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Analysis of Hepatitis C Virus Isolates among Healthy Blood Donors and Drug Addicts in Chiang Mai, Thailand

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Hepatitis C virus (HCV) isolates obtained from 25 anti-HCV antibody-positive healthy blood donors and 29 drug addicts in Chiang Mai, Thailand, were analyzed. HCV RNA was detected in 23 blood donor samples (92%) and 24 drug addict blood samples (83%) by PCR for a portion of the NS5 region. Subtype analysis revealed that HCV type 3a (HCV-3a) was the prevailing subtype (30%), which was followed in prevalence by HCV-1a (21%), -1b (13%), -3b (13%), and -6a (2%). Six (13%) of the 47 isolates showed low sequence similarities with known types and subtypes. The sequence variants could be grouped into four branches in a molecular evolutionary phylogenetic tree.

The genome of hepatitis C virus (HCV) exhibits considerable degrees of sequence variation among different isolates (2, 3, 8, 11, 15, 17, 18). On the basis of this variation, Simmonds et al. (22) have proposed that HCV can be classified into at least six major types (HCV-1 to -6), each of which may be further divided into a few subtypes. The prevalence of HCV subtypes differs in different geographical areas. For example, HCV-1b is the most prevailing subtype in the Far East (19) and in some European countries (20), while HCV-1a and HCV-4a are the most prevailing subtypes in the United States (8) and in the Middle East (22, 23), respectively. Likewise, HCV-5a has been reported to be confined to South Africa, and HCV-6a has been found so far only in Hong Kong (22). HCV-3a was first identified in Thailand (15) and was later reported to be common in Europe (22, 23) and Brazil (24). Besides the differences in geographical distribution, some differences in clinicopathological features of HCV infection have been observed among different types and subtypes. For example, HCV-1b is reported to be more resistant to interferon treatment (10, 21) and more closely associated with severe liver damage than other subtypes (20, 21). Recently, we and other investigators have independently identified the presence of a new subtype of HCV (HCV-1d) in Indonesia (5–7, 16), which may be closely associated with severe liver damage (7). Following the strategy that was applied in Indonesia, we have been analyzing HCV prevalence in Thailand. In the present study, we analyzed nonstructural (NS5) region sequences of the HCV genome obtained from healthy blood donors and drug addicts to determine the prevalence of each type and subtype in Chiang Mai, Thailand. We also report that sequence variants that have not yet been reported are circulating in this area.

Sera were collected from healthy volunteer blood donors at Blood Bank Section, Maharaj Nakorn Chiang Mai Hospital, and from drug addicts, either intravenous drug users (IVDU) or opium or heroin (OP/HE) smokers, at Northern Drug Dependent Treatment Center, Chiang Mai, Thailand, and they were tested for anti-HCV antibodies by a second-generation enzyme-linked immunosorbent assay (Abbott Diagnostics, Wiesbaden-Delkenheim, Germany). HCV RNA extracted from anti-HCV antibody-positive sera was reverse transcribed using Rous-associated virus 2 reverse transcriptase (Takara Shuzo, Kyoto, Japan) and the primer no. 167R (4, 12), and the resultant cDNA was subjected to reverse transcription and PCR to amplify a portion of the NS5 region of HCV. Positions and sequences of the primers used in the present study are shown in Fig. 1. The first-round PCR was performed over 35 cycles with a thermal reactor (Hybaid, Teddington, Middlesex, United Kingdom), each consisting of 1 min at 94°C, 1.5 min at 40°C, and 2 min at 72°C using outer primers (no. 166 and no. 167R) (4, 12), and Tth DNA polymerase (Toyobo, Osaka, Japan) in a buffer supplied with the enzyme. With 5 μl of first-round PCR products, the second-round PCR was carried out with the same program. We used different sets of primers in the second round so that we could amplify the HCV genome from as many serum samples as possible, including the following mixtures of primers: (i) HC23, HC24, and HC26 (4, 7); (ii) HC15 and HC16 (4, 7); (iii) HC23 and HC28 (7); (iv) HC23 and HC32; and (v) HC23 and HC34. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and were visualized by UV illumination. Amplified DNA fragments were sequenced by a direct sequencing method with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit and an ABI 373A Autosequencer (Applied Biosystems, Foster City, Calif.). Each sequence result was compared with those of the reported types and/or subtypes (3, 11, 15–18, 22, 25), and sequence results were also compared with each other. A phylogenetic tree was constructed by the unweighed pairwise grouping method (13).

