BIOMEDICAL ENGINEERING

journal homepage: be.ub.ac.id

Detection of hepatitis c virus rna in blood donors with nested pcr technique

Supiana Dian Nurtjahyani[1†], Mochammad Amin[2], Retno Handajani[3]

[1]Department of Biology, Faculty of Teacher Training and Science, PGRI Ronggolawe University, Tuban, East Java, Indonesia

[2]Institute of Tropical Disease, Airlangga University and [3]Department of Biochemistry, Faculty of Medicine, University of Airlangga, Surabaya, East Java, Indonesia

ARTICLE INFO

Article history:
Received
Accepted
Available online

Keywords:
Anti-hepatitis C virus
Virus Hepatitis C
Nested-PCR
Blood donors, Indonesia

†Corresponding author:
diananin39@gmail.com

ABSTRACT

Objective: This study was conducted to detect hepatitis C virus (HCV) RNA among blood donors in Tuban, East Java, Indonesia, using nested Polymerase Chain Reaction (PCR) technique.
Material and methods: Seven blood donors with positives anti-HCV were obtained from 500 blood donors from Red Cross Blood Donor Center (Palang Merah Indonesia = PMI) Tuban, East Java, Indonesia, consisting of 375 men (75%) and 125 women (25%). Detection of anti-HCV antibody prevalence in the blood donors was done by using Enzyme-Linked Immunosorbent Assay (ELISA). The detection of HCV RNA in blood donors sera with positives anti-HCV were performed using nested-PCR technique with three set of primer pairs targeting the NS5B and 5'UTR regions of HCV. Further amplification products were visualized on a 2% agarose gel containing ethidium bromide under UV illumination.
Result: Seven positives anti-HCV blood donors sera (1.4%) were obtained from 500 blood donors sera, in PMI Tuban, East Java, Indonesia. Out of seven blood **donors** sera with positives anti-HCV, 6 sera (85,7%) were positives HCV RNA by using nested PCR technique. Five HCV RNAs were detected with two set of primers based on NS5B HCV region and 1 positive HCV RNA was detected with another set of primer based on 5'UTR HCV region.
Conclusion: HCV RNAs were detected in 85,7% positives anti-HCV blood donors using nested PCR technique, in PMI Tuban, East Java. Further research is needed to determine the genotype and subtype of HCV.

1. Introduction

Infection by Hepatitis C Virus (HCV) has been spreading throughout the world. It is estimated that approximately 130-150 million of the world's population is infected with HCV [1]. Hepatitis C virus infects liver cells and can cause inflammation of the liver with multiple complications for a long time. In some cases, it has been found that the presence of chronic HCV infection could develop into cirrhosis or liver cancer. Hepatitis C virus is an RNA virus (~55 to 60 nm), spherical, shielded, single-stranded, a member of hepacivirus family, has similarities to flaviviruses [2,3,4]. Until now, 7 major genotypes of the hepatitis C virus have been known [5,6].

Hepatitis C virus spreads mainly through blood transfusion (90%). However, lately transmission through blood transfusions decreased dramatically, which is only about 4%. It had been reported that more than 50% cases of HCV were transmitted through intravenous drug abuse and it re-infection or super-infection can occur [4]. Other transmission also occurs through puncture wounds, tattoos, and hemodialysis due to the use of contaminated tools. In addition, HCV can also be transmitted through sexual intercourse, and from mother to her child. Nonetheless, this way is possible when HCV RNA load is very high [7,4]. Based on the mentioned above, blood donors has the opportunity to transmit HCV infection. The transmission power of hepatitis C disease is

very high especially in developing and densely populated countries such as Indonesia. he prevalence of HCV in blood donors in Western Europe and North America varies between 0.1 to 0.3%, while in Japan 1 to 2% and in Indonesia ranges from 0.5 to 3.4% [8]. Kemenkes RI [9] reported that in the screening between 100 Indonesian blood donor samples showed 10 of them were infected with HCV, and a part of them potentially become chronic and progress to liver cancer. It has huge implications on public health problem in Indonesia.

Detection of HCV infection commonly using serology test. Serology test for HCV infection is detection of anti-HCV-antibodies, but unfortunately not all of anti-HCV antibodies positive sera were also positive HCV RNA [10]. Molecular technique can be use for detection viral particles. Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the molecular technique for HCV RNA detection and nested PCR is one of the powerful technique for HCV RNA detection.

The purpose of this study is to confirmed the presence of HCV RNA in blood donors sera by detection of HCV RNA using Nested PCR techniques among blood donor sera with positives anti HCV in Red Cross blood donors center (Palang Merah Indonesia = PMI) in Tuban, East Java, Indonesia.

