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High Prevalence of Hepatitis C Virus Genotype 6 in Vietnam

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SUMMARY This study aimed to update the prevalence of the various Hepatitis C virus genotypes in Vietnamese blood donors. One hundred and three HCV antibody-positive plasma samples were collected from blood donors at the National Institute of Hematology and Blood Transfusion, Hanoi, Vietnam. All specimens were subjected to RT-PCR of the 5' untranslated region (UTR) to confirm the presence of HCV RNA. The core and NS5B regions of the positive samples were subsequently amplified by RT-PCR followed by direct sequencing and phylogenetic analysis. Seventy out of 103 samples (68.0%) were RNA positive. Core and NS5B were successfully amplified and sequences were obtained for 70 and 65 samples, respectively. Phylogenetic analysis revealed that genotype 6a was the most predominant among Vietnamese blood donors with a prevalence of 37.1% (26/70), followed by genotype 1a at 30.0% (21/70) and genotype 1b at 17.1% (12/70). The prevalence of two other genotype 6 variants, 6e and 6l was 8.6% and 1.4%, respectively. Further analysis of recent studies showed that the geographic distribution of genotype 6 covered mainly southern China and the mainland of Southeast Asia including Vietnam, Laos, Thailand, and Myanmar. The GenBank accession numbers for the sequences reported in this study are FJ768772-FJ768906.

Hepatitis C virus infection presents a global burden with a prevalence of an estimated 2.2% of the world population, equivalent to almost 150 million people, with 3 to 4 million persons newly infected each year.[1] It is estimated that 27% of infected individuals develop cirrhosis and 30% of those proceed to hepatocellular carcinoma.[2] The main route of hepatitis C transmission is direct percutaneous exposure through blood transfusion or transplantation from infectious donors.[3] An effective treatment for this infection still remains a challenge, especially for genotypes 1 and 6, which are predominant in Southeast Asia, including Vietnam.

HCV is an enveloped virus and belongs to the family *Flaviviridae*. Its genome comprises single-stranded RNA of positive polarity with one open reading frame of approximately 9,600 nucleotides encoding a single polyprotein. The polyprotein flanked by untranslated regions consists of three structural proteins (core, E1 and E2) and seven non-structural proteins (P7 and NS2-NS3-NS4A-NS4B-

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NS5A-NS5B).[4] The virus is characterized by its genetic diversity that is used for genotyping. Up to now, six genotypes and more than seventy subtypes have been identified and the difference between genotypes may vary in excess of 30% and between subtypes by 20-25%.[5] The 5'untranslated region, which is highly conserved, is used for virus detection while both core and NS5B are more variable and widely applied for genotyping.[6]

In Vietnam, the prevalence of HCV infection varies depending on the geographical location and the target population. HCV prevalence in the general population was 1.0% to 2.0%.[7-9] Blood transfusion still represents one of the predominant risks for HCV infection as almost all blood donors are paid.[10] The genotype prevalence, especially in blood donors, has never been clearly determined although Vietnam is among those countries in which genotype 6 variants were first detected.[11] Furthermore, previous studies on HCV in Vietnam usually collected their samples in the South and the methods applied for genotyping were Line probe hybridization (LiPA), genotype-specific primers or DNA branch technique, which resulted in many isolates remaining unclassifiable.[7] Only limited data and very small sample sizes on HCV genotype prevalence have so far been reported from North Vietnam.[11] Moreover, they were obtained 15 years ago when there was no consensus on the HCV nomenclature.[5] Therefore, we conducted this study with the aim to update the genotype prevalence of HCV in Vietnamese blood donors.

MATERIALS AND METHODS

Study population

The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand and by the Thai Binh Medical University, Vietnam. The specimens were collected with the permission of the Director of the National Institute of Hematology and Blood Transfusion, Vietnam.

A total of 103 HCV antibody-positive samples were collected from blood donors at the National Institute of Hematology and Blood transfusion, Hanoi, Vietnam, from January to July 2008. The ratio of male to female was 5:1, and the donors' age was on average 28 years (ranging from 19 to 49). Plasma samples were divided into aliquots and stored at -70^{o}C until further testing.

Hepatitis C virus RNA extraction and RT-PCR

Viral RNA was extracted from 100 µl of each plasma sample applying the guanidinium-isothiocyanate method as described elsewhere[12] and used for cDNA synthesis by means of random hexanucleotide primers.

