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Detection Of Hepatitis C Virus (Hcv) Infection And Its Genotype In Patients At Hepatology Outpatient Clinic, Dr Soetomo General Hospital, Surabaya.

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Abstract. Hepatitis C virus (HCV) is an RNA virus that can cause liver inflammation (hepatitis) and has the potential to become chronic and can progress to liver cirrhosis and hepatocellular carcinoma. Detection of HCV RNA infection, and genotype/subtype of HCV was performed on 70 blood sera of patients at the Hepatology Outpatient Clinic of Dr Soetomo General Hospital, Surabaya, Indonesia. Detection of HCV infection was carried out by Anti-HCV determination using enzyme immunoassay (EIA) technique, detection of HCV RNA by Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique based on genome regions NS5b and 5'UTR, followed by electrophoresis with agarose gel. In positive PCR results, HCV genotype/subtype was determined by direct sequencing method using ABI 310 sequencer and sequencing results were analyzed by comparing the products with previously published HCV nucleotides. Sera were obtained from 41 (58.6%) male and 29 (41.4%) female patients. Anti-HCV was found positive in 17/70 (24.29%) patients and 16/17 (94.1%) was proved to contain HCV RNA when determined by RT-PCR technique. Patients with positive HCV RNA have the potential to transmit HCV infection. From the genotype/subtype analysis of sequencing results we obtained 2/16 (12.5%), 3/16 (18.75%) and 6/16 (37.5%), 1/16 (6.25%), 1/16 (6.25%), 2/16 (12.5%), 1/16 (6.25%) HCV genotypes 1, 2, and HCV subtypes 1b, 1c, 2a, 3a, 3k respectively. Conclusion: In patients who went to the Hepatology Outpatient Clinic, Dr. Soetomo General Hospital Surabaya, we found positive Anti-HCV was 24.29%. In 94.1% of patients with positive Anti-HCV, HCV RNA was still detected and HCV genotype 1 with subtype 1b were still dominant HCV subtypes.

Keywords: HCV infection, Anti-HCV, HCV-RNA, Genotype / Subtype

1. Introduction

Hepatitis C virus (HCV) infection is still a health problem in the world including in Indonesia and can be a cause of liver inflammation (hepatitis). Hepatitis C virus has been shown to have a worldwide distribution, occurring among persons of all ages, ethnicity, genders, and regions of the world [1]. Eighty-five % (85%) HCV infections become chronic [2] and have the potential for liver cirrhosis and are subsequently at risk for hepatocellular carcinoma (HCC) [2, 3, 4, 5], liver failure, and death [4]. The other publication also mentioned that chronic HCV infection has been linked to the development of HCC in many areas of the world [1].

Hepatitis C virus infection has parenteral transmission pathway, for example through blood transfusion and blood product, through sexual contact, sharing needles or HCV-contaminated devices, and from mother to child [6]. The population who are classified as having role a potential risk of HCV transmission, such as medical procedures, injections for medications and immunizations, injections applied outside of medical settings, tattooing, and scarification techniques, have shown wide geographical variations [1].

Antibodies to HCV (anti-HCV) are the most commonly available marker of HCV infection at present [4]. Detection of positive anti-HCV is also a sign that someone is infected with HCV. Individual with positive anti-HCV does not always negative for HCV RNA in the blood, so it is necessary to check



the presence of HCV RNA in the blood. A previous study showed that 95% of patients with positive anti-HCV still have HCV RNA in their blood [7].

Hepatitis C virus is a single-stranded RNA virus, positive-sense, and classified into Hepacivirus genus [5] of Flaviviridae family [4, 8]. Hepatitis C virus with a length of 9.6 kb, consists of the 5' UTR, genes for structural proteins (Core, E1 and E2) and genes for non-structural proteins (NS1, NS2, NS3, NS4A, NS4B, NS4A, NS5B) and 3'UTR [3, 9]. Hepatitis C virus shows high genetic heterogeneity and can be classified into seven major genotypes (HCV 1–7) including 67 confirmed and 20 provisional subtypes, on the basis of phylogenetic analysis of genome sequences [3, 8]). Hepatitis C virus (HCV) genome sequence is highly variable. The nucleotide sequence differs by 31% to 33% among genotypes and by 20% to 25% among subtypes [4, 10, 11, 12]. HCV genotype distributions show different geographic patterns [8] and patients infected with HCV 1b subtype have a greater risk of progression to HCC than do those infected with other subtypes [13].

