RESEARCH ARTICLE

Epidemiology of Hepatitis C Virus Genotypes in Northeastern Thai Blood Samples

Sahapat Barusrux[1]*, Chatchawan Sengthong[2], Yupa Urwijitaroon[1]

Abstract

Background: Hepatitis C virus (HCV) infection is an important cause of liver cancer in Thailand. The highest prevalence of anti-HCV positive among Thai blood donors is found in the northeastern region. The present analysis of the genotype distribution among anti-HCV positive northeastern-Thai blood donors was conducted to provide a base for the epidemiological pattern of HCV infection in this region. Materials and Methods: A total of 112 HCV seropositive healthy blood donors were randomly selected and tested for the presence of HCV-RNA by RT-PCR. HCV-RNA positive samples were genotyped by direct sequencing at core region genomes and confirmed by phylogenetic analysis. Results: HCV viremia was found in 94.6% (106/112) of HCV seropositive blood donors. There were 3 major genotypes distributed among this population. HCV genotype 3a was the most prevalent (71.7%) followed by genotypes 1a (7.5%), 1b (7.5%), 6i (3.8%), 6f (2.8%) and 6n (1.9%). Conclusions: HCV genotype 3a in asymptomatic infections in northeastern Thailand is significantly higher than other previous reports. Subgenotype 6 prevalence is less than in neighboring countries and distribution patterns differ. The findings are relevant as predictors for using interferon therapy in this population.

Keywords: HCV - genotype - northeastern Thai - asymptomatic cases - blood samples

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer and the fifth most frequent among men and the seventh among women worldwide (Jemal et al., 2011). Hepatitis C virus (HCV) and Hepatitis B virus (HBV) are the most common (63.3% and 33.3% respectively) of viral hepatitis-associated liver cancer cases on the island of Guam (Haddock et al., 2013). The population attributable fraction (PAF%) studied in 2005 in China showed that HCV was responsible for 27.7% of liver cancer deaths, while aflatoxin was estimated to be 25.0%. Alcohol drinking was responsible for 15.7% with smoking responsible for 13.9% (Fan et al., 2013). The differentially expressed genes study between cirrhosis and HCC samples showed the upregulating of ECM-receptor interaction, focal adhesion, cell adhesion molecules and other cancer-related pathways, and downregulating of complement and coagulation cascades pathways involved in HCV-induced hepatocarcinogenesis (Cheng, et al., 2012). They also found a significant hypermethylation of tumor suppressor genes (P14, P15, and P73), and a mismatch repair gene (O6MGMT) in HCV related chronic liver disease and HCC. The methylation processes were not just early events in hepatocarcinogenesis but accumulated with progression to cancer. This evidence showed the progression of chronic hepatitis C to cirrhosis and hepatocellular carcinoma is associated with increasing DNA promoter methylation (Zekri et al., 2013). The risk of HBV (OR=17.31) and HCV (OR=28.57) for HCC was demonstrated by multivariate analysis. Habitual betel quid chewing with chronic HBV or HCV correlated with adverse hepatic fibrosis and more severe liver damage that contributes to a higher risk for HCC (Jeng et al., 2014).

The major route of HCV transmission is direct percutaneous exposure through blood transfusion or transplantation from infected donors. To reduce the risk of acquiring HCV infection from transfusion, the screening of anti-HCV in blood donors has been implemented in Northeastern Thai since 1993 (Urwijitaroon et al., 1993). The prevalence of antibodies to HCV in the Thai population varies from 0.89 to 5% (Chainuvati et al., 1991; Urwijitaroon et al., 1993; Songsivilai et al., 1997). HCV and HBV, but not human imminodeficiency virus (HIV), or human T-lymphotropic virus type I (HTLV-I) are highly prevalent in northeastern Thailand (Barusrux et al., 1995). Co-infection of HCV has been reported at about 18.0% (13/72 cases) with HIV infected (Barusrux et al., 1997) and about 11.0% (13/118 cases) with HGV infected (Barusrux and Urwijitaroon, 2006) in blood donors.

