JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 201–207 Vol. 35, No. 1
0095-1137/97/$04.00+0

New Hepatitis C Virus (HCV) Genotyping System That Allows for Identification of HCV Genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a

TOMOYOSHI OHNO,[1,2]* MASASHI MIZOKAMI,[3] RONG-RONG WU,[3] MOHAMED G. SALEH,[4] KEN-ICHI OHBA,[3] ETSURO ORITO,[3] MOTOKAZU MUKAIDE,[5] ROGER WILLIAMS,[4] AND JOHNSON Y. N. LAU[2]

Department of Public Health[1] and Second Department of Medicine,[3] Nagoya City University Medical School, Kawasumi, Mizuho, Nagoya 467, and Center for Molecular Biology and Cytogenetics, SRL, Inc., Hachioji, Tokyo 192,[5] Japan; Section of Hepatobiliary Diseases, Department of Medicine, University of Florida, Gainesville, Florida[2]; and Institute of Liver Studies, King's College Hospital, London, United Kingdom[4]

Received 13 May 1996/Returned for modification 26 August 1996/Accepted 30 September 1996

Recent studies have focused on whether different hepatitis C virus (HCV) genotypes are associated with different profiles of pathogenicity, infectivity, and response to antiviral therapy. The establishment of a simple and precise genotyping system for HCV is essential to address these issues. A new genotyping system based on PCR of the core region with genotype-specific PCR primers for the determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a was developed. A total of 607 samples (379 from Japan, 63 from the United States, 53 from Korea, 35 from Taiwan, 32 from China, 20 from Hong Kong, 15 from Australia, 6 from Egypt, 3 from Bangladesh, and 1 from South Africa) were tested by both the assay of Okamoto et al. (H. Okamoto, Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyamura, and M. Mayumi, J. Gen. Virol. 73:673–679, 1992) and this new genotyping system. Comparison of the results showed concordant results for 539 samples (88.8%). Of the 68 samples with discordant results, the nucleotide sequences of the HCV isolates were determined in 23, and their genotypes were determined by molecular evolutionary analysis. In all 23 samples, the assignment of genotype by our new genotyping system was correct. This genotyping system may be useful for large-scale determination of HCV genotypes in clinical studies.

Serological tests detecting antibody to hepatitis C virus (HCV) have shown that HCV is the major etiological agent for both transfusion-acquired and sporadic non-A, non-B hepatitis (1, 15, 21). Since the first report of the HCV genome by the Chiron research group (5), numerous complete or partial nucleotide sequences of HCV isolates have been reported worldwide (6, 10, 13, 29–31). Comparison of these sequences revealed marked genetic heterogeneity of the HCV genome. On the basis of sequence variation in both the coding and noncoding regions, several classification systems have been proposed. Enomoto et al. (7) classified HCV into two major types and noted that each genotype could be further classified into two subtypes. Later, Mori et al. (20), Simmonds et al. (38), Stuyver et al. (39), and Bukh et al. (2, 3) reported several additional genotypes and proposed their own classification and nomenclature schemes.

A number of HCV genotyping systems were also developed. Nakao et al. (22) and McOmish et al. (17) genotyped HCV by restriction fragment length polymorphism. Okamoto et al. (32) and Chayama et al. (4) genotyped HCV by PCR with genotype-specific primers. However, current classification of HCV involves at least six major types and a series of subtypes (3, 25). None of the reported systems was able to determine the HCV genotype in all six major types and the common subtypes on the basis of the core region of HCV. Here, we report a convenient genotyping system, based on PCR of the core region with genotype-specific primers, which allows for the determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a.

* Corresponding author. Mailing address: Section of Hepatobiliary Diseases, Division of Gastroenterology, Hepatology, and Nutrition, Department of Medicine, University of Florida, Gainesville, FL 32610. Phone: (352) 392-7353. Fax: (352) 392-7393.

MATERIALS AND METHODS

Subjects. Serum samples used in this study were obtained from 607 patients chronically infected with HCV, all of whom were seropositive for anti-HCV by second-generation enzyme immunoassay (Dinabot Laboratories, Tokyo, Japan, or Abbott Laboratories, North Chicago, Ill.) and seronegative for hepatitis B virus, human immunodeficiency virus, and autoimmune markers. Of the 607 samples, 379 were from patients in Japan, 63 were from the United States, 53 were from Korea, 35 were from Taiwan, 32 were from China (Nanjing), 20 were from Hong Kong, 15 were from Australia, 6 were from Egypt, 3 were from Bangladesh, and 1 was from South Africa.

Serum HCV RNA was detected by reverse transcription-PCR based on the highly conserved 5′ untranslated genomic region as reported elsewhere (12).

