

Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology* 20, 23–31.

Bradley, D. W. & Balayan, M. S. (1988). Virus of enterically transmitted non-A, non-B hepatitis. *Lancet* 331, 819.

Emerson, S. U. & Purcell, R. H. (2003). Hepatitis E virus. *Rev Med Virol* 13, 145–154.

Emerson, S. U., Anderson, D., Arankalle, A., Meng, X. J., Purdy, M., Schlauder, G. G. & Tsarev, S. A. (2005). *Hepevirus*. In *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses*, pp. 853–857. Edited by M. A. M. C. M. Fauquet, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier/Academic Press.

Favorov, M. O., Kosoy, M. Y., Tsarev, S. A., Childs, J. E. & Margolis, H. S. (2000). Prevalence of antibody to hepatitis E virus among rodents in the United States. *J Infect Dis* 181, 449–455.

Guu, T. S., Liu, Z., Ye, Q., Mata, D. A., Li, K., Yin, C., Zhang, J. & Tao, Y. J. (2009). Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. *Proc Natl Acad Sci U S A* 106, 12992–12997.

Hirano, M., Ding, X., Li, T. C., Takeda, N., Kawabata, H., Koizumi, N., Kadosaka, T., Goto, I., Masuzawa, T. & other authors (2003). Evidence for widespread infection of hepatitis E virus among wild rats in Japan. *Hepatol Res* 27, 1–5.

Huang, C. C., Nguyen, D., Fernandez, J., Yun, K. Y., Fry, K. E., Bradley, D. W., Tam, A. W. & Reyes, G. R. (1992). Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). *Virology* 191, 550–558.

Jameel, S. (1999). Molecular biology and pathogenesis of hepatitis E virus. *Expert Rev Mol Med* 1999, 1–16.

Johne, R., Heckel, G., Plenge-Bönig, A., Kindler, E., Maresch, C., Reetz, J., Schielke, A. & Ulrich, R. G. (2010a). Novel hepatitis E virus genotype in Norway rats, Germany. *Emerg Infect Dis* 16, 1452–1455.

Johne, R., Plenge-Bönig, A., Hess, M., Ulrich, R. G., Reetz, J. & Schielke, A. (2010b). Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. *J Gen Virol* 91, 750–758.

Kabrane-Lazizi, Y., Fine, J. B., Elm, J., Glass, G. E., Higa, H., Diwan, A., Gibbs, C. J., Jr, Meng, X.-J., Emerson, S. U. & Purcell, R. H. (1999). Evidence for widespread infection of wild rats with hepatitis E virus in the United States. *Am J Trop Med Hyg* 61, 331–335.

Korkaya, H., Jameel, S., Gupta, D., Tyagi, S., Kumar, R., Zafrullah, M., Mazumdar, M., Lal, S. K., Xiaofang, L. & other authors (2001). The ORF3 protein of hepatitis E virus binds to Src homology 3 domains and activates MAPK. *J Biol Chem* 276, 42389–42400.

Li, T. C., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M. A., Uchida, T., Takeda, N. & Miyamura, T. (1997). Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol* 71, 7207–7213.

Li, T.-C., Zhang, J., Shinzawa, H., Ishibashi, M., Sata, M., Mast, E. E., Kim, K., Miyamura, T. & Takeda, N. (2000). Empty virus-like

particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. *J Med Virol* 62, 327–333.

Li, T.-C., Chijiwa, K., Sera, N., Ishibashi, T., Etoh, Y., Shinohara, Y., Kurata, Y., Ishida, M., Sakamoto, S. & other authors (2005a). Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis* 11, 1958–1960.

Li, T.-C., Takeda, N., Miyamura, T., Matsuura, Y., Wang, J. C., Engvall, H., Hammar, L., Xing, L. & Cheng, R. H. (2005b). Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. *J Virol* 79, 12999–13006.

Meng, X.-J. (2010). Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol* 140, 256–265.

Meng, X.-J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. (1997). A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci U S A* 94, 9860–9865.

Takahashi, K., Iwata, K., Watanabe, N., Hatahara, T., Ohta, Y., Baba, K. & Mishiro, S. (2001). Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. *Virology* 287, 9–12.

Takahashi, K., Kitajima, N., Abe, N. & Mishiro, S. (2004). Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. *Virology* 330, 501–505.

Tei, S., Kitajima, N., Takahashi, K. & Mishiro, S. (2003). Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* 362, 371–373.

Wang, Y., Zhang, H., Ling, R., Li, H. & Harrison, T. J. (2000). The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. *J Gen Virol* 81, 1675–1686.

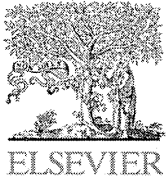
Wickham, T. J. & Nemerow, G. R. (1993). Optimization of growth methods and recombinant protein production in BTI-Tn-5B1-4 insect cells using the baculovirus expression system. *Biotechnol Prog* 9, 25–30.

Xing, L., Li, T.-C., Mayazaki, N., Simon, M. N., Wall, J. S., Moore, M., Wang, C.-Y., Takeda, N., Wakita, T. & other authors (2010). Structure of hepatitis E virion-sized particle reveals an RNA-dependent viral assembly pathway. *J Biol Chem* 285, 33175–33183.

Yamashita, T., Mori, Y., Miyazaki, N., Cheng, R. H., Yoshimura, M., Unno, H., Shima, R., Moriishi, K., Tsukihara, T. & other authors (2009). Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. *Proc Natl Acad Sci U S A* 106, 12986–12991.

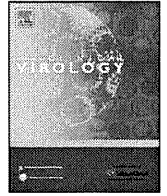
Zafrullah, M., Ozdener, M. H., Panda, S. K. & Jameel, S. (1997). The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. *J Virol* 71, 9045–9053.

Zhao, C., Ma, Z., Harrison, T. J., Feng, R., Zhang, C., Qiao, Z., Fan, J., Ma, H., Li, M. & other authors (2009). A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. *J Med Virol* 81, 1371–1379.



Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

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

Koji Ishii^{a,*}, Tomoko Kiyohara^a, Sayaka Yoshizaki^a, Takaji Wakita^a, Tomoe Shimada^b, Naomi Nakamura^b, Kazutoshi Nakashima^b, Yuki Tada^b, Mamoru Noda^c

^a Department of Virology II, National Institute of Infectious Diseases, Japan

^b Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Japan

^c Division of Biomedical Food Research, National Institute of Health Sciences, Japan

ARTICLE INFO

Article history:

Received 8 October 2011

Received in revised form

16 November 2011

Accepted 21 November 2011

Keywords:

Hepatitis A virus

Diffuse outbreak

Phylogenetic analysis

Genotype

ABSTRACT

Background: Hepatitis A virus (HAV) is still one of the most common causative agents of acute hepatitis in Japan. Although a relatively small number of annual acute hepatitis A cases (approximately 100–150, 0.78–1.17 per million) were recently reported, a larger number of cases (346, 2.71 per million) were reported in 2010.

Objectives: To investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses.

Study design: HAV specimens were obtained from 61 cases from 22 different prefectures. These viral specimens were genotyped by PCR amplification and sequencing of the VP1/2A region of HAV genome.

Results: Phylogenetic analysis revealed that 61 HAV strains could be divided into three genotypes: IA (44 cases), IB (1 case) and IIIA (16 cases). The IA genotype consisted of two genomic sub-lineages. The sequences of one of the two IA sub-lineages (corresponding to 31 cases) were very similar, 26 of these 31 isolates had 100% identity. The other IA sub-lineage corresponded to strains endemic to Japan. The sequences of Japanese IIIA strains were similar to those of strains that caused a large epidemic in the Republic of Korea from 2007 to 2009.

Conclusions: The resurgence of HAV in 2010 can be attributed to importation of two newly emerged HAV genotypes.

© 2011 Elsevier B.V. All rights reserved.

1. Background

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

tion), Madagascar and the USA,^{1,5,9–12} and subgenotype IIIB was responsible for some cases of HAV infection in Denmark and Japan.^{1,10,11,13}

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

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

* Corresponding author at: Department of Virology II, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. Tel.: +81 42 848 7037; fax: +81 42 561 4729.

E-mail address: kishii@nih.go.jp (K. Ishii).

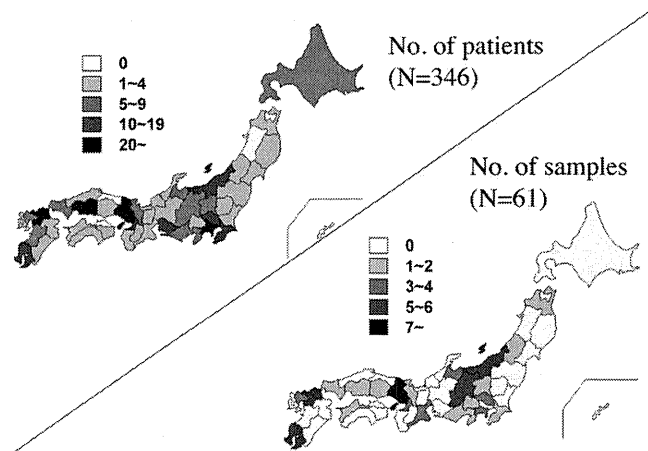


Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

2. Objectives

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

3. Study design

3.1. Data collection

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

3.2. RNA extraction, RT-PCR and phylogenetic analysis

A 10% fecal suspension (wt/vol) was prepared with phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 10,000 × g for 10 min. Viral RNA was extracted from the fecal suspension or sera by using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT) was performed with the SuperScript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Seven microliters of the purified RNA was added to a reaction mixture (final volume, 20 μl) containing 50 pmol of random hexamer, 25 mM MgCl₂ buffer, 10 mM deoxynucleotide triphosphates, 10× RT buffer, 0.1 M dithiothreitol, and 200 U SuperScript III RT. The mixture was incubated at 42 °C for 1 h, after which 10 U of RNase H was added at 37 °C for 20 min.

