GENOTYPIC DISTRIBUTION OF HEPATITIS C VIRUS IN VOLUNTARY BLOOD DONORS OF NORTHERN THAILAND

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Abstract. The purpose of this study was to determine the prevalence and distribution of HCV genotypes among voluntary blood donors in northern Thailand. From 1998 to 2000, 167 serum samples which tested positive for anti-HCV antibodies in the screening of voluntary blood donors from 5 provinces in northern Thailand were selected for genotyping. Viral RNA was extracted from the sera. The core-E1 region of the HCV-RNA genome was amplified using a OneStep RT-PCR kit. The core-E1 amplicon was sequenced and the HCV genotype was assigned by comparing with the reference sequences available in the GenBank database. Of 167 anti-HCV positive serum samples, 126 (75.4%) contained HCV RNA as detected by PCR. HCV genotype 3 was the most predominant genotype (39.6%), of which 33.3% belonged to genotype 3a and 6.3% to 3b. Genotype 6 was detected in 31%, and genotype 1 was detected in 27.8%. Of the genotype 1 isolates, 14.3% were 1a, 12.7% were 1b, and 0.8% were 1c. Two HCV isolates detected in the present study were untypeable. About 75% of anti-HCV positive blood donors had chronic HCV infection. In northern Thailand, genotype 3a was the most predominant genotype, while genotype 6, 1a and 1b were also commonly found. The genotypic distribution of HCV isolates from various regions of Thailand were more or less similar. Nevertheless, in this study, the prevalence of HCV genotype 6 (31%) was higher than previously reported by others (8-18%). Phylogenetic analysis of the HCV isolates detected in the present study was also performed.

INTRODUCTION

Hepatitis C viruses (HCVs) have been proven to be a major etiologic agent of parenterally transmitted non-A, non-B hepatitis. About 80% of newly infected persons progress to chronic infection (WHO, 2000). The WHO estimated a worldwide prevalence of about 3% or 170 million people are chronically infected with HCV (WHO, 1999). Cirrhosis develops in about 10% to 20% of persons with chronic infection, and liver cancer develops in 1% to 5% of persons with chronic infection over a period of 20 to 30 years (WHO, 2000). Prevalence rates across the world have changed as a reflection of the awareness of transfusion-related hepatitis C infection and more evidence supporting intravenous drug use (IDU) as the leading risk factor for the spreading of HCV (Sy and Jamal, 2006).

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HCV is an enveloped virus with a positive-stranded RNA genome of approximately 9,400 bp in length. Most of the genome forms a single open reading frame (ORF) that encodes three structural (core, E1, E2) and seven non-structural (p7, NS2-NS5B) proteins. Short untranslated regions (UTRs) at each end of the genome are required for the replication of the genome, a process that has recently been found as an additional requirement for a *cis*-acting replication element in the coding sequence of NS5B (Simmonds, 2004; You *et al*, 2004).

HCV has evolved by distinct selective pressures that are associated with hypervariable regions of the envelope glycoprotein E2 to prevent recognition by antibodies that are induced by infection. HCV also accumulates sequence changes as a result of "neutral" sequence drift over time. This process, rather than adaptive changes, accounts for much of the sequence diversity that is observed between different genotypes (Simmonds, 2004). Based on the high level of sequence variation, HCV is classified into 6 major genotypes (genotypes 1-6), and within each genotype, closely related variants are grouped into a number of subtypes. Each of the six major genetic groups of HCV contain a series of closely related subtypes that differ from each other by 20-25% in nucleotide sequences, compared with >30% divergence between genotypes (Simmonds, 2004; Simmonds *et al*, 2005).

Available evidence indicates that some HCV genotypes are distributed worldwide, while others are confined to more restricted geographic areas. Genotype 1b has a worldwide distribution and is often found to be the most common genotype (Mellor *et al,* 1995; Simmonds, 2004). Genotypes 2a and 2b are also widely distributed and are particularly common in Japan and northern Italy (Mondelli and Silini, 1999; Huy and Abe, 2004). Genotype 3 is the most common on the Indian subcontinent, while genotype 4 is the most common genotype in Africa and the Middle East (Mellor *et al,* 1995; Simmonds, 2004). Genotypes 5 and 6 are limited to specific geographic areas; genotype 5 was found in South Africa and genotype 6 was found in Southeast Asia, having been identified in Vietnam, Thailand and Indonesia (Huy and Abe, 2004).

