Prevalence and genotypic distribution of GB virus C and torque teno virus among patients undergoing hemodialysis

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Abstract. Patients undergoing hemodialysis are at increased risk of infection with blood-borne viruses, including GB virus C (GBV-C) and torque teno virus (TTV). However, the prevalence and genotypic distribution of these viruses in the assessed patients undergoing hemodialysis remains unclear. The present study investigated these issues and the possibility of nosocomial transmission among patients undergoing hemodialysis in a unit in Yogyakarta, Indonesia. GBV-C RNA was detected in 92/161 patients (57.1%) by nested reverse-transcription polymerase chain reaction. Phylogenetic analysis of the 5'-untranslated region (UTR) classified the GBV-C isolates into genotypes 6 (85%), 2 (8%), 4 (6%), and 3 (1%). TTV DNA was detected in all patients by the amplification of the 5'-UTR and open reading frame-1 (ORF1) by nested and semi-nested polymerase chain reaction. Phylogenetic analysis based on the ORF1 revealed that genotype 1 was dominant (84%), followed by genotypes 2 (10%) and 3 (6%). The greater prevalence of GBV-C genotype 6 in patients undergoing hemodialysis compared with the general population and the identical sequences observed in multiple isolates provided strong evidence of patient-to-patient transmission. The prevalence of TTV in hemodialysis patients was similar to that observed in the general population, and only one pair of TTV isolates was identical. These results indicated that nosocomial infection was not the main cause of the high prevalence of TTV in patients undergoing hemodialysis. In conclusion, GBV-C and TTV infections are common in patients undergoing hemodialysis in Yogyakarta, Indonesia, and transmission is likely to be nosocomial in the case of GBV-C infection.

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Introduction

Patients undergoing hemodialysis are at increased risk of acquiring GB virus C (GBV-C) and torque teno virus (TTV; also known as transfusion transmitted virus) infection as a result of their impaired immune system and frequent contact with blood, blood products, equipment and surfaces contaminated with these viruses. A high prevalence of GBV-C infection in patients undergoing hemodialysis has previously been documented, with rates ranging from 3.9-26.5% in Iran, Egypt, Turkey and Brazil (1-5). Based on the results of two older studies, however, the prevalence in Indonesian patients undergoing hemodialysis is greater. Handajani *et al* (6) reported a prevalence of 29% among patients undergoing hemodialysis in Surabaya, and Tsuda *et al* (7) reported a prevalence of 55% in Yogyakarta. In general, the prevalence of GBV-C is greater in patients undergoing hemodialysis than in low-risk populations, including blood donors or healthy individuals.

Multiple GBV-C genotypes (1-7) have been identified based on the genetic diversity of full or partial genome sequences. However, genotypes 4, 6 and 7 are highly similar and can be classified as one group. Thus, a simpler classification comprising of 5 groups of GBV-C genotypes has been recommended (8). In Indonesia, genotype 4 was reported to be predominant (55.5%) in blood donors, followed by genotypes 3 (22.2%), 2 (11.1%) and 6 (11.1%) (6). In contrast, genotype 6 was predominant among patients with chronic liver disease and patients undergoing hemodialysis, being detected in 60% and 83.3% of patients, respectively (6). These data suggest that genotype 6 is most common in Indonesian patients undergoing hemodialysis.

Notably, a previous study identified that the prevalence of GBV-C infection was 88.8% in patients with human immunodeficiency virus (HIV) in Yogyakarta, Indonesia (9). This prevalence was greater than expected, and genotype 2 was predominant (58.3%), followed by genotypes 6 (28.4%) and 3 (12.6%). The distribution of GBV-C genotypes in patients with HIV differed from that observed in blood donors, and may reflect a change in the prevalence and genotypic distribution of GBV-C infection in Yogyakarta, Indonesia.

TTV is also common in patients undergoing hemodialysis in certain countries, with prevalence rates ranging from 27.8-68.8% in Iran, India, Italy and Brazil (10-14). However, only two previous studies have examined the prevalence of TTV infection in Indonesia. In a healthy population, TTV was detected in 95% of individuals. The isolates were primarily classified into genotypes 1, 2 and 3 (98%), which were prevalent worldwide. However, genotype 22 and 23 were found to be unique in Indonesia. Genotype 22 was more common in Indonesia than in Japan, whereas genotype 23 was restricted to an isolated area, Kutai on Kalimantan Island (15). Irian Jaya, an area in the east part of Indonesia, had a different pattern of genotype distribution from Java Island and other areas in Indonesia (16). There are currently no data available regarding hemodialysis patients.

A previous study of patients undergoing hemodialysis in Yogyakarta, Indonesia, demonstrated high prevalence rates of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, which may have occurred via nosocomial infection (17). It is important to know whether other blood-borne viruses circulate in patients undergoing hemodialysis. Therefore, in the present study, the prevalence and genotypic distribution of GBV-C and TTV were investigated in patients undergoing hemodialysis. The possibility of nosocomial infection was also assessed by molecular analysis.

Materials and methods

Patients undergoing hemodialysis. The present study enrolled 161 patients undergoing hemodialysis, who were tested for HBV and HCV infection by blood chemistry, serological and molecular examination in a previous study (17). The patients underwent hemodialysis at a unit in Yogyakarta, Indonesia, between January and February 2010. The age ranged from 12-79 years (mean ± standard deviation; 48±13 years). There were 93 male patients (57.8%) and 68 female patients (42.2%). Almost all of the patients (97.5%) were Javanese. Blood samples (5 ml) were collected prior to starting hemodialysis. The blood samples were allowed to clot, and then centrifuged at 1,500 x *g* for 10 min at room temperature. The sera were separated and stored at -80˚C for further use. Sociodemographic factors, risk factors, alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) concentrations, and markers of HBV and HCV infection were obtained as described previously (17).

The present study was reviewed and approved by the Ethics Committees at Kobe University (Kobe, Japan) and at Gadjah Mada University (Yogyakarta, Indonesia). All subjects provided written informed consent.

