Genotype Determination of Hepatitis C Virus Strains in Malaysia

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Abstract: Out of 12 patient sera obtained from Malaysia, seven sera (7/12) were found to contain hepatitis C virus (HCV) genome by nested RT-PCR with universal HCV specific primers which were derived from 5' noncoding region (5' NCR). Genotype determination of these 7 Malaysian HCV strains were carried out both by subtype-specific core amplification system (Okamoto et al., 1992 and 1993) and sequencing in the 5' NCR. Four Malaysian strains were major genotype 1 (two of subtype II/1b, one of subtype I/1a and one of co-infection with I/1a+ II/1b). The genotypes of 3 remaining strains were determined as major genotype 3 by nucleotide sequences in the 5' NCR. The results showed that HCV major genotype 1, 2 and 3 are common in the world, especially in Asian countries including Malaysia.

Key words: HCV genotype determination, subtype-specific core amplification, sequencing in 5'NCR, Malaysian strains

INTRODUCTION

Hepatitis C virus (HCV) is classified under a genus of the family *Flaviviridae* which possesses a positive-sense, single-stranded RNA genome of approximately 9500 nucleotides. HCV is the major causative agent of blood-borne non-A, non-B hepatitis worldwide (Choo et al., 1991). According to the HCV sequence variation, HCV genotype can be determined and classified by its entire genome or partial sequence. As the numbers of HCV strains increasing, many HCV major genotypes and subtypes have been identified in the world. Until now, HCV have been classified into 11 major genotypes (or groups). Each of the 1, 2 and 3 major genotypes can be divided into several subtypes which were called I/1a, II/1b and Ic (genotype 1); III/2a and IV/2b (genotype 2); V/3a, VI/3b, 3c, 3d, 3e and 3f (genotype 3) (Simmons et al., 1993b; Sakamoto et al., 1994; Tokita et al., 1994a; Tokita et al., 1994b).

Among several HCV genotype detection methods, the most common ones are nucleotide

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sequence analysis and subtype−specific core amplification system (Okamoto et al., 1992 and 1993). We used sequence analysis of 5'NCR to determine the HCV genotype, because this region possesses certain sequence variation, in spite of high conservation as well as its easiness to amplify by polymerase chain reaction (PCR) with specific primers for identical length of fragment. (Altamirano et al 1995; Kleter et al., 1994; Simmonds et al., 1993a; Smith et al., 1995). Since 1992, another HCV genotype detection method was reported by Okamoto et al. (1992 and 1993), which was called subtype−specific core amplification system. By using this nested PCR with mixed subtype specific primers which were derived from core protein region, HCV genotype can be determined for Ⅰ/1a, Ⅱ/1b, Ⅲ/2a, Ⅳ/2b and Ⅴ/3a subtypes.

We determined genotypes of Malaysian HCV strains which were found in patient's sera both by sequencing and subtype−specific core amplification methods, in order to understand HCV variants distribution and epidemiology of HCV in Malaysia.

Materials and Methods

HCV RNA extraction: Twelve hepatitis patients sera were selected out of the test specimens sent to the Department of Virology, Institute for Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia in 1995. The standend HCV subtype Ⅱ/1b patient serum was kindly provided from WHO Collaborating Center for Reference and Research on Viral Hepatitis, Institute for Clinical Research, Nagasaki Chuo National Hospital, Japan. Each 50 μl patient serum was mixed with 500 μl of GTC−2ME (guanidinium thiocyanate−β mercaptoethanol) by vortex for 3 sec. Then it was added with 50 μl of 2M sodium acetate (pH 4), 500 μl of phenol and 100 μl of chloroform/isoprophy alcohol (49:1), mixed well by vortex for 10 sec again and kepted on ice for 15 min. After centrifugation (15,000 rpm at 4℃ for 20 min), upperphase was mixed with 4 μl of glycogen (10㎎/ml) and 600 μl of 2−propanol, and stored overnight at−20℃. After centrifugation and drying, RNA pellet was dissolved in 10 to 15 μl H_2O with 0.25 μl of RNase inhibitor (Takara Co.). Each 5 μl of RNA was prepared for each reaction of nested reverse transcriptase PCR (RT−PCR).

