from Vietnam, Thailand, Myanmar, China, Hong Kong and HBV/Ce from Japan, Korea. The subtypes (subgenotypes) of the 16 strains were confirmed by a phylogenetic analysis of the complete genome (Fig. 2a). Taking advantage of the five SNPs of T1041, C1044, A1050, A1053 and C1155, a RFLP method with three endonucleases was developed for distinguishing between HBV/Cs and HBV/Ce. PCR products of 309 bp in size (nt 964–1272), amplified on HBV/Cs strains, were split by *Ase*I digestion into two fragments of 88 and 221 bp and/or *BstE*II digestion into two fragments of 76 and 233 bp (Fig. 3), while those on HBV/Ce strains were not. In contrast, the products of 309 bp, amplified on HBV/Ce strains, were broken down by *Nci*I digestion into two fragments of 192 and 117 bp, while those on HBV/Cs strains were not.

Total 49 HBV/C samples, consisting of 24 in Hong Kong and 25 in Japan, were examined for the specificity of the novel PCR-RFLP method. Based on the PCR-RFLP, the 24 strains from Hong Kong were classified into HBV/Cs, and the 25 from Japan were HBV/Ce. To confirm the reliability of the PCR-RFLP method, the precore region plus core gene was sequenced directly on all 49 samples. All the 24 HBV/Cs and 25 HBV/Ce samples determined by PCR-RFLP were

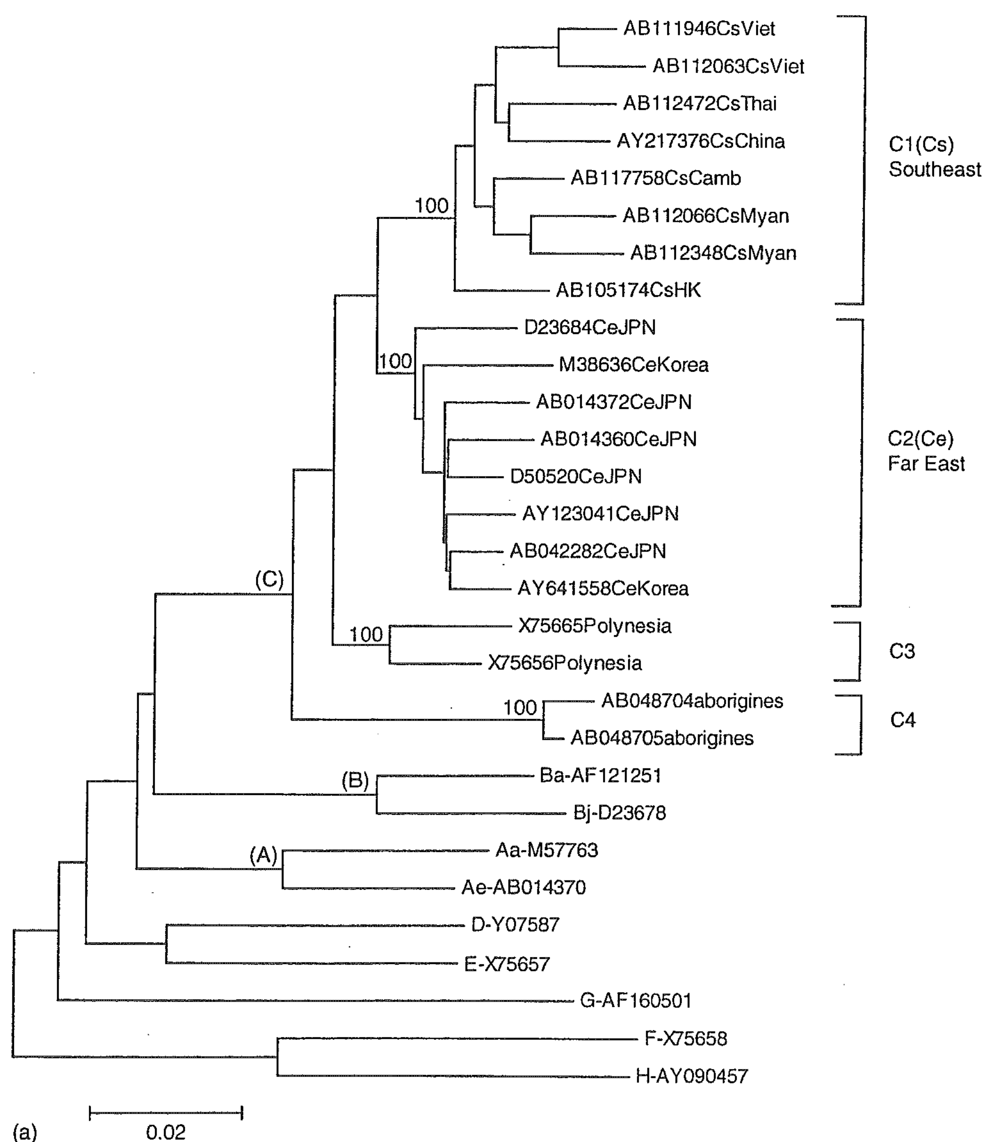

Fig. 2. (a) A phylogenetic tree constructed on the complete genome sequences of 29 HBV strains. Eight HBV/C1 (Cs) and eight HBV/C2 (Ce) strains (shown in Fig. 1) are compared along with four other HBV/C (C3 and C4) and nine HBV strains representative of the other seven genotypes (Aa, Ae, Ba, Bj, D–H). (b) A phylogenetic tree constructed on the X gene, precore and core gene sequences spanning 398 bp. Together with the above 29 representative sequences retrieved from database, 24 HBV/C1 (Cs) strains determined by PCR-RFLP belong to HBV/C1 (Cs) and 25 HBV/C2 (Ce) strains by PCR-RFLP had a cluster with the representative HBV/C2 (Ce) strains from database. All strains in this study are shown in bold. Each representative strain from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet). The length of the horizontal bar indicates the number of nucleotide substitution per site.

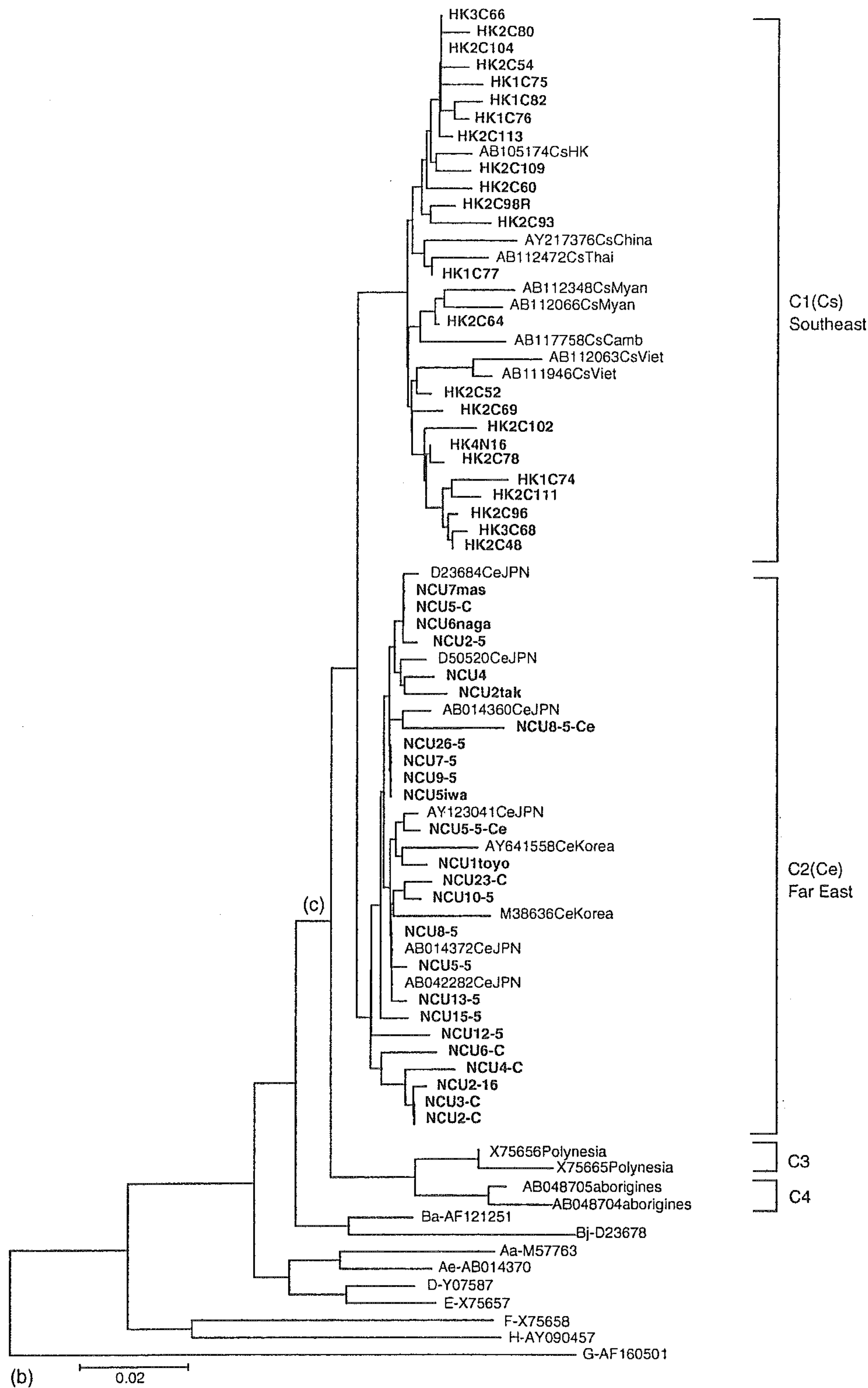

Fig. 2. (*Continued*).

completely classified into each subtype (subgenotype) by sequencing. To evaluate the sensitivity of the method, serial dilution of each HBV/Cs and HBV/Ce clones was used for the hemi-nested PCR, and its detection limit was five copies per assay.