Detection of GBV-C RNA. RNA was extracted from 140 µl sera using an RNA extraction kit (QIAamp Viral RNA Mini kit; Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The RNA was then converted into cDNA using a SuperScript III One-Step Reverse Transcription Polymerase Chain Reaction system (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and outer reverse primers in E1 and 5'-untranslated region (UTR). The cDNA was used as a DNA template for analysis by polymerase chain reaction (PCR), with primer pairs designed to amplify the 5'-UTR and E1 region of the GBV-C genome.

The partial 5'-UTR of the GBV-C genome was amplified using the following outer primer sequences: Forward 5'-GCCAAAAGGTGGTGGATGGG-3', reverse 5'-CGGAGC TGGGTGGCCCCATGC-3'; and the following inner primer sequences: Forward 5'-TGGTAGGTCGTAAATCCCGG-3', reverse 5'-TGGTCCTTGTCAACTCGCCG-3' in a nested PCR to obtain an amplicon of 262 nucleotides (nt; nt 134-395). A portion of the E1 gene was amplified using the following outer primer sequences: Forward 5'-ATCATGGCAGTCCTT CTGCT-3', reverse 5'-TCARTCCATCTCCAAAACTC-3'; and the following inner primer sequences: Forward 5'-GGGCAA TATTTSCTCACAAA-3', reverse 5'-CAAAACTCACTTTCC CACTT-3' in a nested PCR, to obtain an amplicon of 347 nt (nt 630-976). The nt numbers refer to the PNF2126 isolate under accession no. U44402. The first and second round PCRs were run under the same conditions for 35 cycles, with each cycle consisting of 1 min at 94˚C, 1 min at 45˚C, and 2 min at 72˚C (6,18).

Detection of TTV DNA. TTV DNA was extracted from 200 µl sera using a DNA extraction kit (QIAamp DNA Blood Mini kit; Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. The 5'-UTR of the TTV genome was amplified by nested PCR using the following outer primer sequences: Forward 5'-GTAAGTGCACTTCCGAATGGC TGAG-3', reverse 5'-GAGCCTTGCCCATRGCCCGGC CAG-3', where R = A or G; and the following inner primer sequences: Forward 5'-CTGAGTTTTCCACGCCCGTCC GC-3', mixed with an equal amount of the primer with the underlined 4 nt replaced by ATGC, and reverse 5'-CCCATR GCCCGGCCAGTCCCGAGC-3'. The amplicon obtained in the first round of PCR was 162 nt long (nt 91-252) while the amplicon obtained in the second round was 134 nt long (nt 111-244). PCR comprised of 35 cycles at 95˚C for 30 sec plus 9 min in the first cycle for the first round, and 25 cycles in the second round at 72˚C for 40 sec, plus 7 min in the last cycle (19,20).

The open reading frame-1 (ORF1) region of the TTV genome was amplified by semi-nested PCR. The first round of PCR was comprised of 35 cycles (94˚C for 30 sec; 60˚C for 45 sec; 72˚C for 45 sec, plus 7 min in the last cycle) using the following primer sequences: Forward 5'-ACAGACAGAGGA GAAGGCAACATG-3', reverse 5'-CTGGCATTTTACCAT TTCCAAAGTT-3'. The second round of PCR was comprised of 25 cycles under the same conditions as the first round, with the following primer sequences: Forward 5'-GGCAACATG YTRTGGATAGACTGG-3', where Y=T or C; R=A or G, and reverse as above. The amplicon obtained in the first round of PCR was 286 nt long (nt 1900-2185), while that obtained in the second round was 271 nt long (nt 1915-2185). The nt positions are based on the TA278 isolate under accession number AB017610 (20-22).

Table I. Characteristics and possible risk factors of GBV-C and TTV infection.

Variable	GBV-C RNA			TTV DNA		
	Positive (n=92)	Negative (n=69)	P-value	ORF1-positive (n=68)	ORF1-negative (n=93)	P-value
Age, mean ± standard deviation	48.4±12.3	47.6±14.2	0.7	48.8±12.6	47.6±13.4	0.5
Male/female ratio	50/42	43/26	0.3	43/25	50/43	0.2
Hemodialysis duration ≥1 year, n (%)	56 (60.8)	47 (68.1)	0.3	43 (63.2)	60 (64.5)	0.9
History of blood transfusion, n (%)	87 (94.6)	66 (95.6)	1.0	66 (97.1)	87 (93.5)	0.5
Number of blood transfusions, >5 times, n (%)	37 (40.2)	29 (42.0)	0.8	32 (47.0)	34 (36.5)	0.2
History of kidney transplantation, n (%)	0 (0)	1 (1.4)	0.3	1 (1.5)	0 (0)	0.4
History of multiple sexual partners, n (%)	0 (0)	2 (2.9)	0.1	1 (1.5)	1 (1.1)	1.0
History of suffering sexually transmitted disease, n (%)	0 (0)	0 (0)	-	0 (0)	0 (0)	-
History of injecting drug use, n (%)	0 (0)	1 (1.4)	0.2	0 (0)	1 (1.1)	1.0
Elevated ALT level, n (%)	10 (10.9)	13 (18.8)	0.1	10 (14.7)	13 (14.0)	0.9
Elevated GGT level, n (%)	61 (66.3)	41 (59.4)	0.4	45 (66.2)	57 (61.3)	0.5
HBV positive, n (%)	27 (29.3)	12 (17.4)	0.08	14 (20.6)	25 (26.8)	0.4
HCV positive, n (%)	79 (85.9)	55 (79.7)	0.3	55 (80.9)	79 (84.9)	0.5

Elevated ALT levels were defined as values >40 IU/l. Elevated GGT levels were defined as values ≥70 IU/l for males and ≥30 IU/l for females. Patients who had HBV DNA in their sera were considered as HBV-positive. Patients who were either anti-HCV or HCV RNA positive were considered as HCV-positive. P-values were determined by the χ^2 test or independent sample *t*-test. P<0.05 was considered to indicate a statistically significant difference. GBV-C, GB virus C; TTV, torque teno virus; ORF1, open reading frame-1; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus.

