratory testing was performed.

Table 1 Background of study patients and hepatitis virus markers

	Total subjects	Chinese staying in Japan	Indigenous Japanese	*P*-value*
No. of patients	623	568	55	
Age, years	47 ± 14	47 ± 14	45 ± 15	NS
Sex (M/F)	292/331	264/304	28/27	NS
ALT (IU/L)	26 ± 44	26 ± 46	25 ± 19	NS
Platelets ($\times 10^4$/mm^3)	22 ± 5.4	22 ± 5.8	24 ± 5.1	0.013
HBsAg (+/−)	63/556	62/502	1/54	0.031
Anti-HBs (+/−)	258/362	259/305	5/50	<0.0001
Anti-HCV (+/−)	11/607	10/553	1/54	NS
Anti-HAV (+/−)	518/100	510/53	8/47	<0.0001
Anti-HEV IgG (+/−)	128/493	120/446	8/47	NS

**P*-value between Chinese subjects staying in Japan and indigenous Japanese subjects.
+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

Data analysis

Data were expressed as mean ± standard deviation. Differences were evaluated by Student *t*-test or χ^2-test. $P < 0.05$ was considered statistically significant. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the Excel statistics program for Windows ver. 7 (SSRI, Tokyo, Japan) and DA Stats software (O. Nagata, Nifty Serve: PAF01644).

RESULTS

Chinese subjects staying in Tokyo have more immunity against HAV and HBV

AMONG 623 STUDY subjects, 549 (88%) had normal ALT levels (ALT ≤40 IU/L). HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were determined in 619 (99%), 620 (99%), 618 (99%), 618 (99%) and 621 (99%), respectively. The overall prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG in the present study was 10%, 45%, 1.7%, 90% and 21%, respectively (Table 1). The prevalence of HBV and HAV markers of Chinese staying in Japan was higher than that of indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%), but there were no differences in anti-HCV and anti-HEV IgG between the two groups (Table 1). These results suggest that Chinese have more immunity against HAV and HBV than indigenous Japanese. A greater proportion of Chinese subjects was HBsAg positive compared to indigenous Japanese subjects.

Sex differences in hepatitis virus markers

Next, we examined the sex differences in the two groups (Table 2). There were no sex differences concerning HBsAg, anti-HBs, anti-HCV and anti-HAV in each of the two groups. Among Chinese subjects staying in Japan, men with anti-HEV IgG were predominant, but this predominance was not seen in the Japanese group (Table 2).

Age differences in relation to prevalence of hepatitis virus markers

Among Chinese subjects staying in Japan, the HBsAg positive rate under 30 years was higher than in those in their 30s ($P = 0.0018$), 40s ($P < 0.0001$) and over 50 years ($P < 0.0001$), and the HBsAg positive rate of those in their 30s was also higher than those over 50 years ($P < 0.053$) (Fig. 1a). Only one HBsAg positive Japanese subject was a 53-year-old man. There were no differences in each age group between Chinese and Japanese subjects (Fig. 1a).

Positive rates of anti-HBs in those under 30 years, in their 30s, 40s and over 50 years in Chinese subjects staying in Japan were higher than those in indigenous Japanese ($P = 0.0037$, 0.0020, 0.0065 and 0.0034,

Table 2 Background of study patients and hepatitis virus markers according to sex differences

	Chinese staying in Japan			Indigenous Japanese		
	Male ($n = 264$)	*P*	Female ($n = 304$)	Male ($n = 28$)	*P*	Female ($n = 27$)
Age, years	47 ± 14	NS	47 ± 13	46 ± 13	NS	43 ± 18
ALT (IU/L)	29 ± 43	NS	23 ± 49	31 ± 19	0.0083	18 ± 16
Platelets ($\times 10^4/mm^3$)	21 ± 5.7	<0.0001	23 ± 5.9	24 ± 5.4	NS	24 ± 4.9
Length of stay (days)	103 ± 78	NS	104 ± 75			
Family of liver diseases (+/−)	13/248	NS	24/273	2/26	NS	0/27
Transfusion (+/−)	5/259	NS	6/297	1/27	NS	1/26
Surgery (+/−)	24/240	0.017	49/254	4/24		4/23
Drug abuse (+/−)	0/264	NA	0/303	0/28	NA	0/27
Tattoo (+/−)	0/264	NS	1/302	0/28	NA	0/27
HBsAg (+/−)	30/232	NS	32/270	1/27	NS	0/27
Anti-HBs (+/−)	119/144	NS	140/162	2/26	NS	3/24
Anti-HCV (+/−)	3/259	NS	7/292	1/27	NS	0/27
Anti-HAV (+/−)	235/28	NS	275/25	3/25	NS	5/22
Anti-HEV IgG (+/−)	68/195	0.015	52/251	2/26	NS	6/21