One of molecular determination method performed to prove the presence of HCV RNA is Reverse Transcription Polymerase Chain Reaction (RT-PCR). The HCV exhibits global genotypic diversity [8]. With RT-PCR technique on HCV nucleotides and sequencing on positive PCR products, HCV genotypes/subtypes can be identified [7, 14, 15, 16, 17].

There are several methods used to determine HCV genotypes, for example by using the direct sequencing of specific PCR amplified portion products of the virus (NS5, Core, E1 and 5' UTR regions), often in combination with the phylogenetic analysis [4]. Sequence analysis of NS5B for genotyping HCV provides precise genotype and subtype identification and an accurate epidemiological representation of circulating viral strains [12]. The highly conserved 5' UTR is also an ideal region for genotyping of HCV [12]. Primers for determining HCV genotype based on NS5B HCV genome [15], NS5B and 5'UTR of HCV have also been proven success in subsequent studies [7, 16, 17].

This study was performed the detection of HCV infection and its genotype in patients at Hepatology Outpatient Clinic, Dr. Soetomo General Hospital, Surabaya.

2. Experimental Methods

The study protocol was reviewed and approved by the Ethics Committees of Dr Soetomo General Hospital, Surabaya, Indonesia and informed consent was obtained from all the patients.

Sera were collected from 70 patients at Hepatology Outpatient Clinic, Dr Soetomo General Hospital, Surabaya, Indonesia. All sera were stored at 80°C until they were used. All of the sera were tested for anti-HCV antibodies by enzyme immunoassay (EIA) technique for the quantitative detection of IgG antibodies against HCV in serum, using a kit from the Foresight HCV EIA Antibody Test Kit REF 1231-1031, according to the manufacturer's instruction. The cut-off value which was used as a level limit of positive and negative result was determined every time the test was done, based on the calculation attached to the kit instructions.

Blood retrieval at Hepatology Outpatient Clinic, Dr Soetomo General Hospital, Surabaya, Indonesia. Sera separation and laboratory work were carried out at the Hepatitis Laboratory, Institute of Tropical Disease (ITD), Airlangga University, except anti-HCV antibodies were done in private laboratory..

Reverse transcription and PCR

Sera with positive anti-HCV antibodies were continued for HCV RNA detection. Hepatitis C virus RNA was extracted from 120 µl positive Anti-HCV sera, using a kit for extraction of viral RNA from Qiagen, according to the instruction in the kit, then it was reverse transcribed into cDNA and amplified using SuperScript One-Step RT-PCR (Invitrogen, Tokyo, Japan) and one set of NS5B primer for this first round PCR that was used by Mori [18] previously. The initial reaction was performed at 45°C for 30 min for cDNA synthesis and at 94°C for 2 min for hot start, followed by the first-round PCR over 35 cycles, with each cycle consisting of the temperature at 94°C 1 min, 45°C 1 min, and at 72°C 2 min. At each time of HCV RNA extraction, cDNA synthesis from HCV RNA and PCR for HCV, negative control (distilled water) and positive control (samples that was known to be positive for HCV RNA), were also run and processed together with the sample. In the first round PCR amplification reaction of NS5b region, agarose electrophoresis was not performed. After first round PCR was performed, it was followed by the second-round PCR under the same conditions PCR cycle described above. For the second round PCR, we amplify NS5B HCV region sequences using Fermentas PCR Kit

reagents and various primers from the NS5B region [15], step by step. For the second round PCR, first we used HC23, HC24, HC26 and HC28 primers. At the end of PCR cycle, extension time was extended for 10 minutes at 72° C. If it still gave negative result in electrophoresis after second round PCR, continued with the second round PCR step by step by using HC23, HC32 and HC34 primers, HC15 and HC16 primer respectively. The sequences and positions of the NS5B primers used in this study are shown in Table 1.