Sequencing and phylogenetic analysis. The PCR products were directly sequenced using a BigDye Terminator v3.1. Cycle Sequencing kit and an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences were manually edited and aligned using ClustalX software (version 2.0.12; http://www.clustal.org). The GBV-C genotypes were determined by phylogenetic analysis of the partial 5'-UTR and E1 sequences, whereas the TTV genotypes were determined using the partial ORF1 sequence. Published sequences were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank; accessed on July 18, 2013) and were used as reference sequences. Phylogenetic trees were constructed using the neighbor-joining method based on the Kimura two-parameter distance estimation model (23). To validate the reliability of the tree topologies, bootstrap reconstruction was performed 1,000 times, and bootstrap values of >70% were considered statistically significant. Analyses were conducted using Molecular Evolutionary Genetics Analysis software version 4.0.2 (http://megasoftware.net). Sequences were also compared in order to identify identical sequences.

Nt sequence accession numbers. The GBV-C and TTV sequences described in the present study were submitted to the DNA Data Bank of Japan under accession numbers LC034595-LC034680 for the 5'-UTR sequences of GBV-C, LC034681-LC034741 for the GBV-C E1 region sequences, and LC034742-LC034809 for the TTV ORF1 sequences.

Statistical analysis. Statistical analyses were performed using χ^2-tests or Fisher's exact tests for categorical variables, and independent Student's *t*-tests or Mann-Whitney U tests for continuous variables using PASW Statistics, version 18.0.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference. Patients were defined as GBV-C or TTV positive if they were RNA positive in at least in one region (5'-UTR or E1 for GBV-C; 5'-UTR or ORF1 for TTV).

Results

Prevalence of GBV-C. GBV-C RNA was detected in 92/161 patients (57.1%). The 5'-UTR and E1 region sequences were both amplified in samples from 55 patients (34.2%), the 5'-UTR sequence only was detected in 31 patients (19.3%), and the E1 region sequence only was detected in 6 patients (3.7%). These results suggest that detection of GBV-C RNA by amplification of the 5'-UTR region is more sensitive than amplification of the E1 region.

GBV-C positivity was not associated with age, sex, or any of the other risk factors analyzed in this study (Table I). A total of 27 patients (29.3%) were co-infected with GBV-C and HBV, while 79 patients (85.9%) were co-infected with GBV-C and HCV (Table I). Most patients from both groups had normal ALT concentrations (≤40 IU/l), with mean ALT concentrations of 20.6±20.1 and 29.2±39.2 IU/l observed in the GBV-C positive and GBV-C-negative groups, respectively (Table I). GBV-C-positive patients tended to have higher GGT

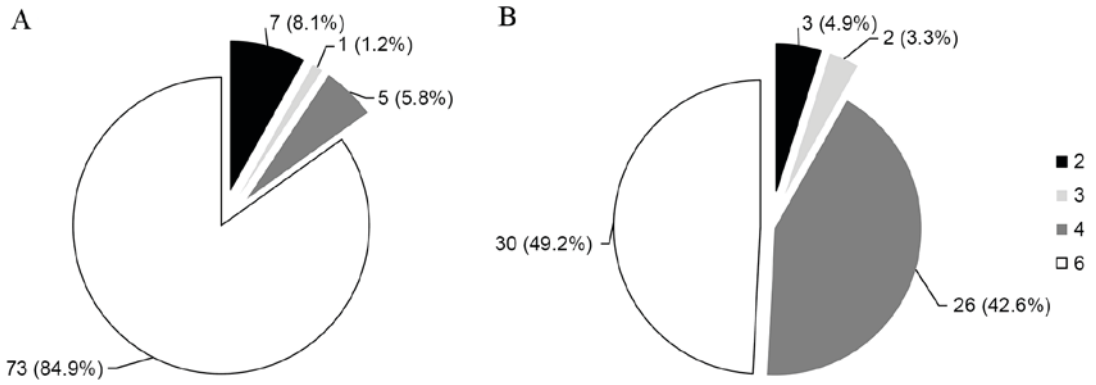

Figure 1. Distribution of GB virus C genotypes based on phylogenetic analysis of the (A) 5'-untranslated region and (B) E1 region.