Synthesis of oligonucleotide primers: All oligonucleotide primers were ordered from Biologica Co. (Nagoya, Japan).

Nested RT−PCR for HCV genome detection: The presence of HCV genome in the patient sera were detected by the nested RT−PCR using universal HCV specific primers which were derived from 5' NCR (Okamoto et al., 1990). The first RT−PCR (20 min at 53℃ for RT, followed by 30 cycles of 1 min at 92℃, 1 min at 53℃ and 1 min at 72℃) was undertaken with sense primer HCV 14.3 (5' −ACTCCACCATAGATCACTCC−3', nucleotide (nt) positions: 7−26) and antisense primer HCV 13 (5'−AACACTACTCGGCTAGCAGT− 3', nt positions: 229−248). The second PCR (1 min at 92℃, 1 min at 53℃ and 1 min at 72℃ for 30 cycles) was undertaken with sense primer HCV 16 (5'−TTCACGCAGAAAGCGT CTAG−3', nt positions: 46−65) and antisense primer HCV 15 (5'−GTTTAT-

CCAAGAAAGGACCC− 3', nt positions: 171−190) (Fig. 1). By this method, positive amplification was obtained in 7 out of the 12 patient sera tested.

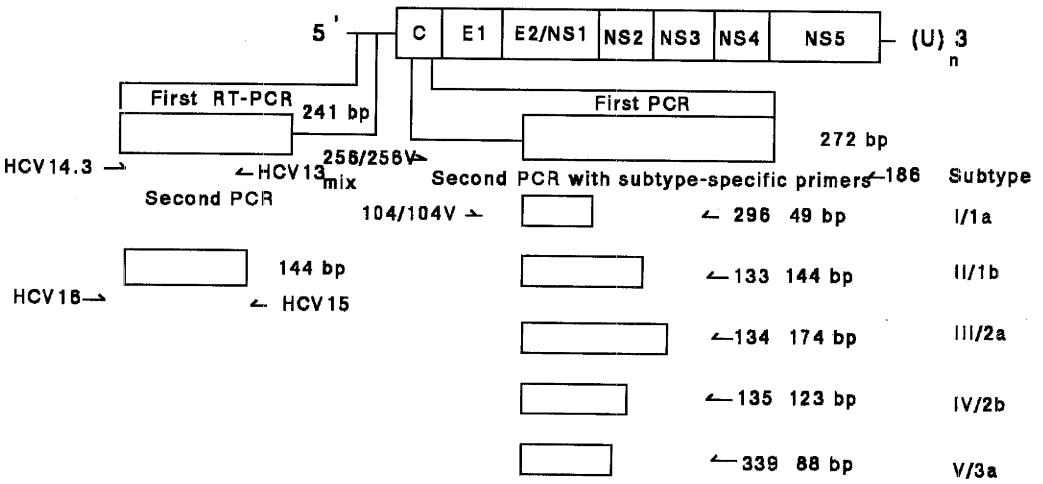


Fig. 1 Physical map of HCV genome. Genotype determination by subtype−specific core amplification system (Okamoto et al., 1993) and nested RT−PCR in 5' non−coding region for HCV detection and cloning.

Genotype determination by Subtype−specific Core Amplification System: For the genotype determination of the 7 HCV patient sera, subtype−specific core amplification method was applied (Okamoto et al., 1993). After RNA extraction, 10 μl of HCV RNA from each specimen was incubated at 65℃ for 5 min and cooled down on ice. Then it was mixed with the same volume of cDNA synthesis mixture [4 μl of 5×RTase buffer, 1 μl of 20 mM dNTPs, 1 μl of 50 pM antisense primer No. 186, 0.5 μl of RNase inhibitor (Takara Co.), 0.5 μl of reverse transcriptase (Life Science Inc.) and 3 μl of RNase free H_2O]. The mixture was incubated at 37℃−42℃ for 60 min. The first PCR (45 sec at 94℃, 45 sec at 55℃, 1.5 min at 72℃ for 35 cycles plus 7 min at 72℃ in final extension step) was done by mixed sense primer (No. 256 and No. 256V) and antisense primer No. 186. The second PCR (30 sec at 94℃, 30 sec at 60℃, 30 sec at 72℃ for 30 cycles plus 7 min at 72℃ in the final extension step) was done by mixed sense primers (No. 104 and No. 104V) and mixed antisense primers (No. 296, No. 133, No. 134, No. 135 and No. 339) (Fig. 1). By this method, genotypes of 4 out of the 7 HCV strains were determined.