Table 1. PCR primers used for NS5b HCV amplification

Primers	Region	Sequence (5' to 3')	Position	Polarity	References
1st PCR					
#166	NS5b	5'-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3'	(8230-8260)	Sense	18
#167R	NS5b	5'-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3'	(8601-8630)	Antisense	18
2nd PCR					
HC23	NS5b	5'-TTTGACTCAACCGTCACTGA-3'	(8256-8275)	Sense	15
HC24	NS5b	5'-CTCAGGCTCGCCGCATCCTC-3'	(8577-8596)	Antisense	15
HC26	NS5b	5'-CTCAGGTTCCGCTCGTCCTC-3'	(8577-8596)	Antisense	15
HC28	NS5b	5'-CACGAGCATGGTGCAGTCCCGGAGC-3'	(8507-8531)	Antisense	14
HC32	NS5b	5'-AGGTAGCACGTCAGCGTGTTC-3'	(8454-8476)	Antisense	15
HC34	NS5b	5'-TAGCACGTCATGGTGTTCCTCAT-3'	(8451-8473)	Antisense	15
HC15	NS5b	5'-ACTGTCACTGAACAGGACAT-3'	(8265-8284)	Sense	15
HC16	NS5b	5'-GCTCTATCCTCATCGACGCC-3'	(8568-8587)	Antisense	15

The second round PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and visualized under UV illumination.

In the case of negative amplification of the NS5B region, 5' UTR sequences were targeted for amplification by PCR. In this analysis, HCV RNA was also reverse transcribed into cDNA and amplified using SuperScript One-Step RT-PCR (Invitrogen, Tokyo, Japan) by using one set of primer 5'UTR1 (sense; 5'-CCGGGAGAGC CATAGTGGTC-3') and 5'UTR2 (antisense; 5'-AGTACCACAAGGCCTTTTCGC-3'), and another set of primer prepared for second round PCR that used previously [16], with the same PCR condition as above. The primers for second round PCR were 5'UTR3 (sense; 5'-TGGTCTGCGGAACCGGTGAG-3') and 5'UTR4 (antisense; 5'-ACCCAACACTACTC GGCTAG-3'). After the first round PCR amplification reaction of 5'UTR region, agarose electrophoresis was performed and it was not followed by second round PCR when electrophoresis gave positive result. The PCR products were electrophoresed in a 3% agarose gel containing ethidium bromide and were visualized under UV illumination.

If the amplification using the primers of the NS5B and 5'UTR regions above were gave negative result, the extracted RNA was reverse transcribed and amplified using the different set of NS5B primers: F1, R1 and F2, R2 that was used in another previous publication [19], as an effort for getting more positive result of HCV cDNA amplification. The PCR products were also electrophoresed in a 2% agarose gel containing ethidium bromide and it were visualized under UV illumination.

Sequencing and genotype/subtype analysis of HCV.

The positive PCR products based on NS5B or 5'UTR containing nucleotide fragments that had been amplified from the target HCV genome were purified using Qiagen gel purification kit according to the kit instructions and checked by electrophoresis. Then the purified nucleotide sequences of the amplified fragments were amplified again with the one of previously PCR primer and sequenced by direct sequencing method using BigDye Terminator Cycle Sequencing kit and an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Each obtained sequence from the NS5B HCV region was compared previously reported subtypes using the BLASTN program from NCBI on the internet. The nucleotide sequences of the 5'UTR HCV

region were determined and compared with the consensus sequence motifs for each of the major genotypes previously reported [20].

3. Results and Discussion

From 70 patients who came to the Hepatology Out Patients Clinic of Dr. Soetomo, Surabaya, 41 (58.6%) male and 29 (41.4%) female patients, 17/70 (24.29%) was proven infected with HCV, marked by a positive Anti-HCV (Table 2).