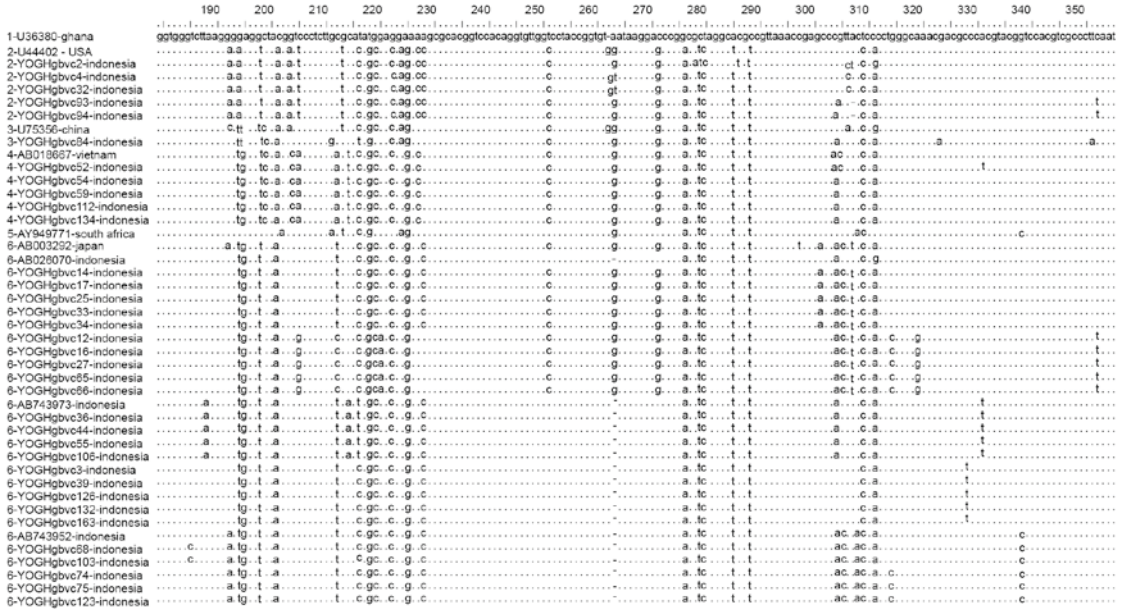

Figure 2. Alignment of the 5'-untranslated region nucleotide sequences of 35 representative GBV-C strains isolated in the present study. The genotype, accession number or isolate name, and the country of origin are provided for each sequence. Dots indicate nucleotides identical to the reference sequence (U36380) and dashes indicate deleted bases. The nucleotide positions are based on the GBV-C PNF2126 isolate (U44402). GBV-C, GB virus C.

concentrations than GBV-C-negative patients, however the difference was not statistically significant, with mean GGT concentrations of 150.3±165.0 and 135.4±144.0 IU/l recorded in GBV-C-positive and GBV-C-negative patients, respectively (Table I). The observed differences in ALT and GGT might be due to the high frequency of co-infection with either HBV or HCV in both groups.

Genotypic distribution of GBV-C. To investigate the genotypic distribution of GBV-C and the possibility of nosocomial infection phylogenetic analysis of the 86-nt 5'-UTR sequence and the 61-nt E1 region sequences was conducted. Phylogenetic trees were constructed for both sequences. Analysis of the 5'-UTR revealed that genotype 6 was the most common genotype (73 patients; 84.9%; Fig. 1A), followed by genotypes 2 (7 patients; 8.1%; Fig. 1A), 4 (5 patients; 5.8%; Fig. 1A), and 3 (1 patients; 1.2%; Fig. 1A). Analysis of the E1 region revealed that genotype 6 was the most common genotype (30 patients; 49.2%; Fig. 1B), followed by genotypes 4 (26 patients; 42.6%; Fig. 1B), 2 (3 patients; 4.9%; Fig. 1B), and 3 (2 patients; 3.3%; Fig. 1B). The difference in genotypic distribution was because 20 strains classified as genotype 6 based on the 5'-UTR were reclassified as genotype 4 and 1 strain classified as genotype 6 was reclassified as genotype 3 based on the E1 region.

Alignment of representative 5'-UTR and E1 region sequences demonstrated that these sequences were identical for some of the isolates, with isolates from Indonesia containing some unique substitutions and deletions which differed from those of isolates from other countries (Figs. 2 and 3). Similar and identical strains were clustered together in the phylogenetic tree with high bootstrap values (Figs. 4 and 5). Multiple strains were completely identical, particularly in the 5'-UTR sequences (YOGHgbvc3, 39, 126, 132 and 163; YOGHgbvc9, 14, 17, 25, 33, 35, 37, 42, 43, 45, 49, 61, 86, 92, 96, 97, 98, 114, 116, 127, 138, 144, 159 and 165; YOGHgbvc12, 16 and 65; YOGHgbvc27 and 66; YOGHgbvc36, 44, 55 and 106; YOGHgbvc46 and 93; YOGHgbvc54, 59, 112 and 134;

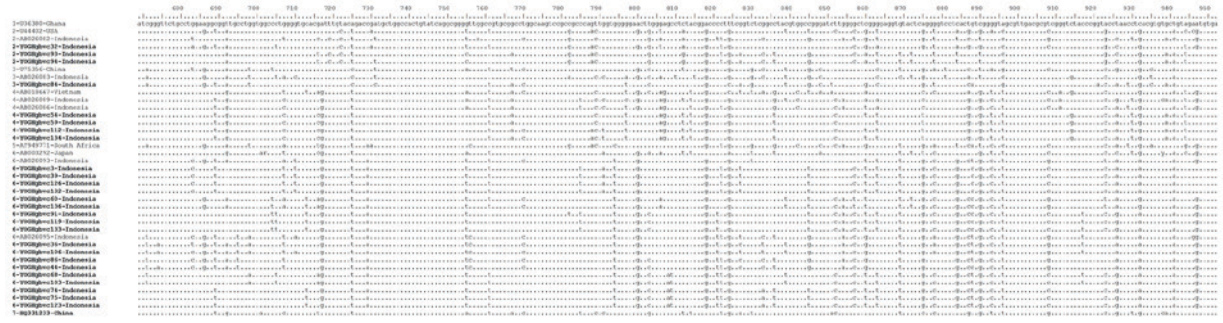

Figure 3. Alignment of the E1 region nucleotide sequences of 26 representative GBV-C strains isolated in this study (in bold font). The genotype, accession number or isolate name, and the country of origin are provided for each sequence. Dots indicate nucleotides identical to the reference sequence (U36380) and dashes indicate deleted bases. The nucleotide positions are based on the GBV-C PNF2126 isolate (U44402). GBV-C, GB virus C.

YOGHgbvc67 and 76; YOGHgbvc68 and 103; YOGHgbvc69 and 73; YOGHgbvc74, 75 123; YOGH91 and 133; YOGHgbvc149 and 162; Fig. 4). Regarding the E1 region, there were fewer identical sequences identified (YOGH3, 39, 126 and 132; YOGHgbvc12 and 76; YOGH16, 66, 67 and 139; YOGHgbvc42 and 89; YOGHgbvc53, 92 and 127; YOGH60 and 136; YOGHgbvc68 and 103; YOGHgbvc74, 75 and 123; YOGHgbvc112 and 134; YOGHgbvc119 and 133; Fig. 5). Overall, 13 isolates were identical in terms of both the 5'-UTR and E1 region sequences (YOGHgbvc3, 39, 126 and 132; YOGHgbvc68 and 103; YOGHgbvc74, 75 and 123; YOGHgbvc92 and 127; YOGH112 and 134; Figs. 4 and 5). A total of 8 of these isolates (61.5%) were obtained from patients who had been on hemodialysis for ≥1 year.