Cloning and sequencing of HCV cDNA products: The genotype of the 3 remaining HCV strains were determined by cloning and sequencing after nested RT−PCR in the 5' NCR. The cDNA of the second PCR products (sense primer HCV 16 and antisense HCV 15) were excised from agarose gel, phosphorylated with T4 DNA polynucleotide kinase (Nippon Gene Co.) and blunted with T4 DNA polymerase (Takara Co.). The modified cDNA fragment

was ligated into *Sma*I site of pUC 19 and transformed into *E. coli* XL−1 Blue Strain. The recombinant pUC 19 was purified with QIA prep Spin Plasmid Miniprep Kit (QIAGEN GmbH, Germany). The sequence of cDNA fragment was determined by ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (U.S.A.). All samples for sequence determination were performed by using 373A DNA Sequencer (Applied Biosystems, U.S.A.). To avoid sequence variability, sequence determinations were carried out for 3 independent clones from each nested RT−PCR product.

Genomic information and analysis: The HCV nucleotide sequences were obtained from GenBank. HCV strains and their accession numbers as follow as: HCV−1: M62321; HCV−BK: M58335; HC−G9: D14853; HC−J6: D00944; HC−J8: D01221; NZL1: D17763; NE137: D16616; NE048: D16612; NE274: D16620; NE145: D16618; NE125: D16614; VN569: D17506; VN540: D17505; VN235: D17498; VN531: D17503; VN405: D17499; VN085: D17497; JK049: D63821; JK046: D63822. The nucleotide sequences of Z4, Z1, Z6, DK13 and SA1 isolates were obtained from Bukh et al. (1992). Sequence analysis was done by DNASIS Mac Version 2.4, NEW CD3 system (Hitachi Co., Japan).

RESULTS

Genotype determination for 7 HCV Malaysian strains were carried out by subtype−specific core amplification using nested PCR (Okamoto et al., 1993). HCV subtype can be identified only for 4 of the 7 HCV−positive patient specimen. Fig. 2 showed the

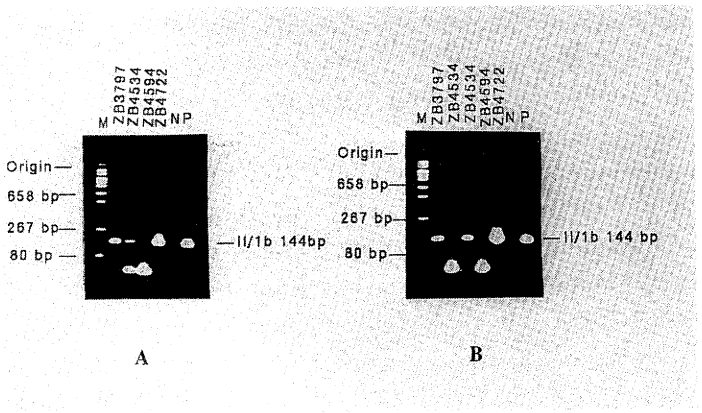


Fig. 2 Genotype determination by using subtype−specific core amplification system after agarose gel [3% agarose gel: 2.8% NuSieve (FMC. Bio Products) + 0.2% Agarose NA (made in Denmark)] electrophoresis. The PCR cDNA bands were visualized by ethidium bromide under U.V light. Photograph A: HCV Subtype were detected by mixed sense and antisense subtype primers during the second PCR. M: PHY Marker (Takara Co. Japan) (Lane 1); Malaysian strains (Lane 2−5); N: negative control (PCR sample was water) (Lane 6); P: positive control (subtype II/1b of HCV sample) (Lane 7). Photograph B: HCV subtype were detected by sense and antisense subtype primers individually during the second PCR.