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

HAV-2799 (5'-ATTCAGATTAGACTGCCTTGGA-3')
 HAV-2907 (5'-GCAAATTACAATCATTCTGATGA-3')
 HAV-3162 (5'-CTTCYTGACCATCTKARTCTTTG-3')
 HAV-3273 (5'-CCAAGAAACCTTCATTATTTTCATG-3')

PCR was carried out using the HAV-2799 and HAV-3273 primer pair, followed by nested PCR with the HAV-2907 and HAV-3162 primer pair. PCR was performed with EX-taq (Takara, Shiga, Japan) according to the manufacturer's instructions. Amplification was performed for 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C

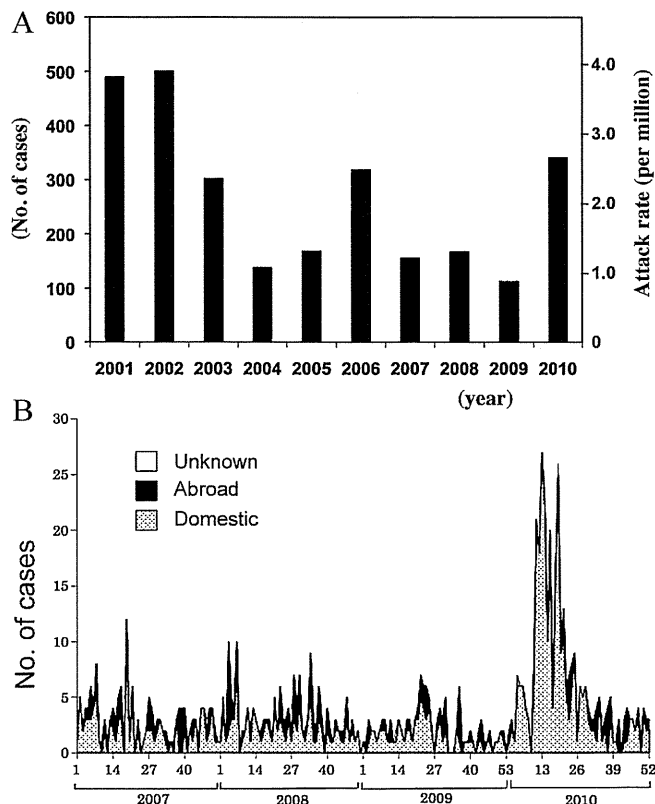


Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t=5.4 \times 10^{-7}$), 2008 ($t=5.6 \times 10^{-5}$) and 2009 ($t=1.8 \times 10^{-5}$). (B) Weekly acute hepatitis A cases from week 1 of 2007 to week 52 of 2010.

for 2 min, and a final extension at 72 °C for 15 min. Three microliters of the PCR product was used as the template for a second round of PCR amplification under the same conditions. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) and used as a template for direct sequencing.

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

4. Results

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

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

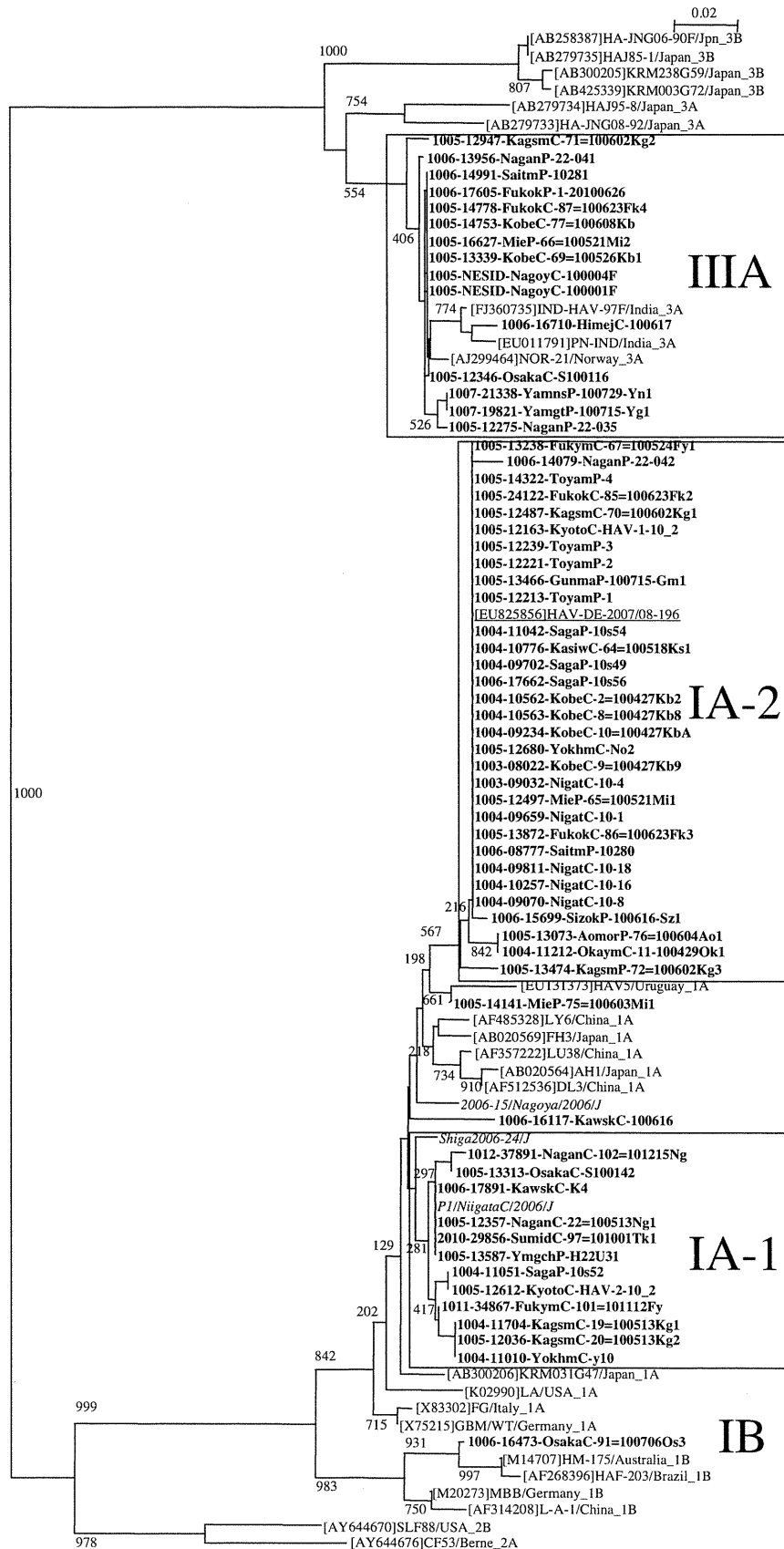


Fig. 3. Neighbor-joining phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus isolates. Reference strains are used in this study and indicated as GenBank accession numbers. Sequences of 61 isolates from this study are shown as YYMM-NESID-KKKKKKKKKK (YYMM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

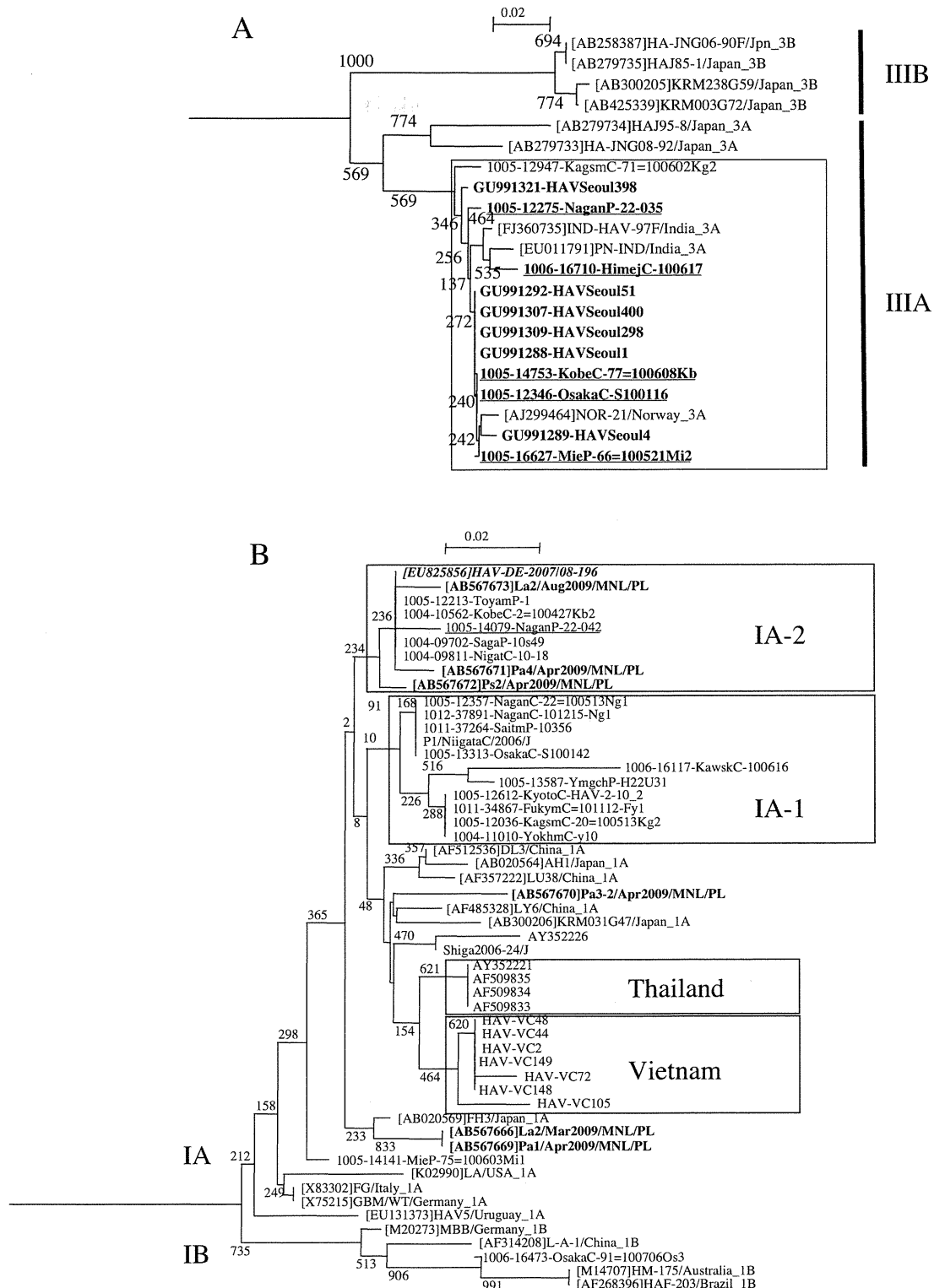


Fig. 4. (A) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from HAV strains (genotype IIIA) isolated from Japan (bold underline) and Korea (bold). Numbers at the branches show bootstrap percentages obtained after 1000 replications of bootstrap sampling. (B) Phylogenetic tree 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 bootstrap 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 Vietnam²⁹ and Thailand,³⁰ formed distinct clusters (Fig. 4B). However, caution is necessary with this result, because

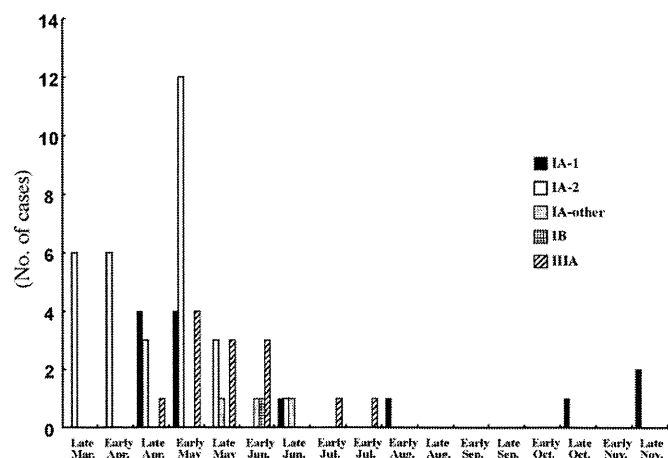


Fig. 5. Temporal distribution of HAV genotypes from late March to late November in 2010.