One controversial issue is whether differences in the clinical and histological severity of liver disease in patients may be explained by infections with different viral genotypes (Mondelli and Silini, 1999). Genotype 1 appears to establish persistence and, in carriers, to be associated with more severe liver disease, compared with genotypes 2 and 3 (Resti *et al*, 2003; Simmonds, 2004). HCV infection can be treated, but is costly and requires long term medical support and follow-up. The HCV genotypes are associated with specific therapeutic responses. Knowing the HCV genotype is helpful for predicting sustained virological response and the choice of treatment duration. Response rates to treatment using the pegylated interferon and ribavirin combination are about 88% for genotypes 2 and 3, and about 48% for genotypes 1, 4, 5, and 6. Subtype determination (*ie*, 1a versus 1b) is not clinically helpful (Poynard *et al*, 2003; Simmonds *et al*, 2005).

We determined HCV genotypes in healthy blood donors who were positive for anti-HCV antibodies. Our HCV genotyping was based on sequence differences in the core-E1 region of the HCV-RNA genome. The results of this study provide dynamic data regarding current subtypes and genotypes of HCV causing infection in this region of the world.

MATERIALS AND METHODS

Samples

Serum samples from voluntary blood

donors in Chiang Mai, Chiang Rai, Lampang, Lamphun and Mae Hong Son Provinces in northern Thailand were screened for blood-transmitted pathogens at the 10th Regional Blood Center Office in Chiang Mai. The blood donors, who donated blood at the mobile units in the community were found to be healthy following National Blood Center criteria. Screening for the presence of anti-HCV was performed by a commercial kit, ORTHO HCV 3.0 ELISA Test System with Enhanced SAVe (Ortho-Clinical Diagnostics, Amersham, Bucks, UK). From 1998 to 2000, samples with repeat positive anti-HCV results were collected and stored at -20ºC. A total of 167 anti-HCV positive serum samples were found: 95 from Chiang Mai, 40 from Chiang Rai, 18 from Lampang, 11 from Lamphun, and 3 from Mae Hong Son. All samples were coded by number; the identities of the patients were unknown to the research team.

Reverse-transcription (RT) and nested PCR amplification

The viral RNA was extracted from 140 µl of patient serum using QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. The viral RNA obtained was used as a template for RT-PCR and first round PCR in a 50 µl reaction mixture using a QIAGEN OneStep RT-PCR Kit, which contained the PCR components that allowed both reverse transcription and cDNA amplification simultaneously in one tube. This reaction mixture contained 10 µl of viral RNA template, 10 µl of 5 x QIAGEN OneStep Buffer (containing 2.5 mM $MgCl_2$), 2 µl of dNTP Mix (containing 10 mM of each dNTP), 0.4 µM of outer forward (C/E1 ESP: 5'-GCAA CAGGGAACCTTCCTGGTTG CTC-3') (positions 834-859), 0.4 µM of outer reverse (C/E1 EAP: 5'-CGTAGGGGACCAGTTCATC ATCAT-3') (positions 1305-1328) primers (Ray *et al*, 2000), 2 µl of GIAGEN OneStep RT-PCR Enzyme Mix, and 24 µl of RNase-free water. With this pair of primers, the expected PCR product size of C/E1 region was 495 bp.

Reverse transcription and DNA amplification were performed with the following thermal cycling conditions: reverse transcription at 50ºC for 30 minutes, then activation of HotStar Taq DNA polymerase and inactivation of reverse transcriptase enzyme at 95ºC for 15 minutes, followed by 35 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 1 minute. The final extension was performed at 72ºC for 10 minutes.

Ten microliters of PCR product from the first-round reaction was used as a template in the second-round reaction with 0.4 mM of the inner nested primers, inner forward (C/E1 ISP: 5'-AACCTTCCTGGTTGCTCTT TCT CTAT-3') (positions 843-868) and inner reverse (C/E1 IAP: 5'-GTTCATCATCA TATCCCATGCCAT-3') (positions 1293-1316) in 100 µl. The components of the reaction mixture included 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 2.5 U of Taq DNA polymerase (Promega Corporation, Madison, WI). The thermal cycling condition was the same as in the first amplification except the step of reverse transcription was omitted. The expected size of the PCR product was 474 bp.

Purification of PCR products

The PCR products from the second-round PCR reaction were purified with QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Briefly, 1 volume of PCR product was added to 5 volumes of PBI buffer and mixed. The mixture was applied onto the QIAquick column and centrifuged at 12,000 rpm for 1 minute. Then, 750 ml of PE buffer was added to the column and centrifuged for 1 minute to wash off the DNA bound to the column. Thirty microliters of EB buffer was added to the center of the QIAquick membrane to

elute the PCR product DNA from the column. The column was allowed to stand for 1 minute and then centrifuged for 1 minute. Finally, the purified PCR product DNA was examined for DNA quantity by agarose gel electrophoresis and spectrophotometry.

