

## Partly 5` Untranslated Region (5` UTR)-Based Phylogenetic Analysis of Three Hepatitis C Virus Isolates from Jakarta, Indonesia: A Preliminary Study

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### Abstract

Currently, we reported results of a nested polymerase chain reaction (PCR) assay specific 5` untranslated region (UTR) region of hepatitis C virus (HCV) genome that showed three different patterns of deoxyribonucleic acid (DNA) fragments (single expected specific DNA band, single DNA band higher in size than an expected band, and multiple DNA bands). Three isolates (Isolate A, B, and C), representing all the three DNA bands, were analyzed by using phylogenetic trees. The results showed that the Isolate A, B, and C were classified into HCV genotypes 2, 1, and 3, respectively. The Isolate A and B were very closely related to viral isolates from Madagascar and Brazil, respectively and were not closely related to other Indonesia isolates. In contrast with the Isolate A and B, the Isolate C was very closely related to another Indonesia isolate. Among all there isolates, the Isolate C was very closely related to an Indonesia isolate detected from a cirrhosis patient, indicating that the Isolate C might be more virulence than the Isolate B and C. However, a complete genome-based comprehensive genetic characterization for all the three isolates needs to be conducted in future research to confirm all findings in this study.

### Abstract

**Analisis Filogenetik Berbasis Region 5` yang Tidak Ditranslasikan Sebagian (*Partly 5` UTR*) terhadap Tiga Isolat Virus Hepatitis C di Jakarta, Indonesia: Kajian Pendahuluan.** Makalah ini adalah laporan hasil pengujian genom HCV dengan metode *nested PCR 5` UTR* spesifik yang menunjukkan adanya tiga pola fragmen DNA yang berbeda (untai DNA spesifik yang diekspektasi tunggal, untai DNA tunggal yang berukuran lebih tinggi daripada untai yang diekspektasi, dan untai DNA majemuk). Tiga isolat (Isolat A, B, dan C) yang mewakili tiga berkas DNA itu dianalisis dengan pohon filogenetik. Hasil penelitian menunjukkan bahwa Isolat A, B, dan C tergolong genotipe HCV 2, 1, dan 3 secara berturut-turut. Isolat A dan B masing-masing berhubungan erat dengan isolat virus dari Madagascar dan Brazil, meskipun keduanya tidak berhubungan erat dengan isolat dari Indonesia. Berbeda dengan isolat A dan B, Isolat C berhubungan erat dengan isolat dari Indonesia. Di antara ketiga isolat, Isolat C memiliki hubungan paling erat dengan isolat Indonesia yang ditemukan pada seorang pasien kirosis. Hal ini menunjukkan adanya kemungkinan bahwa Isolat C lebih berbahaya daripada Isolat B dan C. Bagaimanapun, karakterisasi genetik komprehensif berbasis genom yang lengkap terhadap ketiga isolat perlu dilaksanakan pada kajian-kajian berikutnya untuk mendukung hasil penelitian ini.

*Keywords: 5` UTR of HCV genome, HCV genotype, phylogenetic tree*

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### Introduction

Hepatitis C virus (HCV) infection is an important public health problem. Worldwide, the virus infected 170 million people, and each year, more than 350.000 people die from hepatitis C-related diseases primarily chronic hepatitis, cirrhosis, and cellular hepatocellular carcinoma (HCC).<sup>1,2</sup> The HCV transmission by unapparent precutaneous ex-

posures, that caused by cross-contamination from reused needles and syringes, multiple-use medication vials, and infusion bags, has been reported.<sup>3</sup> In developing countries, unsafe medical injections and transfusions are predominant routes of HCV transmissions.<sup>4</sup>

The HCV belongs to the family of *Flaviviridae* and an envelope-positive sense ribonucleic acid (RNA) virus

with an RNA genome of approximately 9,400 bp.<sup>5</sup> The genome consists of untranslated regions (UTRs) and a single open-reading frame encoding three structural (core, E1, E2) and seven non-structural (p7, NS2-NS5) proteins. Non-structural protein (NS5B) is a moderately variable region and common used for HCV subtyping.<sup>6</sup> Another region, 5' untranslated region (5' UTR), is a highly conserved region and commonly used in HCV genotyping and evolutionary studies.<sup>7,8</sup>

Recently, we reported a nested PCR assay that amplified partly 5' UTR region.<sup>9</sup> Of 39 positive plasma samples, three different patterns of DNA fragments were detected. Pattern 1 was one specific DNA band, pattern 2 was one DNA band higher in size than an expected band, and pattern 3 was multi-DNA bands.<sup>9</sup> Since we detected the three DNA-band patterns, it makes us eager to learn more; therefore, in this preliminary study we performed phylogenetic analysis of three HCV isolates representing all three DNA-band patterns.

