Three different GB virus C/hepatitis G virus genotypes

Phylogenetic analysis and a genotyping assay based on restriction fragment length polymorphism

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Abstract The 5′-untranslated region (5′-UTR) sequences of 33 GB virus C/hepatitis G virus (GBV-C/HGV) obtained from different geographic areas were determined through reverse-transcription polymerase chain reaction and dideoxy chain termination sequencing, the alignment of sequences, the estimation of the number of nucleotide substitution per site, and construction of phylogenetic trees. The 5′-UTR of GBV-HGV was found to be heterogeneous, with 70.9–99.5% homology. Three distinct phylogenetic branches were observed consistently in all phylogenetic trees. GBV-C is the prototype for one, HGV for another, and there is a new branch which consisted of GBV-C/HGV isolates from Asia. Genotype-specific restriction sites for the restriction enzymes, *ScrFI* and *BsmFI*, were identified, and a simple restriction fragment polymorphism analysis was developed for genotyping. These data provide evidence that GBV-C/HGV consists of three different genotypes. Our simple genotyping assay will also provide a tool for epidemiological studies of GBV-C/HGV infection.

Key words: Hepatitis G virus; GB virus-C; Genotype; 5′-UTR; Phylogenetic tree; Restriction fragment length polymorphism; Reverse-transcription polymerase chain reaction

1. Introduction

Recently, two research groups independently discovered a new virus associated with non-A-E hepatitis which they named as GB virus C (GBV-C) [1] and hepatitis G virus (HGV) [2]. Studies in blood product recipients have demonstrated the transmissibility of GBV-C/HGV [2]. Based on clinical and epidemiological profiles, these viruses are believed to be transmitted through parenteral route, including blood and its transfusion products, and sharing of needles among intravenous drug users [3,4]. Comparison of the nucleotide and the deduced amino acid sequences showed good homology between these viruses. In addition, the putative genomic organization also appears to be similar. Hence, they are probably isolates from the same group of viruses. For simplicity, they are referred to as GBV-C/HGV in this paper, as proposed by Dr. Zuckerman [5].

The presence of a GDD domain in the putative NS5B region, together with the similar genomic organization of this putative gene and other flaviviruses, suggests that the GBV-C/HGV *NS5B* gene encodes for a RNA-dependent RNA polymerase. This type of viral enzyme is known to have no proofreading activities during the replication of RNA genome and hence, the GBV-C/HGV genome is expected to be heterogeneous. Recently researchers from Abbott reported independently that the GBV-C/HGV isolates consisted of five genotypes based on the 5′-UTR [6]. However, it is no report on GBV-C/HGV genotyping confirmed with different evolutionary strategies and on simple methods for genotyping of this virus.

In the present study, we have surveyed a large number of GBV-C/HGV samples worldwide and identified 33 GBV-C/HGV RNA positive samples. We have sequenced the putative 5′-UTR of these isolates and determined the phylogenetic relations between these isolates with three different strategies, which suggests the existence of three different genotypes. A restriction fragment length polymorphism (RFLP) for the determination of these genotypes was then designed.

2. Materials and methods

2.1. Serum samples

Thirty-three serum samples positive for GBV-C/HGV RNA as determined by our RT-PCR assays based on the 5′-UTR of GBV-C/HGV were available for this study. These serum samples were collected from patients from Cameroon, Israel, Japan, Korea, Mongolia, Saudi Arabia and the USA.

2.2. RNA extraction and CDNA synthesis

RNA was extracted from 100 μl of serum using a SepaGene-RVR (Sanko, Tokyo, Japan). Extracted RNA was precipitated with 200 μl of isopropanol at room temperature for 5 min, pelleted down by

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centrifugation (12000 rpm, for 15 min, at 4°C), washed twice with 70% (v/v) ethanol, and air-dried. cDNA was then synthesized by incubating in a 25 μl reaction mixture containing the extracted RNA, 300 ng of random hexamer (Life Technologies, Gibco BRL, Gaithersburg, MD), 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 200 μmol/l deoxyribonucleotide triphosphate, 3 U/μl RNasin (Promega, Madison, WI) and 250 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) and incubated at 37°C for 60 min.

2.3. PCR conditions

One-tenth (2.5 μl) of the synthesized cDNA was added to following amplification mixture, 5 μl of 10×AmpliTaq polymerase buffer (100 mmol/l Tris-HCl at 8.4, 100 mmol/l KCl, 15 mmol/l $MgCl_2$, 0.1% gelatin), 2.5 μl of 10 mmol/l deoxyribonucleotide triphosphate, 0.5 μl of AmpliTaq Gold (5 U/μl) (Perkin-Elmer Cetus, Norwalk, CT). 10 pmols of sense primer (5gf2; 5′-GGTTGGTAGGTCGTAAATCCCGGTCA-3′) and antisense primer (5gf6; 5′-GACATTGAAGGGCGACGTRGACCGTAC-3′; where R represent a mixture of A and G) in a 50 μl reaction volume. The amplification profile was 9 min at 96°C, followed by 40 cycles of 94°C for 60 s (denaturation), 60 s at 55°C (annealing), and 60 s at 72°C (extension), using a 96-well cycler (GeneAmp 9600, Perkin-Elmer Cetus). One microliter of the first-round PCR product was then added to a second-round PCR mixture with the same composition but with the set of inner primers (5gf3; sense, TGGTAGCCACTATAGGTGGGT-3′; (5gf4, sense, 5′-GCGACGTGGACCGTACRTGGGCGT-3′). Five microliters of the second-round PCR products was then analyzed by electrophoresis on a 3% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

The PCR primers were designed based on the most conserved regions derived from the known sequences available in GenBank. These primers were confirmed to be specific for GBV-C/HGV through computer search. The primers were prepared with a DNA synthesizer (Model 394, Perkin-Elmer Cetus). Standards precautions for avoiding contamination for PCR were observed. A negative control serum were also included in each run to ensure specificity.