3.3. Mutations in the enhancer, BCP and precore region in patients infected with HBV/C1 and C2

An alignment of sequences covering the BCP and the encapsidation signal (ε) in HBV/Cs and HBV/Ce allowed

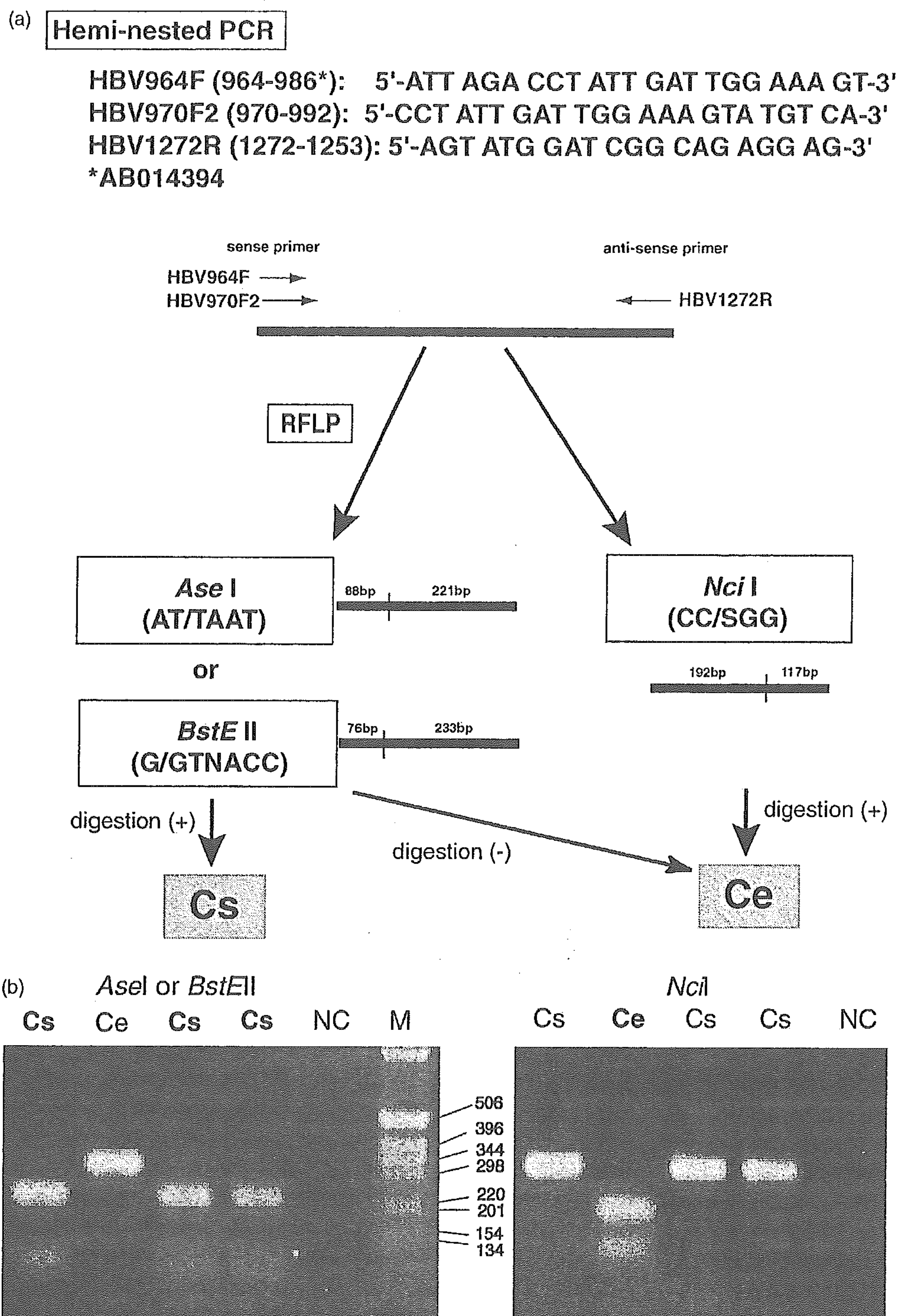

Fig. 3. (a) The strategy of a novel subtyping assay of HBV/C based on PCR-RFLP with *BstE*II, *Ase*I, *Nci*I restriction enzymes. HBV/Cs is digested by *BstE*II and/or *Ase*I, while HBV/Ce is digested by only *Nci*I. (b) Identification of restriction patterns obtained by restriction endonuclease digestion. Using hemi-nested PCR followed by cleavage with three kinds of restrict enzyme, it was possible to distinguish between HBV/Cs and HBV/Ce.

the identification of specific substitutions for HBV/C1 and HBV/C2 strains at nt 1721, 1757, 1775, 1856 and 1858 (Table 2). The prevalence of T1653, A1896 and A1899 substitutions was significantly higher in HBV/Ce than that in HBV/Cs, while the prevalence of A1727 and A1898 substitutions was higher in HBV/Cs. Double mutation in BCP (T1762/A1764) was highly prevalent in both subtypes (subgenotypes). Interestingly, the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found only in HBV/Ce strains (45/162, 28%), whereas no mutation was found in HBV/Cs strains due to C1858. Another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs strains (7/58, 12%) (Table 2).

Table 2
Subtype-specific mutations in basic core promoter and encapsidational signal of HBV/Cs and Ce strains

Nucleotide position	Cs	This study ($n=24$)	Database ($n=34$)	Ce	This study ($n=25$)	Database ($n=137$)	P-value
1653	T	2 (8%)	0	T	7 (28%)	37 (27%)	<.0001
1721	A	22 (92%)	33 (97%)	G	24 (96%)	137 (100%)	<.0001
1727	A	19 (79%)	30 (88%)	A	13 (52%)	59 (43%)	<.0001
1757	A	13 (54%)	10 (29%)	G	25 (100%)	137 (100%)	<.0001
1762/1764	T/A	21 (88%)	13 (38%)	T/A	20 (80%)	65 (47%)	NS
1775	G	15 (63%)	28 (82%)	A	25 (100%)	132 (96%)	<.0001
1856	T	9 (38%)	6 (18%)	C	25 (100%)	137 (100%)	<.0001
1858	C	23 (96%)	23 (68%)	T	25 (100%)	137 (100%)	<.0001
1896	A	0	0	A	6 (24%)	39 (28%)	<.0001
1898	A	7 (29%)	0	A	0	0	<.0001
1899	A	1 (4%)	1 (3%)	A	4 (16%)	19 (14%)	0.029

4. Discussion

Chronic patients infected with HBV/C have a more aggressive clinical course than those infected with HBV/B [3,18]. In this study, we focused on HBV/C because it is prevalent mainly in Asia and seems to contribute to progressive liver disease and poor clinical outcomes in infected patients. Phylogenetic analyses of the complete genome show at least 4 subtypes (subgenotypes) of HBV/C (C1–4) with different geographic distribution (Fig. 2a) [19,20]. HBV/C1 was found only in Southeast Asia including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong and southern China, while HBV/C2 was found in far East Asia including Japan, Korea and northern China. Additionally, two another subtypes (subgenotypes) of HBV/C were named as C3 and C4 [19,20]. C3 was found in a large area of the Pacific from New Zealand to Polynesia, while C4 was isolated from Aborigines in Northeast Australia [17]. However, as C3 and C4 strains were rarely found in most Asian countries, we focused the classification between Cs (C1) and Ce (C2) in the present study.

A total of 118 complete genome sequences of the HBV/C strains isolated in the different geographic regions were analyzed phylogenetically in the recent study [13]; the phylogenetic subclusters within HBV/C were subsequently designated respectively to the geographic regions, i.e. "Cs" for Southeast Asian (Vietnam, Thailand, Myanmar and Southern China), and "Ce" for far East Asia (Korea, Japan, and Northern China). According to this classification, 80% of the patients in Hong Kong were belonged to the Cs and 20% to the Ce [13]. When taken in account both facts, i.e. evident geographic origins of these subtypes (subgenotypes) and the phylogenetic confirmation, the designation using the small letters (indicating possible origins) appears to be logical, similarly to the previously reported Asian "Ba" and Japanese "Bj" [5,7], Africa/Asian Aa and European Ae [8]. Hence, "Cs" and "Ce" designation was applied to the present study.

Based on five SNPs between HBV/Cs and HBV/Ce, we developed a novel PCR-RFLP method for distinguishing between HBV/Cs and HBV/Ce with high reliability. All 49 samples examined were completely classified by the PCR-RFLP. This method allows the classification between these subtypes (subgenotypes) without using expensive, labor- and time-consuming methods such as sequencing and molecular evolutionary analyses. Examining additional 171 complete sequences from database, only 9 sequences of HBV/Ce have exceptional mutations at the restriction site of *Nci*I, indicating that less than 5% of the strains known up to date are unclassified by this method, and require sequencing as previously described [17].

Some specific mutations were detected in the encapsidation signal site; the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found only in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found only in HBV/Cs strains (Fig. 4). These mutations could stabilize the ε loop structure and the former HBeAg-negative mutants bearing a TAG stop codon mutation at codon 28 (A1896) uniformly replicate at least 20-fold better than mutants bearing a TGA stop codon at the same amino acid position enhance viral replication [21]. This C1858 variant was frequntly found in HBV/A and HBV/F [22]. Additionally, A1899 mutation was more prevalent in the HBV/Ce. As previously reported, the effects caused by these two closely linked mutations (A1896 and A1899) on viral replication are not independent each other [21]. The stringent selection for a highly efficient RNA encapsidation element may play a crucial role in the natural occurrence of these two closely linked precore mutations. Our replication model also shows that the combined mutations can induce higher replication in vitro (unpublished data). Hence, these several virological differences between the two subtypes (subgenotypes) might influence clinical outcomes such as fulminant hepatitis or hepatocarcinogemesis.