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

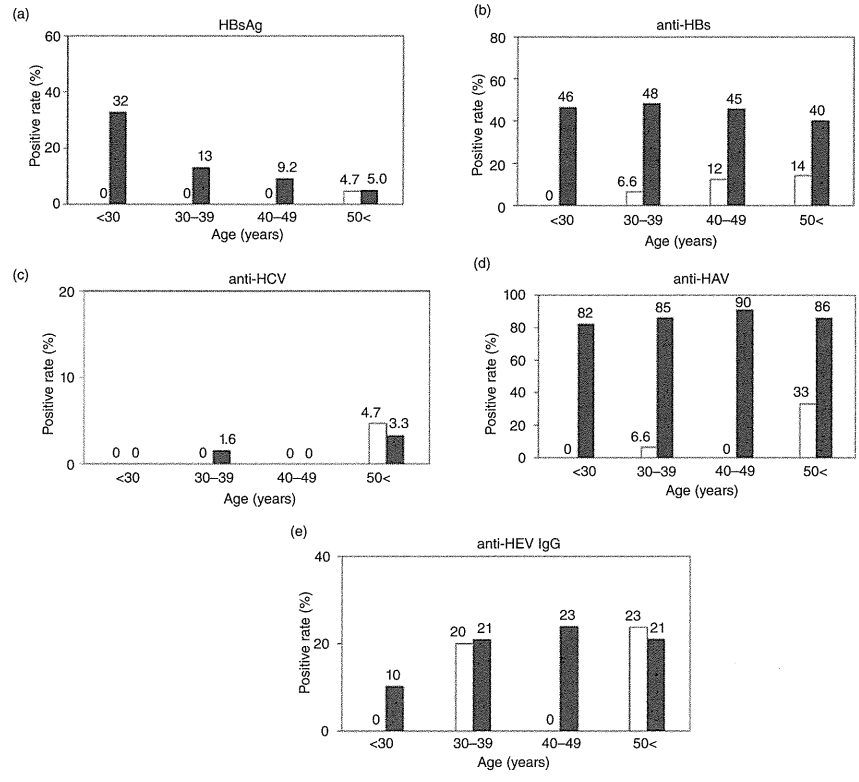

Figure 1 Hepatitis virus markers in study subjects according to age. (a) Hepatitis B surface antigen (HBsAg); (b) anti-HBs antibody; (c) anti-hepatitis C virus (HCV) antibody; (d) anti-hepatitis A virus (HAV) antibody; and (e) anti-hepatitis E virus (HEV) immunoglobulin (Ig)G antibody. White bar, indigenous Japanese; black bar, Chinese staying in Japan. Positive rates (%) are indicated.

respectively). There were no differences between each age group of Chinese subjects and also no differences between each age group of indigenous Japanese subjects in the present study (Fig. 1b).

There were no significant differences in anti-HCV positive rates in each age group of Chinese subjects or in each age group of Japanese indigenous subjects. There were also no significant differences in anti-HCV positive rates of each age group between Chinese and Japanese groups (Fig. 1c).

The positive rate of anti-HAV in subjects under 30 years was lower than in those over 50 years in the indigenous Japanese group ($P = 0.030$) (Fig. 1d). There were no differences among the respective age groups in Chinese subjects. Among the same age groups, the positive rates of anti-HAV in Chinese subjects were higher than those in indigenous Japanese subjects ($P < 0.0001$, each) (Fig. 1d).

There were no significant differences of anti-HEV IgG positive rates in each age group of Japanese indigenous subjects. As for Chinese subjects, there was a difference in anti-HEV IgG positive rate between the groups under 30 years and those in their 40s ($P = 0.029$). There were no significant differences in anti-HEV positive rates of each age group between the Chinese and Japanese groups (Fig. 1e).

