Identification of hepatitis C virus genotypes in volunteer blood donors from blood transfusion center of Tuban, Indonesia

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ABSTRACT

Aims: The most common transmission route of hepatitis C virus (HCV) is via blood transfusion. Therefore, the screening of HCV is necessary to be performed regularly for all the volunteer blood donors. The prevalence of HCV subtypes varies in different geographical areas. The aim of this study is to identify the HCV genotypes of the HCV-RNA positive samples and performed serological and molecular characterization of HCV among blood donors from Blood Transfusion Center of Tuban, East Java, Indonesia collected during the year of 2015.
Methodology and results: All blood donor samples were screened by enzyme-linked immunosorbent assay (ELISA) for anti-HCV. Reverse Transcription - Polymerase Chain Reaction (RT-PCR) was performed to detect the HCV-RNA. Subsequently, the HCV-RNA positive samples were genotyped using direct sequencing followed by subtype/genotype and phylogenetic analysis. Of the 500 blood samples, 7 were positive for anti-HCV antibody (1.4%) and 6 out of 7 (85.71%) were determined to be HCV-RNA positive. Among HCV-RNA carriers, genotyping showed genotypes 1 was the most prevalent. HCV subtypes 1a and 1b were detected in total of 4 out of 6 individuals (66.67%), two individuals for each. HCV subtypes 2a and genotype 1 were the least frequent among blood donors (each counted for 16.67%).
Conclusion, significance and impact for study: The prevalence of HCV found in this study is considerably low. The identification of genotypes 1a and 1b as major HCV genotypes circulating in blood donors in the Tuban city of East Java. This result may contribute in a better medical management towards HCV carriers.

Keywords: Genotypes, Hepatitis C virus, blood donors, phylogenetic tree

INTRODUCTION

The global incident of hepatitis C Virus (HCV) infections increased rapidly over the years. Approximately 170 million of world populations are chronically infected with HCV and it can lead to 350,000 deaths per year (European Association for Study of Liver, 2015). Based on the World Health Organization data, there are 3 to 4 million new HCV infections occurring each year (WHO, 2017).

HCV is categorized as a single stranded RNA virus that belongs to the genus *Hepacivirus*, a member of the family *Flaviviridae* (Choo *et al.*, 1991). According to genetic diversity, HCV is classified into seven distinct genotypes and over 70 different subtypes broadly distributed worldwide (El-Shamy *et al.*, 2014; Smith *et al.*, 2014). Remarkably, those HCV genotypes and subtypes are distinct geographically and thus having their own risk group. The predominant genotypes within the United States, Europe, Australia, and East Asia (Japan, Taiwan, Thailand, and China) are 1, 2, and 3. Genotype 4 is largely enclosed to the Middle East, Egypt, and Central Africa, whereas genotypes 5 and 6 are found mostly in South Africa and Southeast Asia, respectively (Simmond, 1997). The wide variety of HCV genetic profiles is the main advantage for this virus to be resistant to many combinations of antiviral drugs. Previous studies revealed that some specific HCV genotypes might respond better to the antiviral treatments (Holland-Staley *et al.*, 2002) and there is a correlation between the genotype diversities and the risk progress of HCV infections to be hepatocellular carcinoma development (EL-Serag, 2012).

Previous studies indicated that HCV infection represents over 70% of post-transfusion hepatitis C (PTHC), and is one of the causes of chronic liver disease, which frequently results in liver cirrhosis (LC), liver failure and hepatocellular carcinoma (HCC) within 20 to 30 years following infection (Gower *et al.*, 2014; Bennett *et al.*,

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2015). Nowadays, the transmission route for HCV infection was often caused by intravenous drug injections. Drug abuse involving shared syringe injections could cause the infection or super-infection of HCV (Ruta and Cernesco, 2015). Other possibilities for the cause of HCV transmission involved severe lesion cases and non-sterile hemodialysis process. HCV infections could be transmitted sexually and possibly transferred from mother to child. However, for the later case it is only achievable if the RNA level of HCV is relatively high (Allison *et al.*, 2012).

