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ORIGINAL ARTICLE

# Molecular characterization of hepatitis C virus genotype 6 subtypes in Thai blood donors



Anchalee Sistayanarain <sup>a,\*</sup>, Suriya Chaiwong <sup>b</sup>

<sup>a</sup> Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand

<sup>b</sup> Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand

Received 24 April 2014; received in revised form 2 January 2015; accepted 19 January 2015

Available online 30 January 2015

## KEYWORDS

Blood donors;  
Genotype and  
subtype;  
Hepatitis C virus;  
Hepatitis C virus-6  
subtypes

**Abstract** *Background:* Hepatitis C virus (HCV) genotype is important for identifying effective antiviral therapy, evaluating pathogenic severity, and tracking transmission routes. In Thailand, HCV genotypes 3 and 1 are the most common. We have previously demonstrated an increasing appearance of genotype 6 in HCV infections in Thailand. However, only limited epidemiological data on genotype 6 in Thailand are available. This study aimed to characterize HCV genotype 6 among apparently healthy Thai blood donors.

*Methods:* In total, 240 blood samples were collected from Phitsanulok Regional Blood Center, Phitsanulok, Thailand. RNA was reverse transcribed and amplified by the nested polymerase chain reaction. HCV genotyping was performed by direct sequencing and phylogenetic tree analysis of core sequences. Amino acid polymorphism of various subtypes of HCV genotype 6 was investigated.

*Results:* Of the 240 samples, 192 were successfully sequenced for the core region and 84 were determined to be of HCV genotype 6 by phylogenetic analysis. The most prevalent HCV-6 subtypes were 6f > 6n > 6c > 6i. Amino acid sequences of the partial core region among these four subtypes differed by one to seven residues.

*Conclusion:* For HCV-6, the subtype 6f was commonly found in Thai blood donors. Comparison of core protein from various HCV-6 subtypes showed substantial polymorphisms, which may form the basis of future studies using samples from patients with clear HCV histories. This feature can be applied to therapies tailored to particular genotype variants.

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\* Corresponding author. Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Tambon Tha Pho, Muang, Phitsanulok 65000, Thailand.

E-mail address: [sisaya@rocketmail.com](mailto:sisaya@rocketmail.com) (A. Sistayanarain).

## Introduction

A common cause of acute and chronic liver diseases arises from hepatitis C virus (HCV), which is classified into six major genotypes (genotypes 1–6) and more than 80 subtypes. HCV genotyping is considered important for predicting a sustained virologic response. Thus, the HCV genotype is an important determinant of effective treatment methods and vaccine development. Antiviral drugs including pegylated interferon-alpha, ribavirin, boceprevir, and telaprevir are current treatments or are under development.<sup>1,2</sup>

The global HCV incidence rate is about 3%; however, the rate varies by region and by genotype: genotypes 1–3 predominate in Europe, North and South America, and Japan<sup>3</sup>; genotype 4 is most frequent in North Africa and the Middle East; genotype 5 is common in South Africa<sup>4,5</sup>; and genotype 6 is largely confined to East/Southeast Asia.<sup>6,7</sup> However, data based on continents conceal local variations, which might provide more accurate insights about the spread of this virus. There have been several studies demonstrating such localization in, for example, Thailand, where HCV genotype frequencies of 3a > 1 > 6 > 2 are found.<sup>8,9</sup> More recently, another region of Thailand demonstrated the prevalence of genotypes 3a > 6 > 1, with genotype 6 in particular being more prevalent in HCV infections.<sup>7</sup> Akkarathamrongsin et al<sup>8</sup> found a prevalence of HCV-6 subtypes in Thai people residing mainly in the central region of the country and subtypes 6f, 6n, 6i, 6j, and 6e were detected using phylogenetic analysis based on the core and NS5B sequences. Another study demonstrated prevalence of infections due to subtypes 6a, 6f, and 6n in four disparate provinces of Thailand.<sup>9</sup> However, failure to detect genotypes 4 or 5 does not preclude their existence in other parts of Thailand.

HCV genotyping commonly uses restriction fragment length polymorphism or polymerase chain reaction (PCR) using type-specific primers and hybridization with specific oligonucleotide probes. However, the genotype specificity of these methods is sometimes questionable and indeed our previous study failed to genotype several samples. By contrast, direct nucleotide sequencing is more reliable for genotyping HCV samples. Although there has been limited epidemiological data on genotype 6 in Thailand, its increasing emergence needs a more thorough assessment of its regional distribution, which might help in limiting its spread and treatment. Therefore, we sought to apply sequence analysis of the core region to understand the molecular epidemiology and genetic diversification of the HCV in order to study the main genotype 6 and also its subtypes among blood donors from Thailand.