HCV is a single positive stranded RNA virus belongs to the family Flaviviridae and classified into six major

*[1]Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, [2]Medical Technology Laboratory, Bangkrathum hospital, Phitsanulok, Thailand *For correspondence: sahapat@kku.ac.th*

genotypes with more than 74 subtypes within each genotype; subtypes are further broken down into quasi-species (Xavier and Bukh, 1998; Zein, 2000). HCV genotype distribution and HCV infection prevalence differs in different geographical areas. There is a previous reported on HCV genotype in northeastern Thai, but that study reported on healthy children or patients with acute illness who attended the well baby clinic, or, OPD clinic of a provincial hospital in the northern region of northeastern Thailand (Udon Thani) (Sunanchaikarn et al., 2007). However, the HCV genotype distribution in children may not exactly reflect the healthy adult. This study aimed to analyze the distribution of HCV genotypes in healthy northeastern Thai blood donors by direct sequencing of core region. The information will be useful for epidemiological pattern and beneficial for therapeutics of HCV infection in this region.

Materials and Methods

Serum samples and HCV RNA detection

One hundred and twelve Thai blood donors, anti-HCV seropositive by Murex anti-HCV ELISA kit (Abbott, U.S.A.), were randomly recruited to participate in this study at the Blood Transfusion Centre, Faculty of Medicine, Khon Kaen University and Blood Blank Unit, Khon Kaen Regional Hospital, Khon Kaen. The study was carried out in accordance with the guidelines of the Helsinki Declaration of 2008 and approved by the Khon Kaen University Human Research Ethics Committee. The plasma samples were stored at -20°C until analysis. RNA extraction was performed by using TrizolR LS Reagent (Invitrogen, Calif) (Chomczynski and Sacchi, 1996). The cDNA was synthesized by random hexamers with murine myeloblastosis SuperScript™ III and was used for PCR template for HCV genome amplification further.

HCV genome amplification

The core region was amplified by nested PCR as previous reported (Lole et al., 2003). Briefly, total 50µl of outer reaction mixture include of 5µl cDNA, 5µl of 10X PCR buffer, 125mmole of MgCl2 , 10mmole of dNTPs, 2.5U of Taq pol and 10μmole of each CC1 outer sense primer 5'-ACTGCCTGATAGGGTGCTTGC-3', CC2 outer antisense primer 5'-ATGTACCCCATGAGGTCGGC-3'. The outer PCR amplification was performed at 94°C for 5min, 61°C, 1.50min, 72°C, 2min for 35 cycles. Total 50µl of inner reaction mixture include of 2µl of outer PCR product, 5µl of 10X PCR buffer, 125 mmole of MgCl2, 10mmole of dNTPs, 2.5U of Taq pol and 10μmole of each CC3 inner sense primer 5'-AGGTCTCGTAGACCGTGCA-3' and CC4 inner antisense primer 5'-CACGTTAGGGTATCGATGAC-3'. The inner PCR amplification was perform with same as thermal cycle program of outer PCR amplification. The 5'UTR region was amplified by PCR as previous reported (Yong et al., 1993) by using KY80 sense primer 5'-GCAGAAAGCGTCTAGCCATGGCGT-3' and KY78 antisense primer 5'-CTCGCAAGCA CCCTATCAGGCAGT-3'. The amplification was performed at 94°C for 15 sec, 55°C, 30sec min, 72°C, 1min for 40 cycles. The 405 bp of core region and 244 bp of 5'UTR region PCR product target were detected by 2.0% agarose gel electrophoresis. PCR target product DNA in excised gels was purified by using a high pure PCR product purification kit (Roche, Germany) for nucleotide sequence. The direct sequencing was performed either CC3(5'-AGGTCTCGTAG-ACCGTGCA-3') or KY80(5'-GCAGAAAGCG-TCTAGCCATGGCGT-3') as the sequencing primers for core or 5'UTR region sequencing, based on dideoxynucleotide chain reaction by ABI Prim 310 genetic analyzer (MegaBACE™ 1000 DNA Analysis System).

