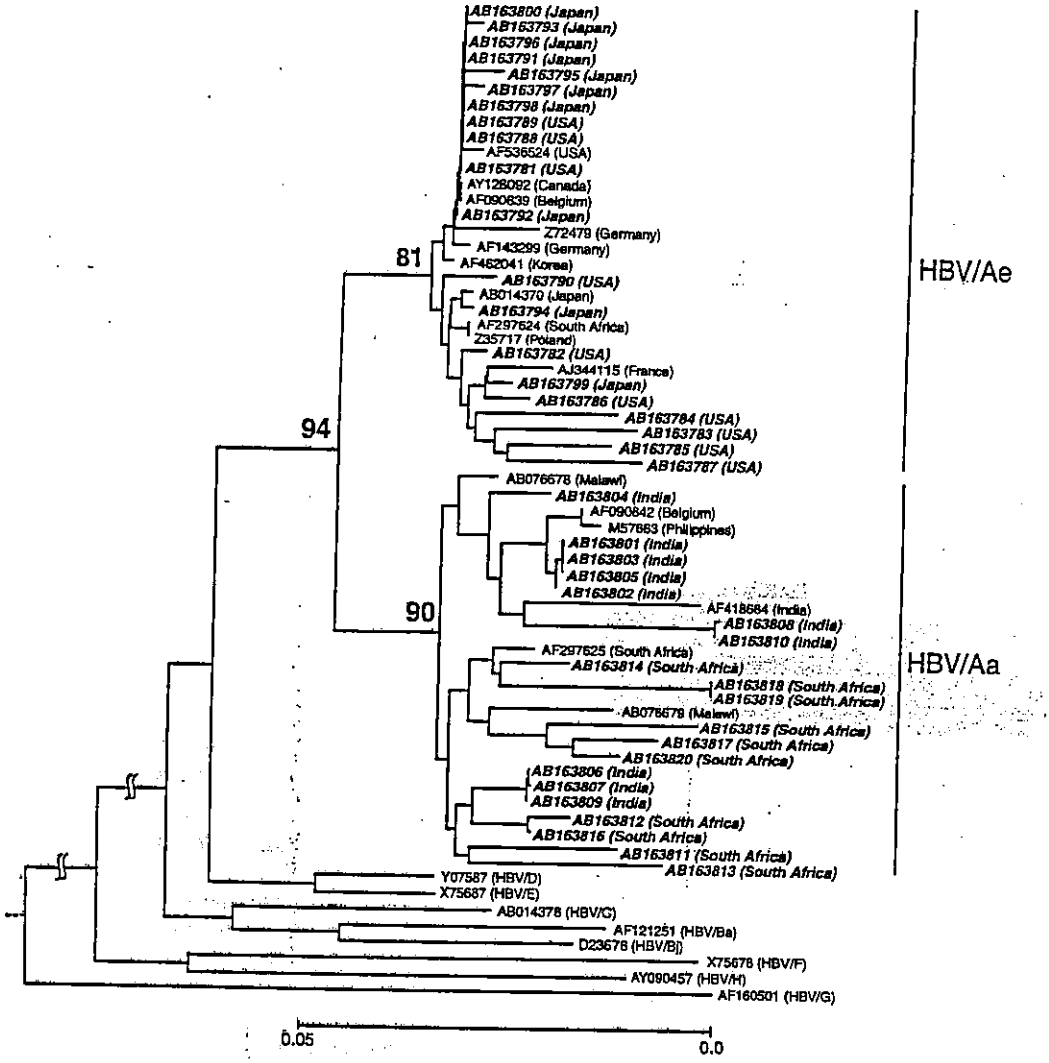

FIG. 2. A phylogenetic tree constructed using HBV X/precore/core sequences (nt 1631 to 2051). Isolates sequenced in the present study from four countries are shown in bold italic. Accession numbers: AB163781 to AB163790 (USA), AB163791 to AB163800 (Japan), AB163801 to AB163810 (India), and AB163811 to AB163820 (South Africa). Ten HBV/Ae and six HBV/Aa reference isolates obtained from the databases are labeled with corresponding accession numbers and countries of origin in parentheses. For the other genotypes (HBV/Ba, HBV/Bj, HBV/C, HBV/D, HBV/E, HBV/F, HBV/G, and HBV/H), reference isolates are also shown by accession numbers. The length of the horizontal bar indicates the number of nucleotide substitutions per site. The numbers on the nodes indicate the percentage of occurrences by bootstrap analysis.

9600; Perkin-Elmer Cetus, Norwalk, Calif). The second-round PCR was performed with the sense primer HB7F and antisense primer HB7R-2 (5′-CCT GAG TGC TGT ATG GTG AGG-3′ [nt 2072 to 2052]; a modification of HB7R) under the same conditions as the first-round PCR. The amplicons were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and observed under UV light.

To confirm the specificity of the amplification, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, Calif.) in the ABI 3100 DNA automated sequencer. For positive controls of HBV/Aa and HBV/Ae isolates, the second-round PCR products were cloned into TOPO TA cloning vector (InVitrogen Corp., Carlsbad, Calif.). Plasmids were amplified in *Escherichia coli* DH5α cells (TaKaRa Shuzo Co. Ltd., Tokyo, Japan) and purified by the QIAGEN procedure.

Molecular evolutionary analyses. A phylogenetic tree was constructed to distinguish the HBV/A subtypes. An alignment of 421 nt (nt 1631 to 2051) of the 40 isolates amplified in this study, together with 24 sequences of HBV/A to HBV/H isolates obtained from DDBJ, EMBL, and GenBank DNA databases, was analyzed. By using the computer program Hepatitis Virus Database Server (http://s2as02.genes.nig.ac.jp/), the number of nucleotide substitutions per site and the genetic distances between the isolates were estimated by the six-parameter method (11), and a neighbor-joining tree was constructed based on these values (23). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1,000 times (10).

HBV/A subtype-specific PCR combined with RFLP assay. A novel HBV/A subtyping assay, based on HBV/A subtype-specific PCR combined with a RFLP assay, was developed and applied to HBV/A isolates only, after the genotype had been determined serologically using HBV Genotypes EIA. The first-round hemi-nested PCR was performed with the sense primer HB7F and the antisense primer HB7R-2 for 5 min at 96°C, consisting of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C (additional 5 min in the last cycle), in a 96-well cycler. The second-round PCR consisted of one of two sets of primers: set I, HBV/A confirmation PCR using the sense primer HBxA (5′-ATA AAT TGG TCT GCG CAC CA-3′ [nt 1789 to 1808]) within conserved X regions (Fig. 1A) and the antisense primer HB7R-2 (for 5 min at 96°C, consisting of denaturation for 45 second at 94°C, annealing for 45 second at 62°C, and extension for 45 second at 72°C [additional 5 min in the last cycle]); and set II, HBV/Ae-specific PCR using the sense primer HBxAe (5′-ATT GGT CTG CGC ACC AGG AC-3′ [nt 1793 to 1812]) with the specific sequences for HBV/Ae at the 3′ end (Fig. 1A) and the antisense primer HB7R-2 under the same conditions as the second-round PCR in set I. In order to distinguish some unusual isolates and take advantage of another subtype-specific restriction site (Fig. 1B), the PCR assay was combined with a RFLP assay using BglII. Restric-

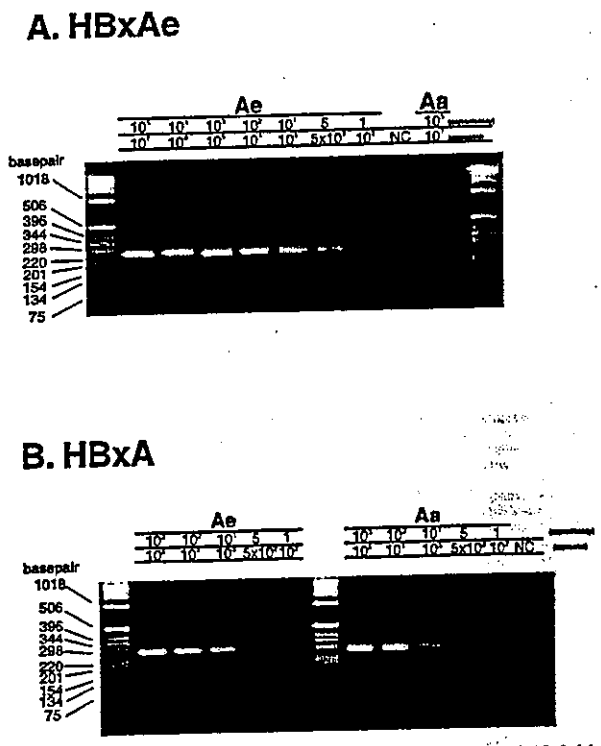

FIG. 3. Sensitivity of subtype-specific PCR assay. Serial 10-fold dilutions containing known numbers of HBV copies of HBV/Ae and HBV/Aa were tested using HBxAe-specific primer (A) and HBxA confirmation primer (B). Molecular weight markers are on the right and/or left lanes.

tion digestions were carried out using 5 μl of the 282-bp amplicon from set II, with 10 U of restriction enzyme BglII (New England BioLabs, Beverly, Mass.) for 3 h. The digested PCR products were electrophoresed on 3% agarose gels and stained with ethidium bromide. The RFLP pattern was examined under UV light; HBV/Ae amplicons were digested into two fragments (221 plus 61 bp), whereas HBV/Aa amplicons were not restricted.

Statistical analyses. Statistical differences were evaluated by Mann-Whitney U test, Fisher's exact probability test, and chi-square test with Yates' correction, where appropriate, with use of STATA software version 8.0 (StataCorp. LP, College Station, Tex.). Differences were considered significant for P values less than 0.05.

Nucleotide sequence accession numbers. Isolates sequenced in this study were submitted to the GenBank/DDBJ/EMBL/ databases under the following accession numbers: AB163781 to AB163790 (USA), AB163791 to AB163800 (Japan), AB163801 to AB163810 (India), and AB163811 to AB163820 (South Africa).

RESULTS

Molecular evolutionary analyses of HBV/A. Based on the alignment of 40 isolates from four countries and 24 reference sequences including HBV/A to HBV/H sequences from the DDBJ, EMBL, and GenBank DNA databases, a phylogenetic tree was constructed for the HBV X/precore/core region (Fig. 2). All 20 HBV/A isolates from the United States and Japan were confirmed to be HBV/Ae, and all 20 isolates from India and South Africa were classified into HBV/Aa with high bootstrap values.

Alignment of HBV/A isolates. Figure 1 shows the alignment of 60 HBV/A sequences (56 sequences from the sequences used for phylogenetic analysis above and 4 additional short sequences from Gambia or India), within the subtype-specific region of the primer (Fig. 1A) and within the core region corresponding to the BglII RFLP site (Fig. 1B). Almost all HBV/Ae isolates had G at nt 1809 and C at nt 1812 (G1809/C1812 pattern), whereas HBV/Aa isolates had T1809/T1812. Taking advantage of these two single nucleotide polymorphisms, two sense primers were designed to distinguish HBV/Ae from HBV/Aa, and two sets of hemi-nested PCRs were developed. Examining available sequences from the databases, we noted exceptions. A few HBV/Aa isolates had G1809/C1812, and therefore may initially be classified as HBV/Ae. In order to distinguish these unusual isolates, a RFLP assay using BglII was developed in a different region of the HBV genome that contains a single nucleotide polymorphism distinguishing HBV/Aa and HBV/Ae. Because the restriction enzyme, BglII, recognizes the nucleotide sequence A/GATCT (nt 1984 to 1989) (Fig. 1B), HBV/Ae isolates were restricted to 221-plus-61-bp fragments and HBV/Aa isolates were not (282-bp fragment). Furthermore, some atypical isolates of HBV/Aa with A1984 were found in the databases; however, these would be excluded initially by the PCR step because of the nucleotide pattern (T1809 or A1809/T1812) (Fig. 1A).