## Materials and methods

### Sample collection

Two hundred and forty serum samples, which were anti-HCV antibody positive, were obtained from blood donors at Phitsanulok Regional Blood Center, Phitsanulok province, Thailand. The blood donors were from Phitsanulok, Phetchabun, Kamphaengphet, Sukhothai, Tak, and Uttaradit provinces in Thailand. Serum samples used in this study

were obtained during the years 2006–2007. The study was approved by the Human Ethics Committee of Naresuan University, Phitsanulok, Thailand.

### RNA extraction and reverse transcription

RNA was extracted from serum using PureLink Viral RNA/DNA Kits (Invitrogen, USA) according to the manufacturer's instructions. RNA was reverse transcribed into complementary DNA by random primer.

### Nested PCR for core gene

The partial core region of HCV was amplified by nested PCR with two sets of oligonucleotide primers using the same optimized condition as described previously.<sup>7</sup> The first-round amplification was carried out with outer sense (Sc2) 5'-GGGAGGTCTCGTAGACCGTGCACCATG-3' and outer antisense primers (Ac2) 5'-GAGMGGKATRTACCCCATGAGRTCGGC-3'. The second-round PCR was amplified with inner sense (Q2) 5'-AGGTCTCGTAGACCGTGCATCATG-3' and inner antisense primers (AQ2) 5'-CAYGTRAGGGTATCGATGAC-3'. The first-round PCR was conducted for 40 cycles according to the following cycling parameters: preliminary 20 cycles at 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute, followed by 20 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The second-round PCR was conducted for 35 cycles according to the following cycling parameters: 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The final extension step was carried out at 72°C for 7 minutes.

### Direct nucleotide sequencing and phylogenetic analysis by amino acid sequence

HCV genotyping was performed by direct nucleotide sequencing and determined by *Basic Local Alignment Search Tool* analysis [National Center for Biotechnology Information site (NCBI)] and phylogenetic tree analysis. Phylogenetic tree of the core amino acid sequences was constructed using the neighbor-joining method by bootstrap analysis with 1000 replicates in MEGA software (version 6.06) ([www.megasoftware.net](http://www.megasoftware.net)). External reference sequences for the phylogenetic construction were retrieved from the Los Alamos HCV database (<http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html>) and NCBI.

All sequences obtained from the core region were submitted to GenBank (accession numbers KF577403–KF577411, KF577413–KF577415, KF577417–KF577433, KF577435–KF577475, KF577477–KF577478, and KF577480–KF577493).