Twenty-five anti-HCV antibody-positive serum samples obtained from healthy blood donors and 29 serum samples from drug addicts (20 from IVDU and 9 from OP/HE smokers) were analyzed by the nested PCR. HCV RNA was detected in 7 (28%) of the 25 blood donor samples with HC23-HC24-HC26 and in 11 serum samples (44%) with HC15-HC16. Of the remaining 7 serum samples that were negative with the above primer sets, 1 became positive by using HC23-HC32 and 4 became positive by using HC23-HC34, most of which gave

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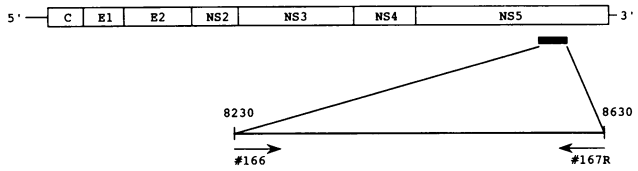

1st PCR

#166	5'-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3'	(8230-8260, +)
#167R	5'-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3'	(8601-8630, -)

2nd PCR

HC23	5'-TTTGACTCAACCGTCACTGA-3'	(8256-8275, +)
HC24	5'-CTCAGGCTCGCCGCATCCTC-3'	(8577-8596, -)
HC26	5'-CTCAGGTTCCGCTCGTCCTC-3'	(8577-8596, -)
HC15	5'-ACTGTCACTGAACAGGACAT-3'	(8265-8284, +)
HC16	5'-GCTCTATCCTCATCGACGCC-3'	(8568-8587, -)
HC28	5'-CACGAGCATGGTGCAGTCCCGGAGC-3'	(8507-8531, -)
HC32	5'-AGGTAGCACGTCAGCGTGTTTCC-3'	(8454-8476, -)
HC34	5'-TAGCACGTCATGGTGTTTCCCAT-3'	(8451-8473, -)

FIG. 1. Positions, sequences, and polarities of the primers for reverse transcription and PCR. All of the primer sequences except that for HC32 and HC34 have been reported elsewhere (4, 7, 12). Sequences are numbered as described by Kato et al. (11). +, sense; −, antisense.

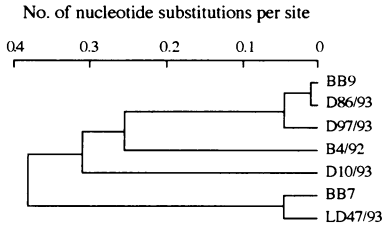

FIG. 2. Phylogenetic analysis of NS5 region sequences of HCV variants in Thailand. On the basis of the sequence alignment, the number of nucleotide substitutions per site was estimated and a phylogenetic tree was constructed by the unweighed pairwise grouping method (13). BB9, B4/92, and BB7 were isolated from blood donors; D86/93, D97/93, and D10/93 were isolated from drug addicts; and LD47/93 was isolated from a patient with chronic hepatitis.

only faint bands. In total, 23 (92%) of the anti-HCV antibody-positive blood donor samples were positive for the NS5 PCR. As for the 29 samples from drug addicts, 13 (45%) and 8 (28%) serum samples showed positive amplification with HC23-HC24-HC26 and HC15-HC16, respectively. Of the 8 remainders, 1 and 2 serum samples became positive with HC23-HC32 and HC23-HC34, respectively. In total, 24 (83%) of the drug addict samples were positive for amplification. The nucleotide sequences of the amplified fragments were determined and compared with those of the reported types and subtypes of HCV. According to the criteria suggested by Simmonds et al. (22), HCV isolates showing sequence similarities of more than 88% at the nucleotide level to any of the reported subtype sequences were assigned to the corresponding subtype. Sixteen (70%) of the 23 isolates obtained from healthy blood donors and 21 (88%) of the 24 isolates from drug addicts were assigned to known subtypes. Six (13%) of the total of 47 isolates showed low sequence similarities (60 to 74%) with known types and subtypes (data not shown) and, therefore, are referred to as new sequence variants. Four isolates from the blood donors gave ambiguous sequence results, probably because of very faint amplification, and, with no more serum samples remaining, they were not analyzed further in this study. Mixed infections with two or more subtypes, if any, were not detected with the present PCR system.