In the course of this study, our group performed 3 distinct polymerase chain reactions. First, the 5'UTR was amplified for confirmation of HCV infection by the following two primer sets: OC1 (5'GCC GAC ACT CCA CCA TGA AT 3' nt 18-37), OC2 (5'CAT GGT GCT CGG TCT ACG AG 3' nt 328-347) and OC3 (5'GGA ACT ACT GTC TTC ACG CAG 3' nt 51-71), OC4 (5'TCG CAA GCA CCC TAT CAG GCA 3' nt 293-313).[13] Secondly, the core and NS5B regions were partially amplified. The primer sets for amplification of the core were 954F (5'ACT GCC TGA TAG GGT GCT TGC GAG3' nt 291-314), 410R (5'ATG TAC CCC ATG AGG ATCG GC3' nt 735-754) for the outer fragment; and 953F (5'AGG TCT CGT AGA CCG TGC ATC ATG 3' nt 324-347), 951R (5'CCA TGT RAG GGR ATC GAT GAC 3' nt 708-729) for the inner fragment.[14] We designed the primers for amplification of the NS5B based on the alignment consensus with reference sequences of different genotypes and subtypes. These primers included NS5B_F1 (5'CAA TWS MMA CBA CCA TCA TGG C 3' nt 8101-8120), NS5B_R1 (5'CCA GGA RTT RAC TGG AGT GTG 3' nt 8805-8825) for the outer fragment and NS5B_F2 (5'GAT GGG HHS BKC MTA YGG ATT CC 3' nt 8159-8181), NS5B R1 for the inner fragment (nucleotide position with reference to the GenBank database accession number M62321). The amplification protocols for the 5'UTR and the core have been described elsewhere.[13,14] The first reaction for amplification of the NS5B gene comprised an initial denaturation step at 94^{o}C for 5 minutes, followed by 40 cycles of denaturation at 94^{o}C for 1 minute, primer annealing at 49^{o}C for 1 minute, extension at 72^{o}C for 1 minute and 30 seconds to be concluded by a final extension step at 72^{o}C for 7 minutes. The second amplification reaction consisted of an initial de-

naturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 52°C for 1 minute, extension at 72°C for 1 minute and 30 seconds and was concluded by a final extension step at 72°C for 7 minutes. The PCR products (5'UTR, 262 bp; core, 405 bp and NS5B, 666 bp) were subjected to electrophoresis in a 2% agarose gel stained with ethidium bromide and subsequently visualized on an UV trans-illuminator (Gel Doc 1000, BIO-RAD, CA).

Sequencing and phylogenetic analysis

Amplicons displaying the size expected for the core and NS5B genes were purified with the Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, NY) according to the manufacturer's manual. The concentration of the collected DNA was measured at 260 nm in a spectrophotometer (Shimadzu UV 160 A, Tokyo, Japan). Between 10-50 ng/µl of each DNA sample were subjected to sequencing with the inner primer sets by the ABI Prism 310 Genetic Analyser (ABI, Fostercity, CA). All sequences were submitted to the GenBank database.

The nucleotide sequences were edited and assembled with CHROMAS LITE v.2.01 (http://www.technelysium.com.au/chromas_lite.html) and SeqMan (DNASTAR, Madison, WI), respectively. Employing the Clustal X 1.8 program, all sequences were aligned with reference sequences of different genotypes and subtypes from the Los Alamos HCV database (http://hcv.lanl.gov/content/hcv-index). Genotype determination was accomplished by BLAST/FASTA (http://www.ncbi.nlm.nih.gov) and by phylogenetic analyses with the MEGA software package version 4.0[15] using the neighbor-joining method. The pair-wise distances between the nucleotide sequences were computed based on the model of the maximum composite likelihood to construct the phylogenetic trees. The phylogenetic trees were tested for their reliability by bootstrap analysis with 1,000 replicates.