Direct sequencing of PCR products and genotype assignment

The purified PCR products of the core-E1 fragment were sequenced using a BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA). Each sequencing reaction consisted of 8.0 µl of premix (PE Applied Biosystems BigDye Terminator RR mix), 1.0 µl of sequencing primer, 100 ng of purified PCR product and H_2O, to yield a total volume of 20 µl. The cycle sequencing was performed in a thermocycler for 25 cycles using the following profile: initial denaturation at 60ºC for 4 minutes. The inner forward primer (C/E1 ISP: 5'-AACCTTC CTGG TTGCTCTTTCTCTAT-3') was used as a sequencing primer. The sequencing product was concentrated by ethanol precipitation and analyzed on an automated DNA sequencer (ABI PRISM, PE Biosystem, Foster City, CA). The sequences obtained were compared to those of HCV reference strains deposited in the GenBank and also available in the Los Alamos HCV database (http://hcv.lanl.gov/content/hcv-index) using BLAST (Basic Local Alignment Search Tool) searches. The genotypes of HCV were then assigned based on the sequence similarity with those of the reference strains.

HCV phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1. The genotypes and GenBank accession numbers of reference strains used in the phylogenetic analysis are as follows: BID-V320/1a (EU155287), H77/1a (NC004102), ThBD-0916/1a (AY739363), BID-V147/1b (EU155220), ThBD-0083/1b (AY739380), HC-J4/1b (D00832), K3A /3a (D28917), ThBD-0230/3a (AY739410), NZL1/3a (NC009824), HCV-Tr/3b (D49374), ThBD-0532/3b (AY 739388), ThK164/3b (D37838), 6a63/6a (DQ 480514), Th846/6c (EF424629), VN540/6e (D88474), ThBD-0198/6f (AY739421), C-0046/6f (DQ835764), JK065/6g (D49751), VN004/6h (D84265), ThKF68/6i (D37851), C-0159/6i (DQ835762), ThBD-0667/6j (AY739431), KM41/6k (DQ278893), VN405/6k (D84264), D33/6l (EF420132), 537796 US/6l (EF424628), B4/92/6m (DQ835767), ThBD-0229/6m (AY 739425), KM42/6n (DQ278894), ThBD-0055/6n (AY739416), QC227/6o (EF424627), QC 216/6p (EF424626), and TV249/6t (EF632070).

GenBank accession numbers for HCV isolates

The core-E1 sequences of 120 HCV isolates included in the present study were deposited in the GenBank database under the accession numbers DQ179116, DQ179143, FJ210654-FJ210676, and FJ687055-FJ687151.

RESULTS

Distribution of HCV genotypes and subtypes

Of 167 serum samples collected from voluntary blood donors from northern Thailand that were positive for anti-HCV by ELISA, 126 (75.4%) were positive for HCV RNA as detected by OneStep RT-PCR. The distributions of the HCV genotypes and subtypes are shown in Table 1. The HCV genotype 3 was the most predominant genotype (39.6%), of which 33.3% were subtype 3a and 6.3% were subtype 3b. HCV genotype 6 was detected in 31.0% and genotype 1 was detected in 27.8%. Among those with HCV genotype 1, 14.3% were subtype 1a, 12.7% were 1b, and 0.8% were 1c. It was interesting to note that two HCV isolates (CMBD 14 and CMBD 86) detected in this study were untypeable since their C/E1 nucleotide se-