2.4. Cloning and sequencing of the PCR products

All the PCR products were cloned into the pGEMT vector (Promega, Madison, WI). Positive clones were identified, expanded, and sequenced bidirectionally using the dideoxynucleotide chain termination method (Dye Terminator Cycle Sequencing FS Ready Reaction Kit, Perkin-Elmer Cetus). The sequences were obtained with a DNA sequencer (ABI PRISM 377 DNA Sequencer, Perkin-Elmer Cetus).

2.5. Molecular evolutionary analysis

Molecular evolutionary analysis was performed to elucidate the molecular evolutionary relationship of the isolates and the previously reported GBV-C and HGV strains. The analyses were performed with the computer program ODEN version 1.1.1 [7]. The number of nucleotide substitutions per site between all possible pairs of these isolates was estimated by the 6-parameter method [8]. Based on these values, phylogenetic trees were constructed by both the unweighed pair grouping method with arithmetic means (UPGMA) [9], and the neighbor-joining (NJ) methods [10]. For more confirmatory evidence, we have also employed the PHYLIP version 3.5 computer program [11] to determine the evolutionary distance. With this program, the genetic distances between pairs of nucleotide sequences were estimated using the DNADIDT program with Maximum Likelihood (ML), and the phylogenetic tree was constructed using the programs NEIGHBOR and DRAWTREE.

The statistical reliability of the phylogenetic trees were assessed using the bootstrap method by Felsenstein based on 100 replicates [12].

2.6. RFLP analysis

RFLP analysis was performed after the genotype-specific restriction sites were identified. Restriction digestions were carried out with 10 μl of the second PCR products for 3 h after adjustment with 10×enzyme reaction buffer according to the manufacturer's recommendations. Reactions were carried out with 10 U of either *ScrFI* (New England BioLabs, MA) at 37°C, or *BsmFI* (New England BioLabs) at 65°C. The digested PCR products were electrophoresed on 3.0% Nusieve GTG (3:1) agarose gel in 1×TBE buffer (134 mmol/l-Tris-HCl, 68 mmol/l boric acid; 2.5 mmol/l EDTA) containing 500 ng ethidium bromide per milliliter. The RFLP pattern was then evaluated under ultraviolet light.

3. Results

The nucleotide sequences of 5′-UTR obtained from all 33 isolates were aligned together with GBV-C which was from a patients from West Africa (accession No. U36380), and HGV from which was from a US patient (accession No. U44402). Phylogenetic trees were then constructed based on all the three well-accepted strategies, UPGMA (Fig. 1a), NJ (Fig. 1b), and PHYLIP (Fig. 1c) methods. Phylogenetic trees of 5′-UTR of GBV-C/HGV genome showed identical phylogenetic relationship based on all three methods. In all trees, the isolates were segregated clearly into three separate phylogenetic branches (or genotypes), which were tentatively named as GB type including GBV-C strain, HG type including HGV strain, and a new type not reported before.

With bootstrap analysis for evaluation of the statistical reliability of the tree, the cluster of the GB, HG and new types had 99%, 95% and 70% of reliability, respectively.

There was good homology between the isolates within a type, 87.4–98.4% for the GB type, 88.0–98.9% for the HG type, and 94.0–99.5% for the new type (Table 1). The homology between different types was significantly lower, with 70.9–84.1% between GBV and HG types, 83.1–91.3% between HGV and the new types, and 79.1–89.0% between the new and GB types (Table 1).

GB type consisted of isolates Af-107, Af-13, Af-26, Af-29, Af-33, Af-42, Af-47, Af-53, Af-87, Af-96, Sa-77 and GBV-C (Table 1 and Figs. 1 and 2A). These were from patients from the African continent (Af for Africa, and Sa for Saudi Arabia). HG type consisted of isolates USA-r11, USA-r119, Sa-97, USA-r90, Sa-29, Ja-S1, USA-r131, USA-r28, Mo-287, Ja-S7, Ja-S4 and HGV (Table 1 and Figs. 1 and 2B). These isolates were from various geographic regions (USA for United States of America, Mo for Mongolia, Ja for Japan). The new type consisted of the isolates Ja-12, Sa-43, Mo-331, Ja-KI, Ja-SN, Mo-345, Mo-351, Is-21, Is-28, Mo-186, and Kor-63 (Table 1 and Figs. 1 and 2C). These isolates were also from patients from various geographic locations (Kor for Korea, Is for Israel).

