# Cloning and Molecular Analysis of HCVP7 Complete Coding Sequence

Yulia Sari [1,2,3], Afiono Agung Prasetyo [2, 3, 4*], Martinus Nuherwan Desyardi [2]

[1] Department of Parasitology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia
[2]Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia
[3]Indonesian Center of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia
[4]Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia

Correspondence to: Afiono Agung Prasetyo, M.D., Ph.D
Department of Microbiology Faculty of Medicine Sebelas Maret University, Jl. Ir. Sutami no 36 A, Surakarta, 57126, Indonesia Tel: +62-271-632489 Fax: +62-271-632489
Email: afieagp@yahoo.com; afie.agp.la@gmail.com

***ABSTRACT***

*The hepatitis C virus (HCV) p7 protein is known essential for virus infection and replication. It exhibits ion-channel activity reported to be specifically blocked by various compounds, making it an attractive drug target. However, at present only little is known about the underlying molecular mechanisms of HCV p7. To predict and more understanding the molecular pattern of HCV p7 we cloned the p7 gene of HCV isolated in Central of Java Indonesia. The sequence results were then aligned with all of HCV 1a p7 complete coding sequences reported in GenBank. In total, 591 sequences were retrieved and aligned by ClustalW. We revealed the nucleotide and amino acid sequence consensus of HCV p7 gene. Overall, only aa 5, 12, 30, 34, 42, 48, 52, 58, and 63 were not have any variation. Amino acid variations may have relevant changes of physicochemical properties so that influence the replication efficiency, therefore, the amino acid variations found in the present report need further study.*

*Key words: HCV, p7*

## Introduction

Hepatitis C Virus (HCV) is known as a major causative agent of liver disease. The incidence of HCV infection in worldwide population reached 3 %, which means 170 million people now infected with HCV (Alter 2007; Holmberg 2009). In addition, some reports show that 70-85 % infection cases of HCV last persistent. Only 20-25 % of patients with HCV infection can be cured completely, while others at high risk to reach a terminal condition (Lemon *et al*., 2007).

HCV genome has a single stranded positive sense RNA along approximately 9600 base pairs. This virus has long Open Reading Frame (ORF) flanked by 5'-untranslated region (UTR) and 3'-UTR. This ORF can be functionally divided into three regions: the N-terminal, center, and C-terminal. N-terminal region associated with the region producing the structural protein: core protein (core/C), envelope glycoprotein 1 and 2 (E1 and E2). Central region consists of proteins p7 and NS2. C-terminal region consists of non-structural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) needed in the replication of RNA (Lemon *et al*., 2007; Chevaliez and Pawlotsky 2006).

Hepatitis C virus (HCV) p7 is an integral membrane protein that forms ion channels in vitro and that is crucial for the efficient assembly and release of infectious virions. The HCV p7 was included in the family of viroporins, which alter membrane permeability and facilitate the release of infectious viruses. The p7 protein is localized in the endoplasmic reticulum (ER) but not associated with mitochondria (Brohm *et al*., 2009; Haqshenas *et al*., 2007; Steinmann *et al*., 2007). The p7 forms a cation-selective ion channels in planar lipid bilayers and at the single-channel level. p7 functions as a H(+) permeation pathway, acting to prevent acidification in otherwise acidic intracellular compartments. This loss of acidification is required for productive HCV infection, possibly through protecting nascent virus particles during an as yet uncharacterized maturation process

(Montserret *et al*., 2010; Wozniak *et al*., 2010).

The amino acid sequence of p7 is largely conserved over the entire range of genotypes, and it forms ion channels that can be blocked by a number of established channel-blocking compounds. Its characteristics as a membrane protein make it difficult to study by most structural techniques, since it requires the presence of lipids to fold and function properly (Cook *et al*., 2010). Circular dichroism analyses revealed that the structure of p7 is mainly α-helical, irrespective of the membrane mimetic medium (Montserret *et al*., 2010). Neither the two trans-membrane helices nor the p7 basic loop individually determines compound sensitivity. In the three hydrophilic regions within the amino-terminal trans-membrane helix, only the conserved histidine at position 17 is important for genotype 1b p7 channel activity. The p7 carboxy terminus may act as a specific sensitivity determinant for the drug amantadine (Griffin *et al*., 2008; StGelais *et al*., 2009).

To predict and more understanding the molecular pattern of HCV p7 gene, we cloned the HCV p7 gene from HCV 1a isolated in Central of Java Indonesia and performed bioinformatics study of all HCV p7 complete genes deposited in GenBank. Here we present our current analysis results of the p7 gene of HCV.

## Methods

**Isolate Selection for Cloning.** Previously, all plasma aliquot with anti-HCV positive from previous study (Prasetyo *et al*., submitted for publication) were performed for nucleic acid extraction followed with nested RT-PCR addressed part of the HCV E1-E2 and HCV NS5B region. The positive PCR products were sequenced and phylogenetic analyzed to role out the predominant HCV in Central of Java Indonesia. All samples having the same genotype with the predominant genotype were further analyzed for its co-infection with another human blood borne viruses (HIV, HBV, HDV, HTLV-1/2, and TTV), HCV RNA titer, and sequencing results.