### Amino acid sequence comparison of genotype 6

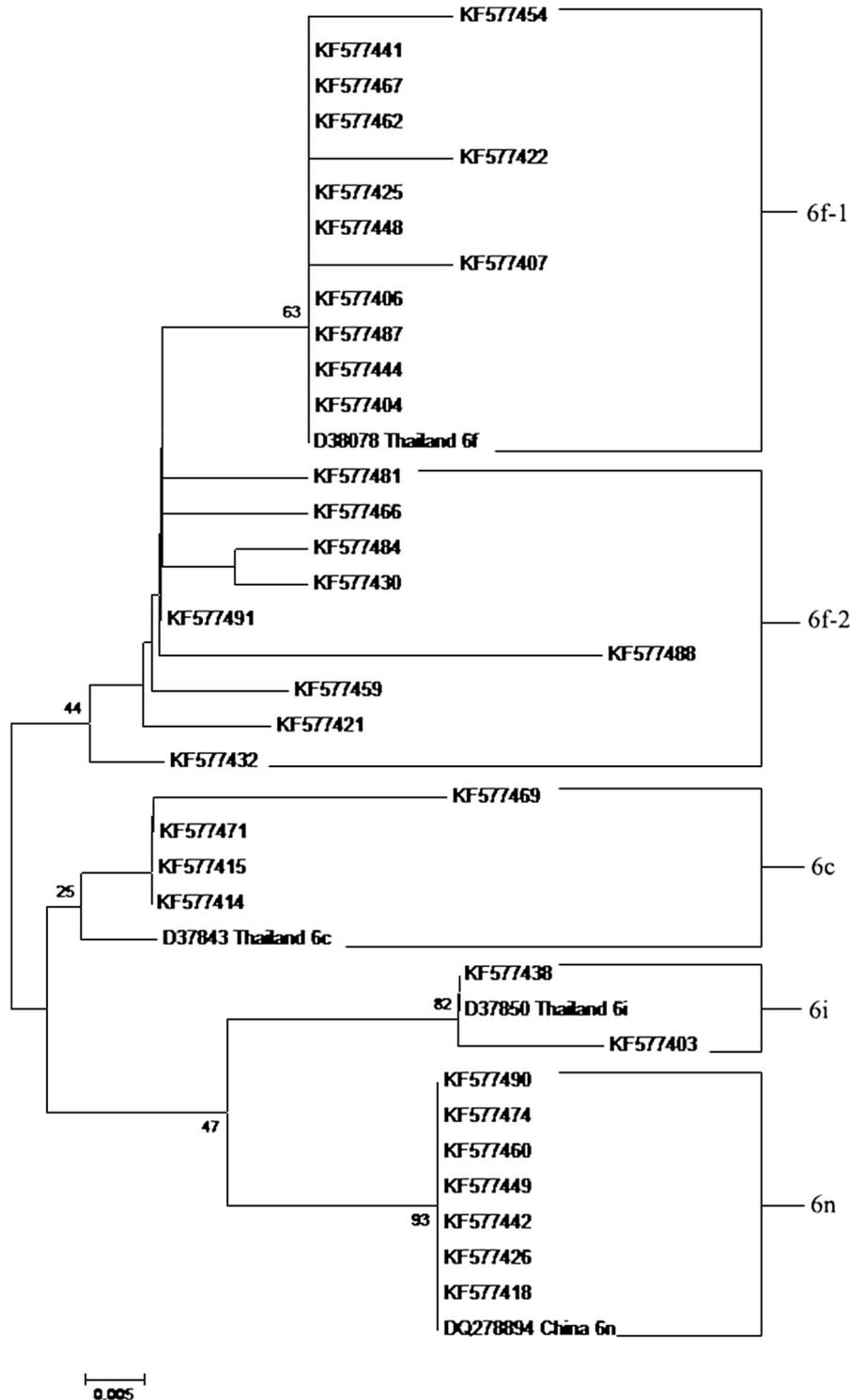
To determine the difference of amino acid residues among various HCV-6 subtypes, amino acid sequences of HCV isolates were aligned based on core region with ClustalW2 and BioEdit software. The prototype sequences of HCV genotype 6 subtypes 6f, 6n, 6c, and 6i used for comparison were as follows: D38078 (6f), DQ278894 (6n), D37843 (6c), and D37850 (6i).

## Results

Of the 240 anti-HCV antibody-reactive serum samples, 192 were successfully sequenced for the core region. HCV genotypes were determined and phylogenetic analysis was

carried out. The HCV genotype 6 was detected in 84 samples.

HCV-6 isolates were identified as having sequences belonging to either subtype 6f (71, 84.5%), 6n (7, 8.3%), 6c (4, 4.8%), or 6i (2, 2.4%). In this study, 6f was the major



**Figure 1.** Phylogenetic tree of the partial core amino acid sequences of HCV-6 subtypes isolated in this study. The reference sequences are represented in accession numbers, genotype or subtype, and country of origin. Bootstrap values are indicated in the tree. The reference sequences are as follows: D38078, DQ278894, D37843, and D37850. KF577491 is shown as the representative strain for other strains with the same amino acid sequences of HCV subtype 6f-2. HCV = hepatitis C virus.



L97F). For subtype 6i, only one amino acid substitution at position 115 (R115K) was identified. Based on a comparison of amino acid V or A at position 68, the HCV subtype 6f was classified as 6f-1 and 6f-2 (Figs. 1 and 2).