In order to design a simple genotyping assay, an attempt was made to identify specific restriction sites that are unique for each genotypes. Two genotype-specific restriction sequences (*ScrFI* and *BsmFI*) were identified (Fig. 2). Fig. 3 shows the restriction digestion pattern and Fig. 4 shows the schematic diagram for the PCR and restriction digestion pattern. For the GB type, the specific restriction enzyme sites for *ScrFI*

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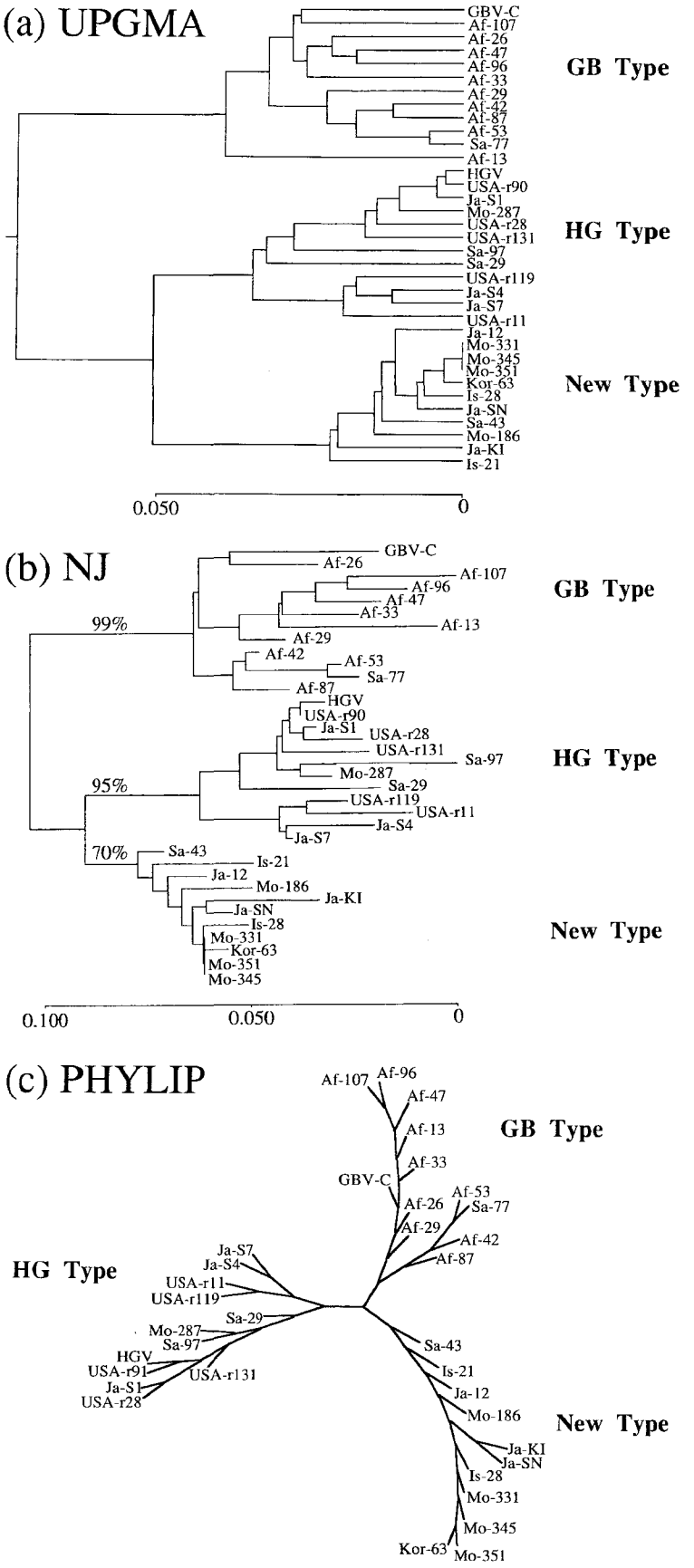
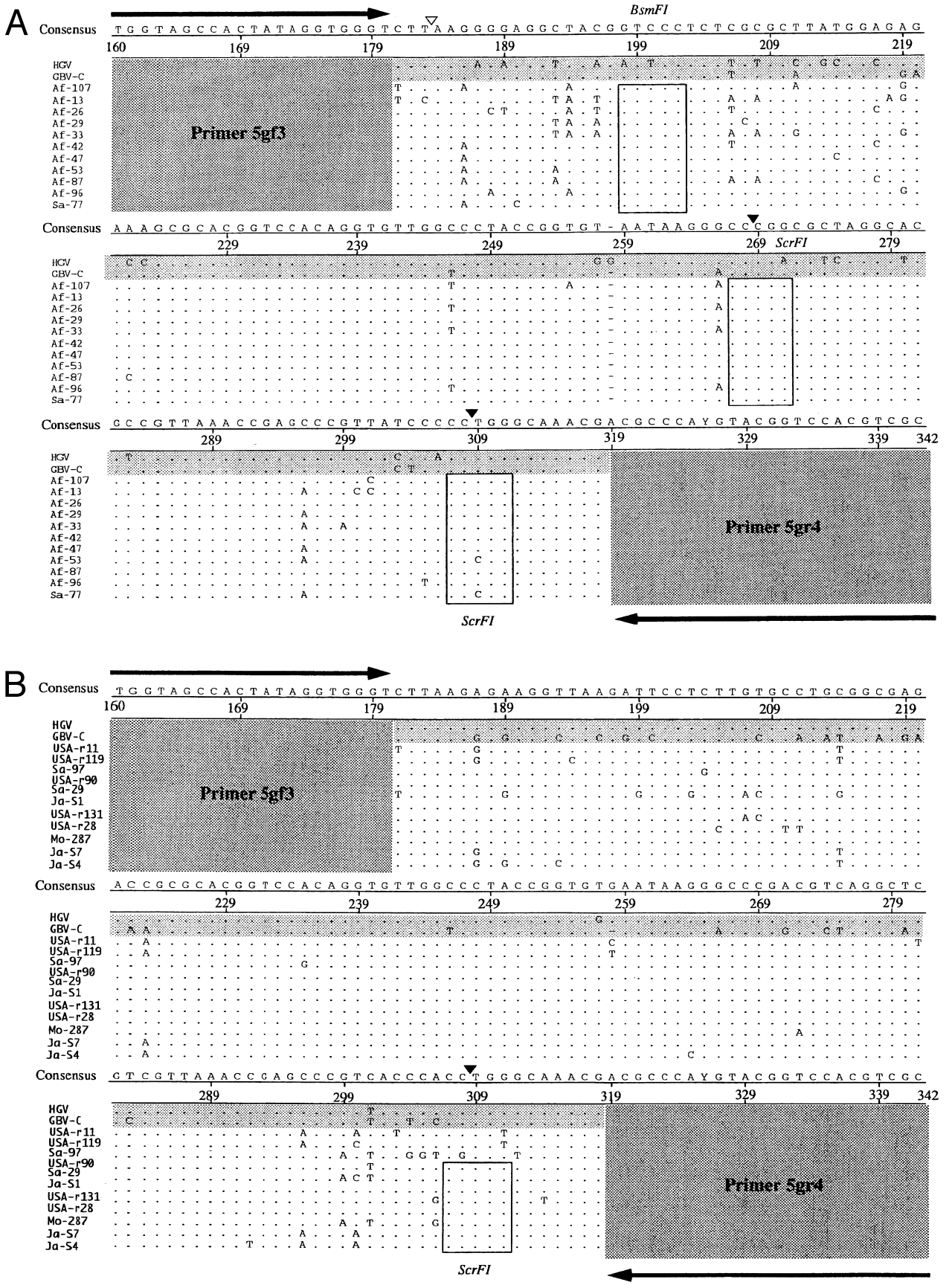
Fig. 1. Phylogenetic trees for the GBV-C/HGV 5′-untranslated region (5′-UTR) as determined by (a) unweighed pair grouping method with arithmetic means (UPGMA), (b) neighbor-joining (NJ), and (c) PHYLIP program. Note that the isolates were segregated into the same branches based on all three methods. To confirm the statistical reliability of the tree obtained, the bootstrap analysis was performed with the method by Felsenstein based on 100 replicates (b). The three major branches had 99%, 95% and 70% reliability, respectively.