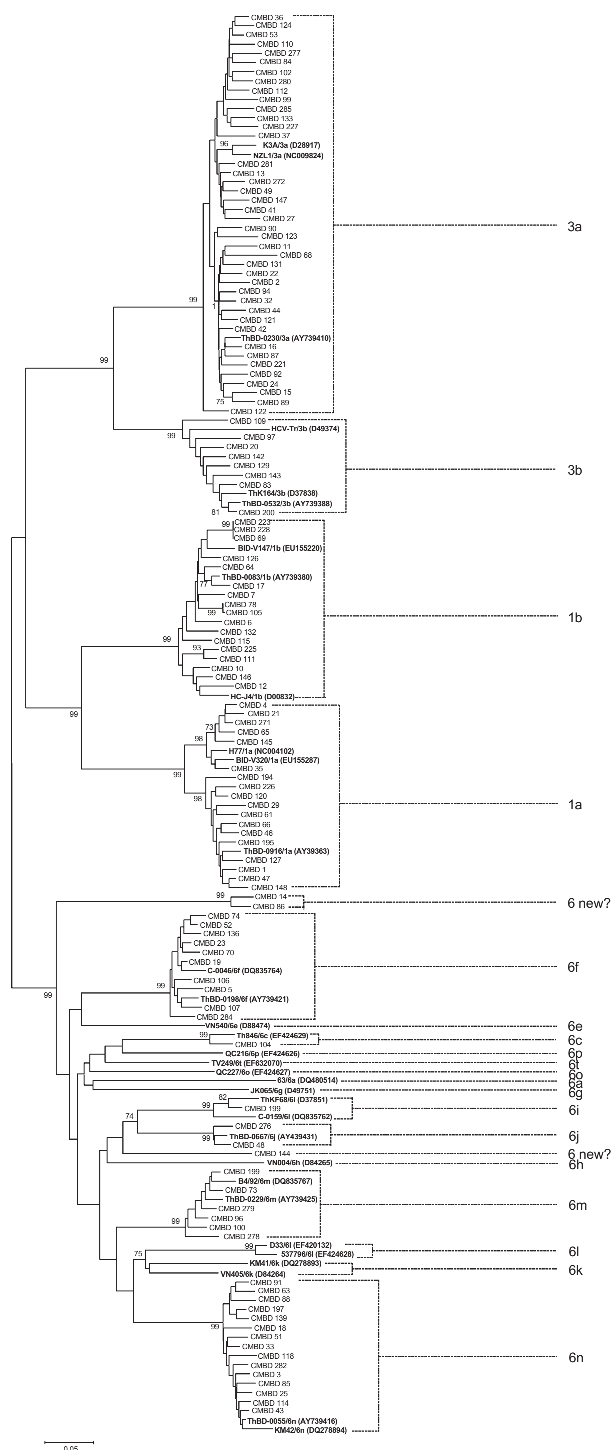

Fig 1–Phylogenetic tree of the core/E1 nucleotide sequence of HCV strains detected in blood donors of northern Thailand. The reference strains are indicated in bold face.

quence showed only 69-70% similarity to known existing genotypes.

HCV phylogenetic analysis

The phylogenetic tree for the C/E1 nucleotide sequences of 120 HCV isolates detected in this study was constructed. The representative HCV reference strains for genotypes 1a, 1b, 3a, 3b, 6a, 6b, 6e, 6f, 6g, 6h, 6i, 6j, 6k, 6l, 6m, 6n, 6o, 6p, and 6t were also included in the tree. The results shown in Fig 1 reveal the HCV isolates described in this study clustered closely together with corresponding reference genotypes. However, two HCV isolates (CMBD 14 and CMBD 86) were clustered in a monophyletic branch separated from the 6 other known genotype reference strains. The genotypes of these two isolates will be determined later.

DISCUSSION

The estimated prevalence of HCV in Southeast Asia is 2.2% (WHO, 2000). In Thailand, the summative percentage for anti-HCV seropositivity in blood donors is 1.4% (Wiwanitkit, 2005), but reaches 85% to 92.5% among intravenous drug users (Hansurabhanon *et al*, 2002; Taketa *et al*, 2003; Jittiwutikarn *et al*, 2006). Anti-HCV positivity rates among blood donors in northern Thailand have been reported to be 0.7% and 1.6% (Jutavijittum *et al*, 1999; Nantachit *et al*, 2007).

In studies of HCV infection among the general population in Thailand, the virus can be detected by PCR in 58-90% of samples which are positive for anti-HCV antibodies (Kanistanon *et al*, 1997; Theamboonlers *et al*, 2002; Sunanchaikarn *et al*, 2007). HCV RNA was detected in 75.4% of anti-HCV positive sera in our study. This suggests that approximately 75% of anti-HCV positive blood donors in northern Thailand are chronically HCV infected and could potentially develop

Table 1
Distribution of HCV genotypes among voluntary blood donors in northern Thailand.

Genotype and subtype	Number (%) of samples
1 (total)	35 (27.8)
1a	18 (14.3)
1b	16 (12.7)
1c	1 (0.8)
3 (total)	50 (39.6)
3a	42 (33.3)
3b	8 (6.3)
6 (total)	39 (31.0)
Unclassifiable (total)	2 (1.6)

complications such as cirrhosis and hepatocellular carcinoma.