2. Materials and Method

2.1 Samples with Positive Anti-HCV

Seven anti-HCV positives sera were obtained from 500 blood donors, 375 men (75%) and 125 women (25%) that collected from January to March 2015 in PMI Tuban, East Java. Blood samples were collected in tubes without anticoagulant. Further separation of samples sera were conducted in the Laboratory of Biology, Ronggolawe University, Tuban, East Java, Indonesia.

Furher laboratory works were conducted in the Hepatitis Laboratory, Institute of Tropical Disease, Airlangga University, Surabaya, East Java, Indonesia. Anti-HCV antibody of blood donors samples were tested by enzyme-linked immunosorbent assay (ELISA) using HCV Antibody EIA kit (Foresight, Acon Laboratories Inc.,San Diego, USA) according to the instructions of the manufacturer.

2.2 HCV RNA extraction and reverse transcription

HCV RNAs were extracted from 140 µl of anti-HCV positives sera using Qiagen RNA extraction kit (QIAGEN GmbH, Hilden, Germany) according to the instructions on the kit. Furthermore, cDNA synthesis was performed using the reverse transcriptase by RT-PCR kit (Toyobo Inc., Osaka, Japan) with random hexamer primer and RNA extraction results as the template. The composition for each tube for cDNA synthesis reaction was 3 µl RNA, 1 µl of random hexamer 100 pmol (Roche Molecular Systems Inc., Branchburg, New Jersey), 3 µl of 5x RT and Master Mix and 3 µl ddH2O (ReverTra Ace®qPCR RT Master Mix, FSQ-201). cDNA synthesis was carried out at 42°C for 50 minutes.

2.3 Nested PCR Amplification for HCV

Hepatitis C Virus RNAs were detected using nested PCR. Three set of primers were used to amplify the region of NS5B and 5'UTR genome of HCV for getting HCV RNA as much as possible. In this study, the usage of the primers from NS5B and 5'UTR region because these region allows for HCV genoyping in the next study.

Initially, the anti-HCV positives sera were amplified using first set of primer for NS5B HCV region, NS5B-F1 / NS5B-R1 as external primers for first round PCR followed by amplification of the first round PCR product using the primers of NS5B-F2 / NS5B-R2 as internal primers for second round PCR. The sequences of the primers were written in Table 1 . The reaction was conducted with a final volume of 20µl, with 40 cycles of PCR. Each PCR cycle consist 60 seconds at 94°C for denaturation, 60 seconds at 56°C for annealing, and 75 seconds at 72°C for extention. Amplification PCR products were visualized by electrophoresis techniques using 2% agarose gel with ethidium bromide staining under UV illumination.

Furthermore, if initial amplification showed negative results, its would be carried out amplification using second set of primers. Primers 166 / 167R and primers HC23 / HC24, HC26, HC28 for NS5B HCV region were used in the first and second round PCR respectively with a temperature of 450C for annealing and another PCR condition as above. Then PCR amplification products were visualized.

Finally, if amplification using first and second set of primers were still showed negatives result, amplification of the 5'UTR HCV region was performed by using UTR1 / UTR2 primers for first

round PCR followed by UTR3 / UTR4 primers for second round PCR. First and second round PCR amplification consist of 40 cycles, each PCR cycle with 25 seconds at94°C for denaturation, 25 seconds at450C (first round) / 500C (second round) for annealing, and 45 seconds at 72°C for extention, then PCR amplification products were visualized.

All of the PCR cycle were performed with hotstart temperature at94°C 5 minutes before first cycle and add the ending temperature 72°C for 7 minutes after last cycle to ensure that the amplified cDNAs were re-naturated perfectly

Table 1. Primers used for nested-PCR

Primer	Polarity	Sequence	Position	References
NS5BF1	Sense	5’-CAATWSMMACBACCATCATGGC-3’	7999-8020	[11]
NS5BR1	Antisense	5’- CAGGARTTRACTGGAGTGTG-3’	8805-8825	[11]
NS5BF2	Sense	5’- ATGGGHHSBKCMTAYGGATTCC-3’	8159-8181	[11]
NS5BR2	Antisense	5’-CATAGCNTCCGTGAANGCTC-3’	8611-8630	[11]
166	Sense	5'-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3'	8230-8260	[12]
167 R	Antisense	5'-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3'	8601-8630	[12]
HC23	Sense	5'-TTTGACTCAACCGTCACTGA-3'	8256-8275	[13]
HC24	Antisense	5'-CTCAGGCTCGCCGCATCCTC-3'	8577-8596	[13]
HC26	Antisense	5'-CTCAGGTTCCGCTCGTCCTC-3'	8577-8596	[13]
HC28	Antisense	5'-CACGAGCATGGTGCAGTCCCGGAGC-3'	8507-8531	[13]
UTR1	Sense	5'-CCGGGAGAGCCATAGTGGTC-3'		[14]
UTR2	Antisense	5'-AGTACCACAAGGCCTTTCGC-3',		[14]
UTR3	Sense	5'-TGGTCTGCGGAACCGGTGAG-3'		[14]
UTR4	Antisense	5'-ACCCAACACTACTCGGCTAG-3'		[14]

