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Hepatitis B and C Virus Infection Among Hemodialysis Patients in Yogyakarta, Indonesia: Prevalence and Molecular Evidence for Nosocomial Transmission

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Hemodialysis patients are at an increased risk of acquiring hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. However, the prevalence of hepatitis viral infection and its genotype distribution among hemodialysis patients in Indonesia are unclear. In order to investigate these issues and the possibility of nosocomial transmission, 161 hemodialysis patients and 35 staff members at one of the hemodialysis unit in Yogyakarta, Indonesia, were tested for serological and virological markers of both viruses. HBV surface antigen (HBsAg) was detected in 18 patients (11.2%) and in two staff members (5.7%). Anti-HCV was detected in 130 patients (80.7%) but not in any staff members. Occult HBV and HCV infection were detected in 21 (14.7%) and 4 (12.9%) patients, respectively. The overall prevalence rates of HBV and HCV infection among patients were 24.2% and 83.2%, respectively. HCV infection was independently associated with hemodialysis duration and the number of blood transfusions. Phylogenetic analysis revealed that 23 of 39 tested HBV strains (59%) were genotype B, 11 (28.2%) were genotype C, and 5 (12.8%) were genotype A. HCV genotype 1a was dominant (95%) among 100 tested HCV strains. Nosocomial transmission was suspected because the genotype distribution differed from that of the general population in Indonesia, and because the viral genomes of several strains were identical. These findings suggest that HBV and HCV infection is common among hemodialysis patients in Yogyakarta, and probably occurs through nosocomial infection. Implementation of strict infection-control programs is necessary in hemodialysis units in Indonesia. ***J. Med. Virol. 85:1348–1361, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: hepatitis B; hepatitis C; hemodialysis; Yogyakarta; Indonesia; nosocomial transmission

INTRODUCTION

Hemodialysis patients are at increased risk of acquiring hepatitis B virus (HBV) and hepatitis C virus (HCV) infection because of frequent contact with blood, supplies, or surfaces contaminated with these viruses [Rahnavardi et al., 2008; Edey et al., 2010]. Consequently, the prevalence of both infections in hemodialysis patients is very high, although it varies among countries and among hemodialysis units within the same country. Nevertheless, the prevalence of infection is generally higher in

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developing countries than in developed countries, and might reflect its prevalence in the general population [Burdick et al., 2003; Rahnavardi et al., 2008; Johnson et al., 2009]. Unfortunately, few data are available for Indonesia. In a study conducted in West Java, the rates of HBV surface antigen (HBsAg) and anti-HCV seropositivity among hemodialysis patients were 6.8% and 73.5%, respectively [Saketi et al., 2003], whereas those in Yogyakarta were 7% and 81%, respectively [Hadiwandowo et al., 1994]. However, as the study by Hadiwandowo et al. [1994] was conducted in 1994, more recent data addressing the rates on infection in Yogyakarta are needed.

The number of blood transfusions and the duration of hemodialysis are established risk factors for HBV and HCV infection in hemodialysis patients [Vladutiu et al., 2000; Busek et al., 2002; Ferreira et al., 2006]. The implementation of blood product screening in blood banks and the use of erythropoietin treatment have decreased the prevalence of infection with these viruses in hemodialysis units. However, outbreaks of both viruses still intermittently occur [Center for Disease Control and Prevention, 2001; Finelli et al., 2005]. Nosocomial transmission might play an important role in such outbreaks, a hypothesis that is supported by the association between hemodialysis duration and risk of infection with these viruses [Soetjipto et al., 1996].

HBV and HCV genotypes show marked geographic distributions. Indonesia is a multiethnic country with as many as 24 HBV subgenotypes, of which HBV subgenotype B3 (HBV/B3) is dominant [Nurainy et al., 2008; Mulyanto et al., 2009, 2010, 2011, 2012; Utsumi et al., 2009]. At least 15 subgenotypes of HCV have been identified throughout Indonesia, of which HCV/1b is the most prevalent [Hotta et al., 1994; Widjaja et al., 1995; Soetjipto et al., 1996; Tokita et al., 1996; Inoue et al., 2000; Utama et al., 2008, 2010; Anggorowati et al., 2012]. Nevertheless, few data exist for hemodialysis patients. Therefore, it is important to gather further information on the prevalence of HBV and HCV infection in hemodialysis settings, as well as identify the prevalent genotypes by performing molecular epidemiological studies using phylogenetic analysis. This tool is very useful in epidemiology studies, and can be used to trace the possible routes of transmission, and provide evidence of nosocomial infection [Kramvis et al., 2005; Kondili et al., 2006].

The objectives of this study were to investigate the prevalence and genotype distribution of HBV and HCV among hemodialysis patients in Yogyakarta, Indonesia. The possibility of nosocomial transmission was also assessed using molecular analysis.

MATERIALS AND METHODS

Patients, Staff Members, and Hemodialysis Unit

A total of 161 patients with end-stage renal disease who were undergoing hemodialysis and 35 staff members (doctors, nurses, technicians, and cleaning and administrative staff) at one of the hemodialysis unit in Yogyakarta, Indonesia, were enrolled in this study, during January and February 2010. After obtaining written informed consent, the demographic and risk factors associated with HBV and HCV infection data were collected using a standardized questionnaire. Risk factors evaluated in this study were hemodialysis duration, frequency of hemodialysis each week, history of blood transfusion, number of blood transfusions, history of renal transplantation, multiple sexual partners, sexually transmitted diseases, injection drug use, and hepatitis B vaccination.

The hemodialysis unit serves approximately 350 patients from Yogyakarta province and the southern region of Central Java province. It provides hemodialysis treatment in morning, afternoon, or evening shifts. The patients were randomly selected from each shift, regardless of age, sex, and race. Before starting their hemodialysis treatment, blood (5 ml) was collected in plain tubes, allowed to clot, and centrifuged at room temperature. The sera were separated and stored at −80°C until used.