(a) UPGMA
GBV-C
Af-107
Af-26
Af-47
Af-96
Af-33
Af-29
Af-42
Af-87
Af-53
Sa-77
Af-13
HGV
USA-r90
Ja-S1
Mo-287
USA-r28
USA-r131
Sa-97
Sa-29
USA-r119
Ja-S4
Ja-S7
USA-r11
Ja-12
Mo-331
Mo-345
Mo-351
Kor-63
Is-28
Ja-SN
Sa-43
Mo-186
Ja-KI
Is-21
GB Type
HG Type
New Type
0.050
0
(b) NJ
GBV-C
Af-26
Af-107
Af-96
Af-47
Af-33
Af-13
99%
Af-29
Af-42
Af-53
Sa-77
Af-87
HGV
USA-r90
Ja-S1
USA-r28
USA-r131
Sa-97
Mo-287
95%
Sa-29
USA-r119
USA-r11
Ja-S4
Ja-S7
70%
Sa-43
Is-21
Ja-12
Mo-186
Ja-KI
Ja-SN
Is-28
Mo-331
Kor-63
Mo-351
Mo-345
GB Type
HG Type
New Type
0.100
0.050
0
(c) PHYLIP
Af-107
Af-96
Af-47
Af-13
Af-33
GBV-C
Af-26
Af-53
Sa-77
Af-29
Af-42
Af-87
Ja-S7
Ja-S4
USA-r11
USA-r119
Sa-29
Mo-287
Sa-97
HGV
USA-r90
Ja-S1
USA-r28
USA-r131
Sa-43
Is-21
Ja-12
Mo-186
Ja-KI
Ja-SN
Is-28
Mo-331
Mo-345
Kor-63
Mo-351
GB Type
HG Type
New Type