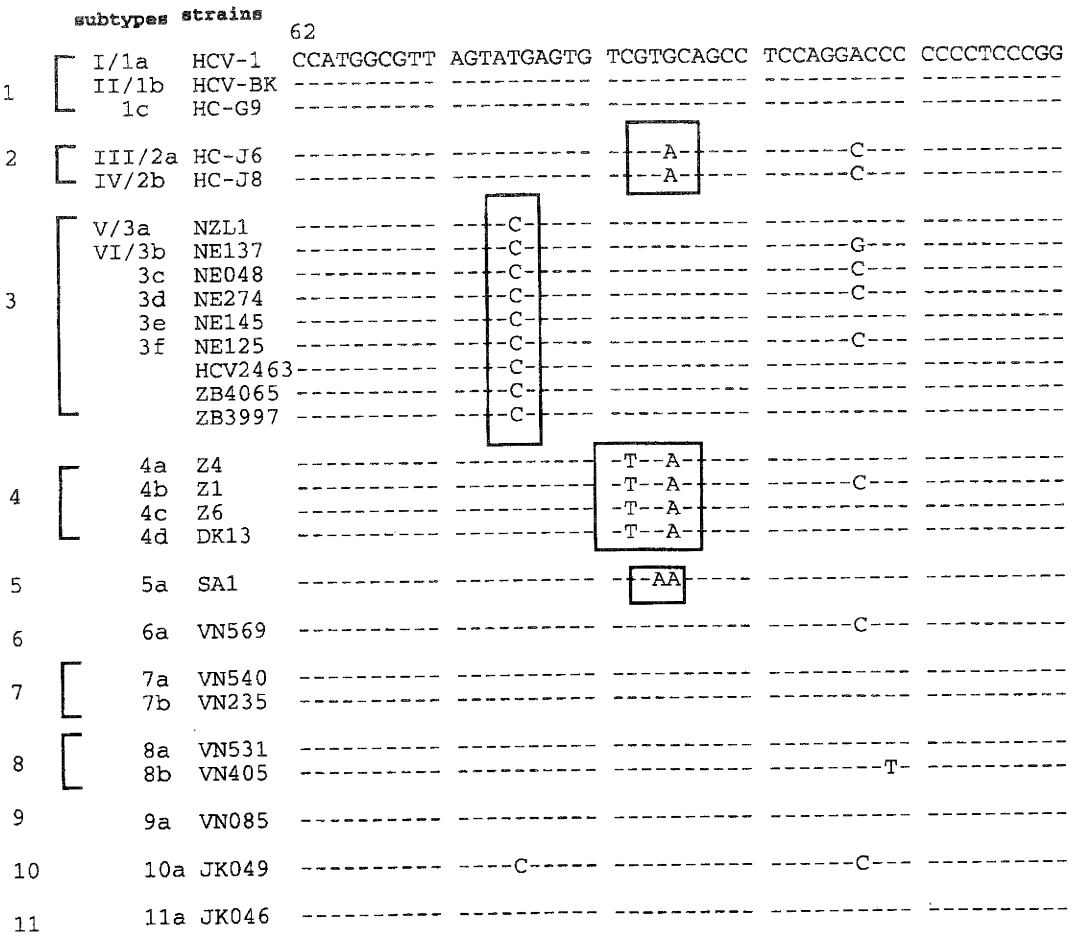
amplified cDNA bands after the second PCR. ZB 3797 strain was HCV subtype Ⅱ/1b because the length of cDNA product was equal to 144 bp same as subtype Ⅱ/1b positive control. ZB 4534 strain was found to be co−infected by HCV subtype Ⅱ/1b and Ⅰ/1a, because two bands of cDNA products were visible at 144 bp and 49 bp. ZB4594 strain was HCV subtype Ⅰ/1a and ZB4722 was subtype Ⅱ/1b, respectively (Fig. 2, A). In order to confirm these results, subtype specific primers were added individually to the specimen before the second PCR. The result were the same as previous ones which were determined by mixed subtype primers (Fig. 2, B).