Thirty machines were available at the hemodialysis unit. Two machines were dedicated for patients with HBsAg seropositivity, 27 were used for patients with HBsAg seronegativity, regardless of anti-HCV status, and one was reserved for emergencies. Separate rooms were only available for patients seropositive for HBsAg. The hemodialysis machine uses a hollow fiber dialyzer with standard acetate and bicarbonate solutions. The dialyzers were cleaned individually, rinsed with sterile saline solution, and disinfected with formaldehyde. Before use, the dialyzers were flushed with 2,500 ml of normal saline solution according to standard procedures. Dialyzers were reused up to a maximum of eight times for all patients.

The staff members did their best to follow the recommendations for HBV and HCV infection control issued by the Indonesian Society of Nephrology. However, because of resource limitation, not all of the patients with HBsAg seronegativity were vaccinated for HBV, and anti-HCV screening was not performed routinely. Assessment of HBV DNA and HCV RNA using PCR assays was not performed regularly because of its high cost. Furthermore, established precautions to avoid infection were not consistently implemented, particularly in emergencies. Some instruments and medications were shared among patients.

The study protocol was reviewed and approved by the Ethics Committees of Gadjah Mada University, Indonesia, and Kobe University, Japan.

Liver Enzymes and Serologic Markers

Serum alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) levels were determined using methods recommended by the Japan Society of Clinical Chemistry. Elevated ALT levels were defined as values >40 IU/L while elevated GGT levels were

defined as values ≥70 IU/L for males and ≥30 IU/L for females.

All samples were screened and confirmed for HBsAg status using reverse passive hemagglutination assays (Mycell II HBsAg; Institute of Immunology, Tokyo, Japan) and chemiluminescent immunoassays (Architect; Abbott Japan, Tokyo, Japan). Patients with HBsAg seropositivity were then tested for hepatitis B virus e antigen (HBeAg). Only patients with HBeAg negativity were further tested for anti-HBe antibody using enzyme immunoassays (Immunis HBeAg and HBeAb EIA; Institute of Immunology). Testing for anti-HBs and anti-HBc was conducted using the passive hemagglutination method (Mycell II anti-HBs and Mycell II anti-rHBc; Institute of Immunology) for all samples. Anti-HCV antibodies were detected using the particle agglutination method (Ortho HCV Ab PA test II; Ortho-Clinical Diagnostics, Tokyo, Japan). All assays were conducted according to the manufacturers' instructions.

Detection of Hepatitis Viruses

HBV DNA was extracted from 200 μl of sera using a DNA extraction kit (QIAamp DNA Blood Mini Kit, QIAGEN Sciences, Germantown, MD) according to the manufacturer's instructions. The HBV genome was amplified by nested PCR as described previously [Sugauchi et al., 2001; Cui et al., 2002; Wong et al., 2011]. The amplified products were visualized on a 2% agarose gel stained with ethidium bromide. Patients and staff members who were negative serologically for HBsAg but positive for HBV DNA, based on PCR assays of at least two regions of the HBV genome, were defined as having occult HBV infection [Raimondo et al., 2007].

HBV DNA was quantified by real-time detection PCR (RTD-PCR) with Taq Man chemistry, as previously described [Abe et al., 1999], using an Applied Biosystems 7500 RT-PCR System (Applied Biosystems, Foster City, CA). The detection limit was 1.8 log copies/ml. A viral load >4 log copies/ml was considered high, whereas a viral load ≤4 log copies/ml was considered low [Moutinho et al., 2006].

HCV RNA was extracted from 140 μl of sera using an RNA extraction kit (QIAamp Viral RNA Mini Kit, QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The NS5B region of the HCV genome was amplified by nested RT-PCR using a SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA) with specific primers, as previously described [Akkarathamrongsin et al., 2010]. Patients or staff members seronegative for anti-HCV but positive for HCV RNA, based on PCR, were defined as having occult HCV infection [Jain and Nijhawan, 2008; Tu et al., 2009; Li and Wang, 2010].

To avoid cross-contamination between samples, standard precautions were used in all procedures. Reagents, samples, and amplified products were stored in separate areas.

Sequencing and Phylogenetic Analysis

The amplified fragments were directly sequenced using a BigDye Terminator v3.1. Cycle Sequencing Kit and an ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems).

HBV genotypes and subgenotypes were determined by phylogenetic analysis based on the Pre-S2 and S region (nucleotides [nt] 1–459). Reference sequences were retrieved from GenBank. Multiple alignments were done using Clustal X software (http://www.clustal.org), and phylogenetic trees were constructed using the neighbor-joining method based on Kimura two-parameter distance estimation. To confirm the reliability of the phylogenetic tree topologies, bootstrap reconstruction was carried out 1,000 times, and bootstrap values >70% were considered significant. Analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.2 (http://megasoftware.net/). Samples with a high viral load were subjected to further PCR amplification to obtain the entire genome sequences. Phylogenetic analysis of HCV was done based on the NS5B region (nt 8,244–8,615) through comparisons with confirmed reference sequences [Simmonds et al., 2005].

Detection of Amino Acid Substitutions in the "a" Determinant Region

Some of the amino acid substitutions in the "a" determinant region are thought to result in undetectable HBsAg in patients with detectable HBV DNA. Sequences of this region were aligned and analyzed to identify amino acid substitutions among HBV strains obtained from patients and staff members positive for HBV DNA. Additional primer pairs, as previously described [Juniastuti et al., 2011], were used to amplify the DNA sequence spanning the "a" determinant region in samples that could not be detected using the primers pairs mentioned above.