The most common genotype in Thailand was 3a (39-51%), followed by genotype 1b (20-27%) and genotype 6 variants (8-18%), depending on the series. Other genotypes (1a, 1c, 3b) are uncommon (Kanistanon *et al*, 1997; Verachai *et al*, 2002; Sunanchaikarn *et al*, 2007). In our study, the major HCV genotypes were 3a (33.3%) followed by genotype 6 variants (31%). Genotypes 1a (14.3%), 1b (12.7%), and 3b (6.3%) were found to be less common, and 1c (0.8%) was relatively rare. Our study shows a relatively high prevalence of genotype 6 (31%) among infected blood donors residing in northern Thailand. HCV genotype 6 is not commonly found worldwide; geographically genotype 6 variants have been identified exclusively in Southeast Asia or in immigrants from this region (Theamboonlers *et al*, 2002; Shinji *et al*, 2004; Thaikruea *et al*, 2004; Noppornpanth *et al*, 2006). Additionally, two HCV isolates, CMBD14 and CMBD86, in the present study were identified as nontypeable strains. Although these two strains have been referred to by Wang *et al* (2009) as strains similar to HCV Chinese strains of the genotype 6v, the similarity is based on a short sequence of 873 bp of C/E1 and NS5B genes out of 9,600 bp for the complete genome sequence. Therefore, the complete genome sequences and phylogenetic analysis of these two HCV isolates remain to be further investigated in order to identify their genotypes.

Genotyping assays are usually based on sequence analysis of an amplified segment of the genome, commonly the 5' untranslated region (5'UTR). This region is highly conserved; a well-characterized set of polymorphisms predicts the genotype and can be conveniently detected by probe hybridization, through changes in restriction sites or by direct sequencing (Simmonds *et al*, 2005). Although several genotype-specific nucleotide changes in the 5'UTR usually allow each of the 6 main genotypes to be differentiated from each other, there are exceptions. Some genotype 6 variants found in Southeast Asia have 5 2UTR sequences identical to those of genotype 1a or 1b (Simmonds *et al*, 2005). Therefore, some 5'NCR-based genotyping methods cannot differentiate genotype 6 variants from genotype 1b (Mellor *et al*, 1996; Kanistanon *et al*, 1997; Chinchai *et al*, 2006). Previous studies in northern Thailand might fail to demonstrate genotype 6 variants and reported them as novel variants of HCV, previously classified as genotypes 7, 8, 9, 10 and 11, genotype 1b/6 group variants, genotype 1, or isolates with unclassified genotype (Apichartpiyakul *et al*, 1994; Sugiyama *et al*, 1995; Simmonds *et al*, 1996; de Lamballerie *et al*, 1997; Kanistanon *et al*, 1997). Our core region-based genotyping was able to discriminate between genotype 6 group variants and genotype 1b and found a high rate of genotype 6 in our study samples.

Reports have indicated that HCV subtype 3a is associated with IDU (Hansurabhanon *et al*, 2002), whereas subtype 1b spreads mainly via blood transfusion (van de

Laar *et al*, 2006). In Thailand, the major risk factors for HCV acquisition are IDU, blood transfusion and tattooing (Chunlertrith *et al*, 2000; Luksamijarulkul *et al*, 2004; Tanwandee *et al*, 2006). A previous study indicated that illicit IDU and past history of blood transfusion are important risk factors for HCV infection in northern Thailand (Thaikruea *et al*, 2004). The distribution of the HCV genotypes in the present study is comparable to a previous report of isolates from drug users in northern Thailand; 168 (82.4%) of 204 specimens that were tested by PCR had detectable HCV RNA, of which 96% were typeable as genotype 3 (39%), 1 (31%), and 6 (26%) by nested RT-PCR of the core/E1 regions of the genome (Jittiwutikarn *et al*, 2006).

HCV circulating in this area were transmitted mainly from IDU (Hansurabhanon *et al*, 2002; Thaikruea *et al*, 2004; Tanwandee *et al*, 2006). Parenteral exposure to blood through the use of contaminated or inadequately sterilized instruments and needles used in medical and dental procedures or activities that break the skin (*eg*, tattooing, circumcision, ear or body piercing) are primary sources of HCV transmission in developing countries (WHO, 2000). The risk of acquisition of HCV by blood transfusion has decreased substantially in recent years because of the screening of donors using serological or nucleic acid amplification methods to identify infected donors and the exclusion of donors with the highest risk (Sy and Jamal, 2006). Sexual and perinatal transmission may occur but are less common (WHO, 2000).

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