Genetic diversity was greater for the E1 region sequences than for the 5'-UTR sequences with an overall mean distance of 0.14 and 0.06, respectively (data not shown). The diversity observed in the E1 region probably occurred prior to the start of hemodialysis in these patients.

Prevalence of TTV. TTV DNA was detected in all patients. The 5'-UTR sequence was amplified in 160 (99.4%) samples, whereas the ORF1 sequence was amplified in just 68 samples (42.2%). This suggests that detection of GBV-C by amplification of the 5'-UTR is more sensitive than amplification of the ORF1 sequence.

As TTV infected all of the patients, the associations between ORF1 positivity and demographics, liver enzyme concentrations and possible risk factors were analyzed. ORF1 positivity was not associated with age, sex or any of the risk factor analyzed in the current study (Table I). A total of 39 patients (24.2%) were co-infected with TTV and HBV, while 134 patients (83.2%) patients were co-infected with TTV and HCV (Table I). The majority of patients in the two groups had normal ALT concentrations, with a mean ± standard deviation of 27.2±33.0 and 22.2±27.5 IU/l recorded in ORF1-positive and ORF1-negative patients, respectively (Table I). ORF1-positive patients tended to have higher GGT concentrations than ORF1-negative patients with a mean ± standard deviation of 138.4±143.8 and 147.9±165.1 IU/l, respectively, although the difference was not statistically significant (Table I). The observed differences in ALT and GGT concentrations might be due to the high frequency of co-infection with HBV and HCV.

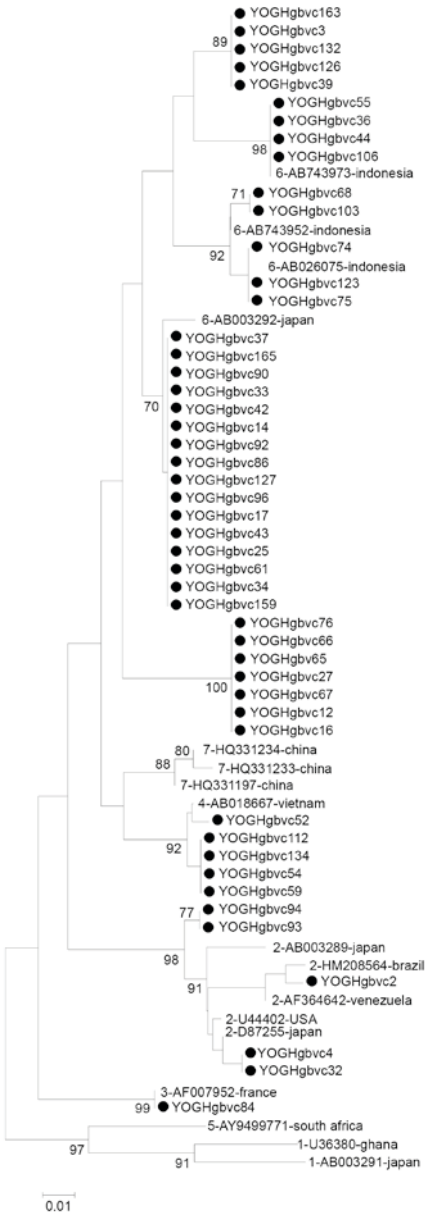

Figure 4. Phylogenetic tree analysis of the 5'-UTR gene sequences of GBV-C strains isolated from patients undergoing hemodialysis. The reference sequences for different GBV-C genotypes were obtained from GenBank. The sequences determined in the present study are labeled with solid black circles and their isolate number, starting with YOGHgbvc. GenBank GBV-C sequences are indicated with their genotype, accession number and country of origin. Bootstrap values are provided at the internal nodes. GBV-C, GB virus C.