Table 1 summarizes the prevalence of each subtype. HCV-3a was the most common subtype in healthy blood donors and showed a high prevalence also among IVDU. Of the total of 47 isolates, 14 (30%) were HCV-3a; this was followed in prevalence by HCV-1a (21%), HCV-1b (13%), HCV-3b (13%), a group of new sequence variants (13%), and HCV-6a (2%). A similar sequence variant was isolated from the serum of a Thai patient with chronic hepatitis and was analyzed together with those of the variants described above. On the basis of the sequences determined, a phylogenetic tree was constructed to observe the relationship among these variants. The results revealed that the variants could be grouped into four branches (Fig. 2). The amino acid sequences of the variants were predicted and compared with those of the reported subtypes (Fig. 3). Residues conserved among all viral RNA-dependent RNA polymerases (3, 9, 17, 18) are also conserved in the variants. In addition to the residues described above, several conserved sequences, which may be necessary for this protein to exert the polymerase activity, were noticed.

Seven serum samples that were negative for the NS5 by PCR were further analyzed by PCR for a portion of the 5′-untranslated region, and five serum samples were determined to be positive. Sequence analysis of this region, which was reported to be successfully used for classification of HCV isolates into major types but not particular subtypes (14), provisionally classified an isolate into major type 3 and another one into type 1 (data not shown). The remaining three isolates were unclassifiable by this method, two of which shared unique sequence motifs with the variants D86/93, D97/93, and B4/92, and the last one of which shared sequence motifs with LD47/93.

Prevalence ratios of anti-HCV antibodies among healthy blood donors, IVDU, and OP/HE smokers in Chiang Mai were 2.4, 97, and 23%, respectively (data not shown). The prevalence ratio among blood donors is comparable with results

TABLE 1. Prevalence of HCV subtypes in healthy blood donors and drug addicts in Thailand

Group	No. of samples	No. of samples (%) assigned to the following subtypes:						
		HCV-1a	HCV-1b	HCV-3a	HCV-3b	HCV-6a	New[a]	U[b]
Blood donor	23	3 (13)	2 (9)	8 (35)[c]	3 (13)	0 (0)	3 (13)	4 (17)
IVDU	17	5 (29)	2 (12)	5 (29)	3 (18)	1 (6)	1 (6)	0 (0)
OP/HE smoker	7	2 (29)	2 (29)	1 (14)	0 (0)	0 (0)	2 (29)	0 (0)
Total	47	10 (21)	6 (13)	14 (30)[d]	6 (13)	1 (2)	6 (13)	4 (9)

[a] New sequence variants.
[b] U, unclassifiable because of ambiguous sequence results.
[c] $P < 0.05$, compared with HCV-1b (Fischer's direct probability test).
[d] $P < 0.05$, compared with HCV-1b or HCV-3b (Fischer's direct probability test).