## Methods

**Clinical specimen and viral RNA extraction.** The blood samples from three patients with chronic disease, obtained from Government Hospital in Jakarta, were used in this study. The plasma samples were prepared by centrifuging the whole blood at 2500 rpm for 10 min. Two hundred micro liter of plasma were extracted to obtain the HCV genome RNA(s) by QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instruction, with 50 µl of final elution and immediately used as template for RT-PCR.

**RT-PCR, Nested PCR, and DNA sequencing.** Primers used for RT-PCR and nested PCR for 5' UTR were previously reported by Garson *et al.*<sup>10</sup> The outer sense and anti-sense primers for RT-PCR were 5'-CCA CCA TAG ATC TCT CCC TGT-3' and 5'-ATA CTC GAG GTG CAC GGT CTA CGA GAC CT-3', respectively. The reaction mixture contained 10 µL viral RNA, 1x one-step RT-PCR buffer (Qiagen), 2.5 mM MgCl<sub>2</sub>, 1x Q solution, 400 µM each of dNTP mix, 0.6 µM each of primers, 2 µL of one step RT-PCR enzyme mix, 10 U RNase inhibitor and sterile distilled water to a total volume of 50 µL. The RT-PCR was performed in a Master Gradient Thermal Cycler (Eppendorf) with the first step at 50 °C for 30 minutes; 35 cycles of 96 °C for 30 seconds, 48 °C for 45 seconds and 72 °C for 1 minute; and extended extension at 72 °C for 7 minutes. The nested primers used for the second PCR were 5'-AGA TCT TCA CGC AGA AAG CGT-3' and 5'-CAC TCT CGA GCA CCC TAT CAG GCA GT-3' as inner sense and anti-sense primers, respectively. The reaction mixture of nested PCR consisted of 1x hot star Taq DNA polymerase buffer (Qiagen), 2.5 mM MgCl<sub>2</sub>, 1x Q solution, 200 µM each of dNTP mix, 0.4 µM each of

primer, 1.5 U hot star Taq DNA polymerase (Qiagen), 2 µL of RT-PCR product and sterile distilled water to a total volume of 50 µL. The reactions were run on thermal cycler using the following conditions: 15 minutes at 95 °C; 30 cycles of amplification 96 °C for 30 seconds, 48 °C for 45 seconds, and 72 °C for 1 minute; and extended extension at 72 °C for 7 minutes. The nested PCR products were analyzed on 1.5% agarose and visualized on ultraviolet transilluminator. The products of nested PCR assays were extracted from gel agarose and purified by QIAquick Gel Extraction Kit (Qiagen). The DNA sequencing was performed by using primers as for nested PCR reaction in Institute of Human Virology and Cancer Biology, University of Indonesia, Jakarta.

**Determination of HCV genotype by phylogenetic tree.** To determine the HCV genotype, we used the following accession numbers from GenBank for phylogenetic analysis, as reported by Idrees *et al.*<sup>11</sup>: genotype 1, M62321; genotype 1, D90208; genotype 2, D00944; genotype 2, D01221; genotype 2, D10075; genotype 3, D14307; genotype 3, D11443; genotype 3, D16612; genotype 4, M84848; genotype 4, M84845; genotype 4, M84862; genotype 4, M84832; genotype 4, M84828; genotype 4, M84829; genotype 5, M84860; and genotype 6, M84827. The Neighbor-Joining method was used to prepare a phylogenetic tree using MEGA.<sup>12</sup>

**Evolutionary analysis of three HCV isolates by phylogenetic tree.** The Neighbor-Joining method was also used to prepare a phylogenetic tree using MEGA5.<sup>15</sup> Three Phylogenetic trees were constructed for evolutionary analysis of HCV Isolate A, B, and C. Sixty eight HCV isolates that were retrieved by GenBank were involved in this analysis. The GeneBank accession numbers of involved HCV isolates are indicated in Figure 4, 5, and 6.