Statistical Analysis

Statistical analysis was performed using χ^2-tests or Fisher's exact test for categorical variables, and independent *t*-tests and the Mann–Whitney test for continuous variables. Risk factors associated with HBV and HCV infection were first evaluated in univariate analyses by determining odds ratios. Risk factors identified in univariate analyses were further analyzed by multiple logistic regression to control for possible confounders. Values of $P < 0.05$ were considered statistically significant. Patients or staff members were defined as HBV positive if they were HBV DNA positive, regardless of serological status. Patients or staff members who were either anti-HCV or HCV RNA positive were considered as HCV positive.

Nucleotide Sequence Accession Numbers

The HBV and HCV sequences described in this study are deposited in the DNA Data Bank of Japan

under accession numbers AB713527 to AB713532, AB713848 to AB713895, AB714147 to AB714242, and AB767304 to AB767307.

RESULTS

Characteristics of the Participants

The characteristics of the participants are shown in Table I. As shown in this table, most of the patients were male and were in their middle adulthood. The patients were mainly Javanese and came from Sleman Regency. Most of the staff members were female and they were generally younger than the patients ($P < 0.001$). They were all Javanese and mainly came from Sleman Regency. Most of them had been working in the hemodialysis unit for a long time (mean ± SD, 14.1 ± 8.0 years; range, 3 months to 42 years).

Chronic glomerulonephritis and hypertensive kidney disease were the main causes of end-stage renal disease in the patients. The duration of hemodialysis treatment was ≥1 year for most patients, and the majority underwent hemodialysis twice a week. Blood transfusion was still commonly performed to treat renal anemia. However, only eight patients had received the HBV vaccine before starting hemodialysis treatment. The staff members were routinely checked for hepatitis markers and were vaccinated. Kidney transplantation was rarely performed to treat patient with end-stage renal disease.

TABLE I. Characteristics of the Hemodialysis Patients and Hemodialysis Unit Staff Members

Characteristics	Patients (n = 161)	Staff members (n = 35)
Gender, n (%)		
Male	93 (58)	13 (37)
Female	68 (42)	22 (63)
Age, mean ± SD	48 ± 13	40 ± 7
Area of origin, n (%)		
Sleman Regency	70 (43)	21 (60)
Gunung Kidul Regency	17 (11)	0 (0)
Kulon Progo Regency	12 (7)	1 (3)
Bantul Regency	12 (7)	7 (20)
Yogyakarta City	22 (14)	3 (9)
Central Java Province	23 (14)	2 (6)
Other regions	5 (3)	1 (3)
Race, n (%)		
Javanese	157 (97)	35 (100)
Sundanese	1 (0.6)	0 (0)
Balinese	1 (0.6)	0 (0)
Batak	1 (0.6)	0 (0)
Gorontalo	1 (0.6)	0 (0)
Cause of end-stage renal diseases, n (%)		
Chronic glomerulonephritis	50 (31)	
Hypertensive kidney diseases	50 (31)	
Diabetic nephropathy	28 (17)	
Obstructive nephropathy	10 (6)	
Polycystic kidney disease	6 (4)	
Lupus nephropathy	1 (0.6)	
Unknown	16 (10)	
Duration of hemodialysis, n (%)		
<1 year	58 (36)	
≥1 year	103 (64)	
Number of dialyses per week, n (%)		
1 time	14 (9)	
2 times	141 (88)	
≥3 times	6 (4)	
History of blood transfusion, n (%)	153 (95)	
Number of blood transfusions, n (%)		
≤5	96 (60)	
>5	65 (40)	
History of kidney transplantation, n (%)	1 (0.6)	
Multiple sexual partners, n (%)	2 (1.2)	
History of suffering sexually transmitted disease, n (%)	0 (0)	
History of injecting drug use, n (%)	1 (0.6)	
Hepatitis B vaccination before hemodialysis treatment, n (%)	8 (5)	

Distribution of Hepatitis Viral Markers

Eighteen of 161 hemodialysis patients (11.2%) and 2 of 35 staff members (5.7%) were HBsAg positive. HBV DNA was detected in all of the patients and staff members who were positive for HBsAg. Twenty-one of 143 (14.7%) patients negative for HBsAg had detectable HBV DNA in at least two regions within the HBV genome, and were defined as having occult infection. In contrast, none of the staff members had occult infection. Thus, the overall rates of HBV infection among patients and staff members were 24.2% and 5.7%, respectively (Table II). Anti-HBs was more frequently detected among staff members than among patients.

Overall, 134 (83.2%) patients had HCV infection. In contrast, none of the staff members were positive for anti-HCV or HCV RNA (Table II). HCV RNA was detected in 96 of 130 seropositive patients (73.8%) and 4 of 31 seronegative patients (12.9%) were classified as having occult infection. Twenty-nine patients (18%) were co-infected with HBV and HCV (Table III).

The overall prevalence of HBV and HCV was significantly higher in hemodialysis patients than in staff members.

Risk Factors Associated With HBV and HCV Infection

The characteristics of patients and possible risk factors for HBV and HCV infection are summarized in Tables III and IV. None of the possible risk factors analyzed in this study were associated with HBV infection (some data are not shown). However, in univariate analysis, hemodialysis duration and the number of blood transfusions were significantly associated with HCV infection. After adjusting for age and gender in multivariate analysis, hemodialysis duration and the number of blood transfusions were independently associated with HCV infection (Table IV, some data are not shown).