A
BsmFI
Consensus
Primer 5gf3
ScrFI
Primer 5gr4
B
Primer 5gf3
Primer 5gr4

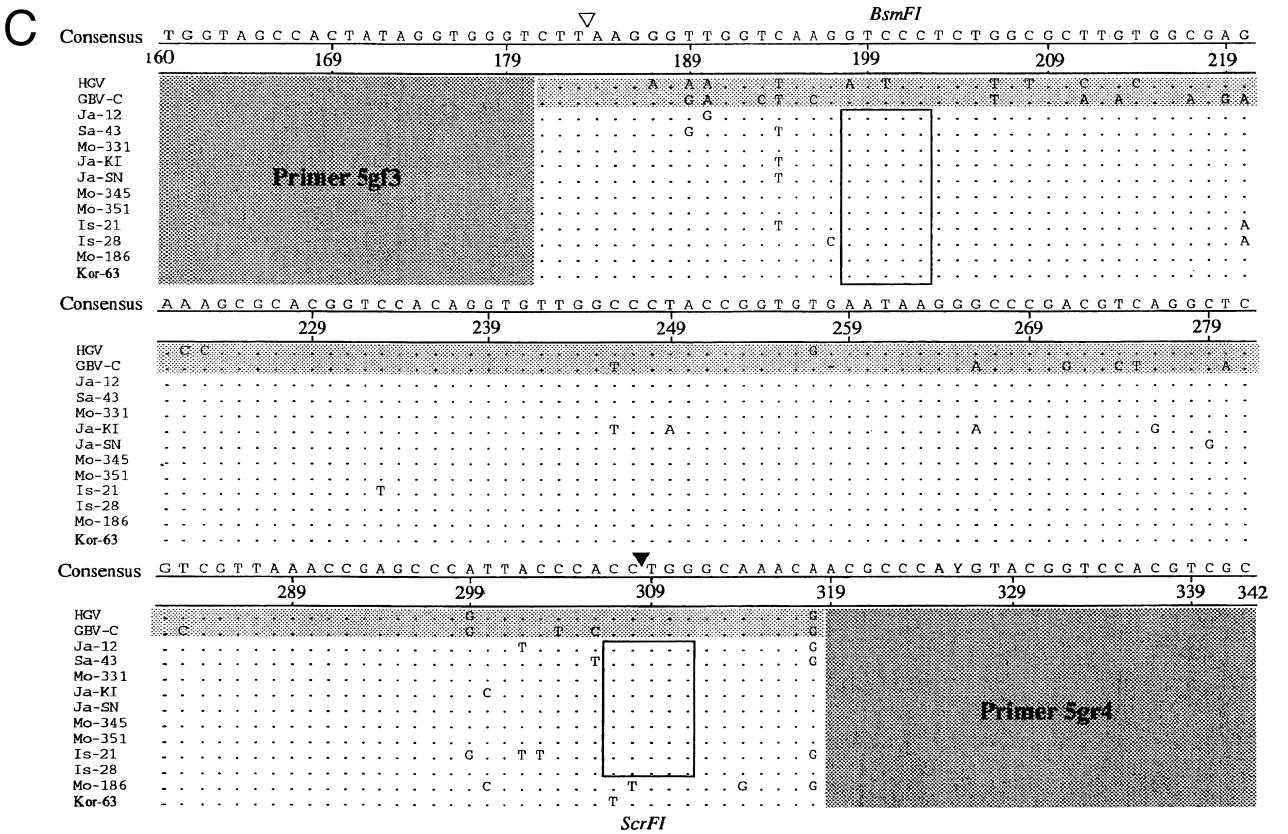


Fig. 2. A: Alignment of the 5′-UTR sequences of the GB type isolate. These nucleotide sequences were derived from African and Saudi Arabian isolates. B: Alignment of the 5′-UTR sequences of the HG type isolates. These nucleotide sequences were from patients from USA, Saudi Arabia, Japan, and Mongolia. C: Alignment of the 5′-UTR sequences of the new type of GBV-C/HGV isolates. These nucleotide sequences were from patients from Japan, Saudi Arabia, Mongolia, Israel and Korea. For all figures, the prototypes GBV-C (Accession No. U36380) and HGV (Accession No. HGU44402) were also included for comparison. Identical nucleotide are shown as a dot. The consensus nucleotide sequences of these isolates for each type are given in the top line. The location of the inner primers are given. The sequences that recognized by the restriction enzymes *ScrFI* and *BsmFI* are boxed. ▼: Cutting site of *ScrFI*, ▽: Cutting site of *BsmFI*. Af, Africa; Sa, Saudi Arabia; USA, United States; Ja, Japan; Mo, Mongolia; Is, Israel; Kor, Korea.

were found at positions 268 and 308 and that for *BsmFI* was found at position 184 in the amplified region for all isolates within this genotype (Figs. 2–4). For the HG type, some strains (e.g., USA-r90, Sa-29, Ja-S1, USA-r131, USA-r28, Mo-287, Ja-S7, Ja-S4, HGV and GBV-C) had a restriction site for *ScrFI* at position 309 while in other isolates (e.g., USA-r11, USA-r119, Sa-97), this restriction site was absent. There was no restriction site for *BsmFI* in all isolates in the HG type (Fig. 2B and Figs. 3 and 4). For the new type, a large proportion of the isolates (e.g., Ja-12, Sa-43, Mo-331, Ja-KI, Ja-SN, Mo-345, Mo-351, Is-21, Is-28, HGV and GBV-C) had a restriction site for *ScrFI* at position 309 in the amplified region while in two isolates (Mo-186 and Kor-63), this restriction site was absent. However, the *BsmFI* restriction site was found at position 184 for all isolates of this new type (Fig. 2C and Figs. 3 and 4). Hence, the pattern of restriction enzyme cutting by *ScrFI* and *BsmFI* allowed the assignment of specific genotypes in these 33 GBV-C/HGV isolates.

4. Discussion

This study showed three important points. First, the 5′-UTR sequence of GBV-C/HGV, though relatively well conserved, is still heterogeneous. Second, phylogenetic trees showed that the isolates clearly segregated into three separate branches, suggesting the presence of three different genotypes. Third, there are genotype-specific restriction sites that may be utilized for the establishment of a simple RFLP-based genotyping assay.

The 5′-UTR sequences for most flaviviruses and pestiviruses, to which GBV-C/HGV is related, are relatively well conserved. This is attributed to the fact that this region is important for both replication and viral protein translation. Recently, an internal ribosomal site [13] has been reported in GBV-C. The predicted secondary, as well as the tertiary and quarternary structures, are likely to be important for viral replication/translation. This poses another constraint to the variability of the nucleotide sequence in this region. However, despite these constraints, this genomic region is still heterogeneous. It is interesting to note that some of the nucleotide substitution are constant for some isolates, suggesting that these isolates are phylogenetically close to each other.