The accession numbers of the reference sequences for genotype 1a were: M62321, AF511950; 1b: AB049087, EF407486; 1c: D14853; 2a: AB047639; 2b: AB030907; 2c: D50409; 2k: AB031663; 3a: AF046866, D28917; 3b: D49374; 3k: D63821; 4a: Y11604; 5a: Y13184; 6a: AY859526, Y12083; 6b:D84262; 6c: EF424629; 6d: D84263; 6e: DQ314805; 6f: DQ835760; 6g: D63822; 6h: D84265; 6i: DQ835762; 6j: DQ835761; 6k: D84264; 6l: EF424628; 6m: DQ835763; 6n: DQ278894; 6o: EF424627; 6p: EF424626; 6q: EF424625; and 6t: EF632069

RESULTS

Proportion of HCV RNA to anti HCV seropositive samples

Out of 103 plasma samples, 70 (68.0%) were confirmed HCV-RNA positive as a result of the successful amplification of the 5'UTR. We obtained 70 and 65 sequences specific for the core and NS5B regions, respectively. The core region of all 5'UTR positive samples could be amplified while the NS5B region could be amplified in 65 samples only. The genotype analysis of the five samples negative for NS5B was therefore based on the core region.

HCV genotype prevalence

BLAST/FASTA and phylogenetic analysis revealed that genotype 6a was the most common in HCV infected Vietnamese blood donors with 37.1% followed by genotype 1a and 1b with 30.0% and

Table 1 HCV genotype determination based on the core and NS5B regions

Regions	Genotype							Total
	1a	1b	3a	3b	6a	6e	6l	
Core (%)	21 (30.0)	12 (17.1)	2 (2.9)	2 (2.9)	26 (37.1)	6 (8.6)	1 (1.4)	70 (100)
NS5B (%)	21 (32.3)	12 (18.5)	1 (1.5)	2 (3.1)	24 (36.9)	4 (6.1)	1 (1.5)	65 (100)

17%, respectively. Two additional genotype 6 variants, 6e and 6l accounted for 8.6% and 1.4% of our specimens. Genotype 3 was the least frequent genotype among Vietnamese blood donors amounting to 2.9 % each for genotype 3a and 3b (Table 1). We did not detect any recombinant or mixed infections of different genotypes. The five samples whose NS5B region could not be amplified were genotyped as 3a (1 case), 6a (2 cases) and 6e (2 cases). Pairwise distance calculation showed that the homology of our sequences with the specific reference sequences based on both core and NS5B region for each subtype ranged from 88.3% to 98.8% (data not shown).

Two phylogenetic trees were constructed based on the sequences obtained for these two regions aligned with reference sequences of different genotypes. The phylogenetic trees based on the core and NS5B displayed a similar result of grouping sequences into genotypes. All genotypes derived from the NS5B region were concordant with those based on the core region (Figs. 1 and 2).