Tuban is historically well known as the biggest seaport as well as the city of commerce in East Java province, Indonesia. The ports are typically full with the merchant ship from abroad, especially neighbor countries. For the past few years, Tuban has been more developed and thus the living customs has been slightly converted into more urban style. This could cause few negative consequences as the cases of illegal drug use and alcohol abuse increased and it might relate to chronic liver diseases. Currently, Tuban is defined as a non-isolated town that has proper facilities to support the efficient traffic between cities and suburbs. This could bring one disadvantage, due to the easy mobility could affect the increase of HCV transmission rates as the infected person could easily travel to the neighbor cities. In order to prevent that, the anti-HCV antibody detection is performed regularly at Tuban Blood Transfusion Center for all the volunteer blood donors. However, the HCV-RNA screening followed by HCV genotyping has not been accomplished in this facility. Based on the different geographical distribution of HCV and the historical background of Tuban as port city where ships from neighbor countries can dock, the aim of this study is to identify the HCV genotypes of the HCV-RNA positive samples from blood donors in Tuban.

MATERIALS AND METHODS

Study populations and sample collection

Voluntary adult blood donors from blood transfusion center of Tuban, East Java were recruited according to standard procedure of the National Blood Transfusion Center of Indonesia. A total of 500 blood samples were collected in 2015 and direct sera separation was conducted in the laboratory of Ronggolawe University in Tuban. The following tests of anti-HCV antibody and the HCV RNA detection in addition to sequencing and genotyping analysis were performed in Institute of Tropical Disease of Airlangga University, Surabaya, East Java.

Anti-HCV antibody detection by ELISA

Detectable anti-HCV was assessed by commercial enzyme-linked immunosorbent assay (ELISA) using HCV Antibody EIA kit (Foresight, ACON Laboratories Inc., San Diego, USA). The assay done based on the ELISA kit's manufacture protocol.

RNA extraction of HCV and reverse transcription

The RNAs were extracted from positives anti-HCV sera, using Qiagen RNA extraction kit (QIAGEN GmbH, Hilden, Germany). Subsequently, 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.

Nucleic acid testing by nested PCR

PCR amplification was done for positive anti-HCV samples obtained from ELISA test. Initially, nested PCR for NS5B region was done for all samples and followed by the nested PCR on 5'UTR region for the negative samples from previous nested PCR. Sets of first round PCR primers used to amplify the NS5B region (Apichartpiyakul *et al.*, 1994) and one set of primers for 5'UTR region (Doi *et al*, 1996). The reference sequences used was H77 (Genebank accession number AF009606).

Direct nucleotide sequencing and phylogenetic analysis

After the PCR amplification, the positive amplicons subsequently were prepared for sequencing. The purification of PCR products was done by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and then directly sequenced by using Big Dye Terminator cycle sequencing Ready Reaction Kit ver. 1.1 (Applied Biosystems, Foster City, CA). Phylogenetic trees based on the NS5B and 5'UTR regions were constructed by using the program GENETYX-Win version 10 (GENETYX, Tokyo, Japan).

RESULTS AND DISCUSSION

Of the 500 blood samples collected, merely 7 samples were positive for anti-HCV but only 6 samples contained detectable HCV-RNA determined by PCR for NS5B and 5'UTR regions of HCV, has been reported by Nurtjahyani *et al.* (2016).

Following the direct sequencing, HCV genotype 1 and 2 were detected in 6 samples. Based on NS5B region of HCV, HCV-1 isolates were identified as having sequences belonging to either subtype 1a (sample BDT-163 and BDT-164, 33.33%) or 1b (sample BDT-394 and BDT-486, 33.33%) and for HCV-2 isolates only one sample was classified as subtype 2a (sample BDT-39, 16.67%). Based on 5'UTR region of HCV, the last sample was only sequenced for its genotype of HCV-1, no subtype (sample BDT-485, 16.67%). The phylogenetic tree of 5 samples was depicted in the Figure 1.