This deduction was confirmed by the phylogenetic trees of the isolates reported here. The isolates, which were collected from six countries in four continents (Africa, America, Europe, and Asia), were found to form three distinct phylogenetic branches. These three major clusters were obtained by three different strategies of molecular phylogenetic analysis and were confirmed with high reliability by bootstrap analysis.

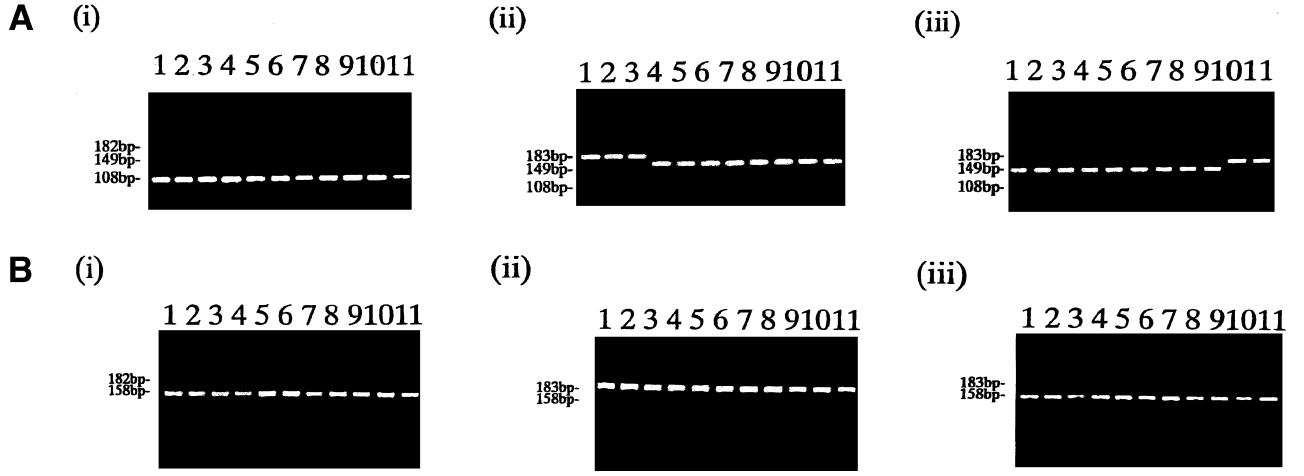


Fig. 3. A: RFLP pattern after *ScrFI* digestion of the 5′-UTR PCR products. (i) GB type of isolates: lanes 1–11, Af-107, Af-13, Af-26, Af-29, Af-33, Af-42, Af-47, Af-53, Af-87, Af-96, Sa-77, respectively. (ii) HG type of isolates: lanes 1–11, USA-r11, USA-r119, Sa-97, USA-r90, Sa-29, Ja-S1, USA-r131, USA-r28, Mo-287, Ja-S7, Ja-S4, respectively. (iii) New type of isolates: lanes 1–11, Ja-12, Sa-43, Mo-331, Ja-KI, Ja-SN, Mo-345, Mo-351, Is-21, Is-28, Mo-186, Kor-63, respectively. B: RFLP pattern after *BsmFI* digestion of the 5′-UTR PCR products. (i) GB type of isolates: same lane order as in (A.i). (ii) HG type of isolates: same lane pattern as in (A.ii). (iii) New type of isolates: same lane pattern as in (A.iii).

Interestingly, it is observed that the distribution of GBV-C/HGV genotypes depends on their geographical distribution. The GB type mainly includes the African isolates with the prototype of GBV-C. The HG type consists of the isolates from the USA, Europe and Asia. The new type consists of the isolates from Asian countries.

There are two issues that need to be addressed. First, this classification needs to be confirmed by studying other parts of the viral genome. Studies on HCV genetic heterogeneity have shown that nearly all HCV isolates segregated together in the same phylogenetic branch [14,15] for all genomic regions studied so far (5′-UTR, core, E1, and NS5). To be scientifically certain, we believe that a similar approach, i.e., to confirm this phylogenetic relationship with other genomic regions, is important for definitive conclusions. The small number of GBV-C/HGV sequences, and the genetic heteroge-