Sensitivity of HBV/A subtype-specific PCR combined with RFLP assay. The sensitivity of the newly developed PCR assay to distinguish HBV/Ae from HBV/Aa was evaluated on serial 10-fold dilutions containing known copy numbers of both HBV/Ae and HBV/Aa clones (Fig. 3). As shown in Fig. 3A, the subtype-specific PCR with sense primer HBxAe could detect 10^2 copies per ml of the isolates which have G1809/C1812, and HBV/Aa isolates with T1809/T1812 were not amplified. On the other hand, the PCR with primer HBxA could detect both HBV/Ae and HBV/Aa, and the detection limits were 10^2 copies per ml (Fig. 3B).

Strategy for the subtyping assay of HBV/A based on HBV/A subtype-specific PCR combined with RFLP assay. Figure 4A shows the final strategy of the subtyping assay of HBV/A based on subtype-specific PCR followed by a RFLP assay. The hemi-nested PCR assay consisted of two sets of second-round PCR, with either sense primer HBxAe (named the HBV/Ae-specific primer) or sense primer HBxA (named the HBV/A confirmation primer), combined with a RFLP assay using BglII, and allowed HBV/Aa to be clearly distinguished from HBV/Ae. In brief, when HBV/A isolates were detected by using both sets of PCR primers and the amplicons were not restricted by the RFLP step (Fig. 4B), the isolates were classified as HBV/Aa. On the other hand, when HBV/A isolates were detected by both sets of PCR primers and the amplicons restricted by BglII, the isolates belonged to HBV/Ae. The isolates positive for the PCR with primer HBxA only and not primer HBxAe were also recognized as HBV/Aa (Fig. 4B).

Application of the HBV/A subtyping assay to U.S. samples. In order to confirm the practical usefulness of this assay, HBV isolates from sera of 109 paid donors from the United States, which had been shown to be HBV/A by enzyme-linked immunosorbent assay, were examined using the subtyping assay. Of the 109 samples obtained from 38 African-American, 16 Asian, 24 Caucasian, and 31 Hispanic subjects, 79 were classified as HBV/Ae and 30 as HBV/Aa. There were no differences between the ethnic groups in the age, gender, and ALT levels in serum of the donors (Table 1). Interestingly, the prevalence of HBeAg in the Asian group tended to be lower than that of the other groups. By examining the ratio of HBV/A subtypes among isolates from each ethnic group with our new assay,

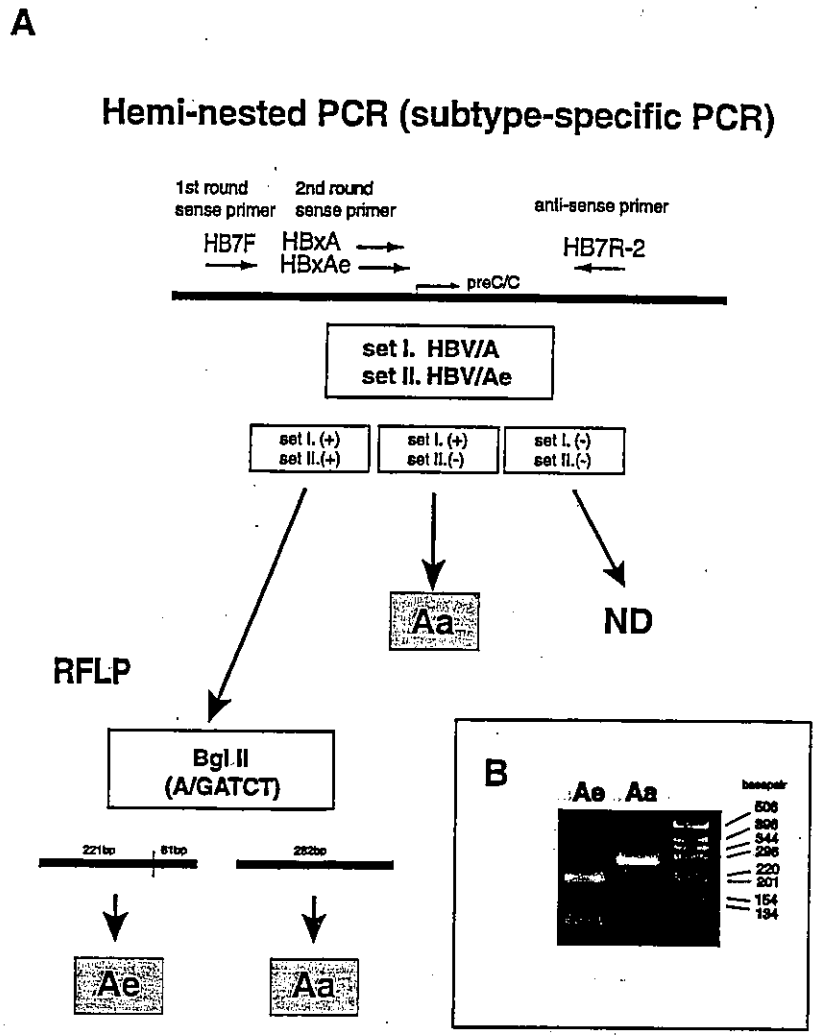

FIG. 4. (A) The strategy of novel subtyping assay of HBV/A based on subtype-specific PCR combined with RFLP assay with BglII. (B) Ethidium bromide-strained 3% agarose gel showing the restriction patterns obtained by BglII digestion. HBV/Ae isolates were restricted to a 221-plus-61-bp fragment and HBV/Aa isolates were not (282-bp fragment). ND, not determined.

84.2% of African-American, 83.3% of Caucasian, and 74.2% of Hispanic isolates were classified as HBV/Ae, whereas 75% of the Asian isolates were HBV/Aa. This indicates that the prevalence of HBV/Aa was significantly higher in Asian isolates than in the other groups (Fig. 5A). As a control study, the subtyping assay was applied to 32 HBV/A sera from South Africa, where subtype Aa is known to predominate (5, 13, 16). A total of 96.9% of HBV isolates belonged to HBV/Aa (Fig. 5B), which was different than the prevalence of HBV/Aa among African-American donors from the United States. Moreover, when the HBeAg positivity of subjects younger than 40 was evaluated, we found that the proportion of carriers infected with HBV/Aa that were positive was lower than with those infected with HBV/Ae (data not shown).

TABLE 1. Clinical backgrounds of blood donors from the United States[a]

Ethnicity (*n*)	Mean age, (yr)	Male sex (%)	ALT median (IU/liter)	ALT range (IU/liter)	HBeAg positive (%)
African-American (38)	34.2 ± 11.4	19 (50.0)	15	6–22	6/33[b] (18.2)
Asian (16)	39.3 ± 17.1	12 (75.0)	22	19–40	2/13[b] (15.4)
Caucasian (24)	40.3 ± 16.3	15 (62.5)	14	6–40	6/24 (25.0)
Hispanic (31)	38.7 ± 14.4	19 (61.2)	18	4–38	5/31 (16.1)

[a] *P* values were not significant for any data.
[b] Five samples in the African-American group and three samples in the Asian group were not available.

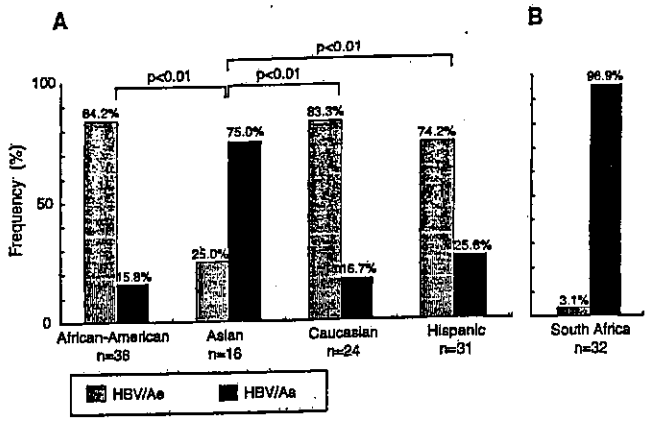

FIG. 5. The prevalence of HBV/Ae and HBV/Aa among different races from 109 U.S. paid donors (A) and 32 black African carriers (B) determined using the HBV/A subtyping assay. The vertical axis indicates percent frequency of HBV/A subtypes. The prevalence was statistically significant between African-Americans and Asians, between Caucasians and Asians, and between Hispanics and Asians.

DISCUSSION

In this study, a subtype-specific PCR combined with a RFLP assay was developed. For HBV/A isolates tested in this study and data extrapolated from sequences in the database, the specificity of this new assay in the X/precore/core overlapping region was 100% and could clearly distinguish HBV/Ae from HBV/Aa.

In previous studies, when HBV isolates showing intergenotypic recombination were excluded, subtype A' (HBV/Aa) was clearly distinguished by phylogenetic analyses from the remaining genotype A (HBV/Ae) in each of the open reading frames except for the small S region (29, 30). Examining all available full HBV sequences from the databases, nucleotide sequences that could distinguish HBV/Aa from HBV/Ae were found in the X/precore/core region. Notably, T1809/T1812 is a nucleotide pattern very specific for HBV/Aa, and almost all HBV/Ae isolates and isolates belonging to the other genotypes have G1809/C1812. Thus, the region of the genome that includes nt 1809 and 1812 was suitable for the subtyping of HBV/A.

In early studies, the individuals infected with HBV in Africa were shown to very frequently seroconvert from HBeAg positivity to negativity in childhood or early adulthood (4, 7), compared with those in the other countries where HBV is endemic (9, 17, 19). Furthermore, HBV-related virus markers were frequently found in young patients with hepatocellular carcinoma in South Africa (12). The unique subtype of HBV/A' (HBV/Aa) was first reported by Bowyer et al. in 1997 (5) and is known to be the major genotype of HBV in South Africa (16). Taken together, these observations allude to HBV/Aa isolates in Africa being different than HBV/Ae isolates in western countries. Therefore, it is useful to distinguish the HBV/Aa with possible different clinical characteristics from the HBV/Ae on the basis of the unique sequences in the X/precore/core region.