The genotype of the 3 remaining Malaysian HCV strains were determined by nucleotide sequences in the 5' NCR, by alignment with other published sequences of HCV genotypes (Fig. 3). These 3 Malaysian HCV strains (HCV2463, ZB4065 and ZB3997) showed two conserved sequences at nucleotide (nt) position 75 and nt position between 153−161, where nucleotide sequences were "C" and "C--TG--GT", respectively. These two nucleotide sequence characteristics were found only in genotype 3 HCV strains, as described previously (Altamirano et al., 1995; kleter et al., 1994; Lee et al., 1992; Stuyver et al., 1994). On the other hand, genotype 2, 4, 5 and 10 showed other sequence characters which were indicated by boxed (nt: 82−85; nt: 153−165 in the 5' NCR. From the alignment of nucleotide sequence among 11 genotypes, genotype 1, 6, 7, 8, 9 and 11 did not possess genotypespecific sequence between nt 61−165 in the 5' NCR.

Discussion

Although the 5' NCR of HCV is highly conserved region, it is an attractive target for genotype classification, because there are obvious genotype−specific sequences in certain regions. Such a character is an advantage to use 5' NCR for genotype identification after sequence alignment. The 3 Malaysian HCV strains analyzed in this report showed characteristic sequence of the major genotype 3 (Fig. 3), but it not yet clear to which subtypes they can be classified. Probably they were not belonging to subtype Ⅴ/3a, because of negative results in subtype−specific core amplification system. Unfortunately, very limited volume of serum was obtained for each Malaysian patient in this study. For determination HCV subtype of genotype 3 in future, sufficient quantity of HCV patient sera is required for cloning and sequencing in other genome region or complete genome.

Among a total 7 Malaysian HCV strains, 4 were identified as major genotype 1, while 3 strains were major genotype 3, respectively (Table 1). Until now, many genotypes of HCV strains have already been classified in the world. It is very important to clarify the relationship between HCV genotypes and geographic area in order to understand the significance of HCV epidemiology and vaccine development in the future. Genotype 1, 2 and 3 are widely distributed around the world, especially subtype Ⅱ/1b of genotype 1 is found quite frequently in Asia (Simmonds et al., 1993b; Tokita et al., 1994b; Wang et al., 1993; Zheng., 1994). HCV genotypes 4, 5 and 6 were found in highly limited geographical areas, apparently confined to Egypt, South Africa, and Southeast Asia (Hong Kong, Thailand, Vietnam and Myanmar),


subtypes strains
62
CCATGGCGTT AGTATGAGTG TCGTGCAGCC TCCAGGACCC CCCCTCCCGG
I/1a HCV-1
II/1b HCV-BK
1c HC-G9
1
III/2a HC-J6
IV/2b HC-J8
2
V/3a NZL1
VI/3b NE137
3c NE048
3d NE274
3e NE145
3f NE125
HCV2463
ZB4065
ZB3997
3
4a Z4
4b Z1
4c Z6
4d DK13
4
5a SA1
5
6a VN569
6
7a VN540
7b VN235
7
8a VN531
8b VN405
8
9a VN085
9
10a JK049
10
11a JK046
11