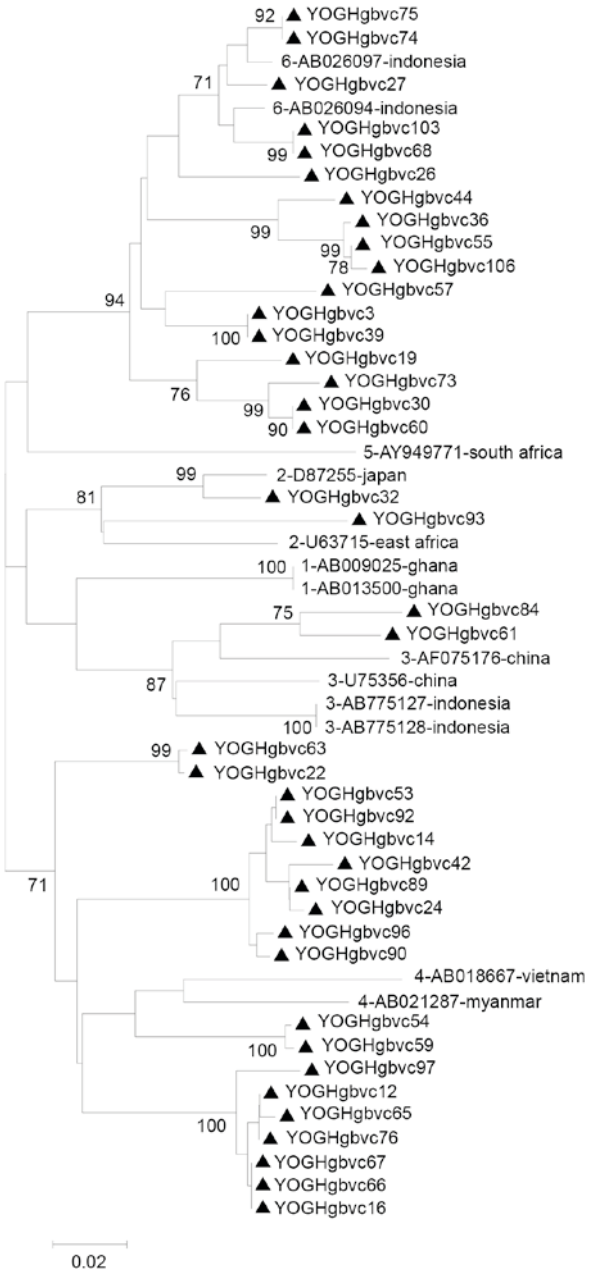

Figure 5. Phylogenetic tree analysis of the E1 gene sequences of GBV-C strains isolated from patients undergoing hemodialysis. The reference sequences for different genotypes of GBV-C strains were obtained from GenBank. The sequences determined in the present study are labeled with solid black triangles and their isolate number, starting with YOGHgbvc. The GenBank GBV-C sequences are indicated with their genotype, accession number, and country of origin. Bootstrap values are provided at the internal nodes. GBV-C, GB virus C.

Genotypic distribution of TTV. Phylogenetic analysis of the ORF1 region identified that genotype 1 was dominant (57 patients; 83.8%; Fig. 6), followed by genotypes 2 (7 patients; 10.3%; Fig. 6) and 3 (4 patients; 5.9%; Fig. 6) respectively. Alignment of representative ORF1 sequences revealed marked variability in these sequences, and several conserved regions belonging to TTV group 1 were identified among genotypes 1, 2, and 3 (Fig. 7). The overall mean distance was 0.31. Certain unique nt substitutions and deletions were detected in the Indonesian genotype 1 isolates, which differentiated these isolates from Japanese genotype 1

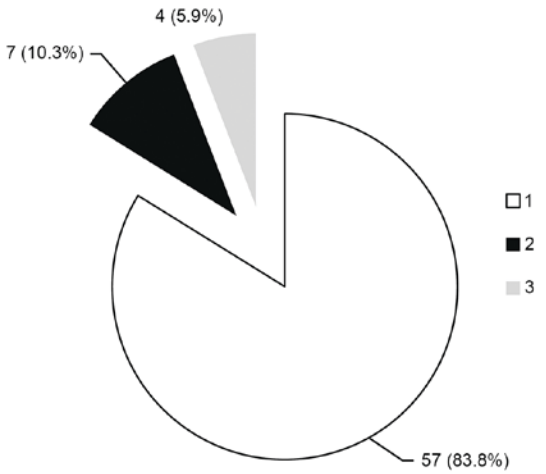

Figure 6. Distribution of torque teno virus genotypes based on open reading frame-1 phylogenetic analysis.

isolates (TA278, AB017610) (Fig. 7). Strains with high similarity were clustered together with significant bootstrap values. The phylogenetic tree demonstrated clear differences among the genotypes, and the sequences YOGHttv1 and YOGHttv63 were identical (Fig. 8).

Discussion

GBV-C, previously known as hepatitis G virus, was discovered in 1995 and is an enveloped single-stranded RNA positive-sense virus (9.4 kb) belonging to the *Flaviviridae* family. Following its discovery, multiple researchers have attempted to determine the properties of this virus and its association with diseases including hepatitis (24) and non-Hodgkin's lymphoma (25). However, no convincing evidence supporting an association between GBV-C infection and any disease exists. In fact, some previous studies have demonstrated beneficial effects of GBV-C in patients infected with HIV or HCV. GBV-C co-infection was associated with an improved prognosis and reduced mortality among HIV-infected patients. GBV-C RNA positivity was also associated with liver function improvement among HCV infected patients (26-30).

GBV-C is predominantly transmitted via the parenteral route. Thus, for epidemiological reasons, GBV-C is of particular interest in patients undergoing hemodialysis who are at risk of parenterally transmitted infection. Some previous studies have used GBV-C as a tool to detect patient-to-patient transmission in hemodialytic settings (31,32).

The prevalence of GBV-C infection is greater among patients undergoing hemodialysis compared with blood donors or healthy individuals in the same region. In several countries, the reported prevalence of GBV-C markers ranged from 0.2-24.6% in blood donors (33-35) and ranged from 3.9-26.5% in patients undergoing hemodialysis (1-5,36). In the present study, the overall prevalence of GBV-C infection was 57.1%, which is similar to the prevalence of 55% reported in Yogyakarta in 1996 (7). This suggests that the prevalence of GBV-C has not changed over the last decade. However, the prevalence was greater than that observed in Surabaya, Indonesia (6). The prevalence of GBV-C infection was greater

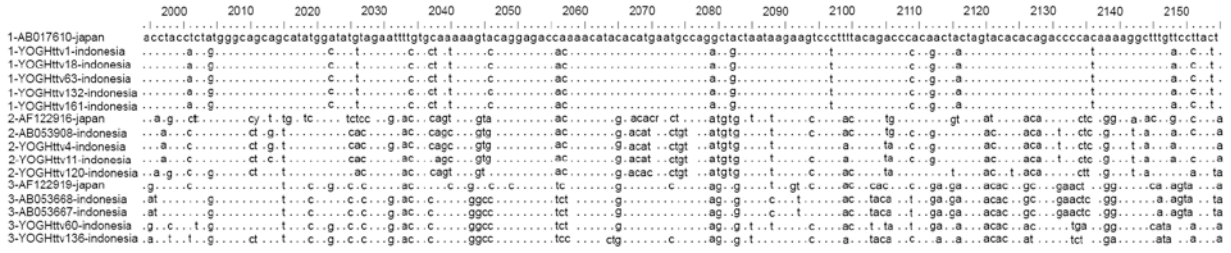

Figure 7. Alignment of the open reading frame-1 nucleotide sequences of 10 representative torque teno virus strains isolated in the present study (in bold font). The genotype, accession number or isolate name, and the country of origin are provided for each sequence. Dots indicate nucleotides identical to the reference sequence (AB017610). The nucleotide positions are based on the TA278 isolate (AB017610).

in Indonesian patients undergoing hemodialysis compared with blood donors, possibly due to the high prevalence in the general population, the lack of screening of GBV-C in blood banks, or patient-to-patient transmission.