Of the 40 original isolates from four countries where HBV is endemic (Japan, the United States, India, and South Africa) and the 28 reference isolates from the database, just one unusual exceptional isolate was found (AF418684 in Fig. 1A). This isolate clustered with subtype HBV/Aa following a phylogenetic analysis but was found to have the characteristics of HBV/Ae according to the subtype-specific PCR assay. When a total of 132 available HBV/Aa sequences deposited in the databases and from our unpublished data (India and South Africa) were investigated in order to determine the frequency of isolates that were exceptions, 14 isolates (10.6%) from South Africa and India (five isolates from South Africa [accession numbers AF297621, AF297622, AF297623, U87742, and U87746] and nine isolates from India [accession numbers AF418684, AF418685, AF418686, AF418687, AF418688, AF418689, AF418690, AF418691, and AF418692]) had the G1809/C1812 pattern, and therefore could not be distinguished from HBV/Ae isolates by using the subtype-specific PCR step. However, because these unusual isolates had G instead of A at position 1984, they were not restricted by BglII and could therefore be correctly classified as HBV/Aa. On the other hand, 22 HBV/Aa isolates (16.7%) from Gambia and India (19 isolates from Gambia [accession numbers AF350080 to AF350098] and 3 isolates from India [accession numbers AF418676, AF418677, and AF418678]) had A1984 at the RFLP site that would allow the amplicons to be digested by BglII, a characteristic of HBV/Ae, but because they had T1809 or A1809/T1812, they would not be amplified by the HBV/Ae-specific primers and therefore would not be classified as HBV/Ae.

The application of this assay for HBV/A isolates from sera obtained from paid donors from the United States and HBV carriers from South Africa showed that the majority of HBV isolates from African-Americans, Caucasians, and Hispanics in the United States were HBV/Ae, whereas isolates from Asians living in the United States and black South Africans were mainly HBV/Aa (Fig. 5). Although almost all HBV/A isolates from black Africans were classified as HBV/Aa, which was consistent with previous reports (5, 13, 16), 84.2% of African-Americans had HBV/Ae. This difference between the subtypes in African-Americans and the Africans may be an indication that the infection may be a consequence of the interaction of African-Americans with other races in the United States as opposed to the introduction of HBV from Africa during the slavery period in the 19th century. However, further studies with a larger number of African-Americans and more detailed sequencing analysis would be necessary to support this assumption. On the other hand, the high prevalence of HBV/Aa in the Asian community in the United States may be a reflection of the high rate of migration from Asia to the United States in recent years.

Infection with HBV/Aa is associated with low prevalence of HBeAg in serum and early seroconversion (14, 15). Recently Ahn and coworkers showed that the T1809/T1812 mutations interfere with the translation of HBeAg in vitro and may be responsible for the early loss of HBeAg seen in southern African black carriers of HBV (1). Because of the low frequency of HBeAg positivity among the paid donors, however, we did not find a significant difference between the prevalence of HBeAg in carriers infected with the two different subtypes of genotype A. A large scale case-control study would be necessary in order to investigate this further.

In conclusion, we have described a novel HBV/A subtyping

assay that can differentiate between subtypes Aa and Ae. The specificity and sensitivity of the HBV/A subtype-specific PCR combined with a RFLP assay were confirmed, and the usefulness of this assay in a practical context was demonstrated. This assay will contribute to further clinical or epidemiological studies that will allow us to clarify the differences between the two subtypes Aa and Ae.

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REFERENCES

1. **Ahn, S. H., A. Kramvis, S. Kawai, H. C. Spangenberg, J. Li, G. Kimbi, M. C. Kew, J. Wands, and S. Tong.** 2003. Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. Gastroenterology 125:1370–1378.
2. **Akuta, N., F. Suzuki, M. Kobayashi, A. Tsubota, Y. Suzuki, T. Hosaka, T. Someya, S. Saitoh, Y. Arase, K. Ikeda, and H. Kumada.** 2003. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J. Hepatol. 38:315–321.
3. **Arauz-Ruiz, P., H. Norder, B. H. Robertson, and L. O. Magnius.** 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J. Gen. Virol. 83:2059–2073.
4. **Botha, J. F., M. J. Ritchie, G. M. Dusheiko, H. W. Mouton, and M. C. Kew.** 1984. Hepatitis B virus carrier state in black children in Ovamboland: role of perinatal and horizontal infection. Lancet 1:1210–1212.
5. **Bowyer, S. M., L. van Staden, M. C. Kew, and J. G. Sim.** 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J. Gen. Virol. 78(Pt. 7):1719–1729.
6. **Chu, C. J., E. B. Keeffe, S. H. Han, R. P. Perrillo, A. D. Min, C. Soldevila-Pico, W. Carey, R. S. Brown, Jr., V. A. Luketic, N. Terrault, and A. S. Lok.** 2003. Hepatitis B virus genotypes in the United States: results of a nationwide study. Gastroenterology 125:444–451.
7. **Dusheiko, G. M., S. M. Bowyer, A. Paterson, E. Song, A. Dibisceglie, and M. C. Kew.** 1985. Clinical and serological events accompanying changes in hepatitis B viral replication: case reports. Liver 5:77–83.
8. **Erhardt, A., U. Reineke, D. Blondin, W. H. Gerlich, O. Adams, T. Heintges, C. Niederau, and D. Haussinger.** 2000. Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. Hepatology 31:716–725.
9. **Fattovich, G., G. Giustina, S. W. Schalm, S. Hadziyannis, J. Sanchez-Tapias, P. Almasio, E. Christensen, K. Krogsgaard, F. Degos, M. Carneiro de Moura, et al.** 1995. Occurrence of hepatocellular carcinoma and decompensation in western European patients with cirrhosis type B. Hepatology 21: 77–82.
10. **Felsentein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.
11. **Gojobori, T., K. Ishii, and M. Nei.** 1982. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. J. Mol. Evol. 18:414–423.
12. **Kew, M. C., and P. Macerollo.** 1988. Effect of age on the etiologic role of the hepatitis B virus in hepatocellular carcinoma in blacks. Gastroenterology 94:439–442.
13. **Kimbi, G., A. Kramvis, and M. Kew.** 2004. Distinctive sequence characteristics of subtype Aa isolates of hepatitis B virus from South Africa. J. Gen. Virol. 85:1211–1220.
14. **Kramvis, A., S. Bukofzer, M. C. Kew, and E. Song.** 1997. Nucleic acid sequence analysis of the precore region of hepatitis B virus from sera of southern African black adult carriers of the virus. Hepatology 25:235–240.
15. **Kramvis, A., M. C. Kew, and S. Bukofzer.** 1998. Hepatitis B virus precore mutants in serum and liver of southern African blacks with hepatocellular carcinoma. J. Hepatol. 28:132–141.
16. **Kramvis, A., L. Weitzmann, W. K. Owiredu, and M. C. Kew.** 2002. Analysis of the complete genome of subgroup A′ hepatitis B virus isolates from South Africa. J. Gen. Virol. 83:835–839.
17. **Lee, W. M.** 1997. Hepatitis B virus infection. N. Engl. J. Med. 337:1733–1745.
18. **Lindh, M., A. S. Andersson, and A. Gusdal.** 1997. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus—large-scale analysis using a new genotyping method. J. Infect. Dis. 175:1285–1293.
19. **Lok, A. S., E. J. Heathcote, and J. H. Hoofnagle.** 2001. Management of hepatitis B: 2000—summary of a workshop. Gastroenterology 120:1828–1853.
20. **Magnius, L. O., and H. Norder.** 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology 38:24–34.
21. **Norder, H., A. M. Courouce, and L. O. Magnius.** 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.
22. **Okamoto, H., F. Tsuda, H. Sakugawa, R. I. Sastrosoewignjo, M. Imai, Y. Miyakawa, and M. Mayumi.** 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J. Gen. Virol. 69(Pt. 10):2575–2583.
23. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
24. **Sanchez-Tapias, J. M., J. Costa, A. Mas, M. Bruguera, and J. Rodes.** 2002. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. Gastroenterology 123:1848–1856.
25. **Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi, and R. Rossau.** 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J. Gen. Virol. 81:67–74.
26. **Sugauchi, F., M. Mizokami, E. Orito, T. Ohno, H. Kato, S. Suzuki, Y. Kimura, R. Ueda, L. A. Butterworth, and W. G. Cooksley.** 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J. Gen. Virol. 82:883–892.
27. **Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. Ueda, Y. Miyakawa, and M. Mizokami.** 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J. Virol. 76:5985–5992.
28. **Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. G. Gish, R. Ueda, Y. Miyakawa, and M. Mizokami.** 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124:925–932.
29. **Sugauchi, F., E. Orito, H. Kato, S. Suzuki, S. Kawakita, Y. Sakamoto, K. Fukushima, T. Akiba, N. Yoshihara, R. Ueda, and M. Mizokami.** 2003. Genotype, serotype, and phylogenetic characterization of the complete genome sequence of hepatitis B virus isolates from Malawian chronic carriers of the virus. J. Med. Virol. 69:33–40.
30. **Sugauchi, F., H. Kumada, S. A. Acharya, S. M. Shrestha, M. T. A. Gamutan, M. Khan, R. G. Gish, Y. Tanaka, T. Kato, E. Orito, R. Ueda, Y. Miyakawa, and M. Mizokami.** 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J. Gen. Virol. 85:811–820.
31. **Usuda, S., H. Okamoto, H. Iwanari, K. Baba, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J. Virol. Methods 80:97–112.
32. **Usuda, S., H. Okamoto, T. Tanaka, K. Kidd-Ljunggren, P. V. Holland, Y. Miyakawa, and M. Mayumi.** 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J. Virol. Methods 87:81–89.
33. **Zhang, X., F. Zoulim, F. Habersetzer, S. Xiong, and C. Trepo.** 1996. Analysis of hepatitis B virus genotypes and pre-core region variability during interferon treatment of HBe antigen negative chronic hepatitis B. J. Med. Virol. 48:8–16.

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Hepatitis B virus genotype G is an extremely rare genotype in Japan

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Abstract

Background: Hepatitis B virus (HBV) has been classified into seven genotypes (A–G). HBV genotypes have a geographically characteristic distribution. Since HBV genotype G (HBV/G) was identified recently, little is known about the distribution of HBV/G in Japan. The aim of this study was to clarify this issue.
Patients and methods: Seven hundred and twenty-one serum samples obtained from patients with HBV in Japan were investigated. The patients included 149 asymptomatic carriers, 325 with chronic hepatitis, 129 with liver cirrhosis, and 118 with hepatocellular carcinoma. Six HBV genotypes (A–F) were determined by restriction fragment length polymorphim targeting to the S region of the HBV genome. Furthermore, HBV/G was investigated by polymerase chain reaction with hemi-nested primers derived from an HBV/G-specific nucleotide sequence.
Results: Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F. Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.
Conclusion: HBV/G was not identified in a large cohort of patients with HBV, either single or dual infection. HBV/G seems to be an extremely rare genotype in Japan.

Keywords: Distribution; Genotypes; Hepatitis B virus; Japan; Polymerase chain reaction; Restriction fragment length polymorphism

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1. Introduction

Hepatitis B virus (HBV) infects approximately 350 million individuals worldwide and can cause a wide spectrum of liver disease [1]. HBV has been classified into seven genotypes based on an entire genome difference of more than 8% [2–4]. HBV genotypes have a geographically characteristic distribution [5]. HBV genotype A (HBV/A) and HBV/D are the most common genotypes worldwide, and account for the majority of cases in Europe and Africa. HBV/B and HBV/C are found in East Asia. HBV/E is confined to Africa, and HBV/F has been identified in indigenous populations of Central and South America. In 2000, a unique strain harboring a 36-base pair (bp) insertion into the core region was identified in France and was phylogenetically classified into the seventh genotype, G [4]. Thereafter, HBV/G was revealed to be distributed in San Francisco [6,7], Germany [8], Mexico [9], and Canada [10], and accounted for 1–5% in these areas. Although little is known about the virological and clinical characteristics of HBV/G, one of its unique characteristics is frequent coinfection with the other genotypes. In San Francisco, eight of the eight HBV/G patients were coinfected with HBV/A [6,7], and all of the HBV/G isolates from Canada were also coinfected with HBV/A, or HBV/A and HBV/C [10].