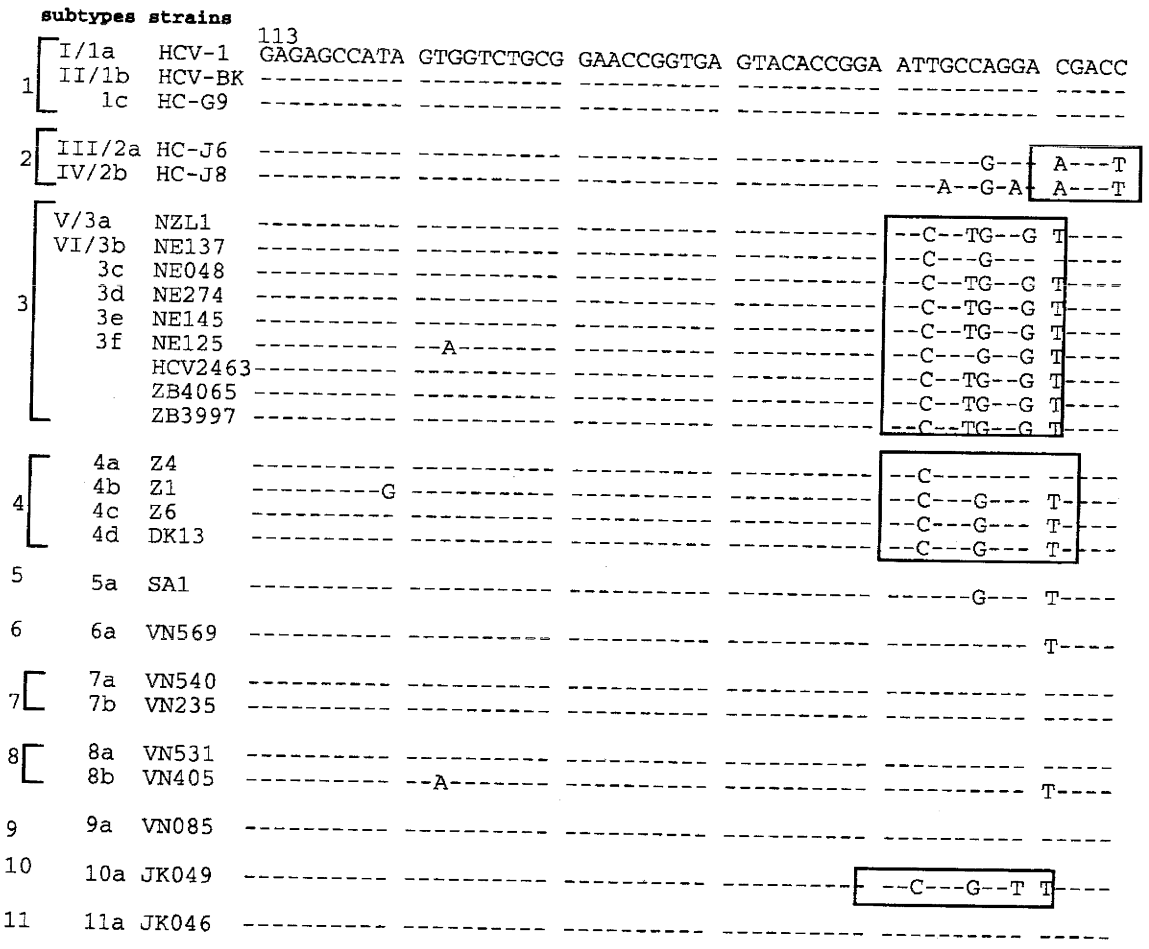


Fig. 3 Alignment of nucleotide sequences in 5' non−coding region. Serial No. 1−11 indicated major genotypes. Hyphens indicated the presence of nucleotides identical to the prototype strains (HCV−1) sequence. The sequence character in different major genotypes were boxed.

Table1 The Results of Genotype Determination with Malaysia HCV strains

Strains	Idetification method	Major genotype or subtype
ZB 3797	subtype-specific core amplification	genotype 1 , II/1b subtype
ZB 4534	as above	genotype 1, I/1a + II/1b subtype
ZB 4594	as above	genotype 1, I/1a subtype
ZB 4722	as above	genotype 1, II/1b subtype
HCV 2463	sequencing in 5 NCR	genotype 3
ZB 4065	as above	genotype 3
ZB 3997	as above	genotype 3

respectively (Mellor et al., 1996; Simmonds et al., 1993b; Tokita et al., 1995). HCV genotypes 7, 8 and 9 have been investigated in Thailand and Vietnam (Tokita et al 1994a and 1995). The newest genotype 10 and 11 have been found in Indonesia (Tokita et al., 1996).

About the relationship between the HCV genotype and interferon therapy, preliminary studies suggested that the sensitivity of HCV to interferon differs according to viral genotypes (Tsubota et al., 1994; Yoshioka et al., 1992). The response to interferon was less in patients infected with HCV subtype Ⅱ/1b (34/85 or 40%) than in those with subtype Ⅲ/2a (22/26 or 85%) or subtype Ⅳ/2b (7/10 or 70%). The influence of HCV genotypes on response to interferon was observed in patients controlled for the severity of liver histopathology (Hino et al., 1994). Further research is to investigate the interferon response to these Malaysian HCV strains with different genotypes.

In conclusion, 7 Malaysian HCV strains belong to major genotype 1 and 3 which are also common genotypes in Asia and in the world. This evidence is very important for the epidemiology of HCV genotype classification and vaccine development.

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