The biologic function of HBeAg remains controversial. Although HBeAg is not required for viral replication, it appears to be necessary for the establishment of chronic infection in animal models [23]. The most common mutation in the precore sequence that abrogates the synthesis of HBeAg is a stop-codon mutation (G1896A). As all HBV/Ce strains possessed T1858 and most HBV/Cs had C1858, the HBV/Cs with C1858 might be responsible for a delayed seroconversion for the loss of HBeAg in the carriers of HBV/Cs. The clinical significance of C1858 and T1858 among HBV/C

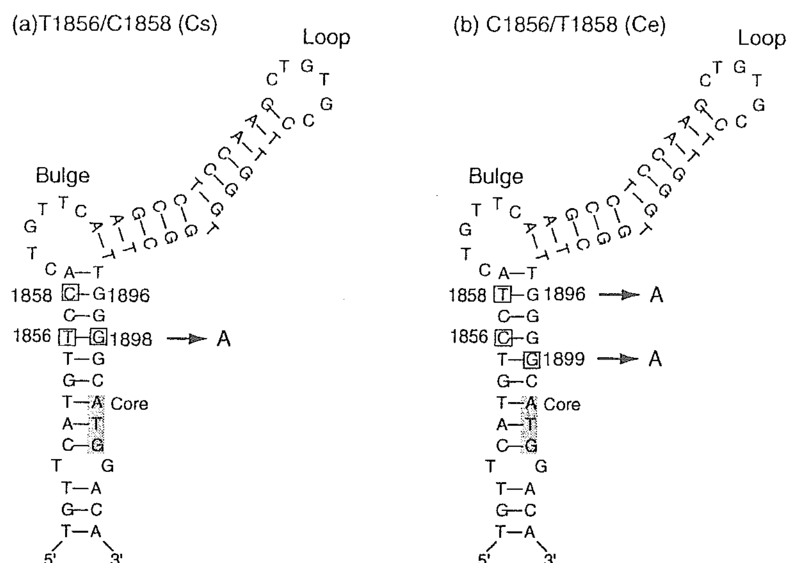

Fig. 4. Conformation of the pregenome encapsidation (ε) signal for (a) HBV/Cs and (b) HBV/Ce. The precore mutation, G1898A accompanied by T1856 forming a base pair with it, was found only in HBV/Cs strains. In contrast, the precore stop mutation, G1896A accompanied by T1858, was found only in HBV/Ce strains. A1899 mutation is significantly predominant in HBV/Ce strains.

is not well known. A previous study among multi-ethnic carriers in Hawaii indicated no significant difference in clinical characteristics between C1858 and T1858 variants [24]. However, as the number of patients was not enough to clarify the significance of this variation, further clinical studies would be required on a case–control study with large-scale cohorts.

A previous study [12] indicated that the amino acid changes specific to HBV/Cs and HBV/Ce were concentrated in the pre-S1, S and P regions, but not in the X and core regions. The pre-S1 region contains the HBV receptor for entering hepatocytes [25] and also has sites for transcriptional factors [26]. Another study [13] showed three amino acids differences in polymerase region. Therefore, the relationship between HBV/Cs and HBV/Ce and their virulence in chronic liver diseases including hepatocellular carcinoma are of great interest, since the prevalence of HBV-related hepatocellular carcinoma is extremely high in Asia compared with other regions.

In conclusion, a new PCR-RFLP method involving 5 SNPs was developed for specifically distinguishing between HBV/Cs and HBV/Ce. The two subtypes (subgenotypes) have distinct geographic distribution and virological characteristics. The novel PCR-RFLP would be useful in evaluating clinical, epidemiological and virological differences between HBV/Cs and HBV/Ce infections in countries where HBV genotype C endemic.

References

[1] Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. J Gen Virol 2002;83:1267–80.

[2] Kidd-Ljunggren K, Myhre E, Blackberg J. Clinical and serological variation between patients infected with different Hepatitis B virus genotypes. J Clin Microbiol 2004;42:5837–41.

[3] 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.

[4] Chan HL, Hui AY, Wong ML, et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 2004;53:1494–8.

[5] 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.

[6] Akuta N, Suzuki F, Kobayashi M, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315–21.

[7] 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.

[8] Sugauchi F, Kumada H, Acharya SA, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811–20.

[9] Kimbi GC, Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. J Gen Virol 2004;85:1211–20.

[10] Tanaka Y, Hasegawa I, Kato T, et al. A case–control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. Hepatology 2004;40:747–55.

[11] Kew MC, Kramvis A, Yu MC, et al. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. J Med Virol 2005;75:513–21.

[12] Huy TT, Ushijima H, Quang VX, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283–92.

[13] Chan HL, Tsui SK, Tse CH, et al. Epidemiological and virological characteristics of 2 subgroups of hepatitis B virus genotype C. J Infect Dis 2005;191:2022–32.

[14] Usuda S, Okamoto H, Tanaka T, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81–9.

[15] Usuda S, Okamoto H, Iwanari H, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 1999;80:97–112.
[16] 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.
[17] Sugauchi F, Mizokami M, Orito E, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 2001;82:883–92.
[18] Kao JH, Chen PJ, Lai MY, et al. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–9.
[19] Norder H, Courouce AM, Coursaget P, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004;47:289–309.
[20] Kramvis A, Kew M, Francois G, Hepatitis. B virus genotypes. Vaccine 2005;23:2409–23.
[21] Yuan TT, Faruqi A, Shih JW, et al. The mechanism of natural occurrence of two closely linked HBV precore predominant mutations. Virology 1995;211:144–56.
[22] Li JS, Tong SP, Wen YM, et al. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. J Virol 1993;67: 5402–10.
[23] Chen HS, Kew MC, Hornbuckle WE, et al. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. J Virol 1992;66: 5682–4.
[24] Sakurai M, Sugauchi F, Tsai N, et al. Genotype and phylogenetic characterization of hepatitis B virus among multi-ethnic cohort in Hawaii. World J Gastroenterol 2004;10:2218–22.
[25] Neurath AR, Kent SB, Strick N, et al. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 1986;46:429–36.
[26] Melegari M, Bruno S, Wands JR. Properties of hepatitis B virus pre-S1 deletion mutants. Virology 1994;199:292–300.

Takehara T, Suzuki T, Ohkawa K, Hosui A, Jinushi M, Miyagi T, Tatsumi T, Kanazawa Y, Hayashi N.

Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication.

J Hepatol. 2006 Feb;44(2):267-74. Epub 2005 Aug 25.

BACKGROUND/AIMS: The lack of small animal models supporting chronic hepatitis B virus (HBV) infection impedes the assessment of anti-viral drugs in the whole animal. Although transgenic mice have been used for this purpose, these models are clearly different from natural infection, because HBV is produced from the integrated HBV sequence harbored in all hepatocytes. METHODS: Balb/cA nude mice were hydrodynamically injected with a plasmid having 1.5-fold over-length of HBV DNA and analyzed for HBV replication. RESULTS: Hydrodynamically injected mice showed substantial levels of antigenemia and viremia for more than 1 year. Covalently closed circular DNA (cccDNA), the template of viral replication in natural infection, was produced in the livers and was critically involved in the long-term HBV production, because disruption of the pol gene of the inoculated DNA resulted in transient expression of HBV genes for less than 2 months. Administration of the IFNalpha gene transiently suppressed HBV DNA replication, but was not capable of eliminating HBV in this model. CONCLUSIONS: In vivo gene transfer of a plasmid encoding HBV DNA can establish chronic viral replication in mice, which involves, at least in part, new synthesis of the HBV cccDNA episome, thus recapitulating a part of human HBV infection.

HEV

Journal of Medical Virology 75:499–503 (2005)

Serological Markers of Hepatitis B, C, and E Viruses and Human Immunodeficiency Virus Type-1 Infections in Pregnant Women in Bali, Indonesia

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Except for hepatitis B virus (HBV), there have been few data on serological markers of hepatitis viruses such as hepatitis C virus (HCV) and E virus (HEV), and human immunodeficiency virus type-1 (HIV) in Bali, Indonesia. During 5 months from April to August 2003, sera were collected from 2,450 pregnant women at eight jurisdictions in Bali, and they were tested for markers of these viruses. Only one (0.04%) was positive for antibody to HCV, but none for antibody to HIV. Hepatitis B surface antigen (HBsAg) was detected in 46 (1.9%) at a prevalence significantly lower than that in 271 of the 10,526 (2.6%) pregnant women in Bali surveyed 10 years previously ($P < 0.045$). The prevalence of hepatitis B e antigen in pregnant women with HBsAg decreased, also, from 50% to 28% during the 10 years ($P < 0.011$). Antibody to HEV (anti-HEV) was examined in 819 pregnant women who had been randomly selected from the 2,450. The overall prevalence of anti-HEV was 18%, and there were substantial regional differences spanning from 5% at Tabanan district to 32% at Gianyar district. Furthermore, the prevalence of anti-HEV differed substantially by their religions. In the Sanglah area of Denpasar City, for instance, anti-HEV was detected in 20 of the 102 (20%) Hindus, significantly more frequently than in only 2 of the 101 (2.0%) Muslims ($P < 0.001$). Swine that are prohibited to Muslims, therefore, is likely to serve as a reservoir of HEV in Bali. In conclusion, HBV is decreasing, HCV and HIV have not prevailed, as yet, while HEV is endemic probably through zoonotic infection in Bali. ***J. Med. Virol. 75:499–503, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: Bali; hepatitis B e antigen; hepatitis B virus; hepatitis C virus; hepatitis E virus; human immunodeficiency virus type-1; pregnancy; zoonosis

INTRODUCTION

Bali is an island in Southeast Asia, between the Bali sea and the Indian Ocean, and has approximately 3 million inhabitants. The prevalence of infection with hepatitis C virus (HCV) has not been examined, as yet, although there are a few reports on serological markers of hepatitis B virus (HBV) and hepatitis E virus (HEV) in Bali [Brown et al., 1985; Wibawa et al., 2004]. Nor is it known whether the population in Bali is affected by human immunodeficiency virus type-1 (HIV), except in commercial sex workers [Ford et al., 2000].