TABLE II. Distribution of Hepatitis Viral Markers Among Hemodialysis Patients and Hemodialysis Unit Staff Members

Hepatitis viral markers	Number (%)		P-value*
	Patients (n = 161)	Staff members (n = 35)	
HBsAg positive	18 (11.2)	2 (5.7)	0.54
HBeAg positive[a]	5 (3.1)	0 (0.0)	1.00
Anti-HBe positive[b]	9 (5.6)	1 (2.8)	1.00
Anti-HBc positive	48 (29.8)	6 (17.1)	0.13
Anti-HBs positive	84 (52.5)	27 (77.1)	**0.008**
HBV DNA positive	39 (24.2)	2 (5.7)	**0.01**
Anti-HCV positive	130 (80.7)	0 (0.0)	**<0.001**
HCV RNA positive	100 (62.1)	0 (0.0)	**<0.001**
Anti-HCV and or HCV RNA positive	134 (83.2)	0 (0.0)	**<0.001**

[a]HBeAg was only tested for HBsAg positive samples.
[b]Anti-HBe was only tested for HBeAg negative samples.
*P-values were determined by the χ^2-test. Significant values are indicated in bold.

Elevated ALT levels were not associated with HBV infection. However, elevated GGT levels were more frequent in patients without HBV infection than in patients with HBV infection ($P = 0.03$), which might be due to the difference in the prevalence of HCV infection. The presence of HBV DNA was associated with anti-HBc positivity ($P < 0.001$; Table III). Elevated ALT levels were not associated with HCV infection. However, elevated GGT levels were more frequent in patients with HCV infection than in

TABLE III. Characteristics of Patients and Risk Factors Associated With HBV Infection

Characteristics and risk factors	HBV DNA positive			HBV DNA negative (n = 122)	Univariate analysis	
	HBsAg positive (n = 18)	HBsAg negative (n = 21)	Total (n = 39)		OR (95% CI)	P-value*
Gender						
Female	6	11	17	51	1	
Male	12	10	22	71	0.9 (0.4–1.9)	0.8
Age						
≤45	5	11	16	49	1	
>45	13	10	23	73	0.96 (0.5–2.0)	0.92
Hemodialysis duration						
<1 year	5	11	16	42	1	
≥1 year	13	10	23	80	0.75 (0.4–1.6)	0.45
Number of blood transfusions						
≤5	11	14	25	70	1	
>5	7	7	14	52	0.75 (0.4–1.6)	0.46
ALT level[a]						
Not elevated	17	20	37	101	1	
Elevated	1	1	2	21	0.3 (0.1–1.2)	0.06
GGT level[b]						
Not elevated	10	10	20	39	1	
Elevated	8	11	19	83	0.4 (0.2–0.9)	**0.03**
Anti-HBs						
Negative	13	10	23	54	1	
Positive	5	11	16	68	0.5 (0.3–1.1)	0.10
Anti-HBc[c]						
Negative	3	14	17	96	1	
Positive	15	7	22	26	4.8 (2.2–10.3)	**<0.001**
Anti-HCV and or HCV RNA						
Negative	3	7	10	17	1	
Positive	15	14	29	105	0.5 (0.2–1.1)	0.08
HBV viral load						
≤4 log copies/ml	12	21	33	—	—	
>4 log copies/ml	6	0	6	—	—	**0.006**

[a]Elevated ALT levels were defined as values >40 IU/L.
[b]Elevated GGT levels were defined as values ≥70 IU/L for males and ≥30 IU/L for females.
[c]The difference was statistically significant if the HBsAg positive and HBsAg negative groups were compared ($P = 0.002$).
*P-values were determined by the χ^2-test. Significant values are indicated in bold.

TABLE IV. Characteristics of Patients and Risk Factors Associated With HCV Infection

Characteristics and risk factors	HCV positive (n = 134)	HCV negative (n = 27)	Univariate analysis OR (95% CI)	Univariate analysis *P*-value*	Multivariate analysis OR[a] (95% CI)	Multivariate analysis *P*-value*
Gender						
Female	56	12	1			
Male	78	15	0.9 (0.4–2.1)	0.79		
Age						
≤45 years	55	10	1			
>45 years	79	17	0.8 (0.4–2.0)	0.69		
Hemodialysis duration						
<1 year	35	23	1		1	
≥1 year	99	4	16.2 (5.2–50.3)	**<0.001**	9.5 (3.0–30.5)	**<0.001**
Number of blood transfusions						
≤5	69	26	1		1	
>5	65	1	24.5 (3.2–185.7)	**<0.001**	11.0 (1.4–88.4)	**0.02**
ALT level[b]						
Not elevated	113	25	1			
Elevated	21	2	0.4 (0.1–1.9)	0.37		
GGT level[c]						
Not elevated	45	14	1			
Elevated	89	13	0.5 (0.2–1.1)	0.07		

[a]Adjusted odds ratio; adjusted for age and gender.
[b]Elevated ALT levels were defined as values >40 IU/L.
[c]Elevated GGT levels were defined as values ≥70 IU/L for males and ≥30 IU/L for females.
*Significant values are indicated in bold.

patients without HCV infection, although the difference was not statistically significant. The mean GGT level was significantly higher in patients with HCV infection than in patients without HCV infection (mean ± SD, 158.0 ± 163.7 vs. 73.7 ± 80.4 IU/L, $P < 0.001$).

Thirty-three (84.6%) of 39 patients with HBV infection had a low (≤4 log copies/ml) or an undetectable viral load. The median titer was 2.8 log copies/ml (range, 1.8–5.5 log copies/ml). Significantly, more patients with overt infection (33.3%) than patients with occult infection (0.0%) had a high viral load ($P = 0.006$, Table III).