Further analysis revealed HCV genotype distribution in gender and age groups. Based on NS5 region of HCV in males blood donors 1 isolate (1/6, 16.67%) identified as HCV subtype 1a, 1 isolate (1/6, 16.67%) identified as HCV subtype 1b, 1 isolate (1/6, 16.67%) identified as HCV genotype 1 and 1 isolate identified as HCV subtype 2 a (1/6, 16.67%). For HCV 5'UTR region detection in

male, 1 isolate (1/6, 16.67%) belonged to genotype 1. In addition, 1 isolate (1/6, 16.67%) belonged to subtype 1a and 1 isolate (1/6, 16.67%) belonged to subtype 1b in females. As for blood donors within age group of 21-30 years, 2 HCV isolates (2/6, 33.33%) identified as subtype 1a based on NS5B region and 1 isolate (1/6, 16.67%) identified as genotype 1 based on 5'UTR region. Furthermore, 2 isolates (2/6, 33.33%) belonged to HCV subtype 1b and 2a in blood donors within age group of 31-40 years. However, only 1 isolate (1/6, 16.67%) belonged to HCV subtype 1b in age group of 41-50 years.

In the HCV NS5B region trees, 2 isolates of subtype 1a formed a small distinct group, closely related to M67463 and M62321 from Japan and America, respectively. Geographical clusters of HCV subtype 1b (2 samples) were proven but demonstrated no strong bootstrap support. Subtype 2a HCV isolates formed one cluster in the tree, closely grouped with reference sequence from Japan (reference sequences D00944 and AB047639). One HCV isolate of subtype 1 from 5'UTR region (Figure 2) directly associated with a reference sequence from America (reference sequence M62321) .
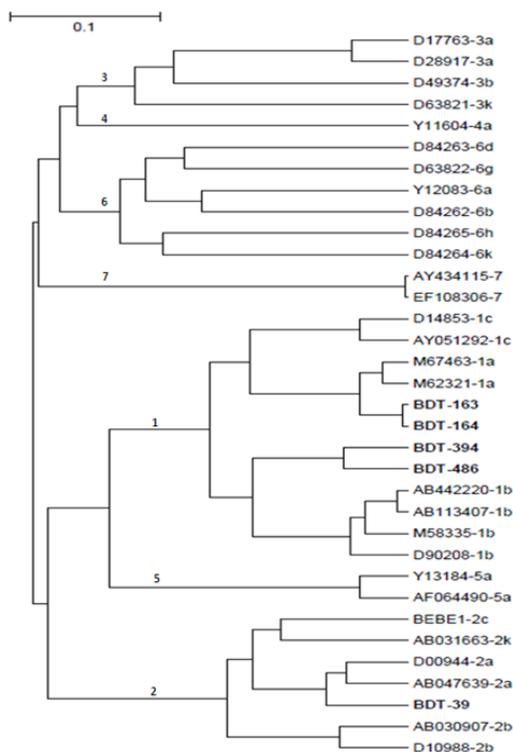
Figure 1: Phylogenetic tree of NS5B nucleotide sequences of HCV-1 and HCV-2 subtypes isolated in this study. The samples and the reference sequences are represented in accession numbers, genotype or subtype. The samples are as follows: BDT-163 and BDT-164 for HCV-1a, BDT-394 and BDT-486 for HCV-1b, BDT-39 for HCV-2a.

The present study revealed a distinction of results between the ELISA screening test for anti-HCV and nested-PCR for HCV RNA amplification. The serological test indicated that 7/500 samples (1, 40%) were positive for anti-HCV. However, after nested-PCR amplification only 6 out of 7 samples are HCV RNA positive. The difference obtained after PCR may be initiated by insufficient HCV viral load that could be detected by PCR.

Previous studies in Surabaya, Indonesia confirmed that only 84% (27/32) of total blood donors with anti-HCV positive sera contained HCV RNA and the most subtype (14/27) is HCV subtype 2a (Soetjipto et al., 1996). The another previous researcher showed that PCR screening is more sensitive and accurate to detect the HCV RNA (Kim et al., 2008). Several anti-HCV false-positive cases have been reported, majorly related to cross reaction with autoimmune hepatitis, also triggered by hypersensitive immune respond to human superoxide dismutase (Larrea et al., 1998; Houldsworth et al., 2014). Thus, utilizing PCR to screen the HCV RNA is considered better as the early detection, especially for asymptomatic HCV cases.

In this study, our results showed that subtype 1a and 1b were the most prevalent (each 33.33%) HCV genotype identified in Tuban, followed by HCV subtype 2a (16.67%) based on NS5B region, and genotype 1 (16.67%) based on 5'UTR region. Phylogenetic trees based on NS5B region of this result showed HCV subtype 1a isolates from blood donor in Tuban located in the same branch with isolates M67463 and M62321 from Japan and America (Inchauspe et al.,1991; Choo et al., 1991). The similar trend depicted for genotype 1 from 5'UTR region directly linked to isolate M62321 from Japan (Choo et al., 1991).