Since 1993, pregnant women in Bali have been tested for hepatitis B surface antigen (HBsAg) in serum, and those positive for HBsAg were examined further for hepatitis B e antigen (HBeAg). Babies born to pregnant women carrying HBsAg along with HBeAg have received the passive and active immunoprophylaxis with hepatitis B immune globulin and vaccine [Tada et al., 1982]; it is found highly efficacious in preventing the persistent HBV carrier state in high-risk babies in Japan [Noto et al., 2003].

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Taking advantage of routine screening for HBsAg of pregnant women in Bali, sera were tested for serological markers of HBV, HCV, HEV, and HIV. The results highlighted decreasing HBV infection, rare infection with HCV and HIV, and a high exposure to HEV that depends on habits and religions of the Balinese.

MATERIALS AND METHODS

Pregnant Women in Bali

During 5 months from April to August 2003, sera were obtained from 2,450 pregnant women, at major hospitals in the eight jurisdictions of Bali (Fig. 1), on routine surveys for HBsAg for preventing the perinatal transmission of HBV. Their mean age was 27 ± 5 (SD) years (range: 16–45 years). The sera were tested for HBsAg, antibody to HCV (anti-HCV) and antibody to HEV (anti-HEV), as well as antibody to HIV (anti-HIV). HBeAg was examined only in sera positive for HBsAg. Sera from all pregnant women were tested for serological markers of these viruses, except for anti-HEV which was examined in approximately 100 each randomly selected in the eight jurisdictions. Anti-HEV was tested in an additional 90 sera from Muslim pregnant women living in the Sanglah area of Denpasar City, in an attempt to find any differences in the prevalence between Hindus and Muslims. The design of the serological survey was in accord with the 1975 Declaration of Helsinki, and approved by Ethics Committee of institutions. Every pregnant woman gave an informed consent.

Serological Tests for Markers of HBV, HCV, HEV, and HIV

HBsAg was tested by hemmaglutination and immunochromatography (Entebe HBsAg RPHA and Entebe HBsAg Strip, respectively: Hepatika Laboratory, Mataram, Indonesia) and HBeAg by enzyme-linked immunosorbent assay (ELISA) (HBeAg ELISA: Institute of Immunology, Tokyo, Japan). Anti-HCV was determined by the dipstick method (Entebe Anti-HCV Dipstick: Hepatika Laboratory). Anti-HEV of IgG class was determined by ELISA with use of a recombinant HEV capsid protein of genotype IV by the method of Mizuo et al. [2002], and anti-HIV by immunochromatography (Entebe Anti-HIV Strip: Hepatika Laboratory).

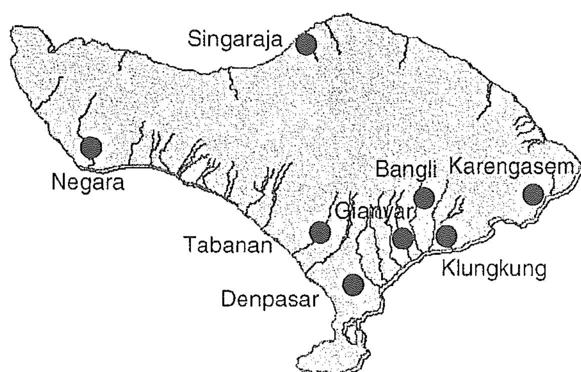

Fig. 1. Map of Bali with eight districts where markers of hepatitis viruses and HIV among pregnant women were surveyed.

HEV RNA was determined by the polymerase chain reaction with primers deduced from the nucleotide sequences in the open reading frame 2 that are preserved irrespective of genotypes [Mizuo et al., 2002].

Statistical Analyses

Categorical variables were compared between groups by the Chi-square test, and continuous variables by the Welch's t-test. Differences with a P value <0.05 were considered significant.

RESULTS

HBsAg in Pregnant Women in Bali

Frequencies of HBsAg, anti-HCV, anti-HEV, and anti-HIV in the eight jurisdictions in Bali are listed in Table I. Overall, HBsAg was detected in 46 of the 2,450 (1.9%) pregnant women during 5 months from April to August 2003. This prevalence of HBsAg was significantly lower than that in 271 of the 10,526 pregnant women in Bali surveyed 10 years before in 1993 (1.9% vs. 2.6%, $P < 0.045$).

The prevalence of HBsAg in Negara in the west (Fig. 1) was by far the highest at 4.5% (6/132), in remarkable contrast to 0.6% (1/161) in Tabanan and 0.8% (1/133) in Singaraja. Differences fell short of being significant, however, due to low numbers of pregnant women examined.

Figure 2 illustrates age-specific frequencies of HBsAg and HBeAg. The prevalence of HBsAg stayed constant in a range from 1.6% to 2.5%, while HBeAg decreased with age; it was most frequent in pregnant women aged younger than 25 years (53% [8/15]). Of 46 pregnant women who carried HBsAg, the 13 with HBeAg in serum were significantly younger than the 33 without HBeAg (24 ± 4 vs. 29 ± 6 years, $P < 0.0190$).

Anti-HCV and Anti-HIV in Pregnant Women in Bali

Infection with HCV or HIV was very infrequent in pregnant women in Bali. Anti-HCV was detected in a single pregnant woman in Denpasar, while anti-HIV was not in any. Thus, the prevalence of anti-HCV was 0.04% and that of anti-HIV less than 0.04%.

Anti-HEV in Pregnant Women in Bali

Anti-HEV was examined in all the 41 pregnant women from Karangasem, and 86–196 randomly selected among those from the other districts. Anti-HEV was detected in 151 of these 819 (18%) pregnant women, producing an overall prevalence of 18%. The mean absorbancy in ELISA on the 151 sera positive for anti-HEV was low at 0.79 ± 0.61. HEV RNA was not detected in any of the 20 sera with a high absorbancy (>1.50).

There were marked regional differences in the prevalence of anti-HEV. It was low in Tabanan (4.7% [4/86])

TABLE I. Serological Markers for HBV, HCV, HEV, and HIV Infections in the Eight Jurisdictions of Bali

Jurisdictions	HBsAg	Anti-HCV	Anti-HEV	Anti-HIV
Bangli	2/115 (1.7%)	0/115	25/93 (27%)	0/115
Denpasar	29/1,594 (1.8%)	1/1,594 (0.06%)	35/196 (18%)	0/1,594
Gianyar	3/151 (2.0%)	0/151	32/101 (32%)	0/151
Karangasem	1/41 (2.4%)	0/41	6/41 (15%)	0/41
Klungkung	3/123 (2.4%)	0/123	19/98 (19%)	0/123
Negara	6/132 (4.5%)	0/133	11/100 (11%)	0/133
Singaraja	1/133 (0.8%)	0/132	19/104 (18%)	0/132
Tabanan	1/161 (0.6%)	0/161	4/86 (4.7%)	0/161
Total	46/2,450 (1.9%)	1/2,450 (0.04%)	151/819 (18%)[a]	0/2,450 (<0.04%)

[a]Anti-HEV was examined in only 819 samples, randomly extracted from among inhabitants from each jurisdiction, except for Karangasem all pregnant women from where were examined.

and high in Gianyar (32% [32/101]) and Bangli (27% [25/93]); the difference between Tabanan and Gianyar was statistically significant ($P < 0.0001$). Frequencies of anti-HEV in the other five districts were much the same and ranged from 11% to 19%. There were no differences in the mean age among pregnant women from distinct religions.

The prevalence of anti-HEV differed with regard to the religion of the pregnant women (Table II). Overall, anti-HEV was detected in 149 of the 769 (19%) Hindus, at a frequency significantly higher ($P < 0.012$) than that in two of the 50 (4.0%) non-Hindus (mostly Muslims). The frequency of anti-HEV higher in Hindus than non-Hindus held in pregnant women from all the eight jurisdictions. In Denpasar where more women were examined than the other seven districts, anti-HEV occurred more often in Hindus than non-Hindus (19% [33/175] vs. 9.5% [2/21]); the difference fell short of being significant due to small numbers examined.

For evaluating the influence of religions on HEV infection, pregnant women living in the Sanglah area of Denpasar City were examined for the prevalence of anti-HEV; inhabitants in this narrow area were surveyed in an attempt to exclude environmental factors such as water quality and sanitation. Anti-HEV was significantly more frequent in Hindus than Muslims there (20% [20/102] vs. 2.0% [2/101], $P < 0.001$).

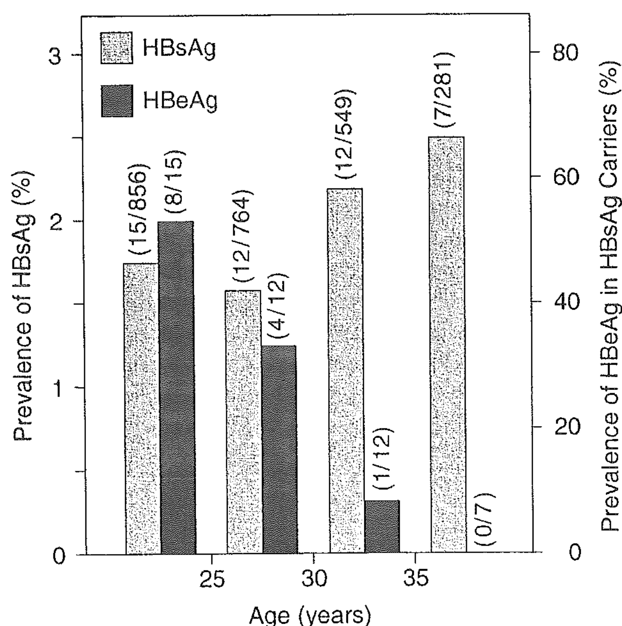

Fig. 2. Age-specific prevalence rates of HBsAg and HBeAg in 2,450 pregnant women in Bali.