HBV and HCV Genotypes

The HBV strains obtained in this study were sequenced and compared with the sequences deposited in GenBank for the pre-S2 and S regions, and a phylogenetic tree was constructed (Fig. 1). Twenty-three of 39 strains (59.0%) isolated from patients belonged to HBV/B. Surprisingly, HBV/C and HBV/A were identified in 11 (28.2%) and 5 (12.8%) strains, respectively. All of the HBV/B strains were classified as HBV/B3. Ten (25.6%) HBV/C strains were classified as C2 and the remaining 1 strain (2.6%) as C7. All of the HBV/A strains were classified as HBV/A2. HBV/C2 was detected mainly in patients with occult infection. HBV/A2 was only detected in patients with occult infection. Two isolates from staff members were classified as HBV/B3. Some of the HBV/A2, HBV/B3, and HBV/C2 strains were identical to the fragment spanning nt 1–459 (YOGHhbv89 and 90; YOGHhbv10, 29, 32, 49, 67, 68, 74, 75, 132, and 149; YOGHhbv54 and 72). These findings strongly suggest the occurrence of nosocomial transmission. Six HBV strains were fully sequenced, and all were HBV/B3.

One hundred HCV strains isolated in this study were compared with 25 strains from the GenBank database, and a phylogenetic tree was constructed (Fig. 2). HCV genotype 1 (HCV/1) was the most common genotype (98%), followed by HCV/3 (2%). HCV/1a, a rare subgenotype among blood donors in Indonesia, was dominant (95%), while HCV/1b, HCV/1c, and HCV/3a were identified in 1 (1%), 2 (2%), and 2 (2%) patients, respectively. The HCV/1a strains showed high similarity (mean ± SD, 96.1 ± 2.0; range, 89.2%–100%). Twenty of them, including two strains isolated from patients with occult infection, were identical to the fragment spanning nt 8,244–8,615 (YOGHhcv13, 46, 57, and 155; YOGHhcv21, 48, 85, 112, 134, and 144; YOGHhcv32, 68, 135, and 136; YOGHhcv42 and 137; YOGHhcv50 and 51; YOGHhcv90 and 93). These findings were also strongly suggestive of nosocomial transmission. To confirm these findings, 95 HCV/1a strains were compared with five local but unrelated HCV/1a strains (AB661787, AB661788, AB661792, AB661794, and AB661816) that were isolated in a previous study [Anggorowati et al., 2012], and with 14 HCV/1a strains isolated in other countries. The phylogenetic tree showed that most of the strains were clustered in the same distinct branches of the tree, but were separated from the five local strains and the strains from the other countries (Fig. 3).

Fig. 1. Phylogenetic tree analysis of the Pre-S2 and S gene sequences of 39 HBV strains isolated from patients, two strains isolated from staff members, and 58 sequences of reference HBV strains from different genotypes (A–J) obtained from GenBank. The GenBank HBV sequences are labeled with their genotype, accession number, and country of origin. The sequences determined in this study are indicated by isolate number, starting with YOGHhbv, and are labeled with black and white diamonds for strains obtained from patients seropositive for HBsAg and patients with occult infection, respectively. HBV strains isolated from staff members are labeled with black circles. Bootstrap values are given at the internal nodes.

Amino Acid Substitution in the "a" Determinant Region

The clinical and molecular data of the patients and staff members with HBV infection, including the distribution of amino acid substitutions in the "a" determinant, are listed in Table V. Amino acid substitutions were more frequently detected in patients with HBsAg seropositivity (67.7%) than in patients with occult infection (47.6%), although this difference was not statistically significant. Four of the HBV/A2 strains were wild-type strains, and one had T126I, N131T, and T143S substitutions. Among the HBV/B3 strains, T131N substitutions were the most frequent. Several specific substitutions (Q129H, Q129R, M133T, C137G, and C137W) were also identified, but less frequently. Among the HBV/C2 strains, I126T substitutions were found in three strains, T131N in one strain, T131P in one strain, and S143T in three strains. The D144G and G145R mutations were both found in one strain. The I126T substitution was detected in the HBV/C7 strain. The T140I substitution was only identified in one HBV/B3 strain isolated from a staff member. None of those substitutions were associated with the occurrence of occult HBV infection. One HBV/A2 strain (YOGHhbv121) had the same amino acid sequence as HBV/C2, one HBV/B3 strain (YOGHhbv65) had the same amino acid sequence as HBV/C2, and one HBV/C2 strain (YOGHhbv36) had the same amino acid sequence as HBV/A2.

DISCUSSION

The prevalence of HBV and HCV infection among hemodialysis patients in Yogyakarta was consistently higher than that among local healthcare workers and blood donors [Hadiwandowo, 1991; Triwibowo, 1993; Hadiwandowo et al., 1994; Rahayujati et al., 2006], suggesting that hemodialysis patients are at greater risk of infection. The high prevalence of anti-HBc seropositivity among patients negative for HBV DNA clearly indicates that hemodialysis patients are susceptible to HBV infection.

This study also confirmed that hemodialysis patients are at greater risk of acquiring HBV and HCV infection compared with staff members at the hemodialysis unit. However, the staff members were still

at risk of HBV infection considering the high prevalence of anti-HBc seropositivity among staff members who were negative for HBsAg. To prevent HBV infection, all staff members in the hemodialysis unit are tested routinely for HBV markers and are vaccinated. Nevertheless, approximately one-fifth of the staff members had lost the vaccine's protective effect, as indicated by undetectable anti-HBs.

Conversely, the patients were not routinely vaccinated before starting hemodialysis because the national health insurance program in Indonesia does not yet cover HBV vaccines. Only eight patients in this study had been vaccinated before starting hemodialysis. However, 84 (52.2%) patients were anti-HBs positive, probably because of the onset of immunity after the resolution of HBV infection.

In this study, the prevalence rates of HBV and HCV infection among hemodialysis patients were similar to those in developing countries, and were higher than those in developed countries [Burdick et al., 2003; Rahnavardi et al., 2008; Johnson et al., 2009]. The differences in prevalence rates among these countries might depend on the differences in the prevalence rates of HBV and HCV among the general population. Indonesia is a country with moderate to high endemic rates of HBV infection [Hasan, 2005], and the prevalence of HCV was reported to be 3.9% [Akbar et al., 1997; Lavanchy, 2011]. Therefore, the prevalence rates of HBV and HCV among hemodialysis patients has tended to increase in recent years. Clearly, infection-control programs should be implemented in developing countries.