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.

Funding

This work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare.

Competing interests

None.

Ethical approval

Not required.

Acknowledgements

Researchers contributing to this study from local institutes of health in Japan include Rika Tsutsui, Yoko Aoki, Tetsuya Saito, Hiroyuki Tsukakoshi, Akimi Yamazaki, Saitama Institute of Public Health, Sachiko Harada, Takayuki Shinkai, Hideaki Shimizu, Shuzo Usuku, Masayuki Onuma, Hiromi Nagaoka, Tetsuya Yoshida, Yuichiro Okamura, Mayumi Obara, Shinichiro Shibata, Hajime Kusuhara, Mayumi Konno, Nobuhiro Iritani, Yoshio Iijima, Shinya Kawanishi, Keiko Sakakibara, Shoji Murao, Reiko Okamoto-Nakagawa, Yasutaka Yamashita, Nobuyuki Sera, Daisuke Kawamoto, Hisato Masumoto and Akihide Kamimura.

References

1. Robertson BH, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegl G, et al. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J Gen Virol* 1992;**73**(6):1365–77.
2. Lu L, Ching KZ, de Paula VS, Nakano T, Siegl G, Weitz M, et al. Characterization of the complete genomic sequence of genotype II hepatitis A virus (CF53/Berne isolate). *J Gen Virol* 2004;**85**(10):2943–52.
3. Nainan OV, Xia G, Vaughan G, Margolis HS. Diagnosis of hepatitis A virus infection: a molecular approach. *Clin Microbiol Rev* 2006;**19**(1):63–79.
4. Costa-Mattioli M, Ferre V, Monpoeo S, Garcia L, Colina R, Billaudel S, et al. Genetic variability of hepatitis A virus in South America reveals heterogeneity and co-circulation during epidemic outbreaks. *J Gen Virol* 2001;**82**(11):2647–52.
5. Costa-Mattioli M, Monpoeo S, Schvoerer C, Besse B, Aleman MH, Billaudel S, et al. Genetic analysis of hepatitis A virus outbreak in France confirms the co-circulation of subgenotypes Ia, Ib and reveals a new genetic lineage. *J Med Virol* 2001;**65**(2):233–40.
6. Cristina J, Costa-Mattioli M. Genetic variability and molecular evolution of hepatitis A virus. *Virus Res* 2007;**127**(2):151–7.
7. Pinto RM, Alegre D, Dominguez A, El-Senousy WM, Sanchez G, Villena C, et al. Hepatitis A virus in urban sewage from two Mediterranean countries. *Epidemiol Infect* 2007;**135**(2):270–3.
8. Normann A, Badur S, Onel D, Kilic A, Sidal M, Larouze B, et al. Acute hepatitis A virus infection in Turkey. *J Med Virol* 2008;**80**(5):785–90.
9. Pina S, Buti M, Jardi R, Clemente-Casares P, Jofre J, Girones R. Genetic analysis of hepatitis A virus strains recovered from the environment and from patients with acute hepatitis. *J Gen Virol* 2001;**82**(12):2955–63.
10. Mitsui T, Tsukamoto Y, Hirose A, Suzuki S, Yamazaki C, Masuko K, et al. Distinct changing profiles of hepatitis A and E virus infection among patients with acute hepatitis, patients on maintenance hemodialysis and healthy individuals in Japan. *J Med Virol* 2006;**78**(8):1015–24.
11. Endo K, Inoue J, Takahashi M, Mitsui T, Masuko K, Akahane Y, et al. Analysis of the full-length genome of a subgenotype IIIB hepatitis A virus isolate: primers for broadly reactive PCR and genotypic analysis. *J Med Virol* 2007;**79**(1):8–17.
12. Yoon YK, Chun BC, Lee HK, Seo YS, Shin JH, Hong YS, et al. Epidemiological and genetic analysis of a sustained community-wide outbreak of hepatitis A in the Republic of Korea 2008: a hospital-based case-control study. *J Clin Virol* 2009;**46**(2):184–8.
13. Totsuka A, Moritsugu Y. Hepatitis A vaccine development in Japan. In: Nishioka K, Suzuki H, Mishiuro S, Oda T, editors. *Viral hepatitis and liver disease*. Tokyo: Springer-Verlag; 1994. p. 509–13.
14. Wasley A, Fiore A, Bell BP. Hepatitis A in the era of vaccination. *Epidemiol Rev* 2006;**28**:101–11.
15. Fiore AE. Hepatitis A transmitted by food. *Clin Infect Dis* 2004;**38**(5):705–15.
16. Mahoney FJ, Farley TA, Kelso KY, Wilson SA, Horan JM, McFarland LM. An outbreak of hepatitis A associated with swimming in a public pool. *J Infect Dis* 1992;**165**(4):613–8.
17. Zhang LJ, Wang XJ, Bai JM, Fang G, Liu LG, Zhang Y, et al. An outbreak of hepatitis A in recently vaccinated students from ice snacks made from contaminated well water. *Epidemiol Infect* 2009;**137**(3):428–33.
18. Urbanus AT, van Houdt R, van de Laar TJ, Coutinho RA. Viral hepatitis among men who have sex with men, epidemiology and public health consequences. *Euro Surveill* 2009;**14**(47).
19. Thompson JD, Higgins DG, Gibson TJ, Clustal W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;**22**(22):4673–80.
20. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;**16**(2):111–20.
21. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;**4**(4):406–25.
22. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;**24**(8):1596–9.
23. Tekeuchi Y, Kobayashi G, Matui Y, Miyajima Y, Tanahashi S, Honma M, et al. Outbreak of food-borne infection with hepatitis A virus. *Jpn J Infect Dis* 2006;**59**(5):346.
24. Hasegawa Y, Matsumoto F, Tanaka C, Ouchi Y, Hayashi K. Outbreak of hepatitis A virus infection caused by food served in a restaurant. *Jpn J Infect Dis* 2007;**60**(2–3):150–1.
25. Kiyohara T, Ouchi Y, Hasegawa Y, Sato T, Yoneyama T, Ishii K, et al. An in-house anti-hepatitis A virus (HAV)-specific immunoglobulin M capture enzyme-linked immunosorbent assay: evaluation and application to an HAV outbreak. *J Med Virol* 2009;**81**(9):1513–6.
26. Cuthbert JA. Hepatitis A: old and new. *Clin Microbiol Rev* 2001;**14**(1):38–58.
27. Kiyohara T, Sato T, Totsuka A, Miyamura T, Ito T, Yoneyama T. Shifting seroepidemiology of hepatitis A in Japan, 1973–2003. *Microbiol Immunol* 2007;**51**(2):185–91.
28. Faber MS, Stark K, Behnke SC, Schreier E, Frank C. Epidemiology of hepatitis A virus infections, Germany, 2007–2008. *Emerg Infect Dis* 2009;**15**(11):1760–8.
29. Hoang PL, Trong KH, Tran TT, Huy TT, Abe K. Detection of hepatitis A virus RNA from children patients with acute and fulminant hepatitis of unknown etiology in Vietnam: Genomic characterization of Vietnamese HAV strain. *Pediatr Int* 2008;**50**(5):624–7.
30. Poovorawan Y, Theamboonlers A, Chongsrisawat V, Jantaradsamee P, Chutsir-omongkol S, Tangkijvanich P. Clinical features and molecular characterization of hepatitis A virus outbreak in a child care center in Thailand. *J Clin Virol* 2005;**32**(1):24–8.
31. Fujiwara K, Yokosuka O, Imazeki F, Saisho H, Saotome N, Suzuki K, et al. Analysis of the genotype-determining region of hepatitis A viral RNA in relation to disease severities. *Hepatol Res* 2003;**25**(2):124–34.
32. Takahashi H, Yotsuyanagi H, Yasuda K, Koibuchi T, Suzuki M, Kato T, et al. Molecular epidemiology of hepatitis A virus in metropolitan areas in Japan. *J Gastroenterol* 2006;**41**(10):981–6.
33. Toyoda H, Kumada T, Kiriya S, Sone Y, Tanikawa M, Hisanaga Y, et al. Clinical and molecular characteristics of hepatitis A virus infections during the years 1992–2003 in Ogaki, a centrally located city of Japan. *J Clin Virol* 2009;**44**(2):145–8.
34. Yun H, Kim S, Lee H, Byun KS, Kwon SY, Yim HJ, et al. Genetic analysis of HAV strains isolated from patients with acute hepatitis in Korea, 2005–2006. *J Med Virol* 2008;**80**(5):777–84.

Original Article

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

Tatsuo Miyamura,^{1*} Koji Ishii,^{2*} Tatsuo Kanda,^{1*} Akinobu Tawada,¹ Tadashi Sekimoto,¹ Shuang Wu,¹ Shingo Nakamoto,¹ Makoto Arai,¹ Keiichi Fujiwara,¹ Fumio Imazeki,¹ Tomoko Kiyohara,² Takaji Wakita² and Osamu Yokosuka¹

¹Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, and

²Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

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

Methods: Hepatitis A virus RNA was detected by strand-specific reverse transcription. Then, HAV VP1/2A regions were amplified by nested polymerase chain reaction (PCR).

Sequences were directly determined and phylogenetic trees were constructed for determining HAV subgenotypes.

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

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

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

INTRODUCTION

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

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

Recent studies on HAV genotypes from South Korea have shown a distinct pattern change in circulating HAV genotypes over the past 10 years.⁷ Until early 2000, almost all isolates tested had been identified as subgenotype IA.⁸ A more recent study showed that subgenotype IIIA has been predominant since 2008.⁷ In addition, a rise in the frequency of hepatitis A outbreaks has recently been observed in South Korea, our immediate neighbor, although the number of hepatitis A

Correspondence: Dr Tatsuo Kanda, Department of Medicine and Clinical Oncology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Email: kandat-cib@umin.ac.jp

*These authors equally contributed to this work.

Received 29 July 2011; revision 3 September 2011; accepted 23 September 2011.

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 μ L 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 $>38.5^{\circ}\text{C}$ 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.