In Japan, HBV/C is the most common genotype, accounting for approximately 85% of all genotypes, and HBV/B follows with 12% [11–13]. However, little is known about the distribution of HBV/G in Japan. We have formerly investigated the 540 sera from patients with hepatitis B collected in and around Nagoya, and found that there were no HBV/G among them [14]. However, the serum samples in the study was obtained from a restricted area, a central part of Japan, therefore, further studies including serum samples collected from the other part of Japan had been required to conclude how often HBV/G distributed in Japan. Moreover, since HBV/G is frequently coinfected with the other genotypes, there is a possibility that HBV/G might exist as a minor population in the sera classified into the other six genotypes (A–F). At this time, to elucidate this issue, we conducted nationwide study of the distribution of HBV/G by analyzing sera obtained from patients with hepatitis B, including those whose genotypes were already known, using hemi-nested polymerase chain reaction (PCR) with HBV/G-specific primers. We also discussed the issues of HBV/G to date.

2. Materials and methods

2.1. Patients

Seven hundred and twenty-one serum samples were collected from patients with HBV in Japan. The patients resided in Hokkaido, Iwate, Yamagata, Niigata, Tokyo, Kanagawa, Nagano, Nagoya, Kyoto, Fukuoka, and Okinawa. The patients in this study were overlapped with some of the previous report [11]. They included 470 (65.1%) males and 251 (34.8%) females. The mean ± S.D. age was 43.6 ± 14.9 years (Table 1).

Table 1
Demographics of the 721 patients in this study

Sample	721
Gender (M:F)	470:251
Age (year)	43.6 ± 14.9
ALT (IU)	78.8 ± 115.8
ALP (IU)	240.8 ± 155.2
γ-GTP (IU)	52.2 ± 96.2
T. bil (mg/dl)	0.99 ± 1.60
HBeAg (%)	45.2
HBV DNA[a] (LGE/ml)	5.69 ± 1.84
Diagnosis	
Asymptomatic carrier	149
Chronic hepatitis	325
Liver cirrhosis	129
Hepatocellular carcinoma	118

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; LGE, log genome equivalents; T. bil, total bilirubin; TMA, transcription-mediated amplification.

[a] Value was calculated using available data of transcription-mediated amplification of 255 subjects.

2.2. Detection of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and HBV DNA level

HBsAg was detected by a particle-agglutination test using a commercial kit (Serodia: Fujirebio, Tokyo, Japan), and HBeAg was detected by ELISA using a commercial kit (Serodia: Kokusai-shiyaku, Tokyo, Japan), following the manufacturer's recommendations. Levels of HBV DNA were determined by the transcription-mediated amplification (TMA) method (Chugai Industry, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per millilitre.

2.3. Determination of six HBV genotypes (A–F) by restriction fragment length polymorphism (RFLP)

DNA was extracted from 100 μl of serum samples using commercial kits (Smitest EX R&D: Genome Science, Fukushima, Japan) under manufacturer's recommendation. The extracted DNA was amplified in a 50-μl reaction mixture containing 0.5 μM of a sense primer MF1 (5′-YCC TGC TGG TGG CTC CAG TTC-3′: nt. 55–75), 0.5 μM of an antisense primer MR2 (5′-AAG CCA NAC ART GGG GGA AAG C-3′: nt. 730–709), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Co. Ltd., Tokyo, Japan), 0.2 mM each dNTPs, 3 mM $MgCl_2$, and 1× AmpliTaq Gold Buffer. The reactions were performed in a GeneAmp PCR system 9600 thermocycler. The sample was denatured at 96 °C for 9 min, and subjected to 40 cycles of PCR (95 °C for 1 min; 60 °C for 1 min; 72 °C for 1 min) followed by 72 °C for 5 min at final extension in a 96-well cycler (GeneAmp 9600; Perkin-Elmer, Norwalk, CT, USA). The amplified product

was subjected to the second round PCR with a sense primer MF2 (5′-GTC TAG ACT CGT GGT GGA CTT CTC TC-3′: nt. 246–271) and MR2 under the same condition as the first round PCR. The second round PCR product with the length of 485 bp was subjected to the digestion with five kinds of restriction enzymes. Genotype B could be distinguished by digestion with *Ear*I because of no recognition site of it was existed. Similarly, genotype C also could be distinguished by digestion with *Alw*I, as no recognition site of it was found within the amplified product. Only genotype E had a recognition site of *Nci*I, and only genotype F had no recognition site of *Hph*I. Finally, the distinction between genotypes A and D were done by digestion with *Nla*IV. Genotype A has a recognition site of *Nla*IV, result in the generation of fragments of 220 and 265 bp. While genotype D had two recognition site of *Nla*IV, result in generation of fragments of 34, 186, and 265 bp. Therefore, genotypes A and D were distinguished by if each of 220 and 186 bp were observed, respectively. The digested amplicon were run on 3% agarose gel stained with ethidium bromide and observed under UV light [15].

2.4. Identification of HBV/G

Nucleic acids extracted from serum were subjected to PCR with hemi-nested primers designed on the 36-bp insertion in the C gene of HBV/G genomes. In brief, the DNA was amplified by the first round of PCR for 40 cycles with HBHKF1 (sense: 5′-ACG GGG CGC ACC TCT CTT TAC-3′ [nt. 1519–1539]) and HBHKR2 that involved the 36-bp insertion characteristic of HBV/G (antisense: 5′-AGC CAA AAA GGC CAT ATG GCA-3′ [nt. 17–37 in the core gene of HBV/G]) in the presence of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The second round of PCR was performed for 40 cycles on the product of the first-round PCR with HBHKF2 (sense: 5′-GCA CTT CGT TTC ACC TCT GCA-3′ [nt. 1581–1601]) and HBHKR2. Then, the products were examined for fragments of 357 bp [15].

3. Results

3.1. Demographics, laboratory findings, and diagnosis of the patients

The mean value of alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase, and total bilirubin in the sera was 78.8 ± 115.8 IU, 240.8 ± 155.2 IU, 52.2 ± 96.2 IU, 0.99 ± 1.60 mg/dl, respectively (Table 1). Three hundred and twenty-six patients (45.2%) were positive for HBeAg. The mean value of HBV DNA measured by TMA was 5.69 ± 1.84 LGE per millilitre. One hundred and forty-nine patients (20.1%) were diagnosed as asymptomatic carriers, 325 (45.1%) with chronic hepatitis, 129 (17.9%) with liver cirrhosis, and 118 (16.4%) with hepatocellular carcinoma.

Table 2
Six genotypes (A–F) and HBV genotype G in 721 subjects from Japan

Genotype	No.	No. of HBV genotype G
A	12	0
A+D	2	0
B	88	0
B+C	5	0
C	610	0
D	3	0
F	1	0

3.2. HBV/G among 721 serum samples

Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F (Table 2). Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.

4. Discussion

Several lines of evidence about the clinical significance of HBV genotypes have been accumulated in recent years. HBV/C causes more severe liver diseases than HBV/B by prolonging active hepatitis accompanying HBeAg production [16,17]. In a Western study, the rate of sustained remission after seroconversion was higher in genotype A than in genotype D hepatitis in patients who seroconverted to anti-HBe, and mortality related to liver disease was more frequent in genotype F than in genotype A or genotype D hepatitis [18]. Clinical data concerning HBV/G are very limited. One previous study analyzed 165 patients living in San Francisco and showed that the ALT level was higher in HBV/G than in HBV/C, and HBeAg was more prevalent in HBV/G than in HBV/C or HBV/D [7]. Further studies with a large sample size are warranted to confirm these findings.

Coinfection with distinct genotypes was seen also in other than HBV/G. In this study, coinfections with HBV/A and HBV/D as well as HBV/B and HBV/C were observed. In the previous study, analyzed 256 sera from the USA, Japan, Uzbekistan, Bangladesh, South Africa, and Cameroon, coinfection with distinct genotypes was identified in 28 subjects (10.9%) [19]. The occurrence of coinfection with distinct genotypes is important in virological aspects. It is reported that genomic recombination between distinct genotypes resulted in hybrid HBV strains, which causes distinct degree of liver diseases [20,21]. In such cases, genomic recombination never occurs without coinfection with distinct genotypes. However, clinical implication of coinfection with distinct genotypes per se still remains unanswered.

Ten years before the classification of HBV/G by Stuyver et al. [4], a unique strain with a 36-nucleotide insertion into the core region, which is known to a characteristic of HBV/G nowadays [22], was isolated from a homosexual man with hu-

man immunodeficiency virus infection [23]. Laboratory findings of his serum showed a few curious values. One was that HBeAg was detected in his serum in spite of a stop codon existing in the precore region of its genome, generally aborting the production of HBeAg at the stage of translation. Stuyver et al. also observed the same phenomenon, detection of HBeAg despite the stop codon in the precore region, and speculated that HBV/G might harbor another mechanism for producing HBeAg. Two years later, the mystery was solved by demonstration of coinfection with HBV/A in four of four sera with HBV/G [6]. It was explained that the HBeAg in the sera was produced by the coinfected precore wild type HBV/A. Furthermore, it was revealed that eight of the eight HBV/G patients from San Francisco were coinfected with HBV/A [7], and three of the three HBV/G patients were coinfected with HBV/A, or HBV/A and HBV/C in Canada [10]. These findings of the high frequency of coinfection of HBV/G with other genotypes give rise to another question, of whether HBV/G is competent to replicate by itself. An inoculation experiment in chimpanzees or an expression study in cultured cells would be required to answer this question.

The entire genome sequence of HBV/G has been reported from France [4,24], the USA [22], and Germany [8] so far. Interestingly, the sequence homology of these strains was surprisingly high. In one study in the USA, 10 HBV/G isolates, including 8 from San Francisco as well as 2 from France (FR1 [4] and B1-89 [24]), had a sequence homology of 99.3–99.8% among themselves [22]. Furthermore, another report from Germany showed that the HBV/G isolate (235/01) was nearly identical (sequence homology of the entire length was 99.7%) to both B1-89 and FR1 [8]. There are a few possible explanations for this finding. One possibility is that there are epidemiological links among French, German, and American HBV/G. A patient with HBV/G from Germany [8] and a homosexual male patient with HBV/G from San Francisco [23] were both positive for human immunodeficiency virus type-1. Thus, HBV/G might spread among a specific population, such as homosexual men or intravenous drug users. This would be also associated with the fact that HBV/G was not found among the patients in the current study, in which homosexual and intravenous drug were not included. The other possibilities are that HBV/G has a high genetic stability or was introduced into humans very recently. The mutation rate of HBV has been estimated to be 4.57×10^{-5} per site per year [25]. Thus, HBV/G might have an exceptionally low mutation rate under specific conditions, or the time since its introduction into humans might not have been long enough to gain a genetic diversity like that of the other six genotypes. To elucidate this issue, more HBV/G isolates from a wide variety of areas should be investigated.