3. Result

As mentioned above, 500 donors participated in this study, consisting of 375 men (75%) and 125 women (25%). In this study, most of the participants were male aged ≤20-70 years. Seven (1,4%) blood donors from 500 blood donors were

Amplification products of HCV using nested-PCR showed that from seven sera donors with positives anti-HCV, six sera indicated the presence of HCV RNAs. positives Anti-HCV by Anti-HCV ELIZA kit test and all were male blood donors. The data showed that the prevalence of HCV was higher in men than in women.

The positives HCV RNA of PCR amplification results were presented in the table 2

Table 2 Nested PCR positive for HCV

Second Round PCR Primer Set		HCV Region	Nested PCR for HCV Positve	Negative
I.	NS5B-F2/NS5B-R2	NS5B	4/7 (57,1%)	3/7 (42,9%)
II.	HC23/HC24,26,28	NS5B	1/7 (14,3%)	2/7 (28,6%)
III.	UTR3/UTR4	5’UTR	1/7 (14,3%)	1/7 (14,3%)
Total		NS5B & 5’UTR	6/7 (85,7%)	1/7 (14,3%)

4. Discussion

The results of the study by using nested-PCR technique on seven blood donors sera with positive anti-HCV showed the presence of HCV RNAs in six blood donors sera. Hepatitis C virus RNAs were detected using two set specific primers and one set spesific primer for targeting NS5B and 5'UTR regions of HCV respectively. In this study HCV RNA were detected in 85, 7% (6/7) blood donors sera with positives anti-HCV, where as 57,1% using first set of primer, 14,3% using second set of primer and 14,3% using third set of primer. NS5B is one of the non-structural protein of HCV which codes polymerase that allow the virus to replicate. NS5B area is also an important target for the development of antiviral therapeutic agents. Several studies have reported that the NS5B region can be used to determine the genotype and subtype of hepatitis C virus and effective for studying the molecular epidemiology of hepatitis C virus [5,15,16]. However NS5B region may be ineffective for detecting HCV recombinants [17] 5'UTR HCV region is conserved region in the HCV genome, and due to high level of of conservation, the 5'UTR is limited in its ability to discriminate some genotype and subtypes (Murphy, 2007)[6], but it can use for detecting the presence or absence HCV RNA in the serum,. [10,13] (Soetjipto, 1996, Apichartpiyakul 1996). The aim of the usage of three set primers in this study were to obtain as many as posible positives HCV RNA. The first set of primer was successful used in the previous study to detect 92% (44/48) HCV RNA in 48 HIV sera with positives anti-HCV[11] . The second and third sets of primers were successful used to detect HCV RNA in 84% (27/32) blood donors sera with positives Anti-HCV[10].

This result showed the difference between the positivity of anti-HCV antibody test with nested-PCR technique. The serology test showed the results of anti-HVC test were positives in 7 sera the HCV RNA determination by nested-PCR it were positives only 6 samples. This results might be caused by HCV infection was over, HCV RNA quantity below the treshold of sensitivity, variability of HCV sequences in the primers' annealing regions, or a serologic test was false positive. Nested PCR examination results using 3 set of primers logically more valid and accurate .Suwarso [18] reveals that some serologic tests are less specific. Some false positive results have been reported, usually cross-react with the case of autoimmune hepatitis, or the presence of an immune response against human- superoxide dismutase (SOD).

5. Conclusion

By using nested PCR technique, HCV RNAs were detected in 85,7% anti-HCV positives blood donors in PMI Tuban, East Java. Further research is needed to determine the genotype and subtype of HCV RNA.

Acknowledgment

Thanks to Kemenristekdikti which has provided a research grant through Kopertis Region VII , Blood Donor Unit PMI Tuban , Tuban Unirow biology laboratory staff and laboratory staff ITD Airlangga University Surabaya, which has provided research facilities and Laboratory staff of ITD.