HCV genotyping by DNA sequencing and phylogenetic construction

The HCV DNA sequences were analyzed for genotype classification by alignment of HCV isolate sequences with representatives of each of the genotype references by using BLAST/FASTA programs and ClustalW program. The highest similarities indicate tentative genotype assignment. The Phylogenetic analysis of HCV sequences was done by unrooted phylogenetic tree type. Phylogenetic analysis for isolates genotyping was focused on 266 nucleotide sequence at the position 399 to 664 nt of the core region by using the ClustalW software package version 1.83. (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was performed using Phylip package version 3.63. based on the genetic base distance method. Kimura 2-parameter algorithm (Gojobori et al., 1982; Felsenstein, 1993) was used to estimate the number of nucleotide substitution between sequences. The trees were constructed by using Neighbor-joining method (Saitou and Nei, 1987). The HCV genotype classification was confirmed by Phylogenetic analysis (Felsenstein, 1985). TreeView 62 version 1.6.6 was used for displaying phylogenetic tree. Reference sequences of all 6 major genotypes from GenBank: genotype 1a(AF011753), 1b(AJ132996), 2a(AF238485), 2b(AF238486), 3a(D17763), 3b(D493740), 4a(Y11604), 4b(U10235), 5a(Y13184), 6a(Y12083), 6b(D84262), 6d(D84263), 6e(D63822), 6f(D37844), 6g (DQ314806),6h(D84265), 6i(D37849), 6k(D84264), and 6n(DQ278849) were retrieved from the GenBank.

Results

HCV RNA in anti-HCV seropositive plasma samples

The average age of the 112 healthy northeastern Thai blood donor anti-HCV seropositive was 35.8±9.30 years (35.9±9.47 years in 99 males and 34.6±8.08 years in 13 females). All plasma samples were confirmed for the presence of HCV RNA by RT-PCR at both (5'UTR) and core regions as shown in Figure 1. HCV RNA was found in 106 cases of the 112 cases (94.6%)- 93 (93.9%) were male and 13 (100%) females. Forty one samples (36.6%) were positive in both 5'UTR/core and 65 (58.0%) positive in core region only. None of the sample gave positive of 5'UTR region alone as summarized in Table 1.

HCV genotyping

The classification of HCV genotypes was based on

Table 1. HCV-RNA Positivity among 112 HCV Seropositive Northeastern Thai Blood Donors

Sex	N	HCV RNA +ve Case (%)	5'UTR/core +ve Case (%)	5'UTR +ve only Case (%)	Core +ve only Case (%)	Negative Case (%)
M	99	93 (93.9)	35 (35.4)	0 (0)	58 (58.6)	6 (6.01)
F	13	13 (100)	6 (46.2)	0 (0)	7 (53.8)	0 (0)
Total	112	106 (94.6)	41 (36.6)	0 (0)	65 (58.0)	6 (5.4)

Table 2. HCV Genotype Distribution among 106 Northeastern Thai HCV Viremia Blood Donors Compare with Previous Reports from Different Regions of Thailand

Genotype	HCV genotype distribution in each Thailand regions					
	Northeast donor (n=106)	4 regions[a] OPD (n=45)	Central[b] donor (n=131)	South[b] donor (n=24)	Northeast[b] donor (n=71)	North[c] donor (n=23)
1a	8 (7.5%)	3 (6.7%)	13 (10.0%)	3 (12.5%)	5 (7.0%)	3 (13.0%)
1b	8 (7.5%)	12 (26.7%)	0 (0)	0 (0)	0 (0)	2 (9.0%)
1b/6	0 (0)	0 (0)	49 (37.4%)	10 (41.6%)	30 (42.3%)	0 (0)
2a	0 (0)	1 (2.2%)	0 (0)	0 (0)	0 (0)	0 (0)
2c	0 (0)	1 (2.2%)	0 (0)	0 (0)	0 (0)	0 (0)
3a	76 (71.7%)	23 (51.1)	51 (38.9%)	7 (29.2%)	30 (42.3%)	8 (35.0%)
3b	0 (0)	1 (2.2%)	5 (3.8%)	1 (4.2%)	2 (2.8%)	3 (13.0%)
6	9 (8.5%)	4 (8.9%)	0 (0)	0 (0)	0 (0)	0 (0)
unidentify	5 (4.7%)	0 (0)	13 (9.9%)	3 (12.5%)	4 (5.6%)	7 (30.0%)