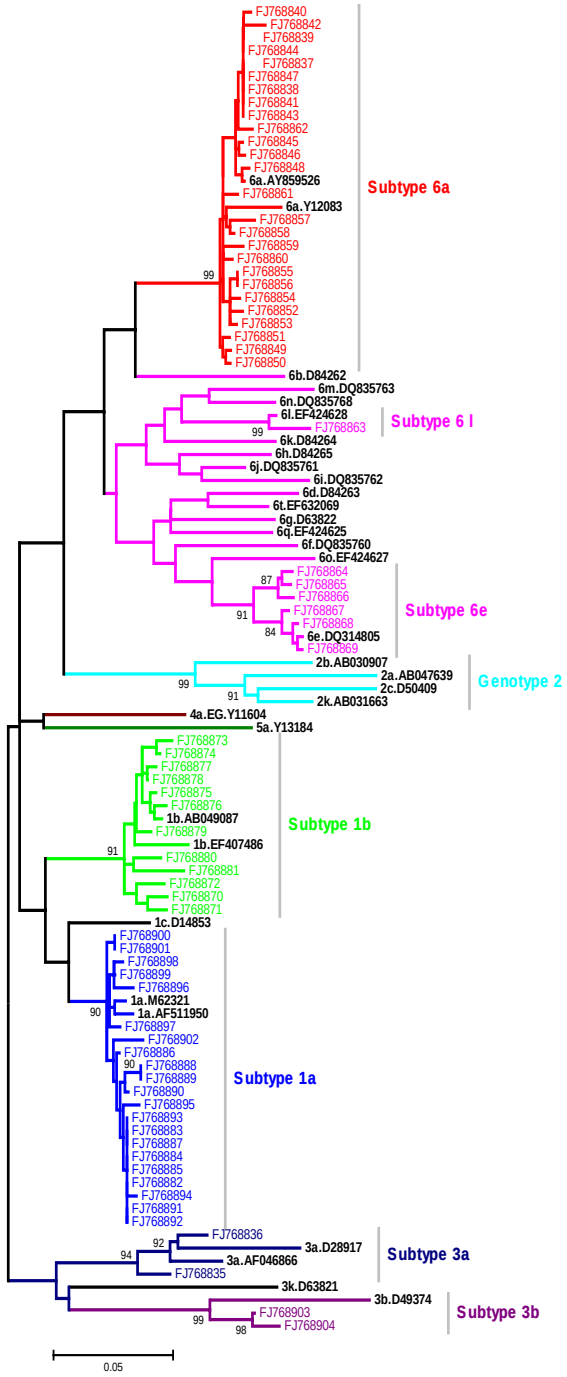

Fig. 1 Phylogenetic tree based on core region sequences. The HCV genotypes are indicated on the roots of the clusters or individuals. The reference sequences are indicated in bold and black color. The numbers shown above the branches represent the percentage of the bootstrap values. The bar at the base indicates the genetic distance per nucleotide site.

DISCUSSION

To confirm the presence of viral RNA in anti-HCV positive samples, we amplified the 5'UTR, a region well documented as highly conserved.[16] We detected viral RNA in 68.0% of the anti-HCV positive samples, a percentage in agreement with the average of 50-90% previously reported by various researchers.[7,17,18] Currently, at the National Institute of Hematology and Blood transfusion, Vietnam, tests for liver function such as the transaminase levels are not performed on blood donors. Some individuals previously infected with HCV may remain anti-HCV positive without displaying viremia, while in some individuals with current HCV infection the viremia may remain undetected due to a low viral load. Also, anti-HCV positive samples with occult infection would neither exhibit viral RNA nor elevated liver enzyme levels.[19]

To determine the HCV genotype, we sequenced both core and NS5B regions an approach previously employed by various research groups.[6,14] Our findings, valid for both core and NS5B sequences, showed that, at the genotype level, genotypes 1 and 6 are common in Vietnamese blood do-

nors (both genotypes accounted for 47.1%). This result was similar to previous studies.[11,20] Interestingly, at the subtype level, we found that subtype 6a was the most prevalent with 37.1% followed by 1a (30.0%) and 1b (17.1%). The pattern of the subtype distribution based on our results was different from previous studies in Vietnam. Although subtype 6a was common, the previous studies showed a higher prevalence of subtype 1b or 1a.[7,11] This might hint at a difference in the geographical distribution of HCV subtypes in Vietnam as all the above studies on the HCV genotypes were performed on specimens collected mainly in the south of Vietnam whereas our samples were mostly obtained from the North. Other aspects that differed between previous studies and ours were the method and the study population. We analyzed specimens from blood donors using sequencing analysis of both core and NS5B regions whereas the previous studies, except for Tokita *et al.*,[11] focused on samples from hepatitis patients and obtained their results by LiPA or 5'UTR analysis.[7,8] Our findings were more consistent with those of Tokita *et al.*[11] who initially investigated HCV infections in Vietnamese blood donors and identified new genotypes subsequently reclassified as genotype 6 variants and at genotype level, genotype 6 was the most common.

Genotype 6 variants in neighboring countries

Subtype 6a has been known to circulate in SEA; however, not all countries in this region have reported its identification. In the present study, we have tried to update the information on HCV genotype distribution (Fig. 3). We found that subtype 6a circulates in the Northwest of SEA including Thailand,[18,22] Myanmar[23] and is especially predominant in Vietnam but not detected in Singapore,[14] Indonesia or the Philippines.[24,25] This suggests a northward distribution of this subtype. Previous studies have identified this subtype in the South-China territories of Hong Kong[26] and Macau.[27] Moreover, various groups have demonstrated a subtype 6a predominance in China's southernmost provinces of Yunnan and Guangxi both of which border on the north of Vietnam.[28-30] Furthermore, in this project, we detected two other genotype 6 variants, 6e and 6l in our samples with a prevalence of 8.6% and 1.4%, respectively. This finding is consistent with that of Tokita *et al*. The circulation of genotype 6 variants has also