## Results and Discussion

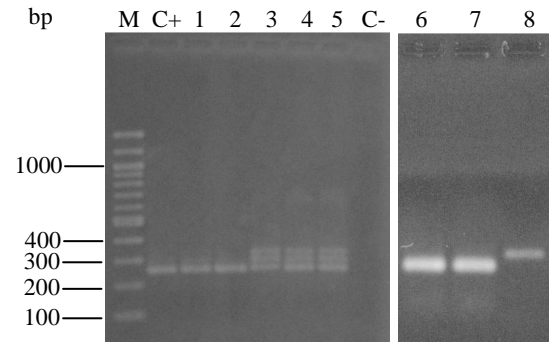
In this study, we performed the 5' UTR region-based phylogenetic analysis of three HCV isolates that were obtained from patients in Jakarta with HCV infected-chronic diseases. All three viral isolates constituted representative isolates of 39 positive plasma samples,<sup>9</sup> that showed three DNA-band patterns amplified by nested PCR (Figure 1). Three samples (Lane 7, 8, and 5 in Figure 1) representing all three DNA-band patterns were named with Isolate A, B, and C, respectively and used for DNA sequencing. The Isolate A showed an expected specific DNA band (259 bp). The Isolate B showed single DNA band higher in size than an expected band, while the Isolate C showed multiple DNA bands. Even though the Isolate C showed multiple DNA bands, one of those DNA bands was an expected DNA band in size (this DNA band was purified from gel agarose for DNA sequencing). Based on DNA

sequencing results, three different DNA lengths (218, 246, and 216 bp) were obtained from Isolate A, B, and C, respectively (Figure 2).

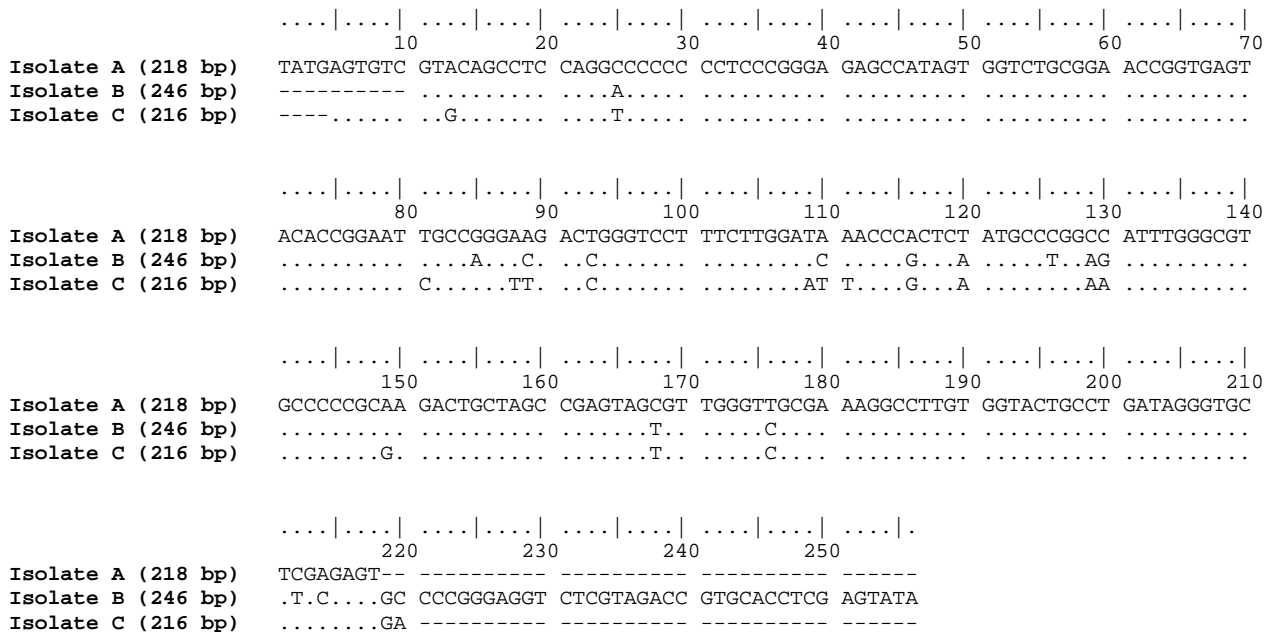
We assumed that all three DNA-band patterns were resulted from different HCV genotypes. To prove it, we conducted phylogenetic analysis to determine the genotypes of Isolate A, B, and C, as reported by Idrees *et al.*<sup>11</sup> Based on this analysis, the Isolate A, B, and C were clustered into groups of HCV genotypes 2, 1, and 3, respectively (Figure 3); therefore, the Isolate A, B, and C were classified into HCV genotypes 2, 1, and 3, respectively. The results are in accordance with those reported by other researchers in Indonesia.<sup>13,14</sup> Soetjipto *et al* reported that prevalence of HCV genotypes from healthy blood donors, patients on maintenance hemodialysis, and patients with HCC were 52% and 29% for the genotypes 2 and 1, respectively.<sup>13</sup> Recently, Utama *et al.* reported the prevalence of HCV genotypes 1 (72.7%), 2 (16%), and 3(11.3%) that were detected from 68 chronic hepatitis (CH), 48 liver cirrhosis (LC), and 34 HCC patients.<sup>14</sup> One limitation in this analysis is that we only can determine the HCV genotypes but not the subtypes, because the 5' UTR region of HCV genome can only be used for determination of the HCV genotyping but not for HCV subtyping. For determination of HCV subtypes, the NS5B region of HCV genome should be used since it is a moderately variable region and common used for HCV subtyping.<sup>6</sup> Hrabec *et al.* reported that the 5' UTR yielded phylogenetic trees with topologies that differ from the HCV polyprotein and complete genome phylogenies,