[a]Sunanchaikarn et al., 2007; [b]Kanistanon et al., 1997; [c]Apichartpiyakul et al., 1994

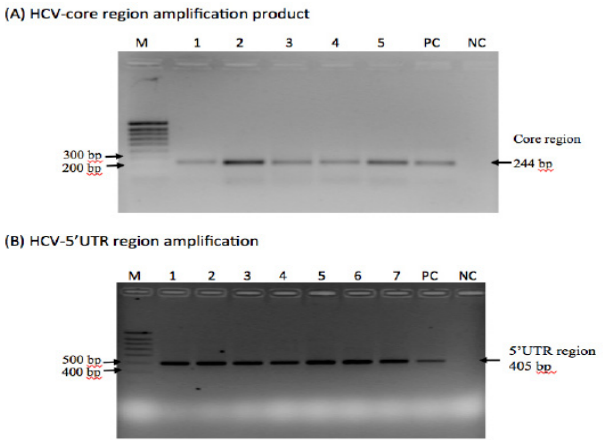

Figure 1. HCV Amplified Products Detection in Agarose Gel Electrophoresis. The positive sample for HCV-core region amplification was showed in lane 1-lane 5 as 244 base pairs **(A)**, and 405 base pairs for HCV-5'UTR region in lane 1-lane 7 **(B)**. The positive control (PC) and negative control (NC) for each HCV-core region and HCV-5'UTR were performed for amplification control. The 1kb DNA ladder (M) was run as DNA size indicator

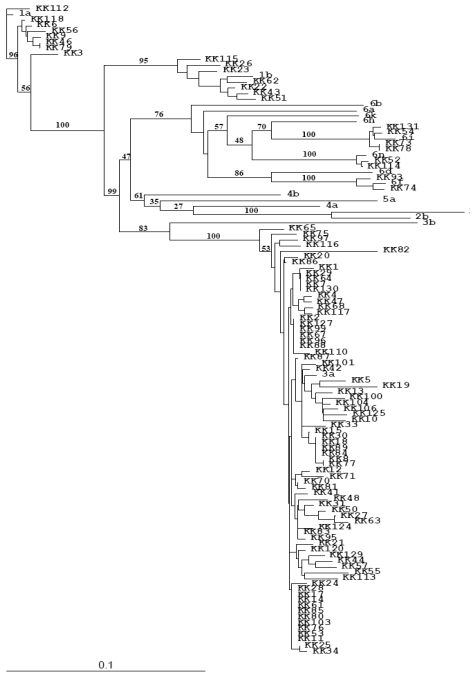

Figure 2. Phylogenetic Analysis of HCV Amplified products. The 266 nucleotides within core region of 106 isolated amplified products was aligned and analyzed for the nucleotide substitution between the sequences by phylogenic construction on the reference sequences of six major genotypes. The accession number of reference sequence: 1a(AF011753), 1b(AJ132996), 2a(AF238485), 2b(AF238486), 3a(D17763), 3b(D493740), 4a(Y11604), 4b(U10235), 5a(Y13184), 6a(Y12083), 6b(D84262), 6d(D84263), 6e(D63822), 6f(D37844), 6g (DQ314806),6h(D84265), 6i(D37849), 6k(D84264), and 6n(DQ278849)

homology of 5'UTR and core region direct sequencing. The 5'UTR composes approximately 340 bps and 573 bps for core region but we were able to sequence 164 bps of 5'UTR at 125 nt to 290 nt and 266 bps of core region at 399 nt to 664 nt. The direct sequencing of each HCV isolate were analyzed for genotype classification by alignment with representative of each genotype references by using BLAST/FASTA programs and ClustalW program with 95 to 100% percent similarities score to define specific subtype matching. The highest percent similarity indicates tentative genotype assignment. The Phylogenetic analysis of HCV sequences was done by unrooted phylogenetic tree for HCV genotype classification confirmed. Phylogenetic tree of 106 isolates were successfully constructed from 266 nucleotides within core region sequences for HCV genotype analysis with reference sequences of all 6 major genotypes from GenBank as shown in Figure 2. Genotype analysis revealed that HCV genotype 3 is the most prevalent (71.7%), followed by genotype 1 (15.0%), and genotype 6 (8.5%). The genoype prevalence showed similar pattern to previous reports in other regions of