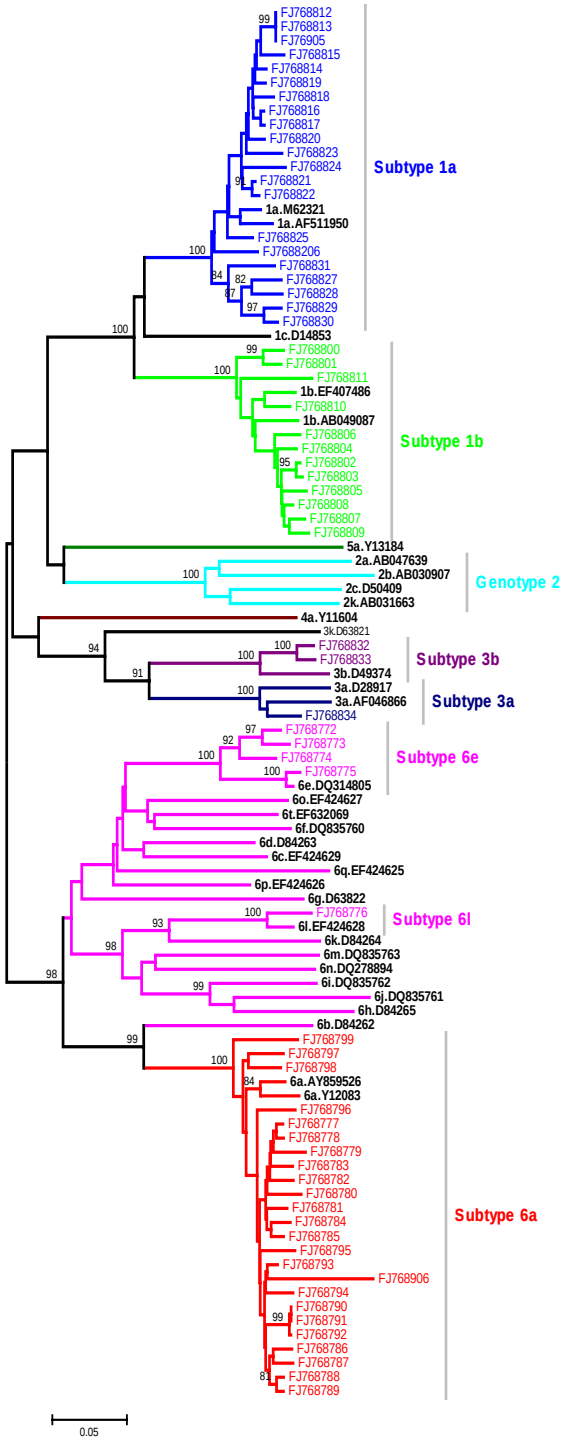

Fig. 2 Phylogenetic tree based on NS5B region sequences. The HCV genotypes are indicated on the roots of the clusters or individuals. The reference sequences are indicated in bold and black color. The numbers shown above the branches represent the percentage of the bootstrap values. The bar at the base indicates the genetic distance per nucleotide site.

been reported from regions such as Thailand,[22,31] Myanmar,[23,32] Laos PDR,[33] and again, for southern China.[28] All research projects referred to have described the geographical distribution of genotype 6 as mainly located in the north of Vietnam, the south of China and spread out over the mainland of SEA.

In the present study, we did not find any genotype 2, which had previously been detected to a limited extent in Vietnam.[8,11,20] This genotype is not common in SEA and has mainly been reported from Japan, South Korea, northern China and Europe.[28,34,35] We could neither detect viral genome recombination nor cases of co-infection with different genotypes. Despite their rarity, cases of mixed infection and recombination have been reported to occur naturally[36,37] or in cell cultures.[38] Our limited number of samples or analysis of only parts of the HCV genome might account for the apparent absence of both recombination and co-infection. Alternatively, these forms of infection might not yet exist in Vietnam.