Conflict of Interest

The authors report no conflicts of interest

References

1. World Health Organization. 2002. Hepatitis C. WHO/CDS/CSR/)LYO/2003
2. Sharma SD. 2010. Hepatitis C Virus: Molecular Biology Current Therapeutic Options. *Indian J Med Res*. 131:17-34.
3. Lavanchy D (2011). Evolving Epidemiology ofHepatitis C Virus. Clin Mcrobiol Infect, 17: 107.
4. Friedman LS. 2013. Liver, Biliary Tract, & Pancreas Disorders. In: Current Medical Diagnosis & Treatment. Fifty- Second Edition. Editors: Papadakis MA, McPhee SJA Rabow MW. New York, pp.662-682.
5. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N. 2005. Consensus Proposals for a Unified System of Nomenclature of Hepatiti C Virus Genotypes. *Hepatology*. 42: 962.
6. Murphy, D. G., Willems, B., Deschenes, M., Hilzenrat, N., Mousseau, R. & Sabbah, S. 2007. Use of Sequence Analysis of the NS5B Region for outine Genotyping of Hepatitis C Virus with Reference to C/E1 and 5'Untranslated Region Sequences. J Clin.Microbiol 45,1102-112.
7. Allison RD, Camtilena CC, Koziol D, Schechterly C, Ness P, Gihble J, Kleiner DE, Ghany MG, Alter HJ. 2012. A 25-Year Study of the Clinical and Histologic Outcomes of Hepatitis C Virus Infection and Its Modes of Transmission in a Cohort of Initially Asymptomatic Blood Donors. The Journal of Infectious Diseases, 206: 654.
8. Lina MR, Bela B, Dadang S. 2004. Uji PCR (Polymerase Chain Reaction) untuk Virus Hepatitis C. Risalah Seminar Ilmiah Penelitian dan Pengembangan Aplikasi Isotop dan Radiasi.
9. Kemenkes RI 2014. Infodatin: Pusat Data dan Informasi Kementrian Kesehatan RI: Situasi dan Analisis Hepatitis. Jakarta.

10. Soetjipto, Handajani R, Lusida MI, Darmadi S, Adi P, Soemarto, Ishido S, Katayama Y, Hotta H, 1996. Differential Prevalence of Hepatitis C Virus Subtypes i healthy blood Donors, Patients on Maintenace Hemodialysis, and Patients with Hepatocellular Carcinoma in Surabaya, Indonesia. J. Clin. Microbiol. 34(12): 2875-2880.
11. Anggorowati N, YoshihikoYano,Didik Setyo Heriyanto, Hanggoro Tri Rinonce, Takako Utsumi, Deshinta Putri Mulya, *et al*. 2012.Clinical and Virological Characteristics of Hepatitis B or C Virus Co-Infection With HIV in Indonesian Patients. J.Med.Virol.84:857-65.
12. Mori, S., N. Kato, A. Yagyu, T. Tanaka, Y. Ikeda, B. Petchlai, P. Chiewsilp, T. Kurimura, and K. Shimotohno. 1992. A new type of hepatitis C virus in patients in Thailand. Biochem. Biophys. Res. Commun. 183:334-342.
13. Apichartpiyakul C, Chittivudikarn C, Miyajima H, Homma M, Hotta H. 1994. Analysis of Hepatitis C Virus Isolates among Healthy Blood Donors and Drug Addicts in Chiang Mai,Thailand.*Journal of Clinical Microbiology*. 32(9): 2276-2279.
14. Doi H, Apichartpiyakul C, Ohba KI, Mizokami M and Hotta H. 1996. Hepatitis C Virus (HCV) Subtype Prevalence in Chiang Mai, Thailand, and Identification of Novel Subtypes Of HCV Major Type 6. J Clin Microbiol 34: 569-574.
15. Nakatani SM. Santos CA, Riediger IN, Krieger MA, Duarte CAB, Debur MdD, Carrilho FJ, Ono SK. 2011. Comparative Performance Evaluation of Hepatitis C Virus Genotyping Based on the 5’ Untranslated Region Versus Partial Sequencing of the NS5B Region of Brazilian Patients with Chronic Hepatitis C.*Virology Journal*. 8: 459.
16. Marascio N, Torti C, Liberto MC, Foca A. 2014. Update on Different Aspects of HCV Variablity: Focus on NS5B Polymerase. *BMC Infectious Diseases*. 14:1471-2334.
17. Juniastuti, Takako Utsumi, Nasronudin, Lindawati Alim Sardjono, Mochamad Amin, Myrna Adianti, Yoshiiko Yano, Soetjipto, Yoshitake Hayashi and Hak Hotta. 2014 High Rate of Seronegative HCV Infection In HIV-Positive Patients. *Biomedical Reports*. 2: 79-84.
18. Suwarso, 1993. Virus Hepatitis-C. *Berkala Ilmu Kedokteran.* XXV (3): 131-137..