Prevalence of hepatitis virus markers in Chinese subjects according to birthplace

Next, we examined the prevalence of hepatitis virus markers in Chinese subjects according to birthplace (Tables 3,4). Although the number of study subjects was limited, the prevalence of anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was quite similar in Chinese subjects independent of their place of birth. Interestingly, the HBsAg carrier rate was higher in the patients from East China than in those from North-East China (Table 4, $P < 0.0001$). In the background between these two areas (Table 3), young age, male dominance and longer term stays from East China were more than those from North-East China ($P < 0.0001$, $P = 0.029$ and $P < 0.0001$, respectively). As for risk factors of hepatitis virus infection, a history of surgery was seen more frequently in those from North China ($P = 0.023$, Table 3). We determined HBV genotypes in 57 of 63 HBsAg positive subjects and revealed that HBV genotype B was more common in those from East China than in those from North-East China ($P = 0.013$, Table 4). We also

Table 3 Background and risk factors of hepatitis virus infection in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Age, years	53 ± 13	40 ± 14	39	41 ± 12	50 ± 13	42 ± 11	60 ± 13	45 ± 15
Sex (M/F)	14/18	8/1	0/1	95/85	140/191	4/6	3/2	28/27
ALT (IU/L)	29 ± 53	22 ± 12	10	26 ± 46	26 ± 47	20 ± 9.8	20 ± 9.8	25 ± 19
Platelets ($\times 10^4/mm^3$)	22 ± 5.2	22 ± 7.3	27	22 ± 5.5	22 ± 6	21 ± 6.5	21 ± 6.5	24 ± 5
Length of their stay (days)	137 ± 90	66 ± 71	46	80 ± 68	113 ± 75	94 ± 76	192 ± 103	
Family history of liver diseases (+/−)	2/30	0/9	0/1	14/162	21/304	0/10	0/5	2/53
History (+/−)								
Transfusion	0/32	1/8	0/1	3/177	6/324	1/9	0/5	2/53
Surgery	7/25	1/8	0/1	14/166	50/280	1/9	0/5	8/43
Drug abuse	0/32	0/9	0/1	0/180	0/330	0/10	0/5	0/54
Tattoo	0/32	0/9	0/1	0/18	1/329	0/10	0/5	0/5

+, Positive; −, negative; ALT, alanine aminotransferase.

Table 4 Hepatitis virus markers in study subjects according to birthplace in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Hepatitis virus markers: +/−								
HBsAg	5/27	1/8	0/1	37/142	17/311	1/9	1/4	1/54
HBV genotype (B/C)	1/4	0/1	0/0	12/20	0/16	1/0	0/1	0/1
Anti-HBs	10/22	4/5	0/1	83/95	158/172	5/5	0/5	5/50
Anti-HCV	0/32	0/9	0/1	3/177	7/320	0/10	0/5	1/54
Anti-HAV	25/7	8/1	1/0	147/29	315/15	9/1	5/0	8/47
Anti-HEV IgG	10/22	2/7	0/1	41/138	60/270	5/5	2/3	8/47

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

determined HCV genotype by direct sequencing HCV core region in two cases from North-East China and one indigenous Japanese, with all three showing genotype 2a (data not shown).

DISCUSSION

THE PRESENT STUDY revealed that Chinese subjects staying in Tokyo have more immunity against HAV and HBV than indigenous Japanese subjects and that approximately 10% of Chinese subjects staying in Tokyo are HBsAg carriers. The HBsAg carrier rate seems to be higher in patients from East China than those from North-East China (Table 4). This might be useful to see the Chinese patients from these areas in clinical practices. There have been several reports about the HBsAg carrier rates of East China[16,17] and North-East China.[13,18] Hepatitis B vaccine was first recommended for routine vaccination of infants in China in 1992.[19] Because of high vaccine prices and user fees charged to parents by local health departments for vaccine purchase and administration, until 2002, infant hepatitis vaccination occurred primarily in large cities of wealthier eastern provinces.[19] In the 2004 survey, estimated vaccine coverage was higher in East China than in North-East China.[19] It is a possible reason why the difference in HBsAg prevalence between these areas was observed in the present study. We do not know the exact reason for this difference, and we consider that further studies will be needed.