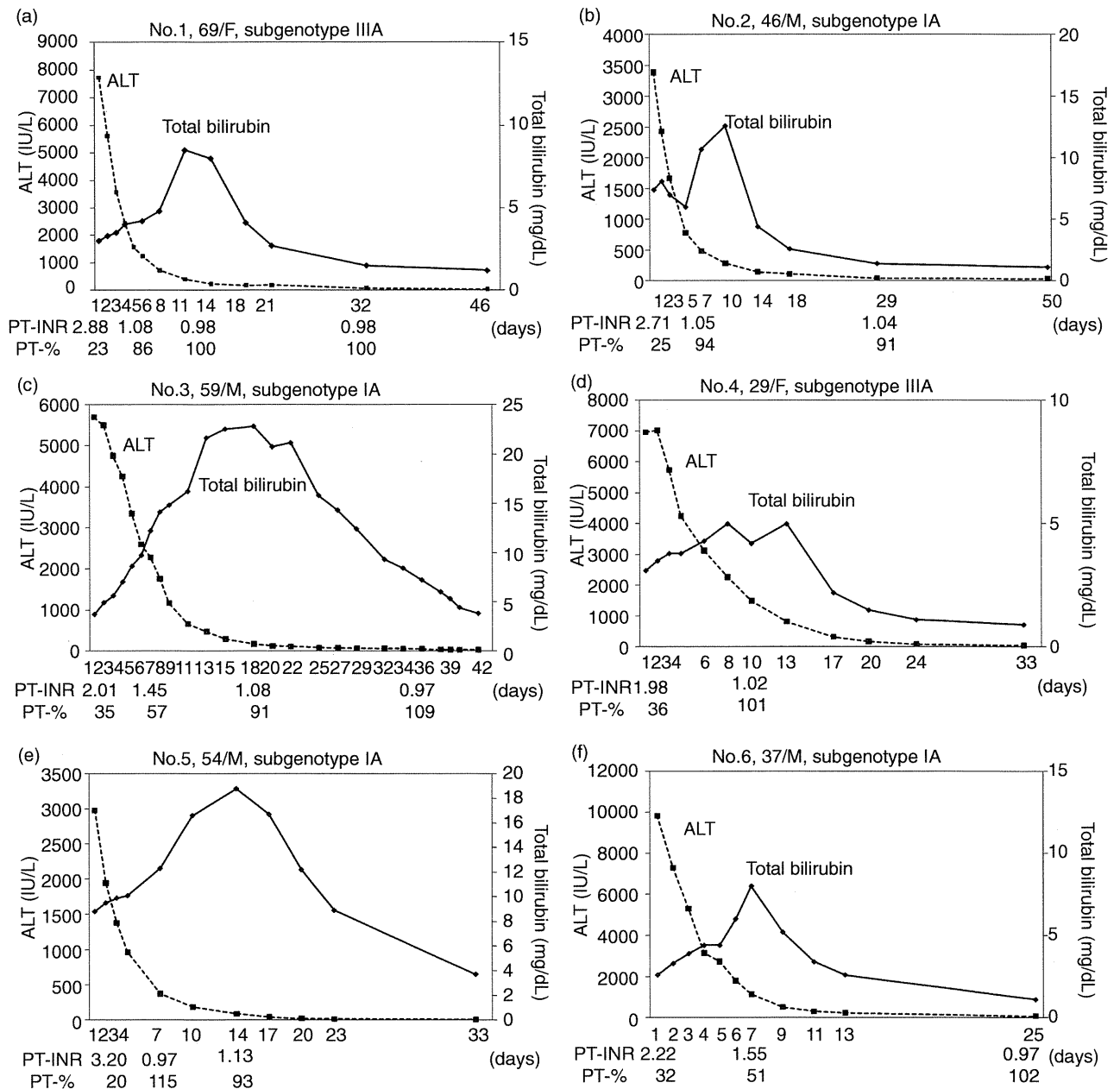


Figure 1 Clinical course of six acute liver failure patients infected with hepatitis A virus (HAV) in Japan. (a), (b), (c), (d), (e) and (f) indicates patient no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6 in Table 1, respectively. All patients presented with acute liver failure with coagulopathy but without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

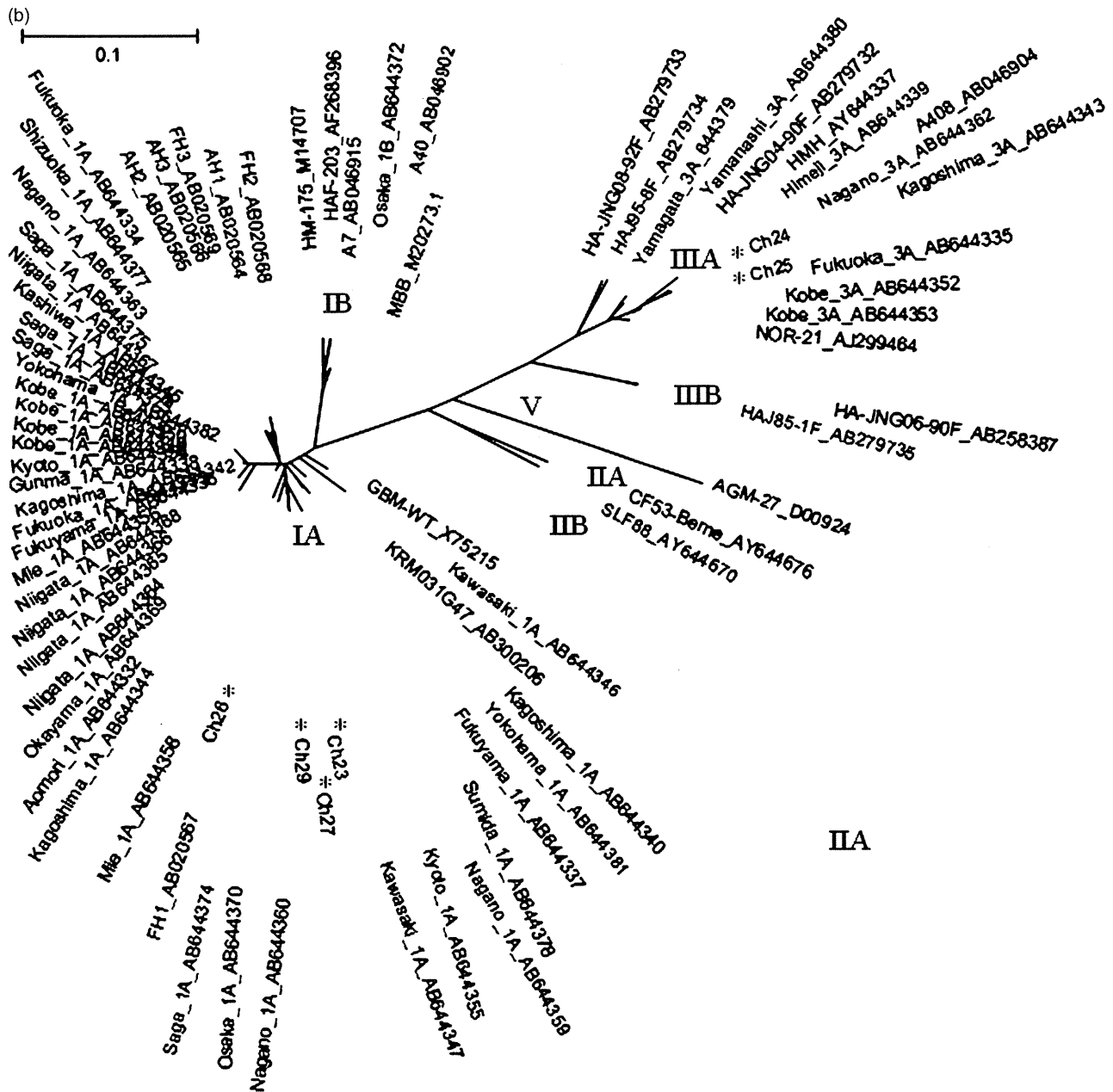


Figure 2 Continued.

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.

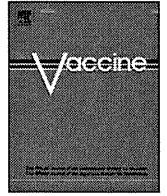
In conclusion, the current study suggested that HAV subgenotype IIIA is also associated with acute liver failure in Japan. We need to make a cautious interpretation of the relation between HAV genotypes and their disease severities.

ACKNOWLEDGMENTS

WE THANK DR Haesun Yun, Korea National Institute of Health, South Korea, and Dr Sook-Hyang Jeong, Seoul National University Bundang Hospital, South Korea for valuable discussions. This work was supported by a grant from the Japan Society of Hepatology (TK), a grant from Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (TK), and a grant from the Ministry of Health, Labour and Welfare of Japan (OY).

REFERENCES

- Daniels D, Grytdal S, Wasley A, Centers for Disease Control and Prevention (CDC). Surveillance for acute viral hepatitis – United States, 2007. *MMWR Surveill Summ* 2009; 127: 158–63.
- Kanda T, Imazeki F, Nakamoto S, Okitsu K, Fujiwara K, Yokosuka O. Internal ribosomal entry-site activities of clinical isolate-derived hepatitis A virus and inhibitory effects of amantadine. *Hepatol Res* 2010; 40: 415–23.
- Robertson BH, Jansen RW, Khanna B *et al.* Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. *J Gen Virol* 1992; 73: 1365–77.
- Fujiwara K, Yokosuka O, Imazeki F *et al.* Analysis of the genotype-determining region of hepatitis A viral RNA in relation to disease severities. *Hepatol Res* 2003; 25: 124–34.
- Takahashi H, Yotsuyanagi H, Yasuda K *et al.* Molecular epidemiology of hepatitis A virus in metropolitan areas in Japan. *J Gastroenterol* 2006; 41: 981–6.
- Toyoda H, Kumada T, Kiriya S *et al.* Clinical and molecular characteristics of hepatitis A virus infections during the years 1992–2003 in Ogaki, a centrally located city of Japan. *J Clin Virol* 2009; 44: 145–8.
- Yoon YK, Yeon JE, Kim JH *et al.* Comparative analysis of disease severity between genotypes IA and IIIA of hepatitis A virus. *J Med Virol* 2011; 83: 1308–14.
- Byun KS, Kim JH, Song KJ *et al.* Molecular epidemiology of hepatitis A virus in Korea. *J Gastroenterol Hepatol* 2001; 16: 519–24.
- Kanda T, Jeong SH, Imazeki F, Fujiwara K, Yokosuka O. Analysis of 5' nontranslated region of hepatitis A viral RNA genotype I from South Korea: comparison with disease severities. *PLoS ONE* 2010; 5: e15139.
- Franco E, Giambi C, Iaiacci R, Coppola RC, Zanetti AR. Risk groups for hepatitis A virus infection. *Vaccine* 2003; 21: 2224–33.
- National Institute of Infectious Diseases (in Japanese). [Cited 30 August 2011.] Available from URL: <http://idsc.nih.go.jp/iasr/rapid/pr3733.html>
- Zimmerman HJ, Lowry CF, Uyeyama K. Infectious hepatitis: clinical and laboratory features of 295 cases. *Am J Med Sci* 1947; 213: 395–409.
- Taylor RM, Davern T, Munoz S *et al.* Fulminant hepatitis A virus infection in the United States: incidence, prognosis, and outcomes. *Hepatology* 2006; 44: 1589–97.
- Kanda T, Yokosuka O, Imazeki F. New antiviral therapies for chronic hepatitis C. *Hepatol Int* 2010; 4: 548–61.
- Sumi H, Yokosuka O, Seki N *et al.* Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; 37: 19–26.