whereas the NS5B gene reliably clustered HCV subtypes and yielded topologies consistent with those of the whole genome and polyprotein.<sup>15</sup>

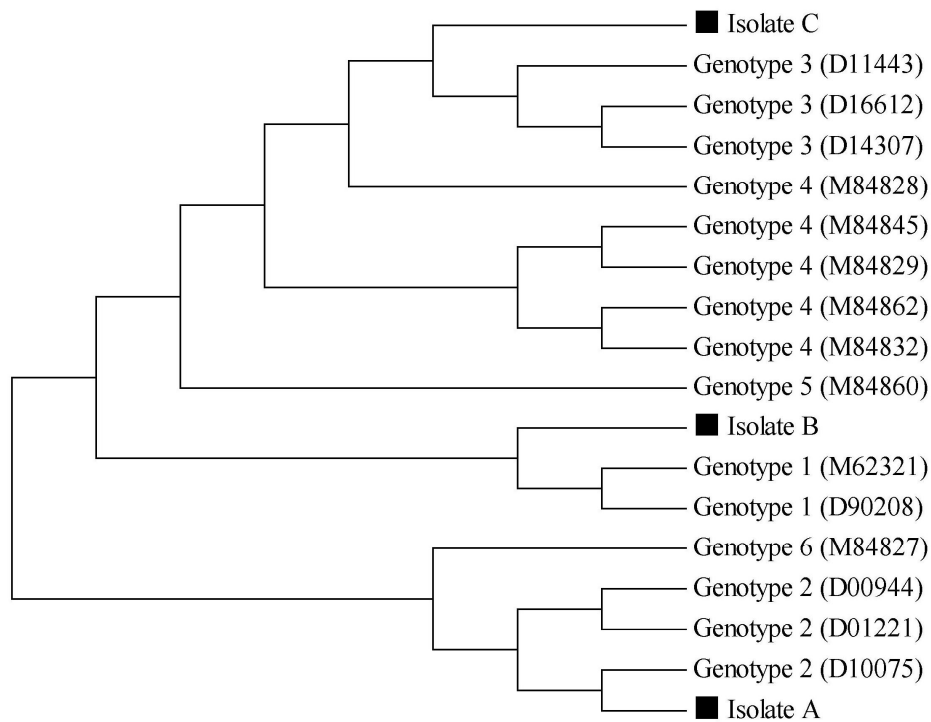
Based on the phylogenetic analysis, Isolate A was very closely related to two viral isolates (Figure 4), from Madagascar (DQ345601/Madagascar/Genotype 2/Unknown Subtype) and Morocco (HQ833236/Morocco/



**Figure 1. Electrophoresis Results of Nested PCR Assays. Positive Nested PCR was Defined by a 259 bp DNA Band Visualized at the Right Position on 1.5% Agarose gel. Three DNA-Band Patterns were Shown. Samples 1, 2, 6, and 7 Showed Pattern 1. Sample 8 Showed Pattern 2. Samples 3, 4, and 5 Showed Pattern 3. M: DNA Ladder. C+ and C-: Positive and Negative Controls, Respectively. bp: Base Pair**