Several medical institutes at which mostly Chinese gather have existed in Japan. Kyowa Clinic, one such facility, is located in Okachimachi, Tokyo, an important juncture of traffic networks. Because Japanese newspapers advertise this clinic, and the doctor sees the patients using both Chinese and Japanese languages, this outpatient-only clinic is known to Chinese subjects' staying in Japan. The patients of this clinic consist of 90% Chinese and 10% Japanese. Most of the less than 65-year-old male Chinese patients are cooks in Chinese restaurants, interior decorators and students, most of the less than 65-year-old female Chinese patients are housewives and students, and most of the Chinese patients 65 years or older are unemployed. Most of the Japanese patients are employees of small businesses and residents near this clinic. The present study has an authentic potential in terms of the clinical practice being different from previous studies, such as those concerning blood donors, in spite of the population selection of the present study seeming unnatural. Although selection biasness of patients with Japanese and Chinese background might exist, we included these Japanese patients, who come to the same clinic as controls to compare with Chinese in the present study. Although the number of hepatitis cases is decreasing, hepatitis is still a major health problem in Japan.[5,8,10,20] In China as well, hepatitis is a major public health burden.[13] As more foreigners take up residence in Japan, we are likely to see more Chinese patients in clinical practice, as approximately one-third of such foreigners come from China.[14] The present study might provide us with important information.

The number of cases of adult hepatitis A has been decreasing in Japan in accordance with socioeconomic and sanitation improvements.[9,10] In 1986, a national prevention program was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg).[21] In 1995, this was extended to babies born to HBeAg negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[21,22] Because there are no universal vaccination programs against HAV or HBV in Japan, HAV and HBV infections are still seen as important issues.[10]

Hepatitis A virus is a single-stranded RNA virus and usually spreads via the fecal–oral route, similarly to HEV. Of interest is the fact that the distribution of anti-HEV IgG among Chinese subjects staying in Tokyo is similar to that of indigenous Japanese subjects, although the prevalence of anti-HAV in Chinese staying in Tokyo is higher than that of indigenous Japanese (Fig. 1d,e). This may be related to differences in infectious routes of transmission of these two viruses or in differences of HAV vaccination between the two countries, as a certain number of HAV-vaccinated young Chinese adults seemed to be included in the present study.[23,24] In any event, a large proportion of Chinese adults seem to be protected by latent infection or immunization against HAV.[13,25]

The positive rate of anti-HEV IgG in the Kanto metropolitan area of Japan was previously reported as 8.6% in qualified blood donors[12] and 6.5% in health checkups.[26] In general, the positive rate of anti-HEV IgG in China has been recognized to be higher than that in Japan,[27] and the same report described a positive rate of anti-HEV IgG of more than 20% in indigenous Japanese aged 70 years or older. In the present study, the mean age of indigenous Japanese was 45 years (Table 1), and anti-HEV IgG positive indigenous Japanese numbered three in their 30s, one in their 50s, three in their 60s and one in their 70s, with the anti-HEV IgG positive rate being higher than in previous reports.[12,25,27] In most areas of

Japan, the positive rate of anti-HEV IgG in males was higher than that in females. We do not know the exact reasons why our anti-HEV IgG patients were not male-dominant (Table 2). The population selection of the present study may not be unbiased. However, as it seems that Japanese females have in recent years developed a taste for broiled pig innards on skewers compared to before, the potential of HEV infection is likely to grow, and greater attention should also be paid to Japanese females.

As HCV is a blood-borne RNA virus, and blood screening for HCV is a standard procedure in Japan, the distribution of anti-HCV of indigenous Japanese subjects is similar to that of Chinese subjects staying in Tokyo. HBV is an incomplete double-stranded DNA virus that infects through blood products and sexual contact as well as mother-to-baby transmission. The differences in the distribution of anti-HBs may be dependent on a different HBV vaccination status or different past HBV infection.

In conclusion, indigenous Japanese subjects have less immunity against HAV and HBV. As the HBV carrier rate is higher in Chinese subjects, this should receive some attention in clinical practice, and it might be important to control hepatitis viruses in Chinese subjects when they are seen by doctors in Japan.

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