In conclusion, HBV/G was investigated in a large cohort of patients with HBV from various areas in Japan, but no HBV/G isolate was identified, in either single or dual infection. The finding of the current nationwide study, the same as that of the previous study investigated the patients in a restricted area, indicates that HBV/G is extremely rare in Japan. Further studies with a large sample size from various areas in the world are required to further reveal the virological and clinical characteristics of HBV/G.

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References

[1] Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733–45.
[2] Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69:2575–83.
[3] Norder H, Ebert JW, Fields HA, Mushahwar IK, Magnius LO. Complete sequencing of a gibbon hepatitis B virus genome reveals a unique genotype distantly related to the chimpanzee hepatitis B virus. Virology 1996;218:214–23.
[4] Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67–74.
[5] Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329–38.
[6] Kato H, Orito E, Gish RG, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. Hepatology 2002;35:922–9.
[7] Kato H, Gish RG, Bzowej N, et al. Eight genotypes (A–H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. J Med Virol 2004;73:516–21.
[8] Vieth S, Manegold C, Drosten C, Nippraschk T, Gunther S. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. Virus Genes 2002;24:153–6.
[9] Sanchez LV, Maldonado M, Bastidas-Ramirez BE, Norder H, Panduro A. Genotypes and S-gene variability of Mexican hepatitis B virus strains. J Med Virol 2002;68:24–32.
[10] Osiowy C, Giles E. Evaluation of the INNO-LiPA HBV genotyping assay for determination of hepatitis B virus genotype. J Clin Microbiol 2003;41:5473–7.
[11] Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–4.
[12] Joh R, Hasegawa K, Ogawa M, et al. Genotypic analysis of hepatitis B virus from patients with fulminant hepatitis: comparison with acute self-limited hepatitis. Hepatol Res 2003;26:119–24.
[13] Lin ZM, Yatsuhashi H, Daikoku M, et al. Hepatitis B virus of genotype C persistence after recovery from acute hepatitis B virus infection in Japan. Hepatol Res 2003;25:244–53.
[14] Kato H, Orito E, Sugauchi F, et al. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Meth 2001;98:153–9.
[15] Mizokami M, Nakano T, Orito E, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66–71.
[16] Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–9.
[17] Orito E, Mizokami M, Sakugawa H, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 2001;33:218–23.

[18] Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. Gastroenterology 2002;123:1848–56.
[19] Kato H, Orito E, Sugauchi F, et al. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. J Virol Meth 2003;110:29–35.
[20] Sugauchi F, Orito E, Ichida T, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985–92.
[21] Sugauchi F, Orito E, Ichida T, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124:925–32.
[22] Kato H, Orito E, Gish RG, et al. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J Virol 2002;76:6131–7.
[23] Bhat RA, Ulrich PP, Vyas GN. Molecular characterization of a new variant of hepatitis B virus in a persistently infected homosexual man. Hepatology 1990;11:271–6.
[24] Tran A, Kremsdorf D, Capel F, et al. Emergence of and takeover by hepatitis B virus (HBV) with rearrangements in the pre-S/S and pre-C/C genes during chronic HBV infection. J Virol 1991;65:3566–74.
[25] Orito E, Mizokami M, Ina Y, et al. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. Proc Natl Acad Sci USA 1989;86:7059–62.

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Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan

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Background/Aims: The mortality due to hepatocellular carcinoma (HCC) has ranged widely in various areas of Japan since 30 years ago and the incidence was particularly high in once *Schistosoma japonicum* (*Sj*)-endemic areas. Our aim was to estimate the spread time of hepatitis C virus (HCV) infection in the past with possible relevance to a higher incidence of HCC in once *Sj*-endemic than *Sj*-nonendemic areas.

Methods: During 2001, 131 strains of HCV-1b were obtained from patients in three previously *Sj*-endemic areas, as well as *Sj*-nonendemic areas in Japan and a cross-sectional study was conducted on them with molecular evolutionary analyses.

Results: A phylogenetic tree reconstructed on HCV-1b sequences in the NS5B region disclosed 2 independent clusters for *Sj*-positive and -negative groups with a high bootstrap value. The estimated effective number of HCV-infections indicated a transition from quiescence to rapid exponential growth in the 1920s among patients with schistosomiasis, which is 20 years earlier than that among patients without schistosomiasis.

Conclusions: The estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* since 1921. A high incidence of HCC there would be attributed to a long duration of HCV infection since 1920s.

Keywords: Hepatitis C virus; *Schistosoma japonicum*; Molecular evolutionary analysis; Hepatocellular carcinoma

1. Introduction

Recently, the molecular clock has been successfully applied to long-term serial serum samples containing hepatitis C virus (HCV) from the US and Japan and estimated the spread time of HCV in the 1930s in Japan, which is 30 years earlier than that in the US in the 1960s [1]. Insofar as a long duration of HCV infection is the most important factor for the development of hepatocellular

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Abbreviations HCV, hepatitis C virus; Anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma; *Sj*, *Schistosoma japonicum*.

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carcinoma (HCC), it can be predicted that the incidence of HCC will increase in the US over the next 2–3 decades. Thus, a combination of classical epidemiological approaches and molecular evolutionary analyses would be particularly useful in the study of contagious diseases, typified by HCV infection.

The way how individuals contracted HCV infection has remained unclear in Japan. Recently, a Japanese report (Ministry of Health, Labour and Welfare: Distribution of age-adjusted mortality rate from liver cancer by prefecture between 1971 and 1975, Tokyo, 2001) indicated that the mortality due to HCC has already varied widely in various areas of Japan since 30 years ago; the incidence of HCC was much higher in Saga/Fukuoka, Hiroshima and Yamanashi Prefectures, which were once endemic for schistosomiasis japonica in the long past. Hence, a high incidence of HCC in the 1970s would be related to HCV transmitted by injection treatment for *Schistosoma japonicum* (*Sj*) conducted since 1921 in these areas. In fact, shared needles and syringes for intravenous injection treatment with antimonyl potassium tartrate or sodium antimony tartrate posed a significant risk for HCV transmission in endemic areas [2]. Indeed, the prevalence of antibody to HCV (anti-HCV) is high (36.5; 95% CI=28.1–44.9%) in patients with chronic schistosomiasis [2] and therefore, HCV infection is considered responsible for the development of HCC in patients with chronic schistosomiasis.

Since, once popular intravenous injection for schistosomiasis was a risk factor for HCV transmission, the spread time of HCV in the areas once endemic for *Sj* in Japan would deserve determination. In this study, molecular evolutionary analyses using principles of both population genetics and mathematical epidemiology [3] were applied to HCV-infected patients with and without a past history of chronic schistosomiasis in once *Sj*-endemic areas.

2. Meterials and methods

2.1. Sample collection

In Japan during 2001, 181 random serum samples positive for anti-HCV were obtained from patients with chronic liver disease in widely separated areas previously endemic for *Sj*, including Kofu in Yamanashi ($n=75$), Katayama in Hiroshima ($n=50$) and Chikugo in Saga/Fukuoka Prefectures ($n=56$). Schistosomiasis was diagnosed by ultrasonographic (US) and/or computer tomographic (CT) modalities or serological examinations [4]. Two kinds of serological tests, which can detect past history of schistosomiasis, were available in this study. In brief, IgG antibodies binding to two different *schistosome antigens, Sj* adult worm antigen and *Sj* egg antigen, were detected using an enzyme-linked immunosorbent assay (ELISA). As it is now accepted that ELISA titer of egg antigen-specific IgG is reliable for case-detection rather than IgG for adult worm antigen [4–6], the results based on the egg antigen-specific IgG were accepted in this study. Samples of more than 0.25 of optical density at 415 nm were determined to be positive, as previously confirmed [4–6]. The serum samples were tested for anti-HCV by Lumipulse II Ortho HCV (Ortho-Clinical Diagnostics K.K., Tokyo, Japan). As patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. For a cross-sectional study, 30 serum samples were obtained from patients infected with HCV in Aichi Prefecture where *Sj* has not been endemic. The age- and sex- matced patients were also selected from the *Sj*-nonendemic areas excluding influece of these factors on HCC incidence. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by Ethic Committees of institutions. Every patient gave a written informed consent to participate in the virological research of HCV. Information of injection treatment for *Sj* was obtained by means of self-administrated questionnaires or structured interviews. None had been treated with interferon therapy for HCV infection. HCC incidence was estimated by historical information from patients themselves and/or medical records during 2001. HCC was diagnosed by liver biopsy or combination of imaging modalities such as US, enhanced CT and angiography.

2.2. Genotyping and sequencing

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. They were reverse-transcribed to cDNA using SuperScript II Rnase H^- Reverse Transcriptase (Invirogen Corp., Carlsbad, California, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) by the method described previously [7].

A sequence spanning 339 nucleotides (nt) in the NS5B region was amplified by polymerase chain reaction (PCR) with primers described previously [1]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, California, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were utilized to confirm HCV genotypes and construct phylogenetic trees.

2.3. Test for clustering between Sj-positive and -negative groups

The phylogenetic tree was first constructed to examine the evolutionary history for *Sj*-positive and *Sj*-negative groups by the neighbor joining method [8]. Furthermore, to test whether either *Sj*-positive or *Sj*-negative group have evolved independently or not, we conducted an interior branch test for the neighbor-joining tree [9]. Thereafter, a *t*-test was conducted for the interior branch length and its standard error, which is computed using the bootstrap procedure.

2.4. Demographic model

A reconstructed tree was built on the NS5B sequence of 339 nt by a heuristic maximum-likelihood topology search with stepwise-addition and the nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85 with the molecular clock enforced by PAUP version 4.0b8.

As estimates of the demographic history, a nonparametric function $N(t)$, known also as the skyline plot, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time [3,10]. A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences [10]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric maximum-likelihood estimates [11,12].

2.5. Statistical method

Data for continuous variables were demonstrated as the mean ± standard deviation. The Fishers' exact test, Chi square test with Yates' correction and one-way ANOVA followed by the Scheffe's multiple comparison test were used to evaluate differences in the mean age, sex ratio

and incidence of HCC between groups, respectively. Differences with P values less than 0.05 were considered significant.

3. Results

Of 181 anti-HCV positive samples, 113 were classified into HCV genotype 1b (HCV-1b), which is predominant in Japan. Fifty-two of 181 samples (29%) were negative for HCV RNA or incomplete for sequencing and the remaining 16 samples (9%) of genotype 2a were excluded in this study due to a minor population. Of the HCV-1b strains, 47 were recovered from patients in Yamanashi, 31 in Hiroshima and 35 in Saga/Fukuoka Prefectures. Along with 18 HCV-1b strains in Aichi Prefecture serving as controls, a cross-sectional study was conducted on them with molecular evolutionary analyses. The patients in areas previously endemic for *Sj* revealed a significantly higher prevalence of chronic schistosomiasis [24/47 (51%) in Yamanashi (Kofu area), 21/31 (68%) in Hiroshima (Katayama area) and 19/35 (54%) in Saga/Fukuoka (Chikugo area)] than that in Aichi Prefecture (0/18 [0%], $P<0.0001$). There were no significant differences in the mean age or sex ratio among patients from these four areas (Fig. 1). Although the mean age of *Sj*-positive patients was just higher than that of *Sj*-negative patients in once *Sj*-endemic areas or matched-control patients in Aichi Prefecture, there were also no significant differences between these groups (Table 1).