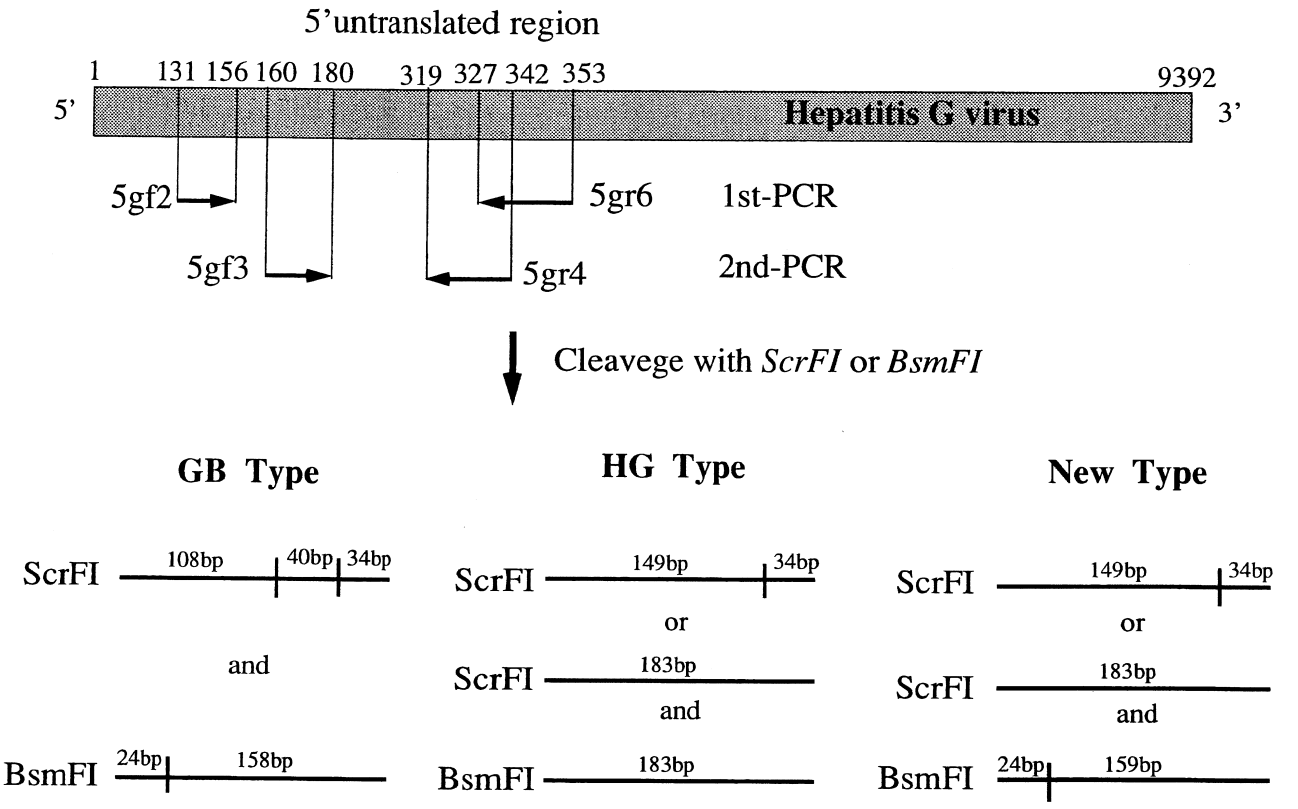


Fig. 4. Schematic representation of the strategy for PCR amplification with nested primer pairs and the RFLP pattern. Comparison of *ScrFI* and *BsmFI* RFLP pattern of the 5′-UTR PCR products from different genotypes is also given.

Table 1
Pair distances of nucleotide sequence alignment of the 5′-untranslated region of 35 GBV-C/HGV isolates