DISCUSSION

In surveys for serological markers of HBV and HCV infections among blood donors performed in 1991 in Jakarta, Indonesia, HBsAg was detected in 5.8% and anti-HCV in 17.7% [Sastrosoewignjo et al., 1991]. HBV and HCV strains indigenous to Indonesia are reported in blood donors and hepatitis patients there [Sastrosoewignjo et al., 1991; Hadiwandowo et al., 1994; Mulyanto et al., 1997]. Data are still inadequate, however, on serological markers of HBV and HCV infections, as well as HIV infection, in the general population in Bali that is isolated from the other Indonesian archipelagos by the sea. Nor are there any data available for the exposure to HEV in Bali, except for a recent report by Wibawa et al. [2004] on 276 family members of chronic liver disease and 797 voluntary blood donors.

Taking advantage of the routine screening for HBsAg, 2,450 pregnant women in Bali were tested for serological markers of HBV, HCV, and HEV infections, and HIV infection. The prevalence of HBsAg examined during 5 months in 2003 was significantly lower than that in 1993 (1.9% vs. 2.6%, $P < 0.045$). It is not certain, however, how the prevalence of HBV markers surveyed

TABLE II. Frequencies of Anti-HEV in Hindu and Non-Hindu Women in Various Districts of Bali

Districts	Hindu	Non-Hindu	Differences
Bangli	25/92 (27%)	0/1	NS[a]
Denpasar	33/175 (19%)	2/21 (9.5%)	NS
Gianyar	32/100 (32%)	0/1	NS
Karangasem	6/39 (15%)	0/2	NS
Klungkung	19/91 (21%)	0/7	NS
Negara	11/90 (12%)	0/10	NS
Singaraja	19/99 (19%)	0/5	NS
Tabanan	4/83 (4.8%)	0/3	NS
Total	149/769 (19%)	2/50 (4.0%)	$P < 0.012$

[a]Not significant.

in pregnant women who give birth to their babies in hospitals is extended to the general population in Bali, where the majority of deliveries are conducted by midwives at home. Wibawa et al. [2004] detected HBsAg in 38 of the 797 (5%) voluntary blood donors and 18 of the 276 (7%) family members of patients with chronic liver disease from Bali. Their results stand at a substantial variance with ours.

Anti-HCV in pregnant women in Bali was low at 0.04%, in contrast to the detection of anti-HCV in 17.7% of voluntary blood donors in Jakarta [Sastrosoewignjo et al., 1991]. Although data are lacking for the prevalence of anti-HCV in the Balinese, it is reasonably expected to be low in the general population of Bali; Wibawa et al. [2004] detected anti-HCV in 6 of the 796 (0.8%) blood donors. With rapid increases of immigrant and tourists into Bali, however, the exposure to HCV may expand in the foreseeable future. In support of this view, the prevalence of anti-HIV among female sex workers in Bali is reported to be higher for immigrants than the Balinese [Ford et al., 2000].

To address possible concerns on the sensitivity of locally produced assays for HBsAg and anti-HCV, the Entebe kits for these viral markers have been used during the past 18 and 8 years since 1986 and 1996 for HBsAg and anti-HCV, respectively, for screening blood units at many blood centers in Lombok and other islands of Indonesia. Indisputable decrease (to practically zero) in the incidence of posttransfusion hepatitis B and C since then would indicate a high sensitivity of these tests.

HIV infection has become very rare in female sex workers in Bali (0.2%), although the frequencies of sexually transmitted disease such as gonorrhoea (60.5%), chlamydia (41.3%), and human papilloma virus (37.7%) remain very high [Ford et al., 2000]. The reasons for such a low exposure to HIV in the Balinese, even in high-risk groups, are not clear. It is a surprise, especially because a pandemic of HIV is expected in Indonesia [Anonymous, 1996]. Isolation from the other areas of Indonesia, surrounded by sea, may have prevented exposure to HIV and HCV that have been introduced more recently than HBV. In addition, heavy punishments imposed on the use of illegal drugs may have prevented the spread of these blood-borne viruses there.

Overall, anti-HEV was detected in 18% of pregnant women living in eight jurisdiction, at a frequency comparable to 18%–20% recently reported in Bali [Wibawa et al., 2004]. Previous findings point to the zoonotic food-borne transmission that may play an important role in HEV infection among Japanese people. For instance, some individuals who ate sashimi prepared from deer caught in the wild [Tei et al., 2003] or feral boar's liver in the raw [Matsuda et al., 2003] developed acute or fulminant hepatitis E. In addition, Yazaki et al. [2003] have suggested the ingestion of pig's liver as a major risk factor for hepatitis E among residents of Hokkaido, Japan. These observations in Japan instigated us to look into whether zoonotic food-borne transmission of HEV also occurs in inhabitants of Bali where anti-HEV has not been surveyed extensively. As the results, the prevalence of anti-HEV was found to be more frequent in Hindu than Muslim residents of Bali. Muslims are strictly prohibited from eating or touching pigs, while Hindus have no such restrictions.

When the prevalence of IgG anti-HEV was compared among pregnant women in eight districts of Bali, significant differences were found among them in a range from 4.7% (4/86) in Tabanan to 32% (32/101) in Gianyar. An even more striking difference was noted in pregnant women between Hindus and non-Hindus (mostly Muslims and a few Christians) (19% [149/769] vs. 4.0% [2/50], $P < 0.012$).

Since the religion of Bali is predominantly Hindu, a random sampling of the Balinese would hardly reflect the anti-HEV status in non-Hindus, as in the study of Wibawa et al. [2004] and ours. Furthermore, the exposure to HEV may be influenced by sanitary conditions and water quality that differ in various areas of Bali. These factors taken into considerations, pregnant women living in a restricted area of Denpasar City (Sanglah) were examined for evaluating the influence of religion on HEV exposure. As the results, anti-HEV was significantly more frequent in Hindus than Muslims (20% [20/102] vs. 2.0% [2/101], $P < 0.001$).

The observed differences in the prevalence of anti-HEV would be attributed to distinct life-styles of the Balinese in association with their religions. Among many differences dependent on religions, those in the dietary habit are prominent. Hindu families in Bali typically keep pigs within the household, as a source of food, and often eat grilled pork that can be undercooked. In contrast, Muslims are rigorously prohibited from tasting or even touching pigs by their religion. Thus, it would be reasonable to implicate close contacts with pigs, along with the ingestion of domestic pork, in a high exposure to HEV among Hindus living in Bali. Although "water-borne" transmission of HEV has been reported in Indonesia [Corwin et al., 1997], the results obtained in this study suggest an alternative mode of HEV transmission in Bali that is "pig-borne." In actuality, pigs in Bali are highly contaminated with HEV; anti-HEV is detected in more than 70% of them [Wibawa et al., 2004]. Furthermore, zoonotic infections are common among children and teenagers in Bali [Chomel et al., 1993].

Women in Bali appear to have been exposed to HEV long before the pregnancy. The absorbancy for anti-HEV in ELISA was mostly low and HEV RNA was not detectable in any of 20 sera with a high absorbancy (>1.50). Hence, the risk of developing fulminant hepatitis by HEV infection during the pregnancy would be lower in Bali than in India [Kar et al., 1997].

REFERENCES

Anonymous. 1996. Indonesia and Vietnam may face AIDS pandemic. AIDS Wkly Plus 28:13.

Brown P, Breguet G, Smallwood L, Ney R, Moerdowo RM, Gerety RJ. 1985. Serologic markers of hepatitis A and B in the population of Bali, Indonesia. Am J Trop Med Hyg 34:616–619.

Chomel BB, Kasten R, Adams C, Lambillotte D, Theis J, Goldsmith R, Koss J, Chioino C, Widjana DP, Sutisna P. 1993. Serosurvey of some major zoonotic infections in children and teenagers in Bali, Indonesia. Southeast Asian J Trop Med Public Health 24:321–326.

Corwin A, Putri MP, Winarno J, Lubis I, Suparmanto S, Sumardiati A, Laras K, Tan R, Master J, Warner G, Wignall FS, Graham R, Hyams KC. 1997. Epidemic and sporadic hepatitis E virus transmission in West Kalimantan (Borneo), Indonesia. Am J Trop Med Hyg 57:62–65.

Ford K, Wirawan DN, Reed BD, Muliawan P, Sutarga M. 2000. AIDS and STD knowledge, condom use and HIV/STD infection among female sex workers in Bali, Indonesia. AIDS Care 12:523–534.

Hadiwandowo S, Tsuda F, Okamoto H, Tokita H, Wang Y, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus subtypes and hepatitis C virus genotypes in patients with chronic liver disease or on maintenance hemodialysis in Indonesia. J Med Virol 43: 182–186.

Kar P, Budhiraja S, Narang A, Chakravarthy A. 1997. Etiology of sporadic acute and fulminant non-A, non-B viral hepatitis in north India. Indian J Gastroenterol 16:43–45.

Matsuda H, Okada K, Takahashi K, Mishiro S. 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis 188:944.

Mizuo H, Suzuki K, Takikawa Y, Sugai Y, Tokita H, Akahane Y, Itoh K, Gotanda Y, Takahashi M, Nishizawa T, Okamoto H. 2002. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J Clin Microbiol 40:3209–3218.

Mulyanto, Tsuda F, Karossi AT, Soewignjo S, Roestamsjah, Sumarsidi D, Trisnamurti RH, Sumardi, Surayah, Udin LZ, Melani W, Kanai K, Mishiro S. 1997. Distribution of the hepatitis B surface antigen subtypes in Indonesia: Implications for ethnic heterogeneity and infection control measures. Arch Virol 142: 2121–2129.

Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, Mito H, Yoshizawa H. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. J Gastroenterol Hepatol 18:943–949.

Sastrosoewignjo RI, Sandjaja B, Okamoto H. 1991. Molecular epidemiology of hepatitis B virus in Indonesia. J Gastroenterol Hepatol 6:491–498.

Tada H, Yanagida M, Mishina J, Fujii T, Baba K, Ishikawa S, Aihara S, Tsuda F, Miyakawa Y, Mayumi M. 1982. Combined passive and active immunization for preventing perinatal transmission of hepatitis B virus carrier state. Pediatrics 70:613–619.

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

Wibawa IDN, Muljono DH, Mulyanto, Suryadarma IG, Tsuda F, Takahashi M, Nishizawa T, Okamoto H. 2004. Prevalence of antibodies to hepatitis E virus among apparently healthy humans and pigs in Bali, Indonesia: Identification of a pig infected with a genotype 4 hepatitis E virus. J Med Virol 73:38–44.

Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H. 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 84:2351–2357.

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Rapid communication

Hepatitis E virus infection in wild mongooses of Okinawa, Japan: Demonstration of anti-HEV antibodies and a full-genome nucleotide sequence☆

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Abstract

Hepatitis E virus (HEV), a single-strand RNA virus, has been recovered not only from human beings but also from various species of animals. Here we report our results suggesting that mongoose should be added to the list of reservoir animals of HEV. Of 100 mongooses we examined in Okinawa, Japan, 21 were thought to be positive for anti-HEV antibodies, among which one was definitely positive for HEV RNA. Full-genome sequencing of the HEV isolate revealed that it segregates to a unique subgroup within genotype III. Interestingly, this mongoose strain was closely related to a swine isolate previously reported from Okinawa, implicating the possibility of interspecies transmission between these animals.

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Keywords: Hepatitis E virus (HEV); Single-stranded RNA virus; Zoonosis; Interspecies transmission; Mongoose

1. Introduction

Hepatitis E virus (HEV), which causes acute sporadic hepatitis as well as outbreaks of so-called "water-borne hepatitis" in human beings, was isolated first from human beings [1], next from swine in the United States [2], then from rat in Nepal [3], wild boar and deer in Japan [4,5], and more recently from horse in Egypt [6]. In addition, it has been reported that other animals worldwide such as monkey, goat, cow, sheep, cat, and so on have antibodies against HEV even though viral RNA has not yet been recovered. Here, we report for the first time an HEV isolate from mongoose, a cat-like carnivore of *Herpestidae* family.

2. Materials and methods

2.1. Antibody assay

IgG class antibodies against HEV in the mongooses' sera were determined using an in-house enzyme-linked immunosorbent assay (ELISA), with some modifications of the previously reported method [7]. Briefly, the solid phase was a recombinant capsid protein of HEV, which was kindly provided by Dr. Li Tian-Cheng, and the tracer antibodies were horse radish peroxidase-labled anti-cat rabbit IgG (MP

☆ The nucleotide sequence reported in this paper will appear in DDBJ/EMBL/GenBank databases under accession number AB236320

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Biomedicals Inc., Ohio, USA). We used this anti-cat antibodies under the presumption that it could surrogate anti-mongoose antibodies (commercially unavailable) because mongooses and cats belong to the same *Feliodea* superfamily.

2.2. Detection and sequencing of HEV genome

Detection and nucleotide sequencing of the HEV RNA in the mongooses' sera were performed by the methods described previously [8,9]. Briefly, HEV RNA from the nucleic acids extracted from the mongoose serum was reverse-transcribed to cDNA with use of the THERMOSCRIPT RT System (Invitrogen Corporation, California, USA), and PCR amplification of several overlapping regions of the HEV genome was carried out in the presence of PLATINUM Taq DNA Polymerase High Fidelity (Invitrogen). The 5′- and 3′-terminal sequences were amplified with 5′-Full RACE Core Set (TaKaRa Shuzo Co., Ltd., Shiga, Japan) and

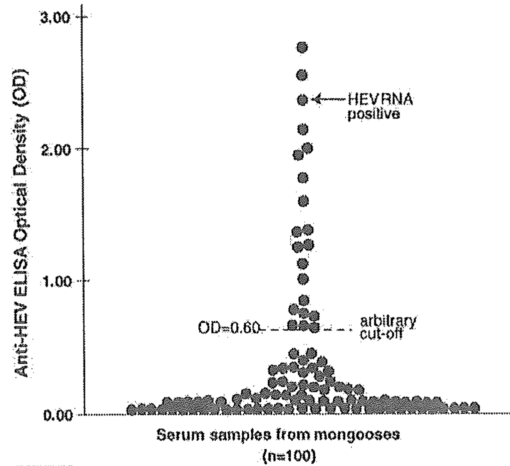

Fig. 1. IgG class antibodies against HEV determined by ELISA.

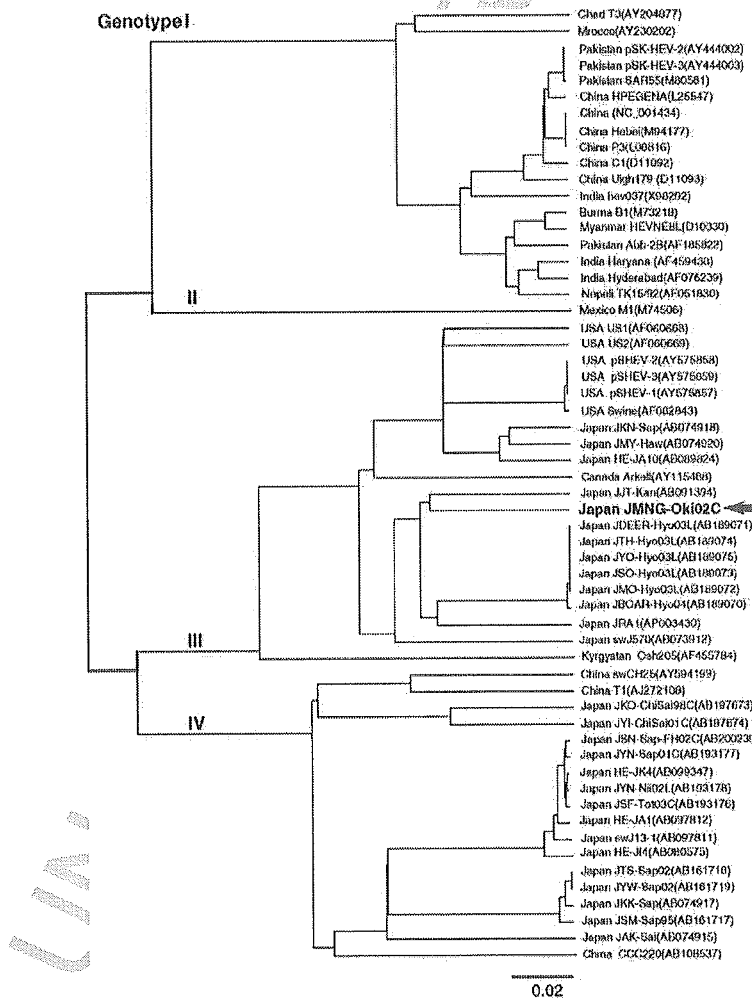

Fig. 2. Phylogenetic tree (UPGMA) based on complete or nearly complete nucleotide sequences of HEV.

3′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen), respectively.

3. Results

3.1. HEV antibodies and RNA in the mongooses' sera

Sera from 100 mongooses captured in 2002 in the mainland of Okinawa prefecture, Japan, were subjected to detection of anti-HEV IgG antibodies and HEV RNA. In an enzyme-linked immunoassay (ELISA) for the antibodies, 21 sera showed optical densities (OD) at greater than 0.600, and were arbitrarily regarded as antibody-positive (Fig. 1). One with the ELISA OD at 2.356 was also positive for HEV RNA by PCR, whose nucleotide sequence was then determined for a partial 412-nt region within ORF2 (isolate name "mnOK1", DDBJ/EMBL/GenBank accession number AB219129), and later for the entire genome (isolate name "JMNG-Oki02C", AB236320).

3.2. The full-genome strain of HEV from mongoose compared to known isolates

The JMNG-Oki02C was comprised of 7236 nucleotides (nt), with the following arrangement of genetic elements from 5′- towards 3′-end: 5′-UTR (nt positions 1–25), ORF1 (nt 26–5137 coding for 1703 amino acids (aa)), ORF2 (nt

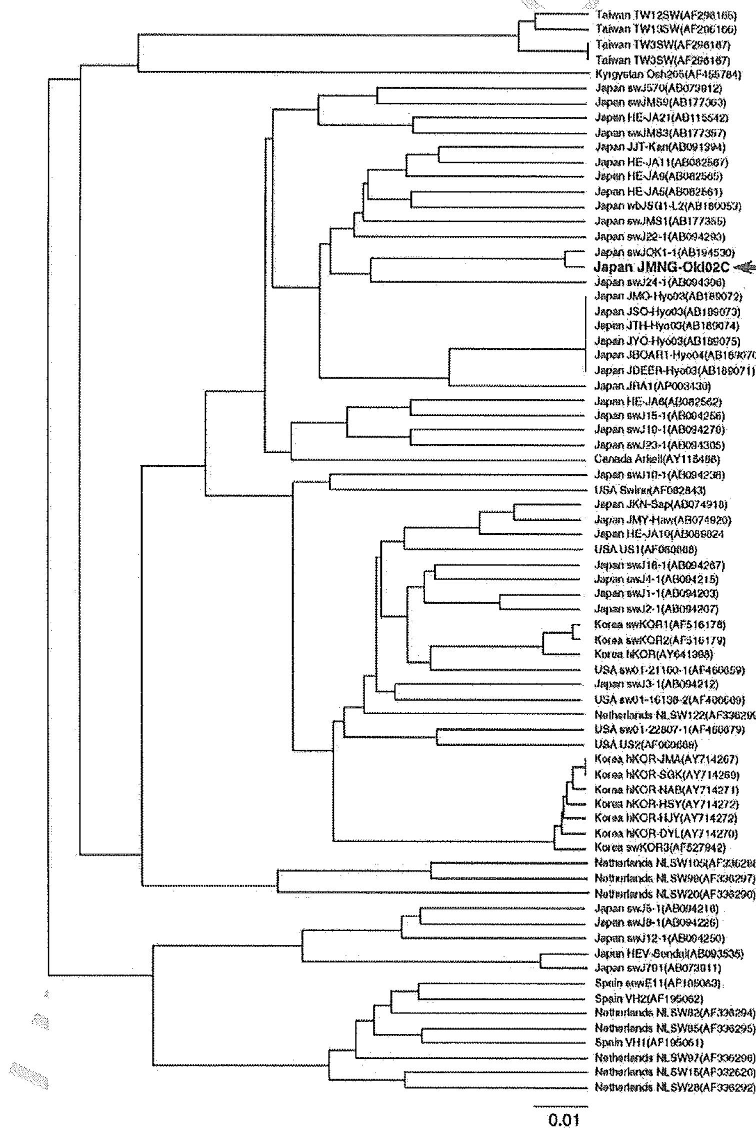

Fig. 3. Phylogenetic tree (UPGMA) based on a partial 412-nt sequence within ORF2. Only isolates of genotype III were included here.