The prevalence of HBV infection in this study was higher than that in another hemodialysis unit in Jakarta, but the prevalence of HCV infection was lower than that in hemodialysis units in Jakarta and Surabaya, where the prevalence rates were 97.9% and 88%, respectively [Budihusodo et al., 1991; Santoso et al., 2010]. However, these findings may simply represent the high prevalence of HBV and HCV in Java, independent of hemodialysis units. In Yogyakarta, the prevalence rates of HBsAg and anti-HCV were reported to be 7% and 81%, respectively, in a study performed by Hadiwandowo et al. in 1994, who examined HBV and HCV infection among hemodialysis patients at the same hemodialysis unit [Hadiwandowo et al., 1994]. The current study

Fig. 2. Phylogenetic tree analysis of the NS5B gene sequences of 100 HCV strains isolated from patients, and 25 reference sequences of HCV strains belonging to genotypes (1–6) retrieved from GenBank. The GenBank HCV strains are labeled with their genotype, accession number, and country of origin. The sequences determined in this study are indicated by the isolate number, starting with YOGHhcv, and are labeled with black and white triangles for strains isolated from patients seropositive for anti-HCV and patients with occult infection, respectively. Bootstrap values are given at the internal nodes.

revealed a higher prevalence of HBsAg but a similar prevalence of anti-HCV to those reported in study by Hadiwandowo et al. [1994]. Although the results might be influenced by differences in the sensitivities and specificities of the techniques used, they do indicate that the current infection-control procedures did not decrease the prevalence of HBV and HCV infection. In these circumstances, nosocomial transmission might play an important role.

In multivariate analysis, multiple blood transfusions and duration of hemodialysis were independently associated with the prevalence of HCV infection among hemodialysis patients. In Indonesia, HCV screening was introduced at blood banks in 1995. However, HCV screening was only started in 2001 in Yogyakarta. Thus, hemodialysis patients that received blood transfusions before 2001 were at increased risk of acquiring HCV infection. Unfortunately, blood transfusion, rather than erythropoietin treatment, is still being used to correct renal anemia in the hemodialysis unit. Consistent with previous studies [Saketi et al., 2003; Santoso et al., 2010], the duration of hemodialysis was independently and positively associated with the prevalence of HCV infection, supporting the hypothesis that nosocomial transmission was responsible for HCV infection within the hemodialysis unit.

Serum aminotransferase levels are generally low in hemodialysis patients because of pyridoxine deficiencies, leading to impaired enzyme synthesis, and/or because of the effects of uremic serum [Yasuda et al., 1995; Arora et al., 2011]. Therefore, the ALT level was not a useful marker for detecting HBV and HCV infection among hemodialysis patients [Pujol et al., 1996; Arora et al., 2011]. To detect viral hepatitis among hemodialysis patients, the cutoff values for ALT should be reduced to 20 IU/L [Yasuda et al., 1995]. In this study, the mean ALT level was 25.6 IU/L in patients with HCV infection, which exceeded this revised cutoff value. Accordingly, this revised cutoff value might be useful for the early detection of HCV infection in Indonesia.

GTT levels were reported to be useful as an indirect marker for hepatitis infection [Fabrizi et al., 2007; Souza et al., 2008]. In this study, the mean GTT level was markedly higher in patients with HCV

Fig. 3. Phylogenetic tree analysis of NS5B gene sequences of 95 HCV/1a strains from hemodialysis patients, 18 unrelated sequences of HCV strains belonging to genotype 1 (1a–1c) retrieved from GenBank, and five unrelated sequences of HCV/1a strains from the same administrative region. GenBank HCV strains are labeled with their genotype, accession number, and country of origin. The sequences determined in this study are indicated by isolate number, starting with YOGHhcv, and are labeled with black and white triangles for strains isolated from patients seropositive for anti-HCV and patients with occult infection, respectively. Unrelated strains from the same administrative region are labeled with black squares. Bootstrap values are given at the internal nodes.

TABLE V. Clinical and Molecular Data of Patients and Staff Members With HBV Infection