## Discussion

HCV genotype 6 is known to circulate in Southeast Asian countries, especially Thailand, Myanmar, Vietnam, and Laos.<sup>6,7,10,11</sup> Studies of HCV genotype have identified genotype 6 in Southern China, Hong Kong, Taiwan, Macau, and Korea.<sup>12–15</sup> Southeast Asian immigrants living in Canada also carry HCV genotype 6 infections.<sup>16</sup> Of all HCV infections, HCV-6 is the most prevalent in Myanmar (49%), Vietnam (47.1%), Hong Kong (27.1%), and Thailand (18–31%). HCV genotype 6 has the highest genetic diversity, forming at least 22 subtypes worldwide (6a–6v).<sup>17</sup> We recently reported that genotype 6 has become more prevalent than genotype 1.<sup>7</sup> There have been several studies on genotype 6 subtypes between 1994 and 2014 from all regions of Thailand.<sup>8,9,18–21</sup> Although subtypes 6a, 6b, 6c, 6e, 6f, 6i, 6j, 6m, and 6n have been identified, the data are insufficiently detailed to detect any time-dependent drift in subtype drift as a whole or by region. The only clear change has been the emergence of type 6v, which is common in northern Thailand.<sup>22</sup> The results of this study show that only four subtypes of HCV-6 (i.e., 6f, 6n, 6c, and 6i) are present in Thailand. In particular, the HCV-6 subtype 6f appears to be exclusive to Thailand.<sup>8</sup> A variant of HCV-6 has also been detected among workers from Cambodia and Myanmar immigrating to Thailand.<sup>23</sup> A new HCV-6 subtype 6n was found in Myanmar mainly within the northern cities bordering China and India.<sup>10,24</sup> Subtype 6n has also been detected in Laos and was found in our samples (8.3%). Very recently, a small number of subtype 6c has been obtained from infected Korean patients by Seong et al<sup>15</sup>; however, in this study, all of the subtype 6c sequences isolated from blood donors were from Uttaradit province, Thailand. Our study also indicated that HCV subtype 6i is rarely found in Thailand. In addition, we found that HCV subtypes 6c and 6i were restricted to areas of Uttaradit and Phetchabun provinces close to Laos where these subtypes are found. Nevertheless, subtype 6f was the most common subtype in our samples, which reaffirms its high frequency in Thailand.

The nucleotide sequence of HCV genotype 6 subtypes differed by 21–29%.<sup>25</sup> We showed 1–8% variations (1–7 amino acid residues). It has been recommended that the entire sequences should be identified for additional information. The HCV core protein, composed of 191 amino acid residues, consists of three domains, namely, an N-terminal hydrophilic domain (positions 1–117), a C-terminal hydrophobic domain (positions 118–170), and the hydrophobic signal sequence.<sup>26,27</sup> A total of 83 amino acid residues of Thai HCV subtype 6f were compared with HCV reference strain (D38078). In this study, we found 13 amino acid substitutions in the HCV subtype 6f, whereas there were three substitutions in subtype 6c. Interestingly, the amino acid at position 68 of HCV subtype 6f showed substitution of V for A in 83% (59/71) of samples. Amino acid V or A at position 68 allowed for further classification of subtype into 6f-1 and 6f-2, respectively (Fig. 2), but there was no

geographic association in this regard. In this study, amino acid sequences of HCV subtype 6n were conserved compared with the corresponding reference strain, which is in accordance with a previous study (amino acid positions 33–115) on individuals in Thailand with subtype 6n.<sup>8</sup> HCV subtype 6n showed no difference in amino acid sequences, suggesting same historic transmission routes. However, historic information about blood donors was unavailable. For subtype 6i, only one amino acid substitution at position 115 (R115K) was identified. The core protein is involved in the association of various pathways that are important to the HCV life cycle.<sup>28</sup> Polymorphisms of amino acid in the core region influences antiviral therapy and the severity of liver disease.<sup>29,30</sup> Mutation of core protein at position 70 (R70Q, R70H) and/or 91 (L70M) of genotypes 1b and 4d is associated with poor interferon treatment outcomes.<sup>29,31</sup> Likewise, HCV-6 subtype 6f is more resistant to combined pegylated interferon and ribavirin treatment than genotypes 1 and 3.<sup>21</sup> However, there are no data relating to drug treatment and residue changes in the core region for genotype 6.

In conclusion, of the four HCV-6 subtypes in this study (i.e., 6f, 6n, 6c, and 6i), the HCV-6 subtype 6f appears to be exclusive to Thailand. There were modest variations in the core region of these four HCV-6 subtypes. However, because the samples were from healthy donors, there was no information to correlate the data, which might predict treatment outcomes. Thus, further study on the epidemiology of the HCV genotype 6 in the whole region of Thailand is important.

## Conflicts of interest

All authors declare no conflicts of interest.

## Acknowledgments

This project was supported by the Annual Research Fund of Naresuan University (the grant number:R2557C001). We are especially grateful to Phitsanulok Regional Blood Center, Phitsanulok, Thailand for collecting the samples.

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