5172–7154 for 660 aa), ORF3 (nt 5134–5502 for 122 aa), 3′-UTR (nt 7155–7226), and a poly-A tail (nt 7227–7236). The residue of nt 6029 was revealed to be Y (both C and T were detected there) in the JMNG-Oki02C isolate (AB236320), whereas we had reported it to be T in the partial sequence mnOK1 (AB219129).

Comparison with full or nearly full-genome HEV isolates so far reported indicated that the mongoose-derived HEV segregates to the genotype III, in particular to a subgroup of those recovered from humans and animals in Japan (Fig. 2). Then when further compared with much wider range of isolates including even those for which only partial sequences had been known, the JMNG-Oki02C sequence showed a strong similarity to one of the swine-derived isolates from Japan (Fig. 3). This swine HEV, swJOK1-1 (AB194530) [10], was an isolate that was obtained from a farm pig in the mainland Okinawa, just the same place where our mongooses were captured.

4. Discussion

Hepatitis E is a zoonosis, supported by direct evidence such as the case where human beings became infected with HEV by eating raw meat (*Sashimi*) of wild deer [4] and by a lot of indirect evidence. Yet we have not known all of the reservoir animals of HEV, unfortunately.

Our present results add to knowledge of the zoonotic aspect of HEV. Since not only antibodies (21%) but also viral RNA were identified, the wild mongoose community we studied in the Okinawa Island is undoubtedly a reservoir, or one of natural hosts, of HEV. Mongoose is a member of the *Herpestidae* family, superfamily of which is *Feliodea*. Of the *Feliodea* animals, only cat has so far been suspected as a reservoir of HEV: presence of antibodies against HEV in pet cat in Japan was once reported [11]. But identification of viral RNA from the *Feliodea* animals has never been successful until our present study.

Another interesting finding in our study was that the mongoose-derived HEV sequence was very homologous to that of a swine isolate (swJOK1-1, AB194530 [10]) which was obtained from a farm pig in the Okinawa Island: nucleotide similarity was 99.5% between JMNG-Oki02C and swJOK1-1 (note: these isolates were analyzed by separate groups of researchers, leaving no chance for laboratory contaminations). Hence, it is possible that an interspecies transmission of HEV might have occurred between pigs and mongooses in Okinawa. Although these animals do not eat each other (mongooses feed on insects, crabs, worms, lizards, and other small creatures), the farm pigs might have been exposed to HEV that was excreted in the feces of mongooses, or vice versa, because mongooses have a cruising radius that is wide enough to intrude the pig farms.

Mongoose was imported into the Okinawa Island in 1910 from India, with an expectation that they might fight and kill the venomous snake *Habu* of Okinawa (because the Indian mongoose was renowned for killing cobras). Although this is merely a speculation, one or some of the imported mongooses might have been infected with HEV at that time, and might transmit it to other will-be-reservoir animals in the Okinawa Island. One of the reasons why we speculate so comes from our unpublished results of molecular clock analyses suggesting that HEV made inroad into Japan around 1900.

Acknowledgments

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References

[1] Reyes GR, Purdy MA, Kim JP, et al. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 1990;247:1335–9.
[2] Meng XJ, Purcell RH, Halbur PG, et al. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA 1997;94:9860–5.
[3] He J, Innis BL, Shrestha MP, et al. Evidence that rodents are a reservoir of hepatitis E virus for humans in Nepal. J Clin Microbiol 2002;40:4493–8.
[4] Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 2003;362: 371–3.
[5] Takahashi K, Kitajima N, Abe N, Mishiro S. 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 2004;330:501–5.
[6] Saad MD, Hussein AH, Bashandy MM, et al. Detection of hepatitis E virus infection in work horses in Egypt, 2005 (published only in DDBJ/EMBL/GenBank databases, accession AY963777–80).
[7] Li TC, Zhang J, Shinzawa H, et al. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J Med Virol 2000;62:327–33.
[8] Takahashi K, Iwata K, Watanabe N, et al. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2001;287:9–12.
[9] Takahashi K, Kang JH, Ohnishi S, et al. Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. Intervirology 2003;46:308–18.
[10] Takahashi M, Nishizawa T, Tanaka T, Tsatsralt-Od B, Inoue J, Okamoto H. Correlation between positivity for immunoglobulin A antibodies and viraemia of swine hepatitis E virus observed among farm pigs in Japan. J Gen Virol 2005;86:1807–13.
[11] Usui R, Kobayashi E, Takahashi M, Nishizawa T, Okamoto H. Presence of antibodies to hepatitis E virus in Japanese pet cats. Infection 2004;32:57–8.

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Short Communication

Molecular tracing of Japan-indigenous hepatitis E viruses

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The ancestor(s) of apparently Japan-indigenous strains of *Hepatitis E virus* (HEV) was probably of foreign origin, but it remains unclear when and from where it made inroads. In this study, 24 genotype 3 and 24 genotype 4 HEV strains recovered in Japan each showed a significant cluster, clearly distinct from those of foreign strains, in the phylogenetic tree constructed from an 821 nt RNA polymerase gene fragment. The evolutionary rate, approximately $0{\cdot}8 \times 10^{-3}$ nucleotide substitutions per site year^{-1}, enabled tracing of the demographic history of HEV and suggested that the ancestors of Japan-indigenous HEV had made inroads around 1900, when several kinds of Yorkshire pig were imported from the UK to Japan. Interestingly, the evolutionary growth of genotype 3 in Japan has been slow since the 1920s, whereas genotype 4 has spread rapidly since the 1980s. In conclusion, these data suggest that the indigenization and spread of HEV in Japan were associated with the popularization of eating pork.

Transmission of *Hepatitis E virus* (HEV) occurs primarily by the faecal–oral route through contaminated water supplies in developing countries (Purcell & Emerson, 2001). Additionally, increasing evidence has indicated that hepatitis E is a zoonosis (Harrison, 1999; Kabrane-Lazizi *et al.*, 1999; Meng *et al.*, 1997, 1998, 2002; Nishizawa *et al.*, 2003; Okamoto *et al.*, 2001; Tei *et al.*, 2003; Yazaki *et al.*, 2003). It has recently been suggested that zoonotic, food-borne transmission of HEV from domestic pigs, wild boars or wild deer to humans plays an important role in the occurrence of domestic infections of hepatitis E in Japan, where people have unique habits of ingesting raw fish (sushi or sashimi) and uncooked or undercooked meat (also organ meats, such as raw liver) (Matsuda *et al.*, 2003; Tamada *et al.*, 2004). Thus, it seems that HEV infection is now autochthonous in Japan. It remains unclear, however, when and from where the ancestral HEV strains made inroads and have spread in

The GenBank/EMBL/DDBJ accession numbers for the HEV nucleotide sequences reported in this paper are shown in Fig. 1.

Supplementary tables are available in JGV Online.