**Figure 2. DNA Sequence Alignment of Partly 5' Untranslated Regions of HCV Isolates from Isolate A, B, and C. bp: Base Pair**



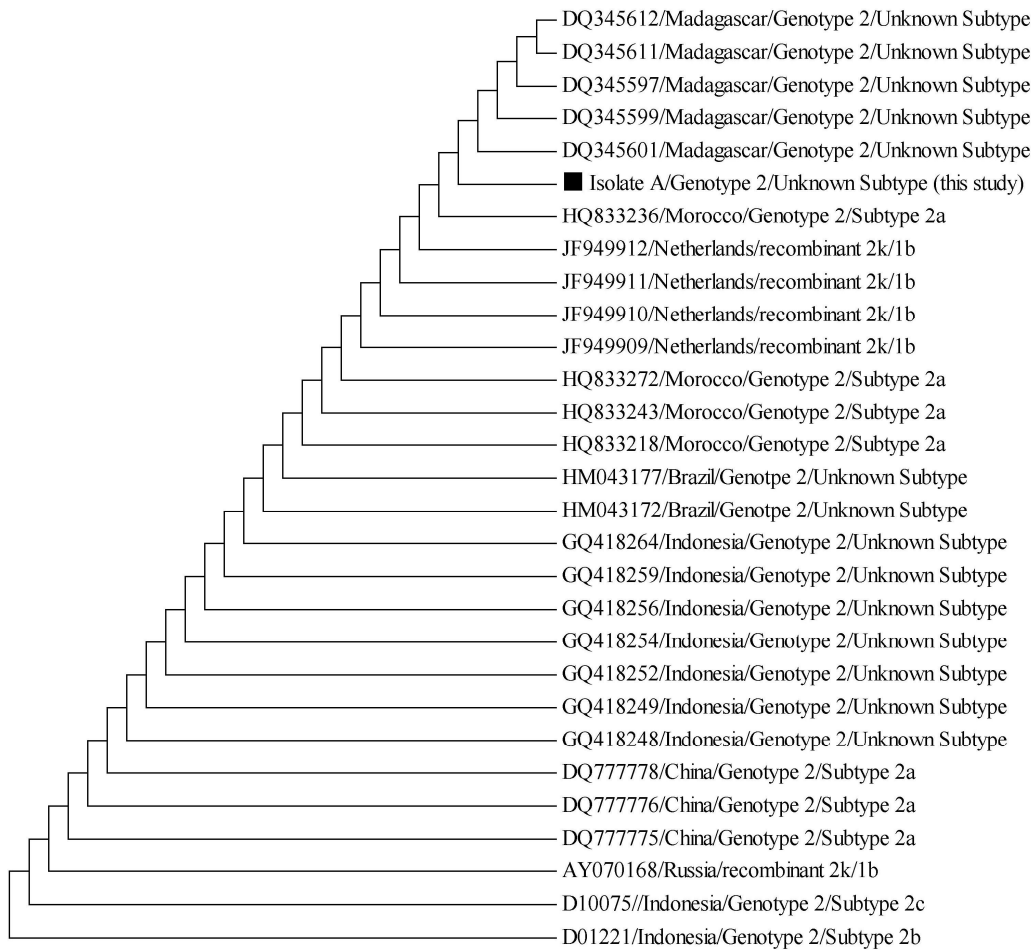
**Figure 3. Determination of HCV Genotypes of Isolate A, B and C Using a Phylogenetic Tree Based on DNA Sequences of Partly 5' UTR Regions. The HCV Genotypes 1-6 are Named in Accordance with Accession Numbers in GeneBank Database**

Genotype 2/Subtype 2a), that were detected in patients with unknown clinical conditions. Compared with Indonesia isolates (unknown subtypes), the Isolate A was not closely related to the isolates from patients with chronic infection (Accession numbers: GQ418248, GQ418248, GQ418252) and cirrhosis (Accession numbers: GQ418254, GQ418256), and HCC (GQ418259, GQ418259). For Isolate B, it was clustered into lineage IV (Figure 5), along with other isolates from Brazil (detected in a chronic patient), Indonesia (detected in a HCC patient), and Canada (detected in a chronic patient). Among all the three isolates, the Isolate B was closely related to an isolate from Brazil, followed by isolates from Indonesia and Canada, consecutively. Other Indonesia isolates were dispersed into lineages I, II and V, in that all the isolates were detected in patients with HCC diseases. The results indicated that the Isolate A and B might be different viral types from other comparable Indonesia isolates in aspect of viral virulence.