Production and characterization of HCV particles from serum-free culture

Daisuke Akazawa^{a,b}, Kenichi Morikawa^{b,1}, Noriaki Omi^{a,b}, Hitoshi Takahashi^{a,b,2}, Noriko Nakamura^a, Hidenori Mochizuki^a, Tomoko Date^b, Koji Ishii^b, Tetsuro Suzuki^{b,3}, Takaji Wakita^{b,*}

^a Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa, Japan

^b Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, Japan

ARTICLE INFO

Article history:

Received 26 October 2010

Received in revised form 8 April 2011

Accepted 19 April 2011

Available online 7 May 2011

Keywords:

Hepatitis C virus

Cell culture

Serum-free

Apolipoprotein

ABSTRACT

Hepatitis C virus (HCV) is a major cause of liver cancer, and it is therefore important to develop a prophylactic strategy for HCV infection. In recent years, a system for cell culture of the infectious HCV particle has been established, and the inactivated particle has potential as an antigen for vaccine development. In this study, we aimed to establish highly efficient HCV particle purification procedures using the following serum-free culture of HCV particles. First, naïve human hepatoma Huh7 cells were grown in serum-free medium that was supplemented with human-derived insulin, transferrin and sodium selenite. Then, *in vitro* transcribed JFH-1 or J6/JFH-1 chimeric HCV-RNA was transfected into the serum-free conditioned Huh7 cells. Infectious HCV was secreted into the culture supernatant with the same efficiency as that from cells cultured in FBS-containing medium. The HCV-core protein and RNA continued to be detected in the culture supernatant when the infected cells were subcultured in serum-free medium. Sucrose gradient centrifugation analyses indicated that the profiles of HCV-core, HCV-RNA and the infectivity of HCV particles were almost identical between HCV from FBS-supplemented and serum-free cultures. We further determined that anti-CD81, anti-SR-BI and anti-E2 antibodies inhibited infection by serum-free cultured HCV to a greater extent than infection by HCV from FBS-supplemented cultures. These HCV particles also differed in the level of associated apolipoproteins: the ApoE level was lower in serum-free cultured HCV. ApoB and ApoE antibody-depletion assays suggested that infection of serum-free cultured HCV was independent of ApoB and ApoE proteins. These data suggest that lipids conjugated with HCV affect infection and neutralization.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. HCV is a human pathogen that is a major cause of chronic hepatitis, liver cirrhosis and hepatic carcinoma. HCV therapy mainly involves treatment with pegylated-interferon and ribavirin; however, these agents are not very effective for patients with high titer HCV-RNA and geno-

Abbreviations: HCV, hepatitis C virus; ITS, insulin-transferrin-selenium; MOI, multiplicity of infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; VLDL, very-low-density lipoprotein.

* Corresponding author. Tel.: +81 3 5285 1111; fax: +81 3 5285 1161.

E-mail address: wakita@nih.go.jp (T. Wakita).

¹ Present address: Institute of Microbiology, University of Lausanne, and Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.

² Present address: Center for Influenza Virus Research, National Institute of Infectious Diseases, Tokyo, Japan.

³ Present address: Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan.

0264-410X/\$ – see front matter © 2011 Elsevier Ltd. All rights reserved.

doi:10.1016/j.vaccine.2011.04.069

type 1. Thus, it is necessary to develop new, more effective therapies and preventive care treatments for HCV. It was discovered that a genotype 2a strain, JFH-1, efficiently replicated in Huh7 cells [1]. Moreover, an *in vitro* culture system that generates infectious HCV has also been successfully developed using the JFH-1 genome [2–4]. Recently, it has been shown to be possible to produce various chimeric HCVs by replacement of the JFH-1 structural protein region with the same region from other strains [5]. These chimeric HCV particles are expected to lead to a HCV vaccine as well as to new pharmaceuticals.

Huh7 is a human hepatoma cell line that was established in 1982 [6]. This cell line can be cultured in serum-free medium supplemented with selenium. Serum-free culture has advantages for the simple purification and preparation of animal-origin-free virus particles. In this study, we successfully produced HCV particles in serum-free culture and compared the properties of these particles to those from FBS-supplemented cultures. Interestingly, serum-free cultured HCV was susceptible to CD81-, SR-BI- and HCV-E2-neutralizing antibodies. It was recently suggested that HCV particles associate with lipids to form viro-lipo particles [7–9], and it has also been shown that HCV particles can associate with

lipids to form exosomes [10,11]. We examined apolipoprotein association of serum-free cultured HCV. We found that this virus had a lower ApoE level than HCV from serum-supplemented cultures and that infection by this virus was apolipoprotein-independent.

2. Materials and methods

2.1. Cell culture

Huh7, Huh7.5.1 ([4], a generous gift from Dr. Francis V. Chisari), Huh7-25 and Huh7-25-CD81 [12] cell lines were cultured in 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (DMEM-10). Our previous FACS analysis indicated that Huh7-25 cells do not express CD81, and that Huh7-25-CD81 constitutively express CD81, on the cell surface [12]. For serum-free culture, the cells were conditioned and cultured in DMEM/F-12 supplemented with Insulin-Transferrin-Selenium-X (ITS) (Invitrogen, Carlsbad, CA).

2.2. Establishment of serum-free cultured cells

Sub-confluent Huh7 cells that were cultured in DMEM-10 were passaged in 10-cm dishes (Nunc, Rochester, NY) in DMEM containing 5% FBS. The cells were then sequentially passaged in DMEM containing 2, 1 and 0.5% FBS and were ultimately passaged in serum-free medium. The cells were detached for passage in serum-free culture using TrypLE Select (Invitrogen).

2.3. Cell growth assay

Cell growth was assayed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. In brief, 1×10^4 cells were seed into a 96-well culture plate (IWAKI, Tokyo, Japan) in 100 μ L of media, and 20 μ L of the assay solution was added into each well at the appropriate time. After incubation for 1 h at 37 °C, the absorbance of the solution at 490 nm was measured.

2.4. Plasmids

pJFH1 and pJ6/JFH1 were generated as previously reported [2,13].

2.5. RNA synthesis

RNA synthesis was performed as described previously [14]. Briefly, the pJFH1 and pJ6/JFH1 plasmids were digested with *Xba* I and were treated with Mung Bean nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment was then purified and was used as a template for RNA synthesis. HCV-RNA was synthesized *in vitro* using a MEGAscript[™] T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

2.6. RNA transfection

RNA transfection was performed as described previously [15]. Briefly, trypsinized cells were washed with Opti-MEM 1[™] reduced-serum medium (Invitrogen) and were resuspended at a density of 7.5×10^6 cells/mL in Cytomix buffer [1]. RNA (10 μ g) that was synthesized from pJFH1 or pJ6/JFH1 was mixed with 400 μ L of the cell suspension and was transferred into an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950 μ F with the Gene Pulser II[™]

apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to a 6-well plate, in which each well contained 3 mL of culture medium.

2.7. Infectivity titration

Huh7.5.1 cells were employed to determine the infectivity titer using end point dilution and immunofluorescence as described below. Briefly, each sample was serially diluted 5-fold in DMEM-10 and a 100- μ L aliquot was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-Core antibody 2H9 and Alexa 488-conjugated secondary anti-mouse IgG antibody. The infectious foci were counted. The titer was then calculated and is indicated as focus forming units per mL (FFU/mL).

2.8. HCV inhibition assay

To analyze the inhibitory effects of anti-CD81 and anti-SR-BI against virus infection, naïve Huh7.5.1 cells (2×10^4) were seeded into a 48-well plate and were incubated for 1 h at 37 °C with JS-81 or rat anti-SR-BI serum ([16], a generous gift from Dr. H. Barth) that was serially diluted with DMEM. Mouse IgG1 (Sigma, St. Louis, MO) and rat pre-immune serum were used as controls for JS-81 and anti-SR-BI, respectively. Antibodies were removed and the cells were washed once with PBS. The cells were then inoculated with viruses (MOI 0.1) from FBS-supplemented or serum-free culture for 3 h, and were then washed with PBS and cultured with DMEM-10 for 72 h. The cells were washed three times with PBS and 100 μ L of Passive Lysis Buffer (Promega) was added into each well. Cell lysates were collected and HCV-core concentrations were measured as described below.

To analyze the inhibitory effects of anti-E2 against HCV particles, viruses that were purified from FBS-supplemented or serum-free culture (2×10^3 FFU) were mixed with mouse anti-E2 (AP33, a kind gift from Genentech, Inc.) antibody, and were then incubated for 30 min at room temperature. Naïve Huh7.5.1 cells (1×10^4) were seeded into a poly-D-lysine coated 96-well plate, and cells were inoculated with the virus-antibody mixtures, which were serially diluted with DMEM-10, and, after 3 h, the mixtures were removed and the cells were washed once with PBS. DMEM-10 was added into each well, and the cells were cultured for 72 h. The cells were fixed with methanol for 15 min at -20°C , and the infected cells were stained with rabbit anti-NS5A antibody using immunofluorescence as described above [17]. Percentage infection was calculated from the infectious titer of each diluted virus.

2.9. Sucrose density gradient analysis and HCV purification

Supernatants (4 mL) of J6/JFH-1 HCV cells were layered on top of a preformed continuous 10–60% sucrose gradient in 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA (TNE buffer). HCV-core levels, HCV-RNA titer and infectious titers of the media are shown in the supplementary table. The gradients were centrifuged using an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (500 μ L each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100- μ L drop from fractions of a gradient run.

Partially purified HCV was prepared by collecting the peaks of HCV-core and HCV-RNA and was used for the infection assay and for characterization.

2.10. Quantification of HCV-core protein and RNA

To estimate the levels of HCV-core proteins, the concentration of HCV-core proteins was measured. Aliquots of samples were

assayed using the HCV Core ELISA kit (Ortho Clinical Diagnostics, Tokyo, Japan). Viral RNA was isolated from harvested culture media or from sucrose density gradient fractions using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). Copy numbers of HCV-RNA were determined by the real-time detection reverse transcription-polymerase chain reaction (RTD-PCR) using an ABI Prism 7500 fast sequence detector system (Applied Biosystems, Tokyo, Japan) [18].