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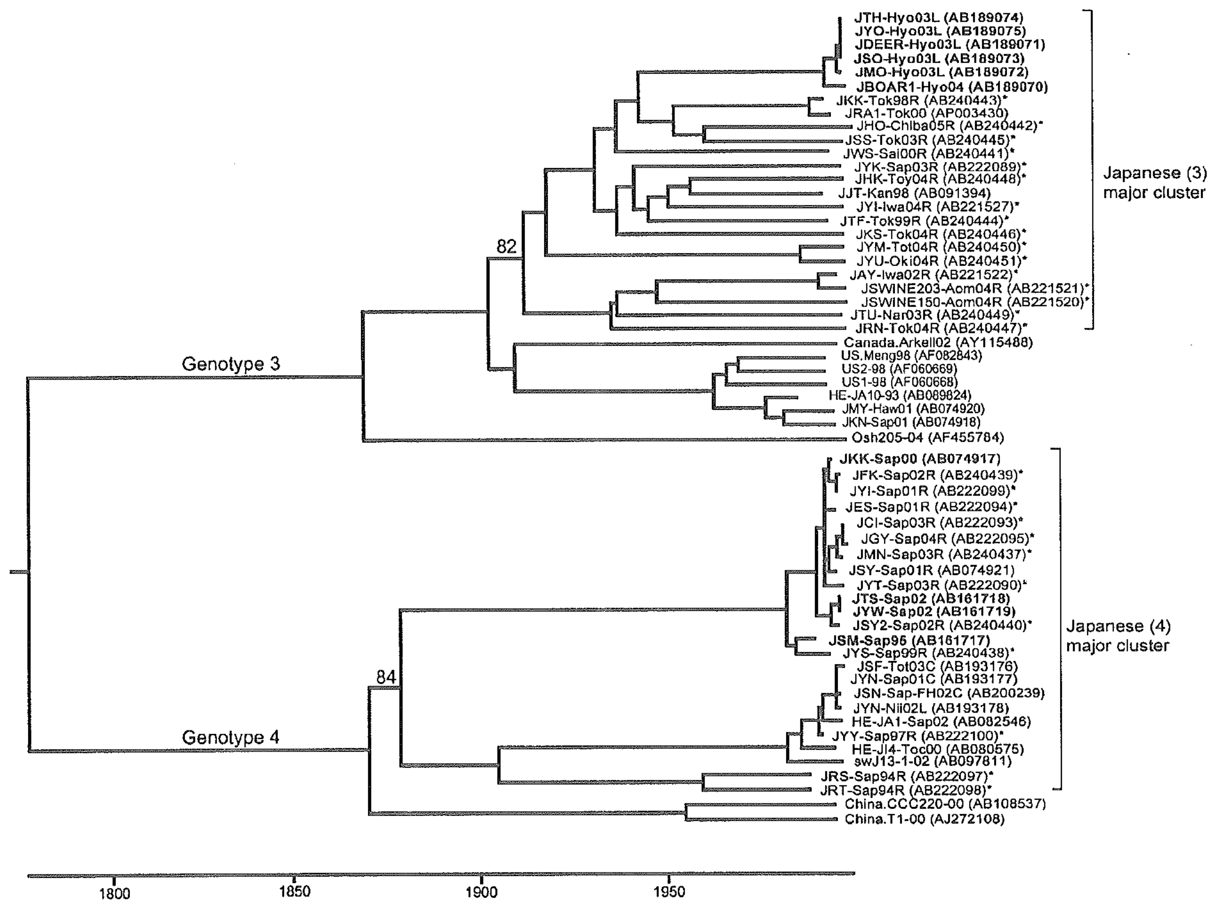

Fig. 1. Phylogenetic tree of the partial RNA polymerase region of the HEV genome. Twenty-four genotype 3 and 24 genotype 4 strains in Japan showed each significant cluster to have a high bootstrap value and to be distinct from other reference sequences (USA, Canada and Japanese minor strains in genotype 3; Chinese strains in genotype 4). Genetic distances have been transformed into a time scale of years by using estimates of the molecular clock ($0{\cdot}84 \times 10^{-3}$ nucleotide substitutions per site $year^{-1}$). Ten strains in bold are used for linear regression in Fig. 2. Strain names are followed by prefecture or city names in Japan: Tok, Tokyo; Sai, Saitama; Sap, Sapporo; Iwa, Iwate; Kan, Kanagawa; Oki, Okinawa; Aom, Aomori; Nar, Nara; Tot, Tottori; Nii, Niigata; Toc, Tochigi; Toy, Toyama. Asterisks indicate strains that were newly sequenced in this study.

Japan. In this study, we first estimated the evolutionary rate of HEV by using Japan-indigenous genotype 3 and genotype 4 strains, which were phylogenetically distinct from the other strains in foreign countries. Then, based on this evolutionary rate, we traced the demographic history of HEV in Japan.

For linear-regression analyses within significant clusters, two independent datasets were applied: one was a Hyogo cluster (genotype 3) with JMO-Hyo03L, JTH-Hyo03L, JSO-Hyo03L, JYO-Hyo03L, JDEER-Hyo03L (these five isolates were obtained in April 2003) and JBOAR1-Hyo04 (April 2004) (Takahashi *et al.*, 2004a), and another was a Sapporo cluster (genotype 4) with JSM-Sap95 (March 1995), JKK-Sap00 (November 2000), JYWSap02 (August 2002) and JTS-Sap02 (September 2002) (Takahashi *et al.*, 2004b). GenBank accession numbers for these strains are given in Fig. 1. To elucidate the epidemiological history of the HEV population in Japan, 48 known and newly sequenced HEV strains ($n=24$ for each of genotype 3 and 4) were used for molecular-evolutionary analyses. The nucleotide sequences of 28 strains for the molecular-clock analyses were determined in this study (the other 20 sequences dealt with in this paper were available from GenBank).

Nucleic acids were extracted from serum samples (50 μl) by using a commercial Smitest EX-R & D kit (Genome Science) and precipitated in a 2 ml tube. The pellet was air-dried for 15 min and then suspended in 10 μl autoclaved distilled water containing 10 U RNase inhibitor ml^{-1} (TaKaRa

Shuzo). A sequence spanning 821 nt in the RNA-dependent RNA polymerase region (corresponding to nt 3961–4781 of the prototype Burmese HEV strain; GenBank accession no. M73218), including the GDD motif, was amplified by PCR in three overlapping regions with 20-mer primers deduced from known HEV sequences. Reverse transcription was performed at 50 °C for 60 min with the Thermo-Script RT system (Invitrogen), and the first- and second-round PCRs were carried out in the presence of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). The final products were sequenced in an ABI 377 DNA sequencer (PE Biosystems) with an ABI Prism BigDye kit (Applied Biosystems). The sequences determined were utilized to confirm HEV genotypes and to construct phylogenetic trees. The reliability of the phylogenetic tree was assessed by bootstrap-resampling tests.

A reconstructed tree was built on the RNA polymerase region by using a heuristic maximum-likelihood (ML) topology search with stepwise addition and nearest neighbour-interchange algorithms. Tree likelihood scores were calculated by using the HKY85 model (Hasegawa *et al.*, 1985) with the molecular clock enforced, using PAUP version 4.0b8. Using the estimated topology, all possible root positions were evaluated under a single-rate dated-tips (SRDT) model with the computer software TipDate v1.2 and the root that yielded the highest likelihood was adopted (Rambaut, 2000). The program provided an ML estimate of the rate and also the associated date of the most recent common ancestor of the sequences, using a model that assumed a constant rate of nucleotide substitution. The molecular clock was tested by a likelihood-ratio test between the SRDT model and a general unconstrained branch-length model [different-rate (DR) model].

For estimates of demographic history, a non-parametric function $N(t)$, also known as a skyline plot, was obtained by transforming the coalescent intervals of an observed genealogy into a piecewise plot that represented an effective population size through time (Pybus *et al.*, 2001; Pybus & Rambaut, 2002). A parametric ML 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 from sampled DNA sequences (Pybus & Rambaut, 2002). 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 ML estimates (Lemey *et al.*, 2003; Pybus *et al.*, 2003; Tanaka *et al.*, 2005). Approximate 95 % confidence intervals for the parameters were estimated by using the likelihood-ratio test statistics.

A phylogenetic tree in the partial RNA polymerase region of the HEV genome is represented in Fig. 1. A functional gene, such as the RNA polymerase gene, is suitable for molecular-evolutionary analyses based on the neutral theory, because the substitution of functional genes is based on the neutral theory. The 24 genotype 3 and 24 genotype 4 strains in Japan showed a significant cluster with a high bootstrap value, which was the major Japanese cluster distinct from other strains found in foreign countries by molecular-evolutionary analyses. Such a significant cluster is suitable for the following coalescent analysis. Additionally, the tree topology based on the RNA polymerase region, including functional genes, was quite similar to that based on complete genomes (data not shown).

To determine the evolutionary rate of HEV, the 48 Japan-indigenous HEV strains (Fig. 1) were subjected to further molecular-evolutionary analyses. The molecular-evolutionary rate was estimated by two independent methods. In brief, linear-regression analyses using highly similar strains, i.e. six genotype 3 strains in Hyogo and four genotype 4 strains in Sapporo, indicated that a molecular-evolutionary rate was $(0{\cdot}81–0{\cdot}88) \times 10^{-3}$ nucleotide substitutions per site year^{-1} (Fig. 2). Second, TipDate (v1.2) was used to compare the DR model with the single-rate (SR) and SRDT models. The SRDT model provided an adequate fit to the data ($P > 0{\cdot}05$; see Supplementary Table S1, available in JGV Online). Based on the SRDT model, the mean rate of nucleotide substitutions was estimated to be $(0{\cdot}81–0{\cdot}94) \times 10^{-3}$ nucleotide

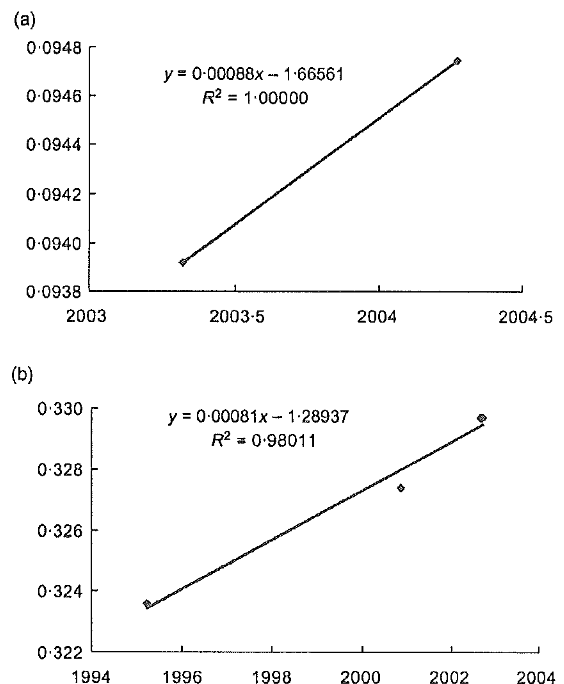

Fig. 2. Linear-regression analyses within the partial RNA polymerase region for evolutionary rate of HEV. (a) The evolutionary rate of genotype 3 in the Hyogo cluster is estimated to be $0{\cdot}88 \times 10^{-3}$ nucleotide substitutions per site year^{-1}; (b) the evolutionary rate of genotype 4 in the Sapporo cluster is estimated to be $0{\cdot}81 \times 10^{-3}$ nucleotide substitutions per site year^{-1}.