Table 3. HCV Subgenotypes Distribution among 106 Northeastern Thai HCV Viremia Blood Donors Compare with Previous Reports in Neighboring Countries

Sub Genotype	Thailand Northeast donor (n=106)	Thailand Central[a] OPD (n=71)	Vietnam Hanoi[b] donor (n=70)	Cambodia immigrant[c] worker (n=25)	Myanmarimmigrant[c] worker (n=15)
1a	8 (7.5%)	no data	21 (30.0%)	0 (0)	1 (6.7%)
1b	8 (7.5%)	no data	12 (17.1%)	6 (24.0%)	1 (6.7%)
2a	0 (0)	no data	0 (0)	0 (0)	0 (0)
2c	0 (0)	no data	0 (0)	0 (0)	0 (0)
3a	76 (71.7%)	no data	2 (2.9%)	4 (16.0%)	4 (26.7%)
3b	0 (0)	no data	2 (2.9%)	1 (4.0%)	5 (33.3%)
6a	0 (0)	0 (0)	26 (37.1%)	0 (0)	0 (0)
6e	0 (0)	1 (0.3%)	6 (8.6%)	5 (20.0%)	0 (0)
6f	3 (2.8%)	39 (10.4%)	0 (0)	2 (8.0%)	2 (13.3%)
6i	4 (3.8%)	8 (2.1%)	0 (0)	0 (0)	0 (0)
6j	0 (0)	7 (1.9%)	0 (0)	0 (0)	0 (0)
6l	0 (0)	0 (0)	1 (1.4%)	0 (0)	0 (0)
6m	0 (0)	0 (0)	0 (0)	0 (0)	2 (13.3%)
6n	2 (1.9%)	16 (4.3%)	0 (0)	0 (0)	0 (0)
6p	0 (0)	0 (0)	0 (0)	1 (4.0%)	0 (0)
6r	0 (0)	0 (0)	0 (0)	5 (20.0%)	0 (0)
Unidentify	5 (4.7%)	0 (0)	0 (0)	1 (4.0%)	0 (0)

[a]Akkkarathamrongsin et al., 2010; [b]Pham et al., 2009; [c]Akkkarathamrongsin et al., 2011

Thailand as summarized in Table 2. The genotype 6 is commonly found in northeastern Thai blood donor, but with prevalence lower than for Vietnamese blood donors. This study found that subgenotype 6i (3.8%) was more widely distributed than other subgenotype 6 among northeastern Thai blood donors. Comparison showed different subgenotype 6 distribution patterns between Thai and neighboring countries such as Vietnam, Cambodia and Myanmar which had higher prevalence of genotype 6 as summarized in Table 3.

Discussion

Based on 266 nucleotide sequences of the core region amplified fragments were determined and analyzed with the reported sequences in databases. Based on more than 95% nucleotide identities, HCV 3a distribution was similar to other reports in other parts of Thailand (Apichartpiyakul et al., 1994; Kanistanon et al., 1997; Hotta et al., 1997; Apichartpiyakul et al., 1999; Hansurabhanon et al., 2002; Theamboonlers et al., 2002).