Table 2 : Anti-VHC level

Anti-HCV	Male	Female	Total
Negative (%)	32/41 (78,05%)	21/29 (72,41%)	53/70 (75,71%)
Mean	0,027	0,025	
Range	(-0,015) – (-0,102)	(-0,011) – (-0,049)	
Positive (%)	9/41 (21,95%)	8/29 (27,59%)	17/70 (24,29%)
Mean	2,874	2,881	
Range	(2,775 - 2,939)	(2,814 - 2,928)	
Total	41/70 (58,57%)	29/70 (41,43%)	70/70 (100%)

When of antibodies to HCV were detected in individual (positive anti-HCV), indicates that the person has had previously infected or has current active infection with HCV [21]. However, when it was only anti-HCV positive, it is not yet known whether the person is in viremia condition / still has HCV or not, and we still need to prove whether HCV RNA is in his blood.

By using RT-PCR technique, HCV RNA detection from positive Anti-HCV sera has been carried out based on a part of NS5B HCV gene region, which is if necessary continued with PCR based on 5'UTR HCV. The NS5B and 5'UTR primers are quite sensitive and have been used in previous HCV studies [7, 15, 16, 17]. The results of HCV RNA detection on samples with positive Anti-VHC in this study are shown in Table 3 below.

Table 3. Primers and HCV PCR result in HCV RNA detection.

PCR Round and Pair of Primers used	Primer Position in HCV region	Positives HCV on PCR
First Round and #166, #167R		
Second Round and HC23, HC24, HC26, HC28	NS5b	7
First Round and 5'UTR1 and 5'UTR2	5'UTR	5
First Round and F1 and R1		
Second Round and F2 and R2	NS5B	4
All primer gives negative results	NS5B and 5'UTR	1
TOTAL		17

Detection of HCV RNA was performed in 17 positive anti-HCV samples and it was found that 94,12% (16/17) samples had positive HCV RNA. In the one of the sample with positive anti-HCV, HCV RNA remains negative although for this detection we had used another NS5B primer pairs F1, R1, F2 and R2 [19] outside the NS5B previous #166, #167R and HC-primers, that was not proposed before. It was reported that 5'UTR based assays are reasonably accurate with more than 95% concordance with genotypes that have been identified by nucleotide sequencing of the NS5B region or other coding regions of the HCV genome [11, 12]. This results is in line with our previously results study in Surabaya, which suggested that 95% of samples with positive anti-VHC still contained positive HCV RNA [7]. The high variability of HCV genome in Surabaya was proven that for getting 94,1% RNA HCV positive in anti-HCV positive sera in this study, it was needed the different NS5B primers [19] out of primers using by Soetjipto [7] in the same city.

In this study, one sample with positive anti-HCV still remain negative for HCV RNA. From a similar study, the detection of HIV in the blood that got anti-HIV positive data but HIV RNA was negative, it were suggested the possibility of nucleotide mutations in the primers annealing regions, reducing the melting temperature, or low levels of virus in the blood below the threshold of sensibility, can cause detection of HIV RNA is negative in the samples with positive anti-HIV [22]. This statement

may also happen in HCV infection in this study, considering the HCV genome sequence is highly variable [12] and the other possibility is HCV RNA disappeared after seroconversion [21].

In 16 sera with RT-PCR positive for HCV, sequencing tests were carried out and 16 HCV sequencing results were obtained. One negative sample on RT-PCR was not sequenced. Sequencing results of these 16 HCV samples were then performed HCV genotype/subtype analysis. Nucleotide sequences from HCV NS5B region were analyzed using the BLASTN program from NCBI on the internet and the nucleotide sequences of the 5'UTR region were determined and compared with the consensus sequence motifs for each of the major genotypes previously reported [20]. Genotyping methods based on the 5'UTR of HCV were important tools for routine clinical purposes because they were sufficiently precise at the genotype level [12]. The principle for determining the HCV genotypes/subtypes in the above way was to compare the nucleotides of samples to various nucleotides from various HCV genotypes/subtypes published previously. In Table 4 below will be shown the results of the HCV genotypes/subtypes analysis.