2.11. Immunoprecipitation of HCV particles

Protein G-Sepharose (GE Healthcare, Little Chalfont, UK) was mixed with DMEM-10 for 1 h at 4°C, and was spun down by centrifugation for 1 min at 5000 rpm (TOMY, Tokyo, Japan). HCV particles (1×10^7 copies HCV-RNA) were mixed with the resin and were incubated overnight at 4°C with rotation. The sample was centrifuged for 1 min at 5000 rpm, and the supernatant was then collected. A 7.5 μ L aliquot of anti-human ApoB (AB742, Millipore, Billerica, MA) or anti-human ApoE polyclonal antibody (AB947, Millipore) was added into the pre-cleared virus fluid (100 μ L), and the mixture was incubated overnight at 4°C. Mouse IgG (5 μ g, Sigma) was used as a control. The mixture was mixed with the resin and incubated for 1 h at 4°C, with rotation. The supernatants were collected following centrifugation and the pellets were then washed twice with PBS and suspended in DMEM-10. Viral RNA was eluted from both the supernatants and the suspended pellets using the QIAamp Viral RNA mini kit (Qiagen). The HCV-RNA titer present in each total RNA from the supernatant and the pellet was evaluated, and the infectivity of the supernatant was measured by inoculation of naïve Huh7.5.1 cells.

3. Results

3.1. Establishment of serum-free cultured Huh7 cells

Huh7 cells are routinely maintained in our laboratory by culture in 10% FCS-supplemented medium. To examine HCV particles produced from infected cells cultured under serum-free conditions, we first established a serum-free culture system which allowed the proliferation of Huh7 cells. It was previously reported that Huh7 cells could be cultured in serum-free media that contains selenium [6]. We therefore examined the growth of Huh7 cells following gradual reduction of the level of FBS and ultimately culture in completely serum-free, selenium-supplemented (ITS-containing) media. The cells could be passaged and cultured over a long period in this medium, although the observed growth, as assayed using an MTS assay, was slightly lower than that of FBS-supplemented cultures for all the cell lines used in this study (Fig. 1 and Supplementary Fig. S1). Based on this result, we used ITS-supplemented media for the evaluation of serum-free cultured HCV.

3.2. Production of HCV particles from serum-free cultures

We next tested the efficiency of HCV particle production in serum-free culture. *In vitro* transcribed HCV-RNAs were transfected into the CD81-negative Huh7-25, and the CD81 positive Huh7-25-CD81 cell lines. The re-infection rate is known to be negligible when Huh7-25 is used [19]. When synthetic RNAs of JFH-1 or J6/JFH-1 strains were transfected, the HCV-core protein and HCV-RNA were detected in the culture media, and each medium was infectious for naïve Huh7 cells (Fig. 2, Supplementary Table). The specific infectivity of each medium (the values of the infectivity titer divided by the values of the HCV-core protein or of HCV-RNA) of J6/JFH-1 HCV was higher than that of JFH-1 (Fig. 2C, Supplementary Table). These results showed that infectious HCV was secreted into the

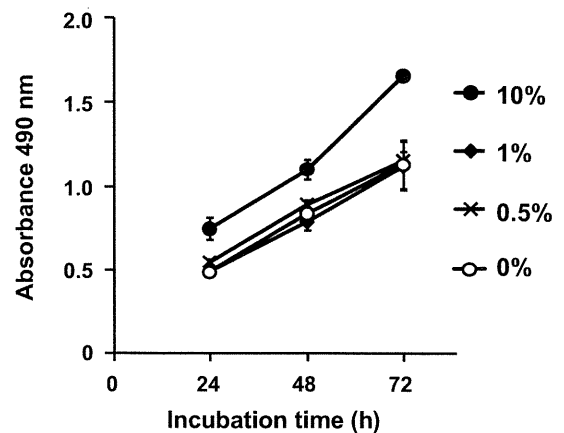


Fig. 1. Cell proliferation assay of serum-free cultured Huh7 cells. Huh7 cells that were seeded into a 96-well plate (1×10^4 /well) were sequentially grown in DMEM/F-12 media containing 10%, 1%, 0.5% and 0% fetal bovine serum. At indicated times, an MTS cell proliferation assay was performed using a commercial kit.

culture medium of both cell lines. The slightly higher HCV production of Huh7-25-CD81 cells may be due to re-infection of secreted virus particles. To determine if HCV-infected cells could be cultured for several passages in serum-free medium, serum-free cultured Huh7 cells were inoculated with infectious J6/JFH-1 chimeric HCV at multiplicity of infection (MOI) of 0.2 and were then cultured for a long period, following which the HCV-core protein and HCV-RNA in the culture medium was analyzed. The HCV-core protein and HCV-RNA were continuously detected in serum-free media, and their level was almost equal to that of infected FBS-supplemented Huh7 culture (Fig. 3).

3.3. Characterization of serum-free cultured HCV by sucrose density gradient analysis

We next compared the characteristics of HCV viruses produced under serum-free and serum-supplemented conditions by density gradient analysis. Each infectious supernatant was layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Eighteen fractions were obtained and HCV-core and RNA titers of each fraction were determined. The detected virus titers in each density fraction were different mainly due to differences in the amount of input virus, as shown in the supplementary table. As previously reported, infectivity of all viruses was observed in fractions of lower density (approximately 1.10 g/mL sucrose) than those in which the peaks of HCV-core and HCV-RNA were detected (Fig. 4), although the specific infectivity of serum-free cultured HCV was slightly lower than that of FBS-supplemented HCV. These results suggested that the infectious HCV produced in the media by serum-free cultures had similar characteristics to those of HCV produced by serum-supplemented cultures. In addition, the virus particles produced from CD81-positive and -negative cells exhibited similar density profiles (Compare Fig. 4A, B vs. C, D).

3.4. Antibodies differentially inhibit HCV from serum-free and serum supplemented cultures

We next examined antibody inhibition of cell infection by HCV derived from serum-free or serum-supplemented cultures. CD81 and SR-BI are candidate cellular receptors for HCV infection. We first determined the inhibitory effect of anti-CD81 and anti-SR-BI antibodies on infection of serum-free cultured HCV. Interestingly, HCV infection by HCV derived from serum-free and serum-supplemented cultures was differently inhibited by these

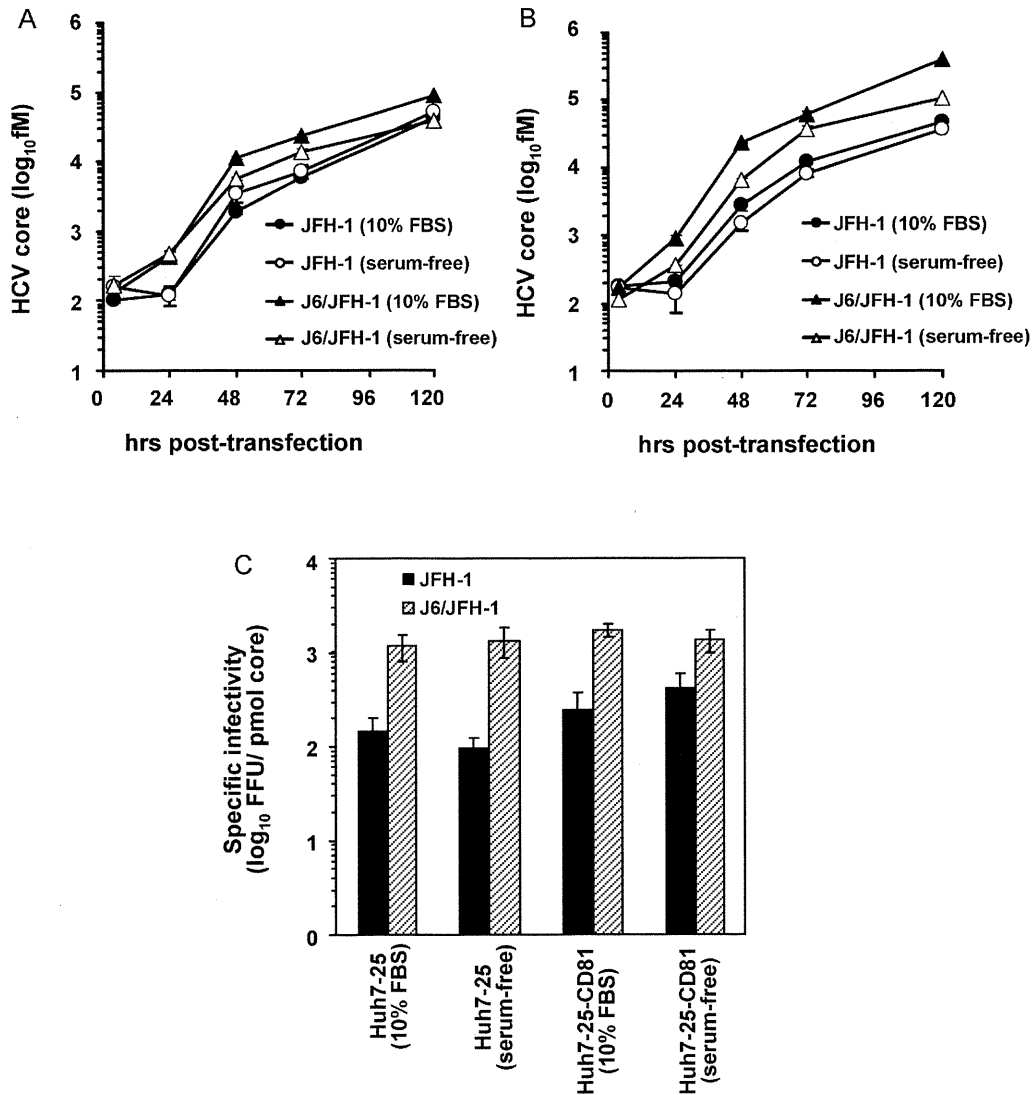


Fig. 2. HCV production from serum-free cultured Huh7 cells transfected with *in vitro* transcribed JFH-1 and J6/JFH-1 RNA. *In vitro* transcribed JFH-1 and J6/JFH-1 RNA was transfected into Huh7-25 (A) and Huh7-25-CD81 (B) cells that were grown under the indicated serum conditions. The culture supernatant was collected 4, 24, 48, 72 and 120 h post-transfection, and the HCV-core protein levels were analyzed using ELISA. All data were measured in triplicate, and are shown as means \pm SD. Infectivity of each supernatant that was collected 120 h post-transfection was analyzed by infectivity titration, and specific infectivity was calculated by dividing the mean value of the infectivity titer by that of the HCV-core protein (C). All data were measured in triplicate, and are shown as means \pm SD. Profiles of HCV-core, HCV-RNA and infectivity are indicated in the Supplementary Table.