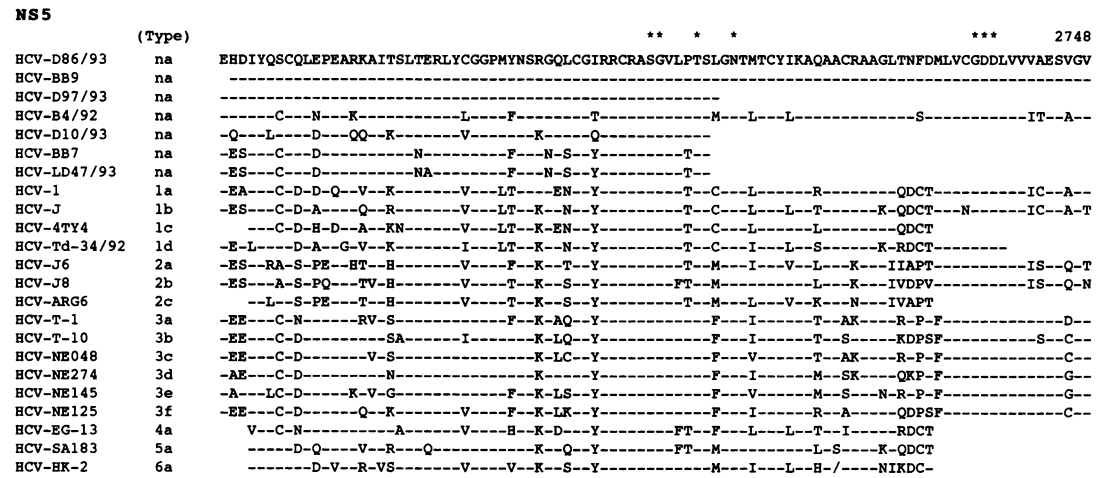

FIG. 3. Alignment of deduced amino acid sequences of a portion of the NS5 protein among various HCV subtypes. The amino acid sequences of HCV-1 (3), HCV-J (11), HCV-Td-34/92 (7), HCV-J6 (18), HCV-J8 (17), HCV-T-1 (15), and HCV-T-10 (15) have been reported elsewhere, and those of HCV-4TY4, HCV-ARG6, HCV-NE048, HCV-NE274, HCV-NE145, HCV-NE125, HCV-EG-13, HCV-SA183, and HCV-HK-2 have been deduced from the nucleotide sequences deposited in the GSDB/DDBJ/EMBL/NCBI DNA databases under the accession numbers, L23447, L23457, D16613, D16621, D16619, D16615, L23469, L23472, and L23475, respectively. Residues identical to those of HCV-D86/93 are indicated by hyphens. A slash in the HCV-HK-2 sequence represents a gap introduced to preserve sequence alignment. Asterisks indicate residues conserved among all viral RNA-dependent polymerases (3, 9, 17, 18). The numeral in the top right corner indicates the position of the most carboxy-terminal residue. na, not assigned.

from a previous report (1). We determined the subtype of HCV obtained from the groups listed above. HCV-3a was the most common subtype in healthy blood donors, which was followed in prevalence by HCV-1a, HCV-3b, a group of new sequence variants, and HCV-1b and which was also frequently found in IVDU. In this connection, it was reported previously that 2, 2, and 1 of 10 anti-HCV antibody-positive patients were infected with HCV-3a, with HCV-3b, and with HCV-1a, respectively, and that the infections in the remaining 5 patients could not be determined because of unsuccessful amplification (15). The high prevalence of HCV-3a in Thailand makes a sharp contrast to the situation in Indonesia, another Southeast Asian country, where HCV-3a is barely found (5–7). Another interesting finding is that 6 (13%) of the 47 HCV isolates had NS5 region sequences that were significantly different from those of hitherto recognized types and subtypes (60 to 74% homologies at the nucleotide level). Phylogenetic analysis of these 6 variants and another variant obtained from a Thai patient with chronic hepatitis revealed that these variants could be further divided into four groups (Fig. 2). At this moment, we are unable to draw a conclusion as to whether the variants reported here represent new types or new subtypes of known types. We are currently analyzing the core and E1 envelope regions of the variants. More detailed phylogenetic analysis based on the sequences not only of the NS5 but also of the regions described above will clarify the relationship among these variants and the hitherto recognized types and subtypes.

The nucleotide sequence data reported in this paper will appear in the GSDB/DDBJ/EMBL/NCBI DNA databases under the accession numbers D28541 to D28547.

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REFERENCES

1. **Boonmar, S., B. Pojanagaroon, Y. Watanabe, Y. Tanaka, I. Saito, and T. Miyamura.** 1990. Prevalence of hepatitis C virus antibody among healthy blood donors and non-A, non-B hepatitis patients in Thailand. Jpn. J. Med. Sci. Biol. **43:**29–36.
2. **Cha, T.-A., E. Beall, B. Irvine, J. Kolberg, D. Chien, G. Kuo, and M. S. Urdea.** 1992. At least five related, but distinct, hepatitis C viral genotypes exist. Proc. Natl. Acad. Sci. USA **89:**7144–7148.
3. **Choo, Q.-L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
4. **Doi, H., S. Yoon, M. Homma, and H. Hotta.** 1994. Identification of hepatitis C virus subtype 3b (HCV-3b) among Japanese patients with liver diseases using highly efficient primers for reverse transcription-polymerase chain reaction. Microbiol. Immunol. **38:** 159–163.
5. **Hotta, H., H. Doi, T. Hayashi, M. Purwanta, M. I. Lusida, W. Soemarto, and M. Homma.** 1994. Sequence analysis of hepatitis C virus obtained from Indonesian patients and identification of novel sequence variants, p. 310–313. *In* K. Nishioka, H. Suzuki, S. Mishiro, and T. Oda (ed.), Viral hepatitis and liver disease. Springer-Verlag, Tokyo.
6. **Hotta, H., H. Doi, T. Hayashi, M. Purwanta, W. Soemarto, M. Mizokami, K. Ohba, and M. Homma.** 1994. Analysis of the core and E1 envelope region sequences of a novel variant of hepatitis C virus obtained in Indonesia. Arch. Virol. **136:**53–62.
7. **Hotta, H., R. Handajani, M. I. Lusida, W. Soemarto, H. Doi, H. Miyajima, and M. Homma.** Submitted for publication.
8. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q.-L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14:**381–388.
9. **Kamer, G., and P. Argos.** 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. Nucleic Acids Res. **12:**7269–7282.
10. **Kanai, K., M. Kako, and H. Okamoto.** 1992. HCV genotypes in chronic hepatitis C and response to interferon. Lancet **339:**1543.
11. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi,**