Name of strains or accession numbers	Age (year)	Sex	ALT level (IU/L)	GGT level (IU/L)	HBsAg	HBeAg[a]	Anti-HBe[b]	Anti-HBc	Anti-HBs	Viral load (log copies/mL)[c]	Genotype	Amino acid substitutions in the "a" determinant region (amino acids 124–147)
Consensus sequence												
AJ012207											A2	CTTPAQGNSMFPSCCCTKPTDGNC
M54923											B3	-------T----------
D23684											C2	--I----T----------S----
AP011104											C7	--I----T----------S----
Patients with HBsAg seropositive												
YOGHhbv11	55	F	18	62	+	-	-	-	+	UD	B3	----------
YOGHhbv22	45	F	5	58	+	-	+	+	-	UD	B3	----------
YOGHhbv23	55	M	4	31	+	-	+	+	-	UD	B3	-----R----------
YOGHhbv30	63	F	14	433	+	-	-	+	-	4.9	B3	-----H----------
YOGHhbv44	34	M	8	37	+	+	NT	+	-	5.0	B3	----------M-----
YOGHhbv68	74	M	6	52	+	+	NT	+	-	4.6	B3	-------N----------
YOGHhbv74	60	F	5	104	+	-	+	+	+	UD	B3	-------N-----G----------
YOGHhbv75	56	M	10	38	+	-	+	+	-	UD	B3	-------N----------
YOGHhbv84	57	M	11	39	+	+	NT	+	-	4.9	B3	-------N-----W----------
YOGHhbv88	40	M	5	64	+	+	NT	+	-	5.5	B3	----------
YOGHhbv123	60	M	11	29	+	-	+	+	+	1.8	B3	-----H-------G----------
YOGHhbv126	57	M	9	61	+	-	-	-	-	UD	B3	-----H----------
YOGHhbv132	23	M	13	73	+	+	NT	+	-	4.8	B3	----------
YOGHhbv149	60	M	31	124	+	-	+	+	-	UD	B3	-------N----------
YOGHhbv162	49	M	9	12	+	-	+	+	-	1.8	B3	-------N----------GR--
YOGHhbv72	73	F	8	27	+	-	+	+	+	2.9	C2	----------
YOGHhbv86	50	F	37	117	+	-	-	-	-	2.4	C2	--T----------
YOGHhbv21	38	M	62	190	+	-	+	+	+	UD	C7	
Patients with occult infection												
YOGHhbv89	42	F	11	20	-	NT	NT	+	+	2.9	A2	----------
YOGHhbv90	54	M	26	192	-	NT	NT	-	-	2.6	A2	----------
YOGHhbv99	53	M	39	36	-	NT	NT	+	+	2.6	A2	----------
YOGHhbv109	12	F	8	42	-	NT	NT	-	-	2.7	A2	----------
YOGHhbv121	52	F	5	19	-	NT	NT	-	-	3.1	A2	--I----T----------S----
YOGHhbv10	25	M	71	346	-	NT	NT	-	-	UD	B3	----------
YOGHhbv14	41	M	4	61	-	NT	NT	-	+	UD	B3	-------N----------
YOGHhbv29	79	F	6	27	-	NT	NT	-	+	2.7	B3	----------
YOGHhbv45	29	M	5	31	-	NT	NT	+	+	UD	B3	-----H---T----------
YOGHhbv46	49	F	10	86	-	NT	NT	-	+	2.6	B3	----------
YOGHhbv49	38	F	4	15	-	NT	NT	-	-	2.3	B3	-------N----------
YOGHhbv65	46	F	5	54	-	NT	NT	-	-	2.7	B3	--I----------S----
YOGHhbv67	50	F	40	80	-	NT	NT	+	+	2.8	B3	----------
YOGHhbv36	33	F	6	48	-	NT	NT	-	+	UD	C2	--T----N----------T----
YOGHhbv54	36	M	8	199	-	NT	NT	+	+	2.8	C2	--T----------T----
YOGHhbv58	49	M	26	581	-	NT	NT	-	+	3.1	C2	----------
YOGHhbv84	36	M	15	60	-	NT	NT	+	-	2.6	C2	----------
YOGHhbv105	40	F	30	81	-	NT	NT	-	-	2.9	C2	--------T----
YOGHhbv107	29	M	21	434	-	NT	NT	+	-	2.6	C2	----------
YOGHhbv118	59	F	3	17	-	NT	NT	-	-	2.8	C2	--T----------
YOGHhbv129	55	M	5	36	-	NT	NT	-	+	UD	C2	-------P----------
Staff members												
YOGHhbv26	33	F	6	12	+	-	-	+	-	2.4	B3	----------I--------
YOGHhbv35	33	M	17	305	+	-	+	+	-	UD	B3	----------

[a,b]NT, not tested.
[c]UD, undetectable (<1.8 log copies/ml).

infection (158 IU/L) than in patients without HCV infection (73 IU/L). In addition, elevated GTT levels were more frequent than elevated ALT levels among patients with HCV infection. These data suggest that GGT is more applicable than ALT as a marker for HCV infection in hemodialysis patients. Clinical measurement of ALT and GGT levels might be useful, providing the cutoff value for the ALT level is reduced.

Occult HBV infection was detected in 14.7% of hemodialysis patients in Yogyakarta. The prevalence of occult HBV infection ranges from 0% to 26.6% among hemodialysis units in different countries [Minuk et al., 2004; Fabrizi et al., 2005; Gwak et al., 2008; Di Stefano et al., 2009; Mina et al., 2010; Motta et al., 2010]. The sensitivities and specificities of the tests used, vaccination rates, healthcare programs, and isolation of hemodialysis for HBV-infected patients could explain these differences in prevalence rates [Hollinger et al., 2010]. Another study showed that occult HBV infection was associated with the presence of isolated anti-HBc and anti-HCV [Di Stefano et al., 2009]. In this study, occult HBV infection was not associated with anti-HBc seropositivity or anti-HCV seropositivity. The small number of samples possibly affected the results of this study.

The prevalence of occult HBV infection in the general population of Indonesia is not well documented. In blood donors and school children, the prevalence rates of occult HBV infection were reported to be 8.1% and 2.2%, respectively [Thedja et al., 2010; Utsumi et al., 2010], which were much lower than the prevalence rate of occult HBV infection determined in the present study. These results suggest that hemodialysis patients are more susceptible to occult HBV infection compared with the general population.

Some amino acid substitutions in the "a" determinant region may affect the antigenicity of HBsAg, resulting in diagnostic failure. The M133L and T143M amino acid substitutions within the "a" determinant region possible altered HBsAg antigenicity among Indonesian blood donors [Thedja et al., 2010], whereas the T126I and T143S substitutions were more frequent among school children [Utsumi et al., 2010]. However, none of the substitutions associated with the disappearance of HBsAg were identified in patients with occult HBV infection in this study. Other mechanisms, such as low viral load and

nucleotide mutations in other regions, might explain the high prevalence of occult HBV infection. The amino acid sequence similarity in the "a" determinant region between genotypes supports the possibility of mixed-genotype infection or inter-genotype recombination in hemodialysis patients. Further studies are needed to address these issues.