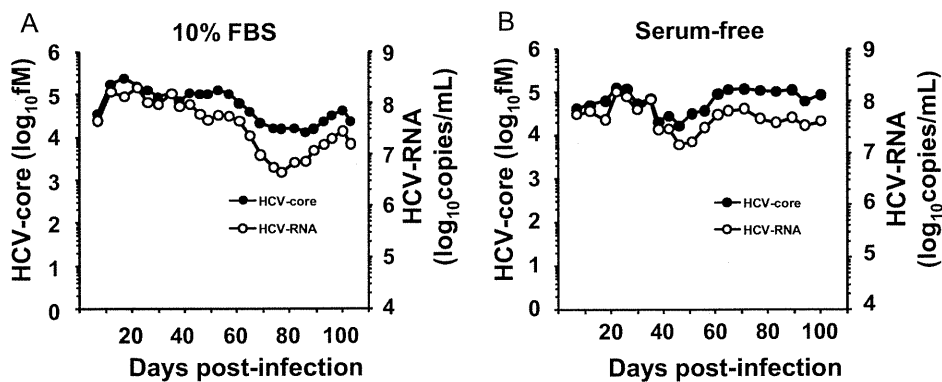


Fig. 3. HCV production from serum-free cultured Huh7 cells infected with J6/JFH-1 HCV. Huh7 cells that were grown in 10% FBS-supplemented (A) or serum-free (B) DMEM/F-12 were inoculated with the J6/JFH-1 virus (MOI, 0.2), and media of sub-cultures were collected. The HCV-core (closed circles) and RNA (open circles) were analyzed using ELISA and RTD-PCR, respectively.

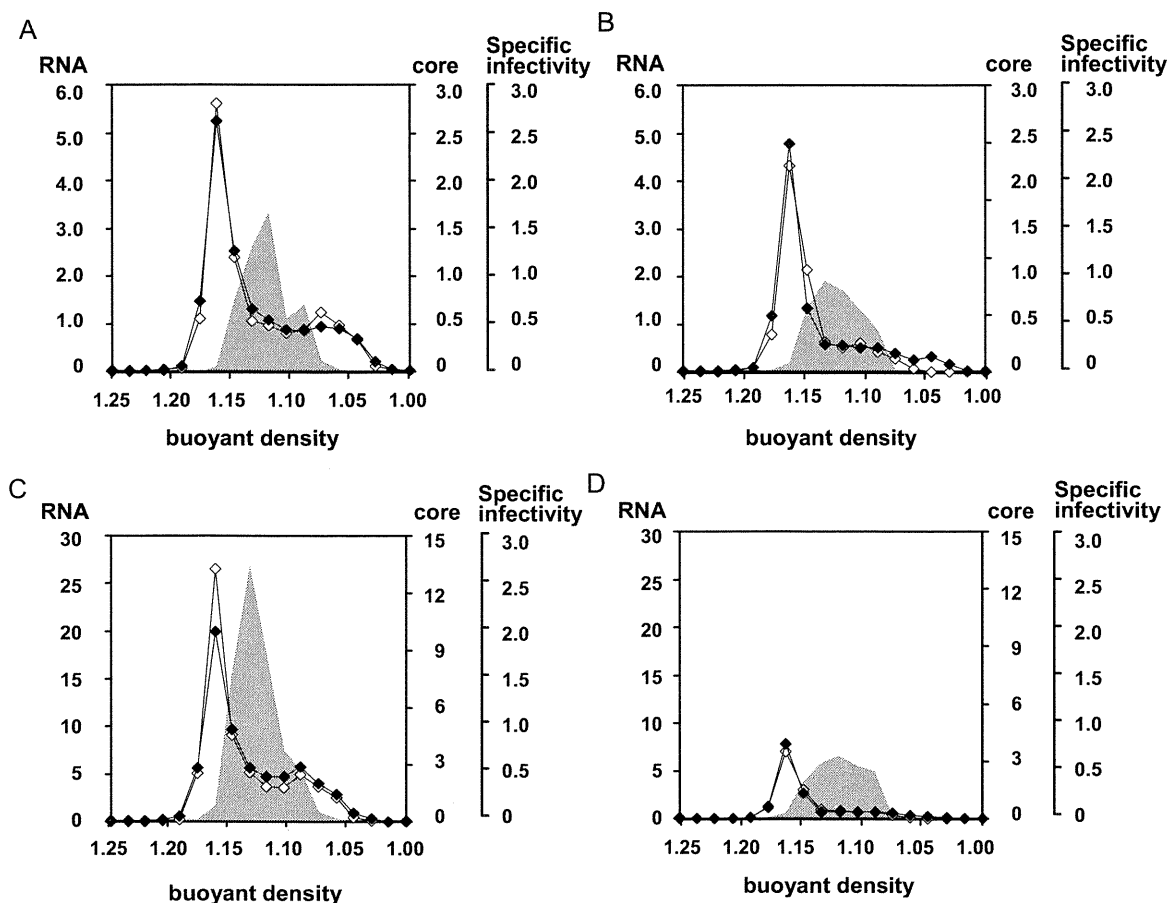


Fig. 4. Density gradient analysis of the supernatants derived from infected serum-free cultured Huh7 cells. *In vitro* transcribed J6/JFH-1 RNA was transfected into Huh7-25 (A and B) and Huh7-25-CD81 (C and D) cells that were cultured under 10% FBS-supplemented (A and C) or serum-free (B and D) conditions. Culture supernatants (4 mL) were collected 5 days post-transfection, and were then layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged using an SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (500 μ L each) were collected from the bottom of the tube. The buoyant density (g/mL, x-axis), the levels of HCV-core ($\times 10^2$ pM, closed diamonds) and HCV-RNA ($\times 10^8$ copies/mL, open diamonds), and the specific infectivity for naïve Huh7.5.1 cells (FFU/pmol core, shown in gray) of each fraction were analyzed as described in Section 2.

antibodies (Fig. 5A and B). Next, to confirm that the anti-E2 antibody, which has been shown to bind HCV particles, inhibits HCV infection, HCV was pre-incubated with the anti-E2 antibody AP33 and inoculated into Huh7 cells. As shown in previous reports [20,21], AP33 inhibited HCV infection. However, its inhibitory effect was different for serum-free and serum-supplemented cultured HCV. Thus, infection of serum-free cultured HCV displayed the highest susceptibility to this antibody (Fig. 5C).

It has also been recently reported that VLDL associates with HCV and affects infectious particle formation and infection [7–9,22,23]. We therefore determined whether apolipoproteins associate with serum-free cultured HCV by immunoprecipitation of apolipoproteins from the culture media with anti-human apolipoprotein antibodies, followed by analysis of the viral titer in the pellet and the supernatant. HCV particles from both serum-free and serum-supplemented cultures were associated with both ApoB and ApoE (Fig. 6A). The percent of HCV from FBS-supplemented and serum-free cultures respectively that was associated with ApoB was $13.22 \pm 0.09\%$ and $16.84 \pm 0.08\%$ ($p < 0.05$, *t*-test) and the percent associated with ApoE was $20.77 \pm 0.33\%$ and $10.04 \pm 0.04\%$ ($p < 0.005$, *t*-test). Thus, serum-free HCV particles had a larger amount of associated ApoB, and a smaller amount of ApoE, than HCV from serum-supplemented cultures. We next determined whether depletion of ApoE affects viral infectivity by measurement of the infectivity titers of the virus in the supernatant following ApoE precipitation. This experiment showed that the infectivity of

HCV from FBS-supplemented cultures, but not of HCV from serum-free cultures, was down-regulated by depletion of ApoB and ApoE (Fig. 6C). These results indicated that apolipoprotein associates differently with viral particles derived from FBS-supplemented and serum-free cultures, and, further, that the infectivity of HCV derived from serum-free culture is only weakly affected by the associated apolipoprotein. These data therefore suggest that, unlike HCV from serum supplemented culture, and in contrast to previous reports regarding HCV infection, infection of HCV derived from serum-free culture may be apolipoprotein-independent. However, further studies are required to confirm this possibility.

4. Discussion

In this study, we established a serum free cell culture system for the production of HCV particles, and compared the characteristics of these particles to those of HCV particles derived from serum-supplemented cultures. The particles derived from serum-free culture were infectious, suggesting that these particles would provide an appropriate antigen for the development of antibodies and vaccines. The serum-free cultured HCV could infect naïve Huh7 cells. Furthermore, sucrose density gradient analysis indicated that the profiles of HCV-core protein and HCV-RNA of serum-free cultured HCV were almost the same as those of HCV from FBS-supplemented cultures. Under serum-free conditions, HCV components (core protein and RNA) tended to be