Our subtyping data is the first report about the distribution of HCV subtypes from volunteer blood donors in Tuban, Indonesia. Previous study showed that 52% of blood donors in Surabaya (a capital city of East Java province) predominantly screened as HCV subtype 2a; this was followed by subtype 1b (15%), HCV-1a (7%), and HCV-1d (7%) (Soetjipto et al., 1996). Another study in the capital city of Indonesia, Jakarta, with a bigger population representing Indonesia in general, revealed that almost 60% among the blood donors subtyped as HCV subtype 1b, followed by subtype 2a and 3b (Utama, et al, 2008). Those results were rather distinct with our findings although the depicted trend is the same regarding to the HCV subtype results. In comparison with previous studies that utilized blood samples from either HCV patients with chronic liver disease. In addition, the distribution of HCV genotypes identified in blood donors in this study is different from that in patients with HCV infection as a report from the previous study, indicated that subtype 1b accounts for 47.70%, followed by subtypes 1c (18.70%), 3k (10.7%), 2a (10.0%), 1a (6.7%), 2e (5.3%), 2f (0.7%) and 3a (0.7%) in HCV patients from Jakarta (Inoue et al., 2000).

In relation to the limitations in this study, the sample population was composed of random blood donors collected from blood transfusion center in a small town Tuban. Thus, our results may not be representative of the

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M62321-Genotipe1 *   1 GGAATTGCCAGGACGACCGGGTCCTTTC--TTGGAT-CAACCCGCTCAATGCCTGGAGATTTGGGCGTG  66
AB047639-Genotipe2   1 .........G...A...T..........--......-A.....A...T.....C..CC..........  66
D63821-Genotipe3     1 .....C...G...TT.............--.....A-.T.............C...A..........  66
Y11604-Genotipe4     1 .....C...G...T..............--......-T.............C...A..........  66
Y13184-Genotipe5     1 .........G...T..............--......-A.............C..........  66
Y12083-Genotipe6     1 .............T..............CA......CA..........  69
QC69-JFH1-Genotipe7  1 .........G...A...T..........--......-A.....A...T.....C..CC..........  66
BDT-485 *            1 ...........................--......-A..........  66
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Figure 2: One sample BDT-485 identified as genotype 1 for 5'UTR region. The table acc number is followed by genotype 1 to genotype 7. The sign "-" indicates that there is similarity with the first row (genotype 1) while in the sample (BDT-485) shows similarity with the first line (genotype 1). In the box (Figure 2) so, all of the BDT-485 samples indicate the similarity with genotype 1 that is marked by a sign "-". (Doi *et al.,* 1996)

Table 1: HCV subtypes and genotype and donor grouping in Tuban.

Genotype	1a (n=2)	1b (n=2)	1 (n=1)	2a (n=1)	Total (n=6)
Men	1	1	1	1	4
Women	1	1	-	-	2
Age range (years)	21-30	31-50	21-30	31-40	21-50
Blood type	O, B	A	O	O	O, A, B

Note: The Table 1 shows that there is 1 sample genotype 1. Whereas the others are 2 samples subtype 1a, and 2 samples subtype 1b. Subtype 2a has only one sample. So, the total samples are 6 that can be typed.

general population in Indonesia. However, data obtained from blood donors could be indirect source of health information of the general population. Additionally, the data in our study are representative of only from 7 Anti-HCV positives out of 500 samples collected from healthy volunteer blood donors from Tuban.

Consequently, those blood donors positive for HCV RNA require immediate medical assistance. This present epidemiological research may help for considering management of HCV related diseases and facilitate better public health control and surveillance, especially in rural area in Indonesia.

CONCLUSION

The HCV sequences of blood donors from Tuban were categorized into 3 subtypes, with the most frequent being subtype 1a and 1b (counted for 33.33% each), followed by 2a based on NS5B and genotype 1 based on 5'UTR region). The classification of subtypes 1a and 1b as a major HCV genotypes distributing in this area represents valuable information for future medical management.

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