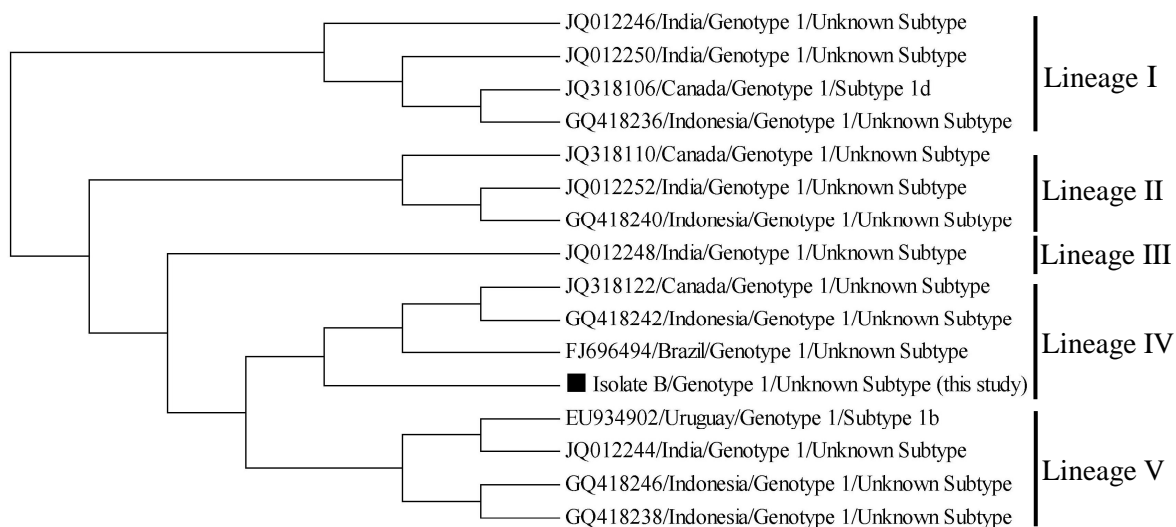
Regarding the HCV virulence associated with genetic variation, there are clinically responsive differences to interferon-based therapy influenced by particular genotypes, for examples, the genotypes 1 and 4 are less responsive than types 2 and 3.<sup>16, 17</sup> The HCV genotypes have also been associated with viral load during interferon treatment and antiviral resistance.<sup>18,19</sup>

Moreover, among subtypes of single HCV genotype showed different amino acid sequences that influenced the efficacy of antiviral drugs and viral clearance by host immune system.<sup>20</sup> A study performed in Surabaya, Indonesia reported that HCV subtypes 1a, 1b, and 1d were frequently found in hemodialyzed patients with normal ALT and elevated ALT levels, whereas subtype 1b followed subtype 1d were the most common subtypes from patients with HCC.<sup>13</sup> From blood donors, HCV subtype 2a was most common subtype and was less pathogenic than other subtypes to cause liver cell injury mediated either by a cytopathic effect and/or through immune mechanisms.<sup>13</sup>