Table 4: HCV Genotypes and subtypes

Primer for sequencing	HCV Sequences	HCV genotype		HCV Subtype				
		1	2	1b	1c	2a	3a	3k
HC23	7			4/16 (25%)	1/16 (6,25%)	1/16 (6,25%)	1/16 (6,25%)	
5'UTR1	5	2/16 (12,5%)	3/16 (18,75%)					
F2	4			2/16 (12,5%)			1/16 (6,25%)	1/16 (6,25%)
TOTAL	16	2/16 (12,5%)	3/16 (18,75%)	6/16 (37,5%)	1/16 (6,25%)	1/16 (6,25%)	2/16 (12,5%)	1/16 (6,25%)

The HCV genotypes/subtypes were vary in various geographic areas [4, 23]. In sequence analysis of positive PCR results of HCV, if the positive results can be obtained from the NS5b region (in this study using sequencing primers HC23 and F2), it will obtain HCV subtype from the sequence analysis results. The use of primers F1, R1, F2 and R2 [19] is intended to get as many as possible HCV RNA from anti-HCV positives samples, considering that HCV genome sequence is highly variable [12]. If the positive results are obtained from the HCV 5'UTR region (in this study using 5'UTR1 sequencing primer), it will be obtain HCV genotype from the sequence analysis results. 5'UTR region of HCV is a conserved HCV region, so that nucleotide variations from various HCV genotypes are relatively low. Of the 16 HCV sequences in this study for HCV genotype/subtype analysis, 12,5% (2/16) is HCV genotype 1, 37,5% (6/16) is HCV subtype 1b, 6,25% (1/16) is HCV subtype 1c, 18,75% (3/16) is HCV genotype 2, 6,25% (1/16) is HCV subtype 2a, 12,5% (2/16) is HCV subtype 3a and 6,25% (1/16) is HCV subtype 3k. In this study, HCV genotype 1 and HCV subtype 1b were still dominant in patients with liver disease in Surabaya, Indonesia, still as stated in the results of our previous study [7], but HCV subtype 3k was found in this study and HCV subtype 3g was found in our previous study. The result study in Jakarta, Indonesia, the bigger city than Surabaya, based on HCV NS5B and 5'UTR region in liver disease patient also found HCV subtype 1b (36,5%) was the most prevalent, followed by subtypes 3k (15,4%), 2a (14,4%), 1a (12,5%), 1c (12,5%), and 2e (4,8%) and subtypes 2f, 3a, 3b, and 4a were also found in some of the samples [24]. Utama [24] found more varying HCV subtypes in Jakarta than we found in Surabaya. It was mentioned in the previous report, genotypes 1, 2 and 3 have a worldwide distribution [1, 6, 8], genotype 1 and 6 account over the 60% of all the genotypes identified (35.2% and 30.8%, respectively) in Southeast Asia, followed by genotype 3 (19.9%) and 2 (11.1%) [4]. Beside HCV genotype and subtype are important indicators for antiviral therapeutic responses [25], HCV mutation in genotype 1b was related to the occurrence of HCC [13]. Subtype 1b of HCV has more

efficient viral replication ability, poor prognosis, possesses major proportion of severe liver disease (LD), and related Liver Cirrhosis and HCC than non-1b subtypes [5, 13].

Considering 85% of HCV infections become chronic with a high risk of hepatocellular carcinoma [2] and HCV subtype 1b is dominant HCV subtype in this study, more attention for consideration and prevention in subsequent management of HCV infection is required. This results of HCV genotype/subtypes study also give additional information to HCV genotype/subtypes diversity data in Indonesia and can be used as consideration for HCV therapy.

4. Conclusion

In patients who went to the Hepatology Outpatient Clinic, Dr. Soetomo General Hospital Surabaya, we found positive Anti-HCV was 24.29% (17/70). In 94.1% (16/17) of patients with positive anti-HCV, HCV RNA was still detected. Genotype/subtype HCV analysis were found 2/16 (12.5%), 3/16 (18.75%) and 6/16 (37.5%), 1/16 (6.25%), 1/16 (6.25%), 2/16 (12.5%), 1/16 (6.25%) HCV genotypes 1, 2, and HCV subtypes 1b, 1c, 2a, 3a, 3k respectively, and HCV genotype 1 and subtype 1b were still dominant HCV subtype in Surabaya. From the results of this study can be suggested further research in the molecular field to better understand the viral genotype distribution that affects the course of infection, mutation patterns, and the progression of the infection to chronic hepatitis, cirrhosis hepatitis and hepatocellular carcinoma.

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