Genotype distribution has a significant impact on the treatment as a genotype dependent response to the combination therapy of IFN-α and Ribavirin has been demonstrated. The sustained virological response (SVR) has been reported to be around 50% for genotype 1 in chronic HCV infections[39,40] and only 25% in patients with cirrhosis[41] while for genotype 6, the outcome was more promising with an SVR exceeding 80%.[42,43] However, pa-

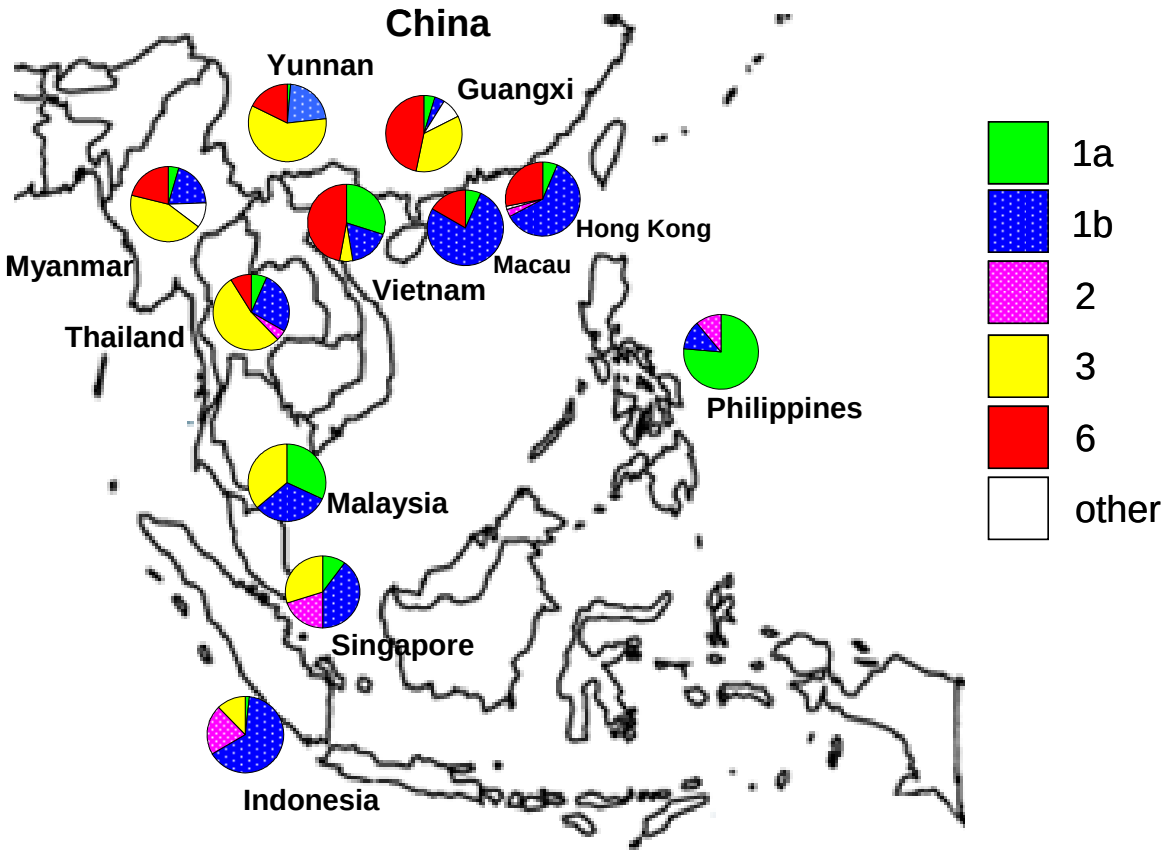

Fig. 3 Map of the HCV genotype distribution in SEA and southern China identified in this study and in previous publications. The genotype 6 is circulating mainly on the mainland of SEA and southern China. The pie charts indicate the proportions of the different genotypes in each region. The data were obtained from references from Thailand,[21] Myanmar,[22] Macau,[26] Hong Kong,[25] Yunnan,[29] Guangxi,[28] the Philippines,[24] Malaysia and Singapore,[14] and Indonesia.[23]

tients infected with either genotype require a prolonged treatment of up to 48 weeks for the best outcome. The present study has demonstrated that genotypes 1 and 6 overlap in Vietnam representing almost 95% of all infected cases which might facilitate hepatitis C management by applying a 48 week therapy for any unknown genotype infection. Nonetheless, additional research will be essential as our analysis focused on blood donors while the genotype prevalence as well as therapy response may well differ in other populations.

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