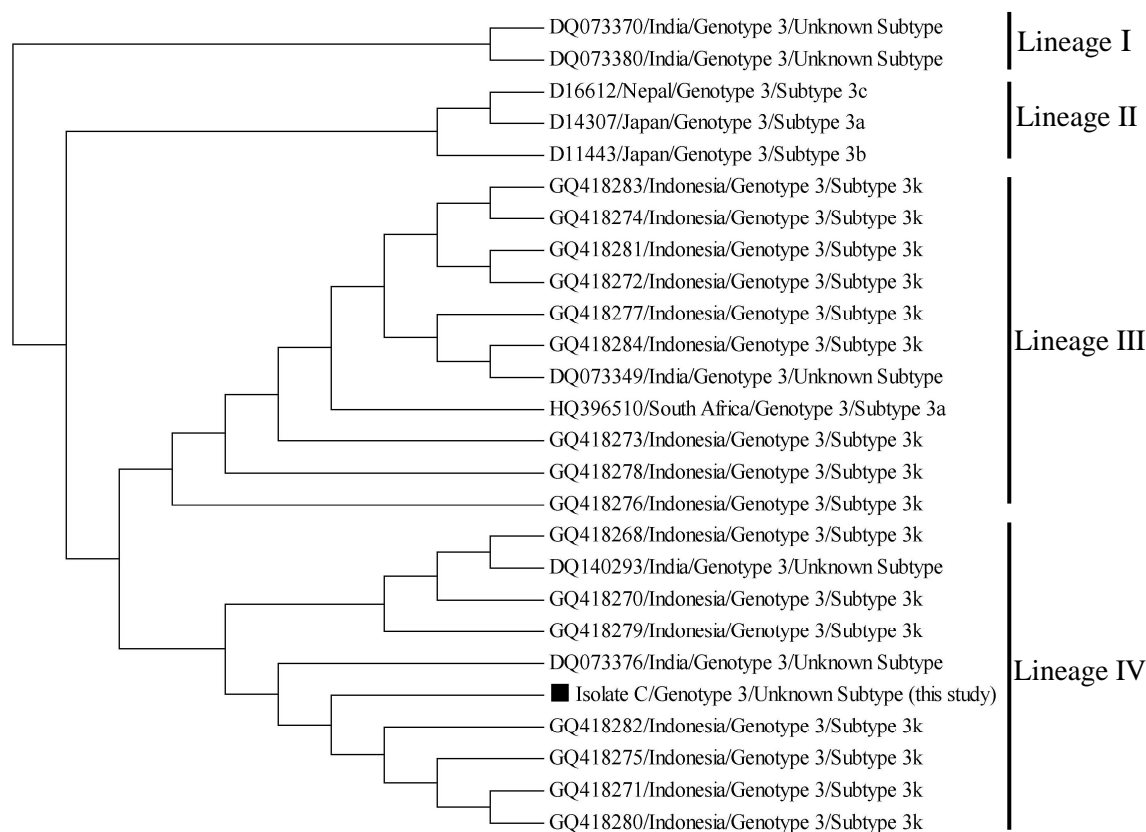
In contrast to Isolate A and B, Isolate C was clustered into lineage IV (Figure 6), consisting of mostly Indonesia isolates that were detected from patients with cirrhosis and chronic infection or asymptomatic. For Indonesia isolates in lineage III, those were detected from patients with HCC, cirrhosis, and chronic infection or asymptomatic. The Isolate C was very closely related to an Indonesia isolate (GQ418282/Indonesia/Genotype 3/Subtype 3k) detected in cirrhosis patient, indicating that the Isolate C might be virulence HCV to cause liver cell injury. This approach, by phylogenetic trees, has been performed to predict the HCV virulence, to obtain evidence of HCV transmission, and to trace an outbreak of HCV and a geographic origin of endemic HCV.<sup>21-23</sup>



**Figure 4. A Phylogenetic Analysis of Isolate A (Genotype 2) based on DNA Sequences of Partly 5' UTR Regions. Other Viral Isolates are Named in Accordance with Accession Numbers in GeneBank Database, Countries where Viruses Detected, Genotypes, and Subtypes. ■: Isolate A Analyzed in this Study**



**Figure 5. A Phylogenetic Analysis of Isolate B (Genotype 1) based on DNA Sequences of Partly 5' UTR Regions. Other Viral Isolates are Named in Accordance with Accession Numbers in GeneBank Database, Countries where Viruses Detected, Genotypes, and Subtypes. ■: Isolate B Analyzed in this Study**



**Figure 6. A Phylogenetic Analysis of Isolate C (Genotype 3) based on DNA Sequences of Partly 5' UTR Regions. Other Viral Isolates are Named in Accordance with Accession Numbers in GeneBank Database, Countries where Viruses Detected, Genotypes, and Subtypes. ■: Isolate C Analyzed in this Study**

## Conclusions

The Isolate A, B, and C, representing a specific DNA band, single DNA band higher in size than an expected band, and multiple DNA bands, respectively amplified by nested PCR (Figure 1), were HCV genotypes 2, 1, and 3, respectively. Compared with other Indonesia isolates, the Isolate A and B were not closely related, while the Isolate C was closely related. Among all three isolates, the Isolate C might be more virulence since the Isolate was closely related to an Indonesia isolate from a cirrhosis patient. However, comprehensively virulence analysis of all three isolates should be addressed in future research, particularly analysis of complete viral genome.

## Acknowledgements

This research was funded by Centre for the Application of Isotopes and Radiation Technology, National Nuclear Energy Agency, Indonesia and supported by Departemen Mikrobiologi, Fakultas Kedokteran Universitas Indonesia.

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