Owing to its parenteral route of transmission, blood transfusions are hypothesized to be the main risk factor for GBV-C infection (37). Therefore, patients undergoing hemodialysis who commonly require blood transfusions are at increased risk of GBV-C infection. However, the results of previous studies that investigated the association between blood transfusion and GBV-C infection in patients undergoing hemodialysis are inconsistent. Certain studies have demonstrated that a history of blood transfusion or a history of multiple blood transfusions are the main risk factors for GBV-C infection (38), while other studies reported negative associations (1,35,39,40). The present study observed no association between the history of blood transfusion and GBV-C infection, possibly as a consequence of the limited sample size and the prevalence of other blood-borne infections, including HBV and HCV. Substantial proportions of GBV-C-negative patients were co-infected with HBV (17.4%) or HCV (79.7%), and this high prevalence of co-infection might mask clinically relevant associations. Further studies regarding patients infected with GBV-C only are required to address this issue.

Previous studies have demonstrated that a longer duration of hemodialysis is a risk factor for GBV-C infection (4), supporting the involvement of patient-to-patient transmission in the high prevalence of GBV-C infection within a hemodialysis unit. However, no association between the duration of hemodialysis treatment and GBV-C infection was observed in the present study, similar to earlier studies (3,5,38). This may be due to the high prevalence of co-infection with other viruses, particularly HCV. HCV co-infection was common in GBV-C-positive and GBV-C-negative patients. The history of GBV-C infection was not assessed using E2 antibodies in the present study; which only measured active GBV-C infection based on viral RNA amplification. Accordingly, the present study potentially underestimated the prevalence of GBV-C. The length of hemodialysis treatment was associated with HCV infection in a previous study regarding the same hemodialysis unit as the present study (17). The other risk factors analyzed in the present study were not correlated with GBV-C infection.

GBV-C and HCV have similar genomic structures and share the same mode of transmission. The prevalence of GBV-C is high in HCV-infected patients (41,42). This high prevalence of co-infection was demonstrated in the present study and in several earlier studies of patients undergoing hemodialysis (4,5) due to the shared mode of transmission. However, it is unclear whether these viruses are transmitted simultaneously or separately. By comparing the sequence alignment and phylogenetic analysis with the HCV sequences from a previous study (8), it was demonstrated that GBV-C and HCV generally infect patients at different times. Only one pair of strains isolated from two patients (no. 112 and 134) were identical in terms of the GBV-C 5'-UTR/E1 region and HCV NS5B sequences, suggesting simultaneous transmission of GBV-C and HCV from one patient to another patient (data not shown). These results indicate that GBV-C and HCV are transmitted independently.

GBV-C and HCV co-infection is not correlated with severity of hepatic disease progression (30,43), as demonstrated by the low or normal liver enzyme concentrations. The present study also demonstrated that GBV-C was not associated with hepatic pathogenic effects, because GBV-C viremia was not associated with ALT or GGT. In fact, previous studies have demonstrated a beneficial effect of GBV-C infection in HCV-infected patients (30,41).

GBV-C is distributed globally, and 7 genotypes have been identified to date (8). The genotypes are widespread, with distinct geographical distributions. Genotype 1 was first described in Africa, and the other genotypes were discovered in Europe (genotype 2), Japan (genotype 3), Southeast Asia (genotype 4), South Africa (genotype 5), Indonesia (genotype 6), and China (genotype 7) (8,44-49). Genotype 4 was reported to be dominant among Indonesian blood donors (55.5%), but genotype 6 was dominant in patients with chronic liver disease (60%) and patients undergoing hemodialysis (83.3%) (6). Thus, genotype 6 appears to be more common among hemodialysis patients compared with other populations. The present study provided data demonstrating that genotype 6 is the most common genotype among patients undergoing hemodialysis. The predominance of genotype 6 may reflect an outbreak of GBV-C infection from a common source. The present study also suggests the involvement of patient-to-patient transmission because several isolates were identical, including some displaying identical 5'-UTR and E1 region sequences. It is feasible that genotype 6 has adapted to be easily transmitted among patients with impaired immunity, including patients undergoing hemodialysis.

Genotype 6 was the predominant genotype observed, based on phylogenetic analyses of the 5'-UTR and E1 region. However, the results were inconsistent, owing to the different