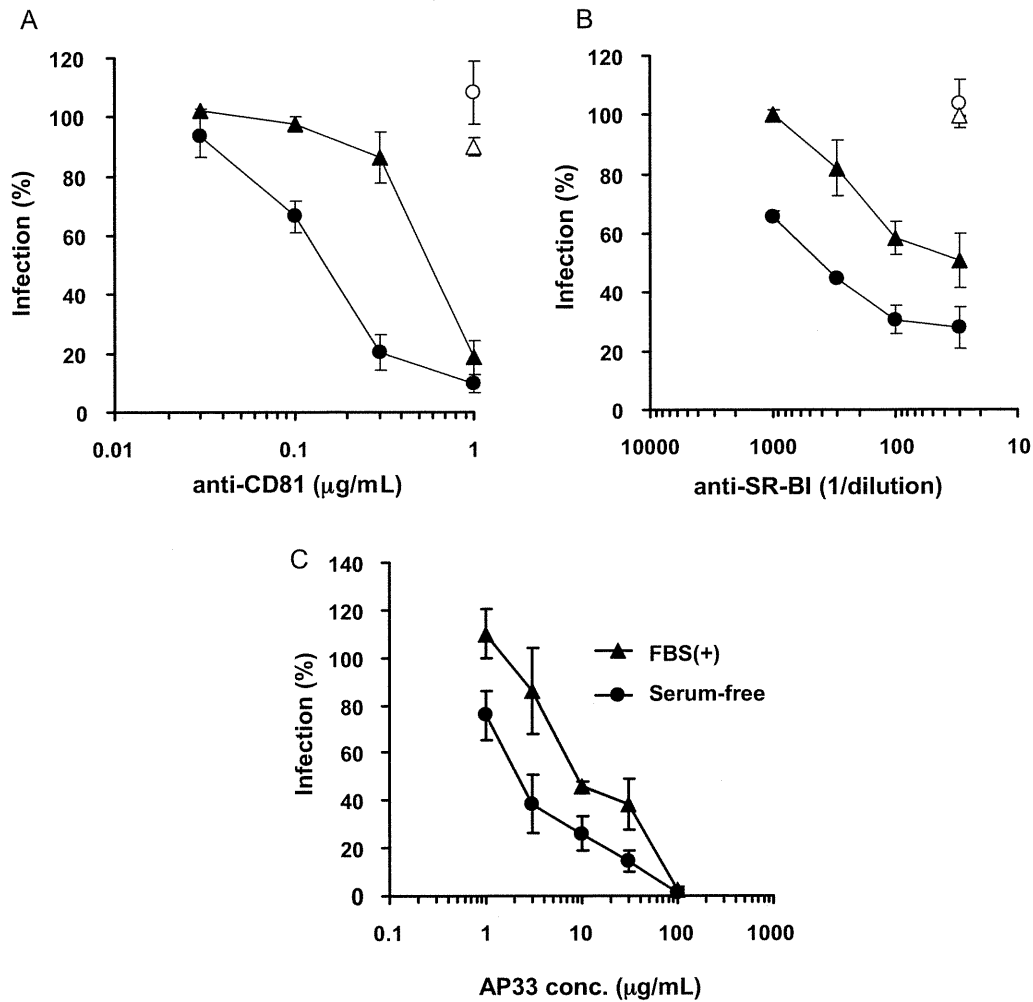


Fig. 5. Inhibition of serum-free cultured HCV infection by anti-CD81, anti-SR-BI and anti-E2 antibodies. Huh7.5.1 cells were pre-incubated with serially diluted anti-CD81 (JS-81, panel A) or anti-SR-BI (panel B) antibody for 1 h, and infectious J6/JFH-1 virus (MOI 0.1) obtained from FBS-supplemented (triangles) or serum-free (circles) culture was inoculated into each well for 72 h. Cells were lysed and HCV-core protein was measured. As controls, 1 µg/mL of mouse IgG (open triangles) and 30-fold diluted rat serum (open circles) were used. For incubation of virus particles with antibody, infectious J6/JFH-1 virus obtained from FBS-supplemented (closed triangle) or serum-free (closed circle) cultures was mixed with the indicated concentration of anti-E2 (AP33, panel C) antibody, and was then inoculated into naïve Huh7.5.1 cells. Infectivity was calculated by titration.

lost from fractions of lower density. Since exosome-like multivesicular particles have been reported to be present in lower density fractions [10], production of multivesicular particles may be different between serum-free and serum-supplemented cultures. When culture supernatants were used, the specific infectivity of serum-free cultured HCV was almost identical to that of FBS-supplemented HCV. However, infectivity of fractions separated on sucrose density gradients was lower for serum-free cultured HCV than for FBS-supplemented HCV cultures. These data may indicate that host factors that contribute to HCV infection differed under different culture conditions. Following infection and subsequent long-term culture under either serum-free or serum-supplemented conditions, HCV-core and RNA levels were identical for the first 40 days, but after this time period the ratio of HCV-core protein:RNA increased in both cultures. This result suggested that long-term culture may induce HCV-core protein which did not have HCV-RNA. As shown in Fig. 3, virus production under serum-free conditions seems to be more stable over 100 days than production under 10% FBS conditions. Fluctuation of viral titer during continuous cultivation of HCV-infected cells has been previously reported [24]. This fluctuation is dependent on the appearance of non-permissive cells with low or no CD81 expression. It is thus

likely that these non-permissive cells appear more rapidly under 10% FBS culture conditions than under serum-free conditions. This result may be due to the higher cellular growth rate of these cells in serum-supplemented culture as shown in Fig. 1 and Supplementary Fig. S1.

Our study highlighted some differences between HCV that was produced under serum-free and serum-supplemented conditions. Thus, interestingly, serum-free cultured HCV showed a higher susceptibility to antibody inhibition of viral infection than HCV from serum-supplemented cultures. The multiplicity of infection (MOI) of partially purified HCV was calculated and an MOI of 0.02 was used for HCV infection of naïve Huh7 cells. Although infection by HCV produced under both culture conditions was inhibited by anti-CD81, anti-SR-BI and anti-E2 antibodies in a dose-dependent manner, the degree of antibody inhibition at each dose was stronger for serum-free cultured HCV than for HCV from serum-supplemented cultures (Fig. 5). The anti-CD81 and anti-SR-BI antibody targets the host cells, since CD81 and SR-BI are candidate cellular receptors for HCV. In contrast, the anti-E2 antibody AP33 is a neutralizing antibody for HCV [20,21], and targets virus particles. The antibody inhibition result obtained therefore suggested that HCV infection was affected by contaminating pro-

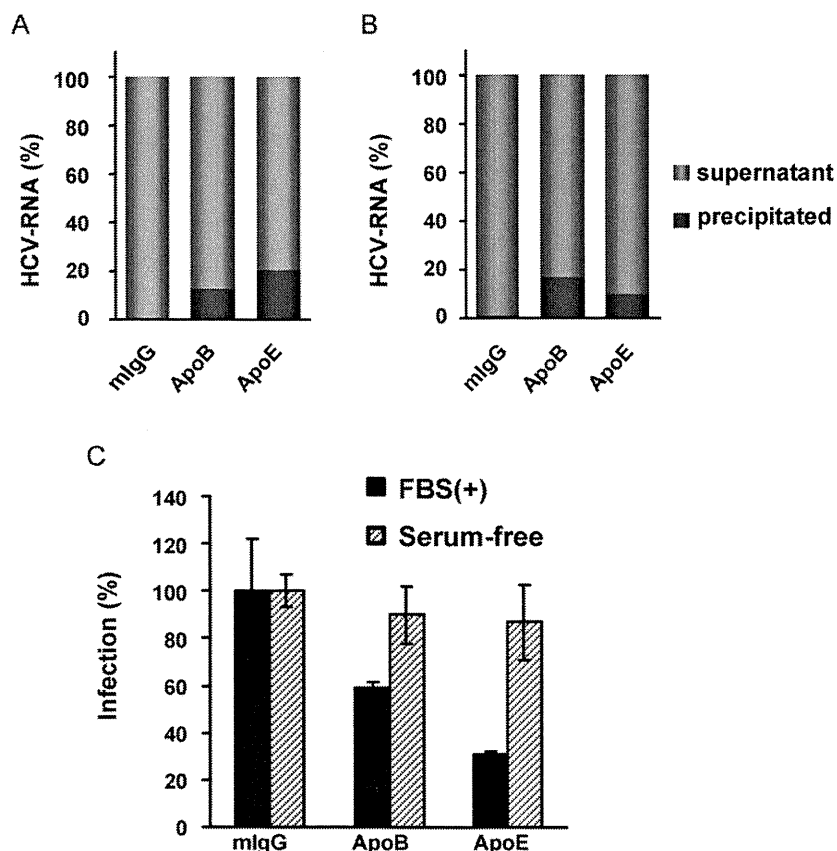


Fig. 6. Immunoprecipitation of cell-cultured HCV with anti-ApoB and ApoE antibodies. 1×10^7 HCV-RNA copies of the J6/JFH-1 virus were incubated with mouse IgG, anti-ApoB or anti-ApoE antibodies, and were then precipitated using protein G-Sepharose. Virus RNA was extracted from the supernatant and the precipitated resin, and HCV-RNA was analyzed using RTD-PCR. All experiments were performed in triplicate, and data are shown as percentages of the mean (A: serum-supplemented, B: serum-free cultured, HCV). Infectivity of the antibody-depleted virus (supernatant) was determined by inoculation into naïve Huh7.5.1 cells. All experiments were performed in triplicate, and data are shown as means \pm SD. All data are indicated as percentage of infection, in which the infectivity (FFU/mL) of the supernatant from the mouse IgG (mIgG) precipitation was designated as 100% (C).

teins or by characteristics of HCV that arose due to the different culture conditions used for HCV production. Since CD81-negative Huh7 cells were also able to produce infectious HCV, and since anti-CD81 and anti-E2 antibodies similarly inhibited infection by HCV produced in serum-free culture (data not shown), it is highly unlikely that viral incorporation of CD81 that is produced by CD81-expressing virus-producing cells modulates anti-CD81-inhibition of viral infection. An alternative possibility was that the different antibody inhibition results may have been due to differences in the level, or type of apolipoprotein associated with HCV. HCV associates with lipoproteins in human sera [25], and it has been reported that the envelope proteins of HCV interact with lipoproteins [26]. Moreover, in a recent study, VLDL was reported to associate with HCV particles and affect viral infection and virus secretion [22,23]. Mature VLDL is composed of the apolipoproteins B, C-II and E, as well as lipid, and ApoB and ApoE have been reported to be important for the infectivity of HCV particles [22,23,27–29]. We found that the content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different (Fig. 6A). Thus, serum-free HCV particles had a greater amount of associated ApoB, and a lesser amount of ApoE, than HCV from serum-supplemented cultures. Since ApoE is usually only found in mature VLDL, the serum-free cultured HCV may associate with immature VLDL. A second difference related to associated apolipoprotein of HCV grown under different serum conditions is that the infectivity of HCV from serum-supplied culture, but not that of serum-free cultured HCV, was down-regulated by antibody depletion of ApoB and ApoE. This result suggested that the infec-

tion of HCV from serum-supplemented culture, but not infection of serum-free cultured HCV, depended on apolipoproteins. It is possible that production of HCV in serum-free culture results in the formation of HCV particles that differ in lipid and lipoprotein composition from particles produced under serum-supplemented conditions. Consequently, the affinity of serum-free cultured HCV for its cellular receptor might have been altered, and its susceptibility to antibody inhibition of infectivity may have been increased. However, further analysis of the lipid content of HCV grown under different serum conditions is necessary to confirm this point.

Production of HCV using a serum-free culture system has advantage for vaccine development because there is low protein contamination, it facilitates simple viral purification procedures, and it does not involve the use of animals. Furthermore, lipoproteins associated with virus particles may shield conserved epitopes. Purified virus particles produced under serum-free conditions may have these shielded epitopes exposed and thus potentially become stronger immunogens for the induction of neutralizing antibodies. In this study, serum-free cultured HCV was infectious *in vitro*, and the viral particles had a form similar to that of native enveloped viruses. However, we have not yet confirmed the antigenic or immunogenetic properties of serum-free cultured HCV, which would be important for its potential use as a vaccine. Nevertheless, we have recently designed a system for the purification of HCV particles (Morikawa, unpublished data) which will allow testing of these parameters. If these purified particles have high antigenicity, then a HCV vaccine that is derived from HCV produced in cell culture may be available in the future.