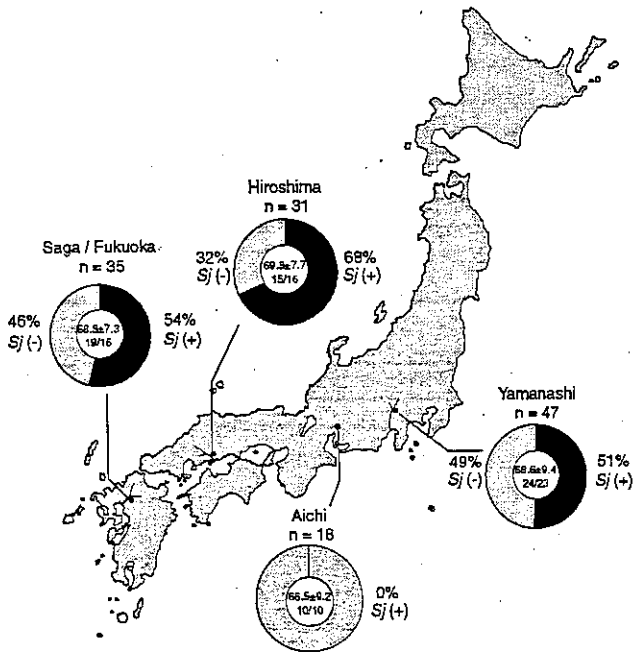

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie graphs include the age (mean± standard deviation) and sex ratio (male/female).

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls
	Positive (n=64)	Negative (n=49)	(Aichi) (n=18)
Mean age			
Total	69.9±7.7	67.4±8.7	66.5±9.2
Yamanashi	69.9±7.2	67.3±11.2	
Hiroshima	71.2±8.7	67.6±6.5	
Saga/Fukuoka	69.0±7.7	67.5±7.1	
Sex (male/female)			
Total	34/30	24/25	9/9
Yamanashi	13/11	11/12	
Hiroshima	10/11	5/5	
Saga/Fukuoka	11/8	8/8	
Incidence of HCC	25/55 (45%)	11/48 (23%)	3/18 (17%)

The incidence of HCC in *Sj*-positive patients was significantly higher than that in *Sj*-negative patients (P=0.0226) or controls (P=0.0488).
Abbreviations: HCC, hepatocellular carcinoma.

For cross-sectional study on the viral population size between HCV-infected patients with and without a past history of schistosomiasis, a phylogenetic tree for HCV-1b strains in the *Sj*-positive and -negative patients was constructed with use of the maximum-likelihood method enforced by the molecular clock as introduced in our previous report [1] and an independent study by Pybus et al. [3]; a substitution rate of 5.3×10^{-4} per site per year [1,3] was assumed for HCV. The phylogenetic tree disclosed 2 independent clusters for *Sj*-positive and -negative groups, with a high bootstrap value (81%) by the interior branch testing (Fig. 2), which is comparative with past epidemiological backgrounds in Japan. From distinct evolutionary histories in the two populations, the effective number of HCV-1b infections through time, $N(t)$, were assessed by the skyline plot. The parameters for several models in Genie v3.5 [3,10] were also examined. Time t was then transformed to year using the same rate, assuming the collecting time (year 2001) as the present. Fig. 3 shows the skyline plots and population growth for *Sj*-positive and -negative patients, according to a specific demographic model in Genie v3.5 with three parameters, piecewise expansion growth model, that was evaluated by the likelihood ratio testing [11,12]. Molecular evolutionary results thus obtained supported our previous study in which the divergence time of the most recent common ancestor of HCV-1b in each area in Japan was estimated before 1850 [1]. Our estimates of the effective number of HCV-infections showed a transition from constant size to rapid exponential growth in the 1920s among patients with chronic schistosomiasis in endemic areas, which is 20 years earlier than that among patients without schistosomiasis in the 1940s. Information on HCC was available for 121 of the 131 patients with HCV-1b. Although they were relatively small in number, the incidence of HCC was significantly higher in *Sj*-positive than -negative patients (P=0.0226) or controls (P=0.0488) (Table 1).

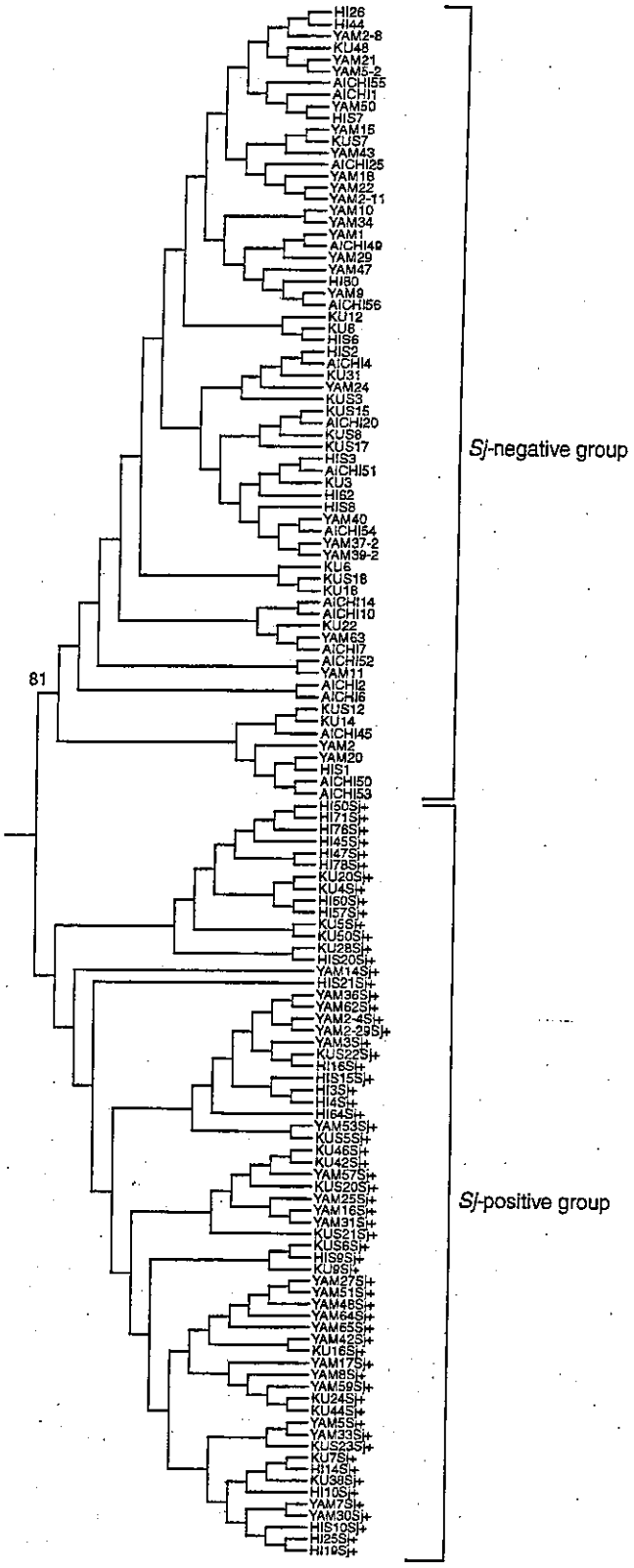

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS; Hiroshima, KU/KUS; Saga/Fukuoka, Aichi; control strains.

4. Discussion

The specific demographic model based on the neutral theory [3,11,12], which has a constant size in the past and changes to exponential growth until the present, is applied to investigate the Japanese endemic of HCV. By means of the molecular evolutionary analyses, the spread time of HCV in *Sj*-positive patients was estimated 20 years earlier than that in *Sj*-negative patients from three areas in Japan where *Sj* was previously endemic (Yamanashi, Hiroshima, Saga/Fukuoka Prefectures). The spread time of HCV much earlier in *Sj*-positive than -negative patients indicates that the previous intravenous injection treatment with antimony compounds (antimonyl potassium tartarate or antimony sodium tartarate) on patients with schistosomiasis since 1921 [2] would have been a significant risk factor for HCV transmission in endemic areas through re-used needles and syringes. Indeed, it might be possible that HCV transmission from *Sj*-positive patients to *Sj*-negative patients occurs in the once *Sj*-endemic areas, but we could not find such strains in this study. One of the reasons is that residents in the village around the river, where schistosomiasis had been the most prevalent, might have been isolated from those in the other areas of the same Prefecture in the past due to the endemic disease 'schistosomiasis'. Interestingly, most Japanese strains from *Sj*-nonendemic areas in the database clustered with the *Sj*-negative group of the present study. Hence, factors other than the injection treatment for *Sj*, such as intravenous stimulants popular during and after World War II [13] and medical treatments including transfusion with blood units from paid donors in the past, would have imposed the risk for HCV transmission in most areas in Japan [14]. In addition, there would have been opportunities for HCV transmission through inadequately sterilized needles and syringes in general practices, which have contributed to a large reservoir of chronic HCV infection in Japan during the 1950s [13]. Such inadequately sterilized medical injections were still common in the less-developed world in the 20th century. WHO estimates that unsafe injections result in 2.3–4.7 million new HCV infections worldwide every year [15].

Although the spread time of HCV in *Sj*-positive group was earlier than that in *Sj*-negative group, there was no significant difference of mean age between the 2 groups. Two possibilities are considered. One is a sampling bias; as patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. Second, the ages that patients had been infected with HCV were different between the 2 groups; the treatments for *Sj* in Japan were mainly conducted among relatively younger people including school children after screening of *Sj* [4,16,17], while the