T. Sugimura, and K. Shimotohno. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87:**9524–9528.

12. **Kato, N., Y. Ootsuyama, S. Ohkoshi, T. Nakazawa, S. Mori, M. Hijikata, and K. Shimotohno.** 1991. Distribution of plural HCV types in Japan. Biochem. Biophys. Res. Commun. **181:**279–285.
13. **Kimura, M.** 1983. Rate of evolution at the molecular level. Comparative studies of protein sequences. The neutral theory of molecular evolution. Cambridge University Press, Cambridge.
14. **Kleter, G. E. M., L.-J. van Doorn, J. T. Brouwer, S. W. Schalm, R. A. Heijtink, and W. G. V. Quint.** 1994. Sequence analysis of the 5′ untranslated region in isolates of at least four genotypes of hepatitis C virus in the Netherlands. J. Clin. Microbiol. **32:**306–310.
15. **Mori, S., N. Kato, A. Yagyu, T. Tanaka, Y. Ikeda, B. Petchlai, P. Chiewsilp, T. Kurimura, and K. Shimotohno.** 1992. A new type of hepatitis C virus in patients in Thailand. Biochem. Biophys. Res. Commun. **183:**334–342.
16. **Okamoto, H., M. Kojima, M. Sakamoto, H. Iizuka, S. Hadiwandowo, S. Suwignyo, Y. Miyakawa, and M. Mayumi.** 1994. The entire nucleotide sequence and classification of a hepatitis C virus isolate of a novel genotype from an Indonesian patient with chronic liver disease. J. Gen. Virol. **75:**629–635.
17. **Okamoto, H., K. Kurai, S. Okada, K. Yamamoto, H. Iizuka, T. Tanaka, S. Fukuda, F. Tsuda, and S. Mishiro.** 1992. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology **188:**331–341.
18. **Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyakawa, and M. Mayumi.** 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J. Gen. Virol. **72:**2697–2704.
19. **Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. **73:**673–679.
20. **Pistello, M., F. Maggi, L. Vatteroni, N. Cecconi, F. Panicucci, G. P. Bresci, L. Gambardella, M. Taddei, A. Bionda, M. Tuoni, and M. Bendinelli.** 1994. Prevalence of hepatitis C virus genotypes in Italy. J. Clin. Microbiol. **32:**232–234.
21. **Pozzat, G., M. Moretti, F. Franzin, L. S. Crocé, C. Tiribelli, T. Masayu, S. Kaneko, M. Unoura, and K. Kobayashi.** 1991. Severity of liver disease with different hepatitis C viral clones. Lancet **338:**509.
22. **Simmonds, P., E. C. Holmes, T.-A. Cha, S.-W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea.** 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol. **74:**2391–2399.
23. **Simmonds, P., F. McOmish, P. L. Yap, S.-W. Chan, C. K. Lin, G. Dusheiko, A. A. Saeed, and E. C. Holmes.** 1993. Sequence variability in the 5′ non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. J. Gen. Virol. **74:**661–668.
24. **Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. V. Heuverswyn, and G. Maertens.** 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. **74:**1093–1102.
25. **Tokita, H., S. M. Shrestha, H. Okamoto, M. Sakamoto, M. Horikita, H. Iizuka, S. Shrestha, Y. Miyakawa, and M. Mayumi.** 1994. Hepatitis C virus variants from Nepal with novel genotypes and their classification into the third major group. J. Gen. Virol. **75:**931–936.