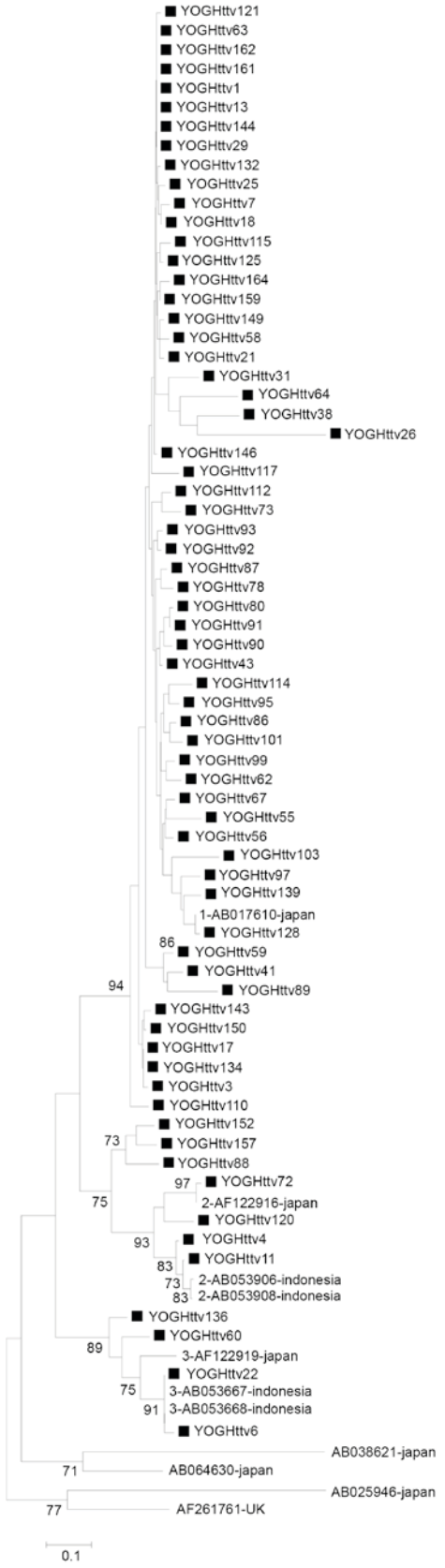

Figure 8. Phylogenetic tree analysis of the open reading frame-1 gene sequences of TTV strains isolated from patients undergoing hemodialysis. The reference sequences for different TTV genotypes were obtained from GenBank. The sequences determined in the present study were labeled with solid black squares and their isolate number, starting with YOGHgbvc. The GenBank TTV sequences are indicated with their genotype, accession number, and country of origin. Bootstrap values are provided at the internal nodes. TTV groups 2, 3, 4 and 5 (AF261761, AB025946, AB038621 and AB064630) were used as outer groups. TTV, torque teno virus.

proportions of each genotype classified by the 5'UTR or E1 region sequences (Fig. 1). A total of 20 isolates (23.3%) classified as genotype 6 based on phylogenetic analysis of the 5'-UTR were reclassified as genotype 4 based on phylogenetic analysis of the E1 region. In addition, 1 isolate classified into genotype 6 based on the 5'-UTR analysis was reclassified into genotype 3 based on the E1 region. However, analyses of the 5'-UTR and E1 region were consistent for genotype 2. There are some possible explanations for these results. For example, the 5'-UTR is more conserved than the E1 region, so analyses based on the E1 region show lower discrimination than analyses based on the 5'-UTR. The possibility of co-infection with ≥2 GBV-C genotypes might also be increased in patients undergoing hemodialysis due to frequent contact with contaminated blood or blood products. In addition, based on full genome analysis, Feng *et al* (8) proposed that GBV-C genotypes could be classified into 5 groups by combining genotypes 4, 6, and 7 into one group, due to their genetic similarities. Thus, genotypes 4 and 6 might represent a single genotype. Genotype 2 was previously revealed to be the most common GBV-C genotype in HIV-infected patients in Yogyakarta, Indonesia (9). This suggests that the difference in genotype distribution in this population is predominantly associated with transmission via drug injection.

TTV is a human non-enveloped single-stranded circular DNA virus, first described in 1997 by Nishizawa *et al* (50), and is a member of the *Anelloviridae* family. TTV infection was previously reported to be associated with a number of diseases, including hepatitis, based on epidemiological data (24,25,50,51). However, there is no strong evidence linking TTV infection to any specific disease. This virus is globally distributed and the prevalence of TTV infection is high in various populations, including patients with liver diseases, patients with HIV, drug users and healthy individuals (51-54). In the present study, the prevalence of TTV infection among patients undergoing hemodialysis, a high-risk population, was determined.

The overall prevalence of TTV infection, based on the 5'-UTR and ORF1 sequences, was 100%. A previous nationwide study of TTV infection in Indonesian healthy individuals revealed a prevalence of 95% (15). Thus, the prevalence of TTV infection among patients undergoing hemodialysis patients in the present study was marginally greater than that observed in the general population. However, on Java Island, where Yogyakarta is located, TTV was detected based on the 5'-UTR in 100% of healthy individuals (15). TTV was detected by amplification of the ORF1 in 68 (42.2%) patients, which was similar to a previous study where the prevalence was reported to be 42% in healthy individuals (15). This suggests that the prevalence of TTV is similarly high among hemodialysis patients and healthy individuals. As with GBV-C, the high prevalence of TTV infection was not associated with any of the risk factors analyzed in the present study. Furthermore, TTV was not associated with hepatic injury.

The genotypic distribution of TTV in the present study was similar to that in healthy individuals. In healthy individuals, genotype 1 was predominant (53%), followed by genotypes 3 (28%) and 2 (18%). These genotypes belong to TTV group 1. TTV group 2 was detected in <2% of subjects (15). In the

present study, genotype 1 was predominant (83.8%), followed by genotypes 2 (10.3%) and 3 (5.9%). These data suggest that the increase in the prevalence of genotype 1 may be due to patient-to-patient transmission. However, it is also possible that the patients were infected with TTV prior to starting hemodialysis, owing to the very high prevalence of TTV in the general population. This hypothesis is supported by a study of sex workers in Papua, Indonesia, which identified that the genotypic distribution of TTV reflected the birth place of the subjects rather than their work environment, and that the infection was more likely to occur during the early period of life rather than via sexual transmission (16). There was substantial genetic diversity of TTV in the present study and only one pair of sequences was identical, suggesting nosocomial infection was unlikely to be responsible for the high prevalence of TTV among patients undergoing hemodialysis.

Although the prevalence rates of GBV-C and TTV infection were high in the present study, screening for GBV-C and TTV infection is not mandatory. GBV-C and TTV do not appear to have any pathogenic properties and do not appear to cause liver disease or other clinical disorders. Furthermore, GBV-C may have beneficial effects in patients co-infected with HCV or HIV. Screening programs are mandatory for other blood-borne viruses, particularly HBV, HCV and HIV. It has previously been reported that the high prevalence of HBV and HCV infection in patients undergoing hemodialysis were associated with nosocomial infection, owing to the failure of hemodialysis units to adhere to strict infection-control procedures (17). Strict adherence to infection-control procedures prevents cross-infection of blood-borne viruses between patients (32).

In conclusion, prevalence rates of GBV-C and TTV infection are high among hemodialysis patients in Yogyakarta, Indonesia. Nosocomial transmission may be involved in infection due to inconsistent implementation of infection-control procedures within hemodialysis units. Hemodialysis units in Indonesia should implement strict infection-control procedures designed to prevent the transmission of blood-borne viruses.

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