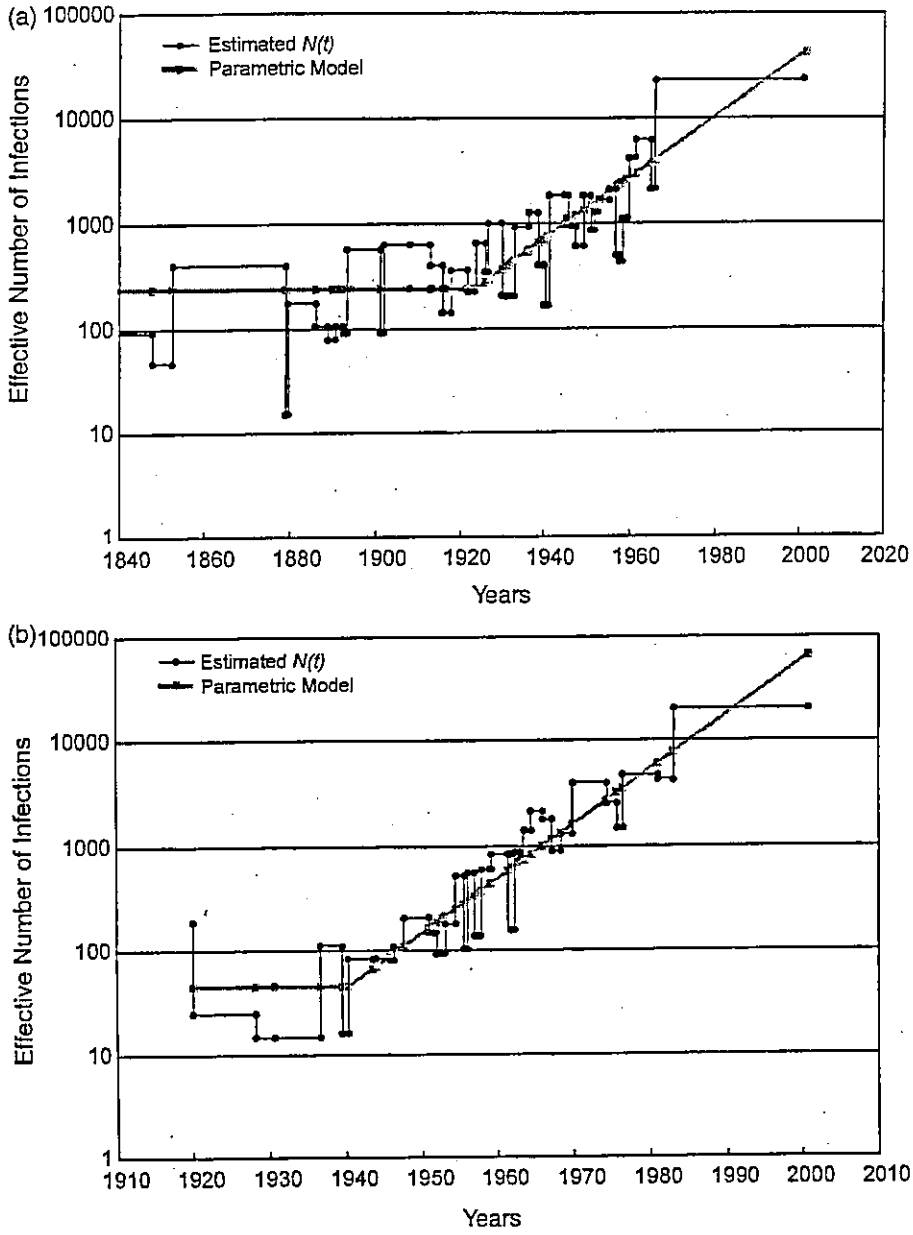

Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and stepwise plots by the black line that represents corresponding nonparametric estimates of $N(t)$ (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

other risk factors such as blood transfusion were found in older people excluding at least children.

A disease possibly caused by schistosomal infection in Japan is documented in a book written some 300 years ago. In 1847, the clinical picture of this disease was precisely described by Yoshinao Fujii in the book 'Katayama-ki' that documented an endemic disease in Katayama area as Katayama's disease (equivalent to schistosomiasis). Water-borne epidemics of schistosomiasis prevailed in inhabitants around rivers (the tributaries of the Fuji river in Yamanashi, the Takaya river in Hiroshima and the Chikugo river in Saga/Fukuoka) in Japan, mediated by small shellfish (Miyairi-kai) serving as the natural host. More than 200,000 individuals were estimated to have been infected with *Sj* in Yamanashi Prefecture alone during 1965 through 1990 [16] and approximately 1,000,000 patients in the entire Japan since 1920s [17]. To cope with these endemics, more than 10 million intravenous injections with antimony compounds had been given in Japan since 1921 [17]. Thus, Japan would have started ahead of any other countries, in terms of HCV spread in association with schistosomiasis, wherein intravenous drugs were invented. Although acute schistosomal infection has disappeared in Japan since long ago, there are still elderly people with

chronic schistosomiasis in previously endemic areas, some of whom are developing HCC [2,14]. Substantial transmission among regions is supported by the lack of regional clustering of HCV sequences in this study.

A similar situation is reported in the Nile delta in Egypt where schistosomiasis once prevailed mediated by small shellfish [18] and the national campaigns for injection treatment with antimonyl potassium tartarate (tartar emetic) from the 1961 until 1986 are suspected to have given rise to the highest endemicity of HCV in the world ever, involving >20% of the national population there [19]. The prevalence of anti-HCV is extremely high (>70%) in patients with schistosomiasis there [18,20,21]. Highly prevalent HCV infection in the general Egyptian population accounts for most HCC cases in Egypt [22]. A question may arise whether schistosomiasis alone is responsible for the development of HCC. Patients co-infected with HCV and *Schistosoma mansoni* (*Sm*) may have a high incidence of viral persistence, accelerated fibrosis and development of HCC [23,24]. A recent population-based study between two large populations with district histories of *Sm* and hepatitis C infections, however, failed to indicate any interaction between *Sm* infection and the prevalence or severity of hepatitis C [25]. Moreover, no significant histological differences were found between anti-HCV-positive Egyptian patients with and without schistosoma [26]. Hence, the long duration of persistent HCV infection would be a more important factor for the development of HCC than the pathogeneticity of *Sm* itself.

Estimating the effective number of HCV infections has been very informative in looking back epidemic spreads of HCV infection in the Unites States [1] and Egypt [12,27]. In addition, it would also be useful in predicting the population size and extent of HCV infection. Studies to foresee future spreads of HCV would be required to cope with and prevent healthcare problems where de novo infections are increasing. The advantage of molecular evolutionary analyses, its ability to accurately estimate the dynamics of HCV based on a limited number of isolates in particular [3], will extend its application anywhere in the world where clinical sequelae of persistent HCV infection pose increasing burdens on the public health of nations.

In conclusion, the evolutionary analyses indicated that the estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* conducted since 1921. The high incidence of HCC in *Sj*-endemic areas is most likely attributed to long duration of HCV infection there transmitted through injection treatments.

Acknowledgements

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References

[1] Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, et al. Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99: 15584–15589.

[2] Iida F, Iida R, Kamijo H, Takaso K, Miyazaki Y, Funabashi W, et al. Chronic Japanese schistosomiasis and hepatocellular carcinoma: ten years of follow-up in yamanashi prefecture, Japan. Bull World Health Organ 1999;77:573–581.

[3] Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. Science 2001;292:2323–2325.

[4] Minai M, Hosaka Y, Ohta N. Historical view of schistosomiasis japonica in Japan: implementation and evaluation of disease-control strategies in yamanashi prefecture. Parasitol Int 2003;52: 321–326.

[5] Matsuda H, Tanaka H, Blas BL, Nosenas JS, Tokawa T, Ohsawa S. Evaluation of ELISA with ABTS, 2-2′-azino-di-(3-ethylbenzthiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. Jpn J Exp Med 1984;54:131–138.

[6] Matsumoto J, Kirinoki M, Kawai S, Chigusa Y, Ilagan EJ, Ducusin BE, et al. Prevalence of schistosomiasis japonica among schoolchildren and animal reservoirs in oriental mindoro, Philippines. Jpn J Trop Med Hyg 1999;27:175–180.

[7] Ohno T, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a. J Clin Microbiol 1997;35:201–207.

[8] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–425.

[9] Dopazo J. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. J Mol Evol 1994;38:300–304.

[10] Pybus OG, Rambaut A. GENIE: estimating demographic history from molecular phylogenies. Bioinformatics 2002;18:1404–1405.

[11] Lemey P, Pybus OG, Wang B, Saksena NK, Salemi M, Vandamme AM. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci USA 2003;100:6588–6592.

[12] Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A. The epidemiology and iatrogenic transmission of hepatitis C virus in egypt: a Bayesian coalescent approach. Mol Biol Evol. 2003;20: 381–387.

[13] Fukui S, Wada K, Iyo M. History and current use of methamphetamine in Japan. Proceedings of Japan–US Scientific

Symposium '90 on Drug Dependence and Abuse; 7–8 September 1990. Tokyo, Japan: Cocaine and methamphetamine behavioral toxicology, clinical psychiatry and epidemiology; 1991 [p. 219–37].

[14] Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.

[15] Drucker E, Alcabes PG, Marx PA. The injection century: massive unsterile injections and the emergence of human pathogens. Lancet 2001;358:1989–1992.

[16] Kitani K, Iuchi M. Schistosomiasis japonica: a vanishing endemic in Japan. J Gastroenterol Hepatol 1990;5:160–172.

[17] Okabe K. Epidemiology of schistosomiasis japonica. Kurume Med J 1975;38:11–55.

[18] Kamel MA, Ghaffar YA, Wasef MA, Wright M, Clark LC, Miller FD. High HCV prevalence in Egyptian blood donors. Lancet 1992;340:427.

[19] Cohen J. The scientific challenge of hepatitis C. Science 1999;285: 26–30.

[20] Hibbs RG, Corwin AL, Hassan NF, Kamel M, Darwish M, Edelman R, et al. The epidemiology of antibody to hepatitis C in Egypt. J Infect Dis 1993;168:789–790.

[21] Frank C, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, et al. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet 2000;355:887–891.

[22] Hassan MM, Zaghloul AS, El-Serag HB, Soliman O, Patt YZ, Chappell CL, et al. The role of hepatitis C in hepatocellular carcinoma: a case control study among Egyptian patients. J Clin Gastroenterol 2001;33:123–126.

[23] Angelico M, Renganathan E, Gandin C, Fathy M, Profili MC, Refai W, et al. Chronic liver disease in the Alexandria governorate, Egypt: contribution of schistosomiasis and hepatitis virus infections. J Hepatol 1997;26:236–243.

[24] Kamal S, Madwar M, Bianchi L, Tawil AE, Fawzy R, Peters T, et al. Clinical, virological and histopathological features: long-term follow-up in patients with chronic hepatitis C co-infected with S. mansoni. Liver 2000;20:281–289.

[25] Blanton RE, Salam EA, Kariuki HC, Magak P, Silva LK, Muchiri EM, et al. Population-based differences in *Schistosoma mansoni*- and hepatitis C-induced disease. J Infect Dis 2002;185: 1644–1649.

[26] Helal T, Danial M, Ahmed H. The relationship between hepatitis C virus and schistosomiasis: histopathologic evaluation of liver biopsy specimens. Hum Pathol 1998;29:743–749.

[27] Tanaka Y, Agha S, Saudy N, Kurbanov F, Orito E, Kato T, et al. Exponential spread of hepatitis C virus genotype 4a in Egypt. J Mol Evol 2004;58:191–195.