Interestingly, genotype 3a prevalence in Northeastern Thailand in this study was markedly higher (71.7%) than previous reports, e.g., about 42.3% (Kanistanon et al., 1997), or about 42.9% (3/7 cases) in cholangiocarcinoma patients in northeast Thailand (Barusrux et al., 2012). However, it was similar to the HCV 3a prevalence among IVDUs found in Southern Thailand, 73.5% (Hansurabhanon et al., 2002). HCV genotype 3a is closely associated with IVDUs in the United States (Zein et al., 1996). The higher prevalence of HCV genotype 3a of this study could probably be due to the genetic diversity of HCV, or, different nature of the subjects e.g., tattooing is common practice among Northeast male. Besides the differences in geographical distribution, some differences in clinicopathological features of HCV infection have been observed among different types and subtypes (Hotta et al., 1997). HCV type 6 variants are common and restricted in the Southeast Asia countries (Apichartpiyakul et al., 1994; Hotta et al., 1997; Kanistanon et al., 1997; Apichartpiyakul et al., 1999; Pham et al., 2009; Akkarathamrongsin et al., 2010; Akkarathamrongsin et al., 2011). HCV genotype 6a was relatively rare among Thai blood donors, 8% to 18% of the HCV RNA positive (Apichartpiyakul et al., 1999; Akkarathamrongsin et al., 2010) and 6% to 20% of IVUDs in northern/northeastern Thailand (Apichartpiyakul et al., 1999).

HCV blood donor isolates in this study found none of HCV genotype 2, 4 or genotype 5. There were 3 distinct subgenotype 6 group variants. Based on nucleotides and amino acids alignment, confirmed by phylogenetic analysis based on core region, 4 cases (4.3%) showed 97-98% homology with genotype 6i (accession number D37849), 3 cases (3.2%) showed 97 to 98% homology with the Thai isolate genotypes 6f (accession number D37844) and 2 cases (1.9%) showed 98% homology with the Kunming, China isolate Km42 of genotype 6n (accession number DQ278894). The subgenotype 6 distribution patterns in Thailand are different between blood donor participants and cholangiocarcinoma (CCA) patients. Thailand and neighboring countries such as Vietnam, Cambodia and Myanmar, which has higher prevalence of genotype 6, are also different. Difference of HCV genotype distribution are seen between healthy persons and Thai adult persons and neighboring country (such as Vietnam, Cambodians and Myanmar).

In previous studies, the novel subtype of HCV major type 6 was reported as clade 6 or type 6 group variants only based on sequences similarities with reported sequences and confirmed by phylogenetic analysis in the subgenomic regions (NS5B, E1 or core region) of HCV genome (Felsenstein, 1985; Robertson et al., 1998). The high rate of viremia found in this study and other previous reports in Thailand should be considered by health policy makers in guidelines for prevention of the disease progression. No vaccine is currently available for prevention and treatment of hepatitis C infection. Genotyping of HCV is clinically significant because it is the single most important predictor of response to HCV treatment. HCV genotype 1b is more resistant to interferon treatment and more associated with severe liver damage than other subtypes. HCV genotype 3a has higher sustained virological response (SVR) rate

in patients than genotypes 1, 4, 5, and 6. HCV genotypes 1 and 4 in infected patients do not respond to interferon treatment as well as genotypes 2 and 3 (Manns et al., 2001; Nguyen and Keeffe, 2004).

It is well recognized that storage temperature can affect HCV-RNA stability and the recommended storage temperature for HCV-RNA is -70°C (Halfon et al., 1996). The samples stored at 25°C maintain their HCV-RNA titer during 14 days and at 5°C were stable for at least 3 months, the stability of HCV RNA in plasma at -20°C for RT-PCR reactivity at least 2.5 years at concentration equal to or higher that 100 IU/ml (Jose et al., 2003). Our study was able to detect HCV RNA in 94.6% of plasma samples stored at -70°C for more than 1 year to 2.3 years. We also found no statistically significant difference in HCV RNA detectable rates between one and two years storage plasma samples.

In conclusion, a significantly higher prevalence of HCV genotype 3a was found among asymptomatic infected patients than previous reports demonstrated. The subgenotype 6 prevalence in northeastern Thailand was less than neighboring countries and the distribution patterns were different. It seems to be a good predictor for using interferon therapy indicator. This study provides information on molecular epidemiology for prognosis of the disease among this group which would be useful for clinical management, and prognosis of the disease in this population.

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