The differences in the definitions, methods, hemodialysis unit conditions, and geographical areas resulted in marked differences in the prevalence rates of occult HCV infection, which ranged from 0% to 90% [Barril et al., 2008; Jain and Nijhawan, 2008; Tu et al., 2009; Li and Wang, 2010]. The present study revealed a high prevalence of occult HCV infection (12.9%). However, this rate might be lower than the actual rate of occult HCV infection based on the new definition comprising the presence of HCV RNA in hepatocytes or peripheral blood mononuclear cells (PBMCs) in the absence of serological or virological evidence of infection [Fabrizi and Martin, 2008]. Further studies are needed to address this issue by using PBMCs and liver tissue obtained from biopsy, because occult HCV infection was reported to be associated with increased mortality and nosocomial transmission within the hemodialysis unit [Li and Wang, 2010].

A nationwide study reported that HBV/B was the most common genotype in Indonesia, where the prevalence in some cities on Java, including in Yogyakarta, ranges from 81% to 98% [Haryanto et al., 2008; Mulyanto et al., 2009]. HBV/C is the second most common genotype and is mainly distributed in the northern part of Sulawesi, North Moluccas, and Papua, and is rarely found in Java. HBV/D is the third most common genotype and HBV/A is the fourth most common genotype place with a prevalence of 0.8%. HBV/A shows a very restricted distribution, being found only in Kalimantan, and has never been identified in Java [Mulyanto et al., 2009; Thedja et al., 2011]. Surprisingly, this study showed significant differences in genotype distribution, as the prevalence of HBV/C and HBV/A has increased since earlier studies. The increased prevalence of HBV/C and HBV/A suggests that cross-infection occurs among patients. To our knowledge, this is the first report to identify HBV/A in patients in Java.

HBV/C2 and HBV/A2 were particularly common in patients with occult infection, and the partial HBV genome (nt 1–459) of several strains was identical (YOGHhbv54 and YOGHhbv72; YOGHhbv89 and YOGHhbv90). Thus, occult hepatitis B cases might play an important role in HBV transmission in our hemodialysis unit. To prevent HBV transmission, HBV-DNA should be examined before hemodialysis treatment, although implementing such programs may be hindered by their cost. Alternatively, based on other studies, screening of anti-HBc and anti-HCV might be useful.

In Indonesia, few studies have documented the distribution of HCV genotypes among hemodialysis patients. In this study, the most prevalent HCV genotype was 1a, differing from that observed in HCV-infected blood donors or patients with liver diseases, where genotype 1b prevailed [Hotta et al., 1994; Widjaja et al., 1995; Soetjipto et al., 1996; Tokita et al., 1996; Inoue et al., 2000; Utama et al., 2008, 2010]. A study conducted in the same hemodialysis unit 16 years before this study showed similar findings, as HCV/1a was dominant (89%) among hemodialysis patients, but was not detected in patients with liver diseases [Hadiwandowo et al., 1994]. The HCV/1a predominance might reflect an outbreak of HCV infection from a common source. To test this hypothesis, the HCV genome of strains obtained from hemodialysis patients was compared, which revealed that many of the strains were similar, or identical. Phylogenetic tree analysis of strains isolated from patients and unrelated strains isolated from the same geographic region and other countries supported this finding. Taken together, the present findings provide convincing evidence for nosocomial transmission of HCV within this hemodialysis unit. As genotype 1 is less responsive to standard therapy than genotypes 2 or 3 [Poynard et al., 1998], clinicians should acknowledge that it will be difficult to treat 98% of the HCV-infected patients, meaning special management approaches are needed.

Several mechanisms may explain the predominance of HCV/1a. This genotype might be adapted for patients with impaired immunity, and may be easily transmitted between patients in a hemodialysis unit. Furthermore, frequent exposure to HCV-contaminated blood, supplies, or surfaces, may favor the emergence of mixed genotype infection. Some evidence suggests that HCV/1a selection occurs following a period of infection. When mixed-genotype infection involving HCV/1a occurs, this subtype tends to prevail and persists as the only subtype during the course of the disease. This phenomenon has been demonstrated in experimental studies using chimpanzees [Okamoto et al., 1994], and has been observed in hemodialysis patients [Qian et al., 2000].

There are many possible routes of viral transmission that may be responsible for nosocomial infection. HBV and HCV cannot pass through the dialyzer membrane to cause cross-infection between patients [de Jong et al., 1992; Hubmann et al., 1995]. However, contaminated blood could be transferred if the dialyzers are not effectively sterilized between patients [Hardy et al., 2000]. Infection should not occur if the dialyzer reuse procedures are followed correctly. Moreover, a large-scale study showed that dialyzer reuse was not a risk factor for HCV seroconversion [Jadoul et al., 1993; Finelli et al., 2005]. The reuse of dialyzers is unlikely to provide a route of nosocomial transmission in the hemodialysis unit. It is suspected that the most likely cause of cross-infection between patients treated in the same hemodialysis unit is cross-contamination of supplies and surfaces (including gloves) through a failure to follow established

infection-control procedures within the hemodialysis unit [KDIGO, 2008]. Unfortunately, the main route of nosocomial transmission could not be determined in the present study. Further studies are therefore needed to assess the possible routes of transmission in this hemodialysis unit.

In conclusion, the prevalence of HBV and HCV infection among hemodialysis patients in Yogyakarta, Indonesia, remains high. Nosocomial transmission might play an important role in infection through a failure to follow infection-control procedures within the hemodialysis unit. Hemodialysis units should implement and adhere to strict infection-control procedures designed to prevent the transmission of HBV and HCV. Such procedures should include hygienic precautions aimed at preventing the transfer of blood or fluids contaminated with blood between patients, either directly or via contaminated equipment or surfaces.

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