地域発生C型肝炎の分子疫学

我が国における地域別HCV罹患状況とその疫学的特徴

Area and age specific prevalence rate of HCV infection in Japan

田中純子　熊谷純子　小宮　裕　吉澤浩司

Key words：HCV抗体陽性率，HCVキャリア数，献血者，肝炎ウイルス検診

はじめに

我が国における肝癌死亡実数は2001年には34,311人(人口動態統計'肝および肝内胆管の悪性新生物')と年間3万人を上回り，人口10万人当たりの死亡率は27.3人(男性38.3人，女性16.7人)となっている．

これまでの血清疫学的調査[1]から，1990年代末における肝癌の約81％はC型肝炎ウイルス(HCV)の持続感染に起因することが明らかになっており，また，新たな感染によるHCVキャリアの発生は特別な場合[2]を除きほぼ止まっている状態にあること[3]，HCVキャリアの母親から出生した児への母子感染率は2.3％程度の低率にとどまること[4]なども明らかとなっている．

これらの成績を背景に2002年4月から開始された肝炎ウイルス検診は，自覚しないまま社会に潜在するHCV・HBVキャリアを見いだし，治療や健康管理を勧奨することによって，最終的には肝癌による死亡の減少を図ろうとするものである．

自覚症状がないまま社会に潜在するHCVキャリア数を推計するためには，まず我が国の健常者集団におけるHCV感染率を知る必要があるが，この成績は，唯一，日本赤十字社血液センターの献血者の資料を基にして算出することができる．日赤血液センターでは，年間約570万本の献血された血液の安全性を確保する目的で，同一の試薬を用い，統一された基準に従った測定が行われている．

今回，日本赤十字社との協力の下に，地域・性・年齢階級別にみたHCV抗体陽性率を算出した．本稿では，この成績を紹介するとともに，国勢調査人口(2000年)を用いて'自覚症状がないまま社会に潜在するHCVキャリア'数を推計したので，あわせて紹介したい．

1. C型肝炎ウイルスの性・年齢・地域別にみた感染率—初回献血者におけるHCV抗体陽性率とその特徴—

厚生労働省のC型肝炎に関する疫学研究班と日本赤十字社とが協力して算出した，年齢・性別にみた献血者のHCV抗体陽性率を図1に示す[5]．データは，1995年1月から2000年12月までの6年間の全献血者の中から，各年ごとに初回献血者総計3,485,648例を抽出し，2000年の時点における年齢に換算してまとめたものである．

HCV抗体陽性率は，全体でみると0.49％であ

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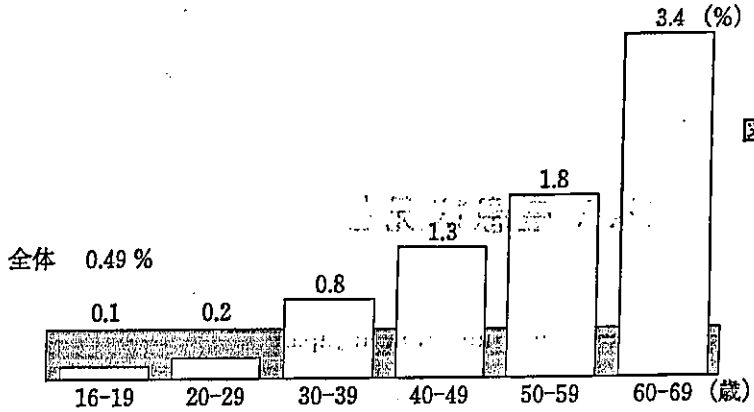
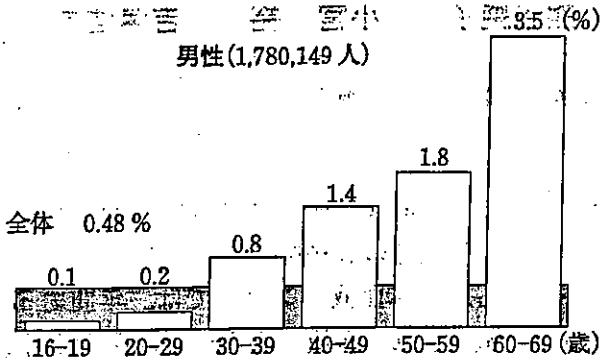
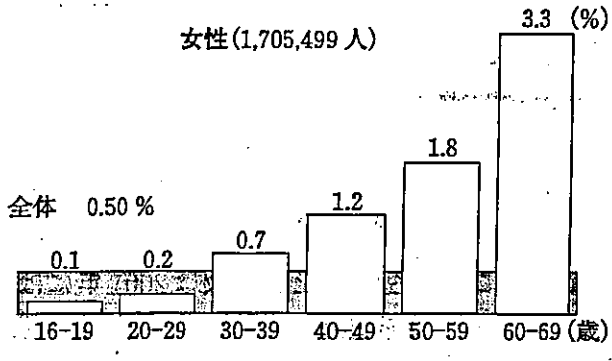
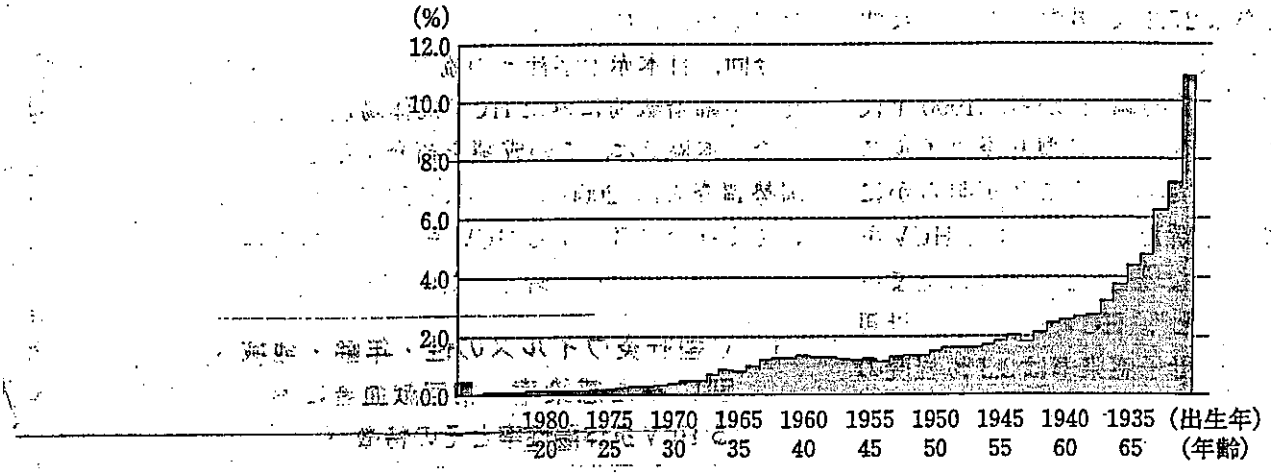


図1 年齢階級別にみたHCV抗体陽性率
(厚生労働省 C型肝炎疫学研究班)
日本赤十字社 初回献血者
1995年1月-2000年12月. 3,485,648人
2000年の時点の年齢に換算







2000年の時点の年齢に換算

図2 1歳刻みの出生年別にみたHCV抗体陽性率
(厚生労働省 C型肝炎疫学研究班)
日本赤十字社 初回献血者
1995年1月-2000年12月. 3,485,648人

り，男性では0.48%，女性では0.50%と男女間での相違は認められない．

これを年齢階級別にみると，10-20歳代では0.2%以下の低い値を示すが，年齢が高い集団ではより高い陽性率を示しており，特に，60歳以上の年齢集団では3%を超える値を示している．

1歳刻みの出生年別(2000年の時点における年齢別)にみたHCV抗体陽性率を図2に示す．1970年以後に出生した若い世代(すなわち2000年時点で30歳以下)ではHCV抗体陽性率は1%以下の低い値を示すが，1965-1950年に出生し

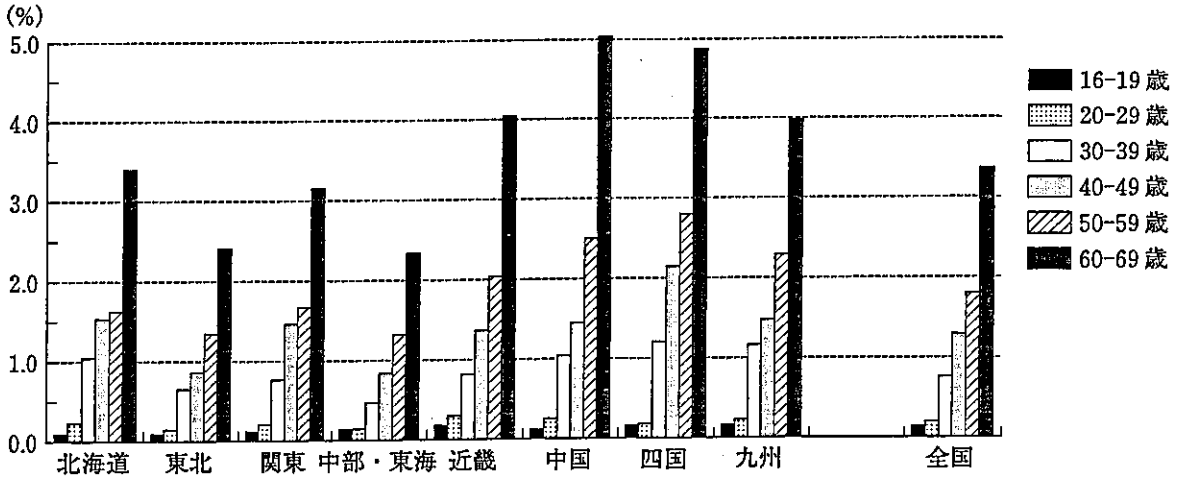


図3　8地域別，年齢階級別にみたHCV抗体陽性率
(厚生労働省　C型肝炎疫学研究班)
日本赤十字社　初回献血者
1995年1月–2000年12月．3,485,648人
2000年の時点の年齢に換算

た集団，すなわち2000年時点の年齢で35–50歳の年齢集団では1%を超え，更に1945年以前に出生した集団(同，55歳以上)では特に高いHCV抗体陽性率を示すことがわかる．

次に，全国を8つの地区に分けて，それぞれの地区における年齢階級別にみたHCV抗体陽性率を示す(図3)．

図には数値を示してはいないが，全体のHCV抗体陽性率を地区別に分けてみると，東北，関東，中部・東海地域におけるHCV抗体陽性率は0.3–0.4%を示していたのに対し，近畿以西の西日本地域，すなわち近畿，中国，四国，九州の各地区および北海道地域では0.6–0.7%とやや高い値を示すことが明らかとなった．

これを年齢階級別にみると，いずれの地区においても，年齢階級が高い集団でHCV抗体陽性率が高い値を示し，特に60歳以上の年齢層では他の年齢層と比べて，際立って高い値を示すという特徴がみられる．一方，29歳以下の若い年齢層では，いずれの地区においても，0.2%以下の低い値を示している．なお，年齢階級に関係なく，PHA法またはPA法によりHCV抗体陽性と判定された集団の約70%がHCVキャリアであることが，過去の検討により明らかになっている．

また，我が国の一般健常者集団ではHCVの新たな感染はほとんど起こっていないことが明らかとなっている．すなわち，献血者集団，定期健康診断受診者および老人福祉施設入所者におけるHCVキャリアの新規発生率について調査した結果[3]，1992–95年あるいは1997年までの期間における献血者集団内でのHCVキャリアの新規発生率は10万人当たり1.8人(広島)から3.5人(大阪)と極めて低率にとどまっていること，会社における健診受診者および福祉施設入所者ではHCVキャリアの新規発生はゼロであったことが報告されている．また，HCVの母子感染についてはHCVキャリアの母親84例から出生した87例(3組は双生児)を前方視的に追跡したところ，2例(2.3%)に感染しているにすぎないことが明らかとなっている[4]．母子感染も含めて，HCVの新たな感染はほとんど起こっていないことは，岩手県において1980年代後半以降に出生した児童のHCV抗体陽性率が，0.1%以下と極めて低い値を示していることによっても裏付けられている．

以上より，年齢階級が高い群ほどHCV抗体陽性率が高い値を示すという現象は，いわゆるコホート効果，すなわち，それぞれの年齢集団が出生してから調査時点に至るまでの間にHCV