**Cloning of HCV 1a p7 Gene from HCV 1a Isolated in Central of Java Indonesia.** The aliquot plasma from "the best candidate for cloning"-isolate were performed for RNA extraction followed with cDNA syntesis. The HCV p7 gene was cloned using HCV 1a p7-F: 5'-ATG GCT YTR GAR AAY CTY RTR DYR CTC-3' and HCV 1a p7-R: 5'-TTA NGC GTA YGC CCG YKG RGG-3' primers. The PCR conditions for cloning was performed with early denaturation for 2 minutes of 95 °C, 16 cycles of 95 °C 15 seconds of denaturation followed with 55 °C 30 seconds of annealing and 68 °C 1 minute of elongation, respectively. The final elongation period was 72 °C 10 minutes. The PCR products were purified, analyzed, and subcloned into pETBlue-1. The recombinant plasmids were then transformed into competent cells. The competent cells were propagated, harvested, and the plasmids were extracted and sequenced. The sequencing results were analyzed using Sequence Viewer and MEGA4.

**Complete HCV p7 Gene Sequences.** First, all HCV p7 gene sequences from all genotype of HCV deposited in GenBank were downloaded. In total, 591 sequences with complete coding sequence were retrieved from GenBank. The sequences then were aligned by ClustalW, for both of nucleotide and amino acid sequences, using CLC Sequence Viewer and MEGA4. From this alignment we revealed the nucleotide and amino acid consensus sequences. The tested sequences were aligned by ClustalW with subsequent inspection and manual modification (Thompson *et al*, 1994; Tamura *et al*, 2007).

## Results and Discussions

Prior of cloning the p7 gene of HCV, we performed phylogenetic analysis of all HCV isolated in Central of Java Indonesia from the previous study. In total, 140 out of 518 plasma samples were positive for anti-HCV. From all positive plasma samples, only 32 isolates were successfully amplified with nested RT-PCR addressed for part of HCV E1-E2 and HCV NS5B region. The phylogenetic analysis retrieved that 43.72% (14/32) isolates were HCV 1a (Prasetyo *et al*., submitted for publication).

To find the best isolate for cloning, all HCV 1a isolates were checked for its purity and RNA titer. After checked the co-infection status with other human blood borne viruses, measured the quantity of the HCV RNA, and based on the prior HCV E1-E2 and HCV NS5B sequencing results, 09IDSKAC-20 was chosen for cloning.

The HCV p7 gene was cloned from 09IDSKAC-20 RNA using standard cloning procedure. Briefly, to design the primers for cloning, we performed alignment of all complete open reading frames for HCV 1a p7 gene. The

motif sequences of the 5'end and the 3'end regions were retrieved and subjected for primer design using FastPCR software. The initiation and stop codon were added into the forward and backward primer, respectively, to ensure the expression of HCV p7 in the pETBlue-1 plasmid. The PCR cloning was performed using Accuprime Pfx Polymerase according its protocol. The PCR products was then subcloned into pETBlue-1, and transformed into competent cells. The recombinant plasmids were extracted and sequenced. The sequences were aligned with all HCV p7 gene retrieved from GenBank.

Different hepatitis C virus (HCV) proteins have been associated with different response to therapy, including that of the drugs resistance. However, the exact mechanisms of virus-mediated resistance are not completely understood. The importance of amino acid (aa) variations within the HCV p7 proteins for replication efficiency and viral decline during the therapy is unknown. To solve this problem we try to perform a bioinformatics study by analyzing the all of HCV p7 gene complete coding sequence deposited in GenBank.

In the three hydrophilic regions within the amino-terminal trans-membrane helix, previous study stated that only the conserved histidine at position 17 is believed important for channel activity. Alignment of different p7 proteins revealed that a HXXXW sequence (positions 17-21) is highly conserved among some HCV genotypes. HXXXW sequence exists in the p7 proteins of some HCV genotypes and that H17 plays an important role in virus replication (Meshkat *et al*, 2009). However, the H17 did not exist in all genotypes of HCV.

In our study, only aa 5, 12, 30, 34, 42, 48, 52, and 63 were found have no variation. The amino acid variations may have relevant changes of physicochemical properties so that influence the replication efficiency and or viral pathogenesis and therapy. For example, the p7 mutations have been found independently increased the amount of virus released (Russell *et al*., 2008). Substitution L20F within HCV p7 may be associated with non-response to combination therapy specifically with amantadine in HCV-1b-infected patients (Mihm *et al*., 2006). The infectivity of several assembly-defective core mutants could be rescued by compensatory mutations identified in p7 and NS2, suggesting genetic interactions with core and highlighting the importance of these nonstructural proteins in infectious virion morphogenesis (Murray *et al*., 2007). A genotype-dependent and subtype-dependent sensitivity of HCV to p7 inhibitors has been identified. The p7 promotes virus particle production in a genotype-specific manner most likely due to interactions with other viral factors. The p7 variants from different isolates deviate substantially in their capacity to promote virus production, suggesting that p7 is an important virulence factor that may modulate fitness and in turn virus persistence and pathogenesis (Griffin *et al*., 2008; Steinmann *et al*., 2007). The p7 can operate independently of an upstream signal sequence, and that a tyrosine residue close to the conserved dibasic motif of p7 is important for optimal virus production in the context of genotype 2a viruses (Brohm *et al*., 2009).

## Conclusions

In our study, only aa 5, 12, 30, 34, 42, 48, 52, and 63 of HCV p7 were found have no variation. The amino acid variations found in the present report need further study.

## Acknowledgments

This study was supported by DIPA Sebelas Maret University No. 2881/H27/KU/2010 and DIPA Faculty of Medicine Sebelas Maret University 2010.

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