			New Type											HG Type												GB Type										Control	
			35	34	33	32	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Control	HGV	1	89.1	88.5	88.0	88.5	89.6	89.6	89.6	87.4	89.6	90.2	89.6	92.9	95.6	97.3	96.7	96.2	98.4	94.0	98.9	94.0	94.0	92.3	79.7	77.5	82.4	80.2	81.3	83.0	79.7	82.4	81.3	76.4	75.3	79.1	
	GBV-C	2	81.9	80.8	82.4	83.5	82.4	82.4	83.0	83.0	82.4	84.6	81.9	79.7	80.2	78.0	77.5	78.6	79.1	78.6	79.7	76.4	78.6	76.9	92.9	95.1	90.7	92.9	93.4	94.0	92.3	91.8	92.9	87.4	94.0		
	Af-107	3	79.7	79.1	79.1	81.3	80.2	80.2	79.7	80.2	80.2	81.3	80.8	78.0	78.6	74.2	75.8	77.5	77.5	76.9	76.4	70.9	78.0	78.0	93.4	95.1	91.2	93.4	94.0	93.4	92.3	92.9	92.3	91.8			
	Af-13	4	80.8	80.2	80.2	81.9	81.3	81.3	80.8	79.1	81.3	82.4	81.9	79.7	80.8	75.3	77.5	77.5	78.0	78.0	76.9	72.0	80.8	80.8	90.1	90.7	91.2	90.7	90.7	90.1	93.4	93.4	90.1				
	Af-26	5	85.2	84.1	84.6	85.7	85.7	85.7	85.2	85.2	85.7	86.3	85.7	80.2	81.3	80.2	80.2	80.2	80.8	79.7	81.9	77.5	80.8	80.2	91.8	95.1	91.8	91.8	92.3	94.5	92.3	94.0					
	Af-29	6	86.8	85.7	86.3	87.4	87.4	87.4	86.8	84.6	87.4	88.5	87.4	83.0	84.1	81.3	81.3	81.9	81.9	82.4	83.0	78.0	83.5	83.0	95.1	94.5	93.4	95.6	95.6	94.5	95.1						
	Af-33	7	83.5	82.4	83.0	83.0	84.1	84.1	83.5	83.5	84.1	85.2	84.1	79.7	81.3	79.7	76.9	78.0	78.6	80.2	80.2	76.4	80.8	80.2	91.2	94.0	91.8	91.8	91.8	90.7							
GB Type	Af-42	8	86.3	85.2	85.7	87.9	86.8	86.8	87.4	85.2	86.8	89.0	86.8	83.5	83.5	81.9	81.9	81.9	82.4	82.4	83.5	79.1	81.9	82.4	96.7	93.4	96.7	96.7	97.3								
	Af-47	9	84.6	83.5	84.1	86.3	85.2	85.2	85.7	83.5	85.2	87.4	85.2	81.9	81.9	80.2	80.2	80.8	80.8	80.8	81.9	76.9	80.2	80.8	97.8	94.0	95.1	97.8									
	Af-53	10	84.1	83.5	84.1	86.3	85.2	85.2	85.7	83.5	85.2	87.4	85.2	81.3	81.9	79.1	79.1	79.7	79.7	80.2	80.8	75.8	79.7	80.2	98.4	93.4	95.6										
	Af-87	11	84.6	83.5	83.5	85.7	85.2	85.2	85.7	83.5	85.2	87.4	85.2	82.4	83.0	81.3	81.3	81.3	81.9	81.9	83.0	78.0	81.3	81.9	94.5	91.2											
	Af-96	12	86.9	81.3	81.9	83.5	83.0	83.0	82.4	82.4	83.0	83.5	83.0	76.9	77.5	76.4	76.4	76.9	76.9	76.9	78.0	74.2	76.9	76.9	92.9												
	Sa-77	13	84.1	83.5	84.1	86.3	85.2	85.2	85.7	83.5	85.2	87.4	85.2	81.3	81.3	78.6	78.6	79.1	79.1	79.7	80.2	75.3	79.1	79.7													
	USA-r11	14	86.9	86.9	85.8	88.0	87.4	87.4	86.9	85.8	87.4	88.0	88.0	94.5	96.7	91.3	92.3	91.8	94.0	89.6	92.9	88.0	96.2														
	USA-r119	15	89.6	89.6	88.5	88.0	90.2	90.2	89.1	87.4	90.2	89.6	90.2	95.1	97.3	92.9	93.4	92.9	95.1	90.2	94.5	89.6															
	Sa-97	16	85.8	84.7	84.2	83.1	85.8	85.8	85.8	83.6	85.8	88.0	85.2	88.5	91.3	94.5	91.8	91.3	93.4	90.7	94.5																
	USA-r90	17	89.6	89.1	88.5	89.5	90.2	90.2	90.2	88.0	90.2	90.7	90.2	93.4	96.2	97.8	97.3	96.7	98.9	94.5																	
	Sa-29	18	89.1	89.6	88.0	86.9	89.6	89.6	89.6	88.5	89.6	91.3	89.1	91.3	91.8	94.0	90.7	94.0	94.0																		
HG Type	Ja-S1	19	88.5	88.5	87.4	88.5	89.1	89.1	89.1	87.4	89.1	89.6	89.1	94.0	96.7	96.7	97.8	97.3																			
	USA-r131	20	88.0	88.0	86.9	88.0	88.5	88.5	88.5	86.9	88.5	89.6	88.5	91.8	94.5	95.6	94.5																				
	USA-r28	21	87.4	87.4	86.3	87.4	88.0	88.0	88.0	86.3	88.0	88.5	88.0	92.3	95.1	95.1																					
	Mo-287	22	89.1	88.0	88.0	87.4	89.6	89.6	89.6	87.4	89.6	90.7	89.6	91.8	94.5																						
	Ja-S7	23	90.2	90.2	89.1	89.6	90.7	90.7	90.7	89.1	90.7	91.3	90.7	97.3																							
	Ja-S4	24	88.5	88.5	87.4	88.0	89.1	89.1	89.1	86.9	89.1	90.2	89.1																								
	Ja-12	25	97.3	96.2	96.7	96.2	97.8	97.8	96.7	94.0	97.8	96.7																									
	Sa-43	26	96.7	95.6	96.2	95.6	97.3	97.3	97.3	95.1	97.3																										
	Mo-331	27	98.9	97.3	98.4	85.6	99.5	99.5	98.4	96.2																											
	Ja-KI	28	95.6	95.1	95.1	92.9	96.2	96.2	96.2																												
	Ja-SN	29	97.8	96.2	97.3	95.6	98.4	98.4																													
New Type	Mo-345	30	98.9	97.3	98.4	95.6	99.5																														
	Mo-351	31	98.9	97.3	98.4	95.6																															
	Is-21	32	95.1	94.0	95.6																																
	Is-28	33	97.8	96.2																																	
	Mo-186	34	96.7																																		
	Kor-63	35																																			

neity of GBV-C/HGV, makes this a difficult task. However, this preliminary data serves as the backbone for further comparison. Second, despite the distinct segregation of the GBV-C/HGV isolates into three separate branches, the diversity of isolates from different branches was still in the range of 8.7–29.1%. Even when the full genome of GBV-C and HGV were compared, the diversity was only around 16%. As GBV-C/HGV is phylogenetically close to HCV [5], the order of classification of HCV genotypes has to be taken into consideration. It appears that the separation of the branches is in the order of subtype difference in the HCV classification. Hence, we are reluctant to call these three branches as representing three major types (including assigning them numerical numbers) since this may imply a similar level of diversity as in HCV. The exact naming of these genotypes awaits further discussion between various research groups for a consensus.

So far, to identify genotypes of GBV-C/HGV, the determination of RNA sequence of each isolates was needed. However, we have developed a simple genotyping method. Using two restriction enzymes (*ScrFI* and *BsmFI*), which recognize genotype-specific site(s) in the product by PCR, three major genotypes are able to be identified by RFLP easily. With our approach, all 33 isolates will be assigned according to the phylogenetic branch. Certainly, this assay will need to be confirmed in prospective studies with GBV-C/HGV isolates from various geographical locations. If this approach is confirmed to be useful in larger studies, this simple method will be useful for the epidemiological survey of GBV-C/HGV. It may also be useful for studying transmission of this virus in selected cases.

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