Primers. Table 1 shows the sequences and nucleotide positions of the primers used in this study. The outer primers and the second-round PCR primers for sequencing were designed on the basis of the conserved nature of these sequences. Sc2 and Ac2 were the sense and antisense outer primers for the core region, respectively. S7 and A5 were the sense and antisense primers, respectively, for the inner PCR used to generate the amplicon for sequencing. Genotype-specific primers were designed on the basis of 91 HCV isolates. Of the 91 HCV isolates that we used for the design of this assay, 90 were classified as genotype 1a, 1b, 1d, 2a, 2b, 3a, 3b, 4, or 5a, as reported in our study (25), and the remaining one, HK2, was assigned genotype 6a (Table 2). A sense primer (S7) based on a conserved region was used for both reaction tubes. The antisense primers were designed on the basis of the conserved nature of those sequences within a genotype and their poor homology with the sequences derived from other HCV genotypes. As there were nine different subtypes that we tried to detect, the detection primers are divided into two different types on the basis of differences in the sizes of the different bands, so that no genotype-specific bands are of similar molecular size in the same gel. For HCV genotype 2a, the antisense primer G2a potentially primes HCV type 4 isolates, based on the sequences available to us. Accordingly, an HCV type 2a-specific sense primer was also added. It should be noted that by deduction from the sequences available, HCV type 2a isolates should have two bands, of 190 and 139 bp, whereas type 4 isolates may occasionally give rise to a 190-bp band. This strategy will enhance the specificity of the assay and aid our interpretation of the data.

TABLE 1. Oligonucleotide primers used for PCR, sequencing, and genotyping[a]

Primer[b]	PCR round[c]	Sequence (5′–3′)[d]	Nucleotide position
Sc2	1	GGGAGGTCTCGTAGACCGTGCACCATG	−24–3
Ac2	1	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	417–391
S7	2s	AGACCGTGCACCATGAGCAC	−12–8
A5	2s	TACGCCGGGGGTCA(TG)T(GA)GGGCCCCA	343–319
Mix 1			
S7	2g	AGACCGTGCACCATGAGCAC	−12–8
S2a	2g	AACACTAACCGTCGCCCACAA	40–60
G1b	2g	CCTGCCCTCGGGTTGGCTA(AG)	222–203
G2a	2g	CACGTGGCTGGGATCGCTCC	178–159
G2b	2g	GGCCCCAATTAGGACGAGAC	325–306
G3b	2g	CGCTCGGAAGTCTTACGTAC	164–145
Mix 2			
S7	2g	AGACCGTGCACCATGAGCAC	−12–8
G1a	2g	GGATAGGCTGACGTCTACCT	196–177
G3a	2g	GCCCAGGACCGGCCTTCGCT	220–211
G4	2g	CCCGGGAACTTAACGTCCAT	87–58
G5a	2g	GAACCTCGGGGGGAGAGCAA	308–289
G6a	2g	GGTCATTGGGGCCCCAATGT	334–315

[a] The expected sizes of the genotype-specific bands amplified by PCR typing are as follows: genotype 1a, 208 bp in size; genotype 1b, 234 bp; genotype 2a, 139 bp and 190 bp (note that, theoretically, the 190-bp amplicon may also be detected in a small proportion of HCV type 4 isolates); genotype 2b, 337 bp; genotype 3a, 232 bp; genotype 3b, 176 bp; genotype 4, 99 bp; genotype 5a, 320 bp; and genotype 6a, 336 bp (Fig. 1).

[b] For the naming of primers, S = sense, A or G = antisense, and c = core region; the notations 1a to 6a are in accordance with the HCV genotype nomenclature proposed by Simmonds et al. (37). Numbering is from the authentic start codon of the open reading frame.

[c] 2s, second-round PCR for sequencing; 2g, second-round PCR for genotyping.

[d] Pairs of nucleotides inside parentheses are degenerate nucleotides.

HCV typing. The HCV isolates of all 607 samples were genotyped by both the method of Okamoto et al. (28) and our system. The former was performed according to the published protocol. The latter was done as follows. Two different primer mixtures, one containing the S7, S2a, G1b, G2a, G2b, and G3b primers (mix 1) and another including the S7, G1a, G3a, G4, G5a, and G6a primers (mix 2), were prepared (Table 1).

Experiments were performed to optimize the PCR. First, the volume of the first-round PCR product added to the second PCR mixture was evaluated. Ten, 5, 2, 0.5, or 0.1 μl was added to the second PCR mixture. It was shown that with 2 to 10 μl of first-round PCR product, a genotype-specific band was seen together with some unanticipated bands, which varied in size, whereas with 0.5 and 0.1 μl of the first-round PCR product, only the genotype-specific band was observed. Hence, in all subsequent experiments, the amount of first-round PCR product used for the second-round reaction was 0.5 μl. Second, the concentrations of primers and magnesium were also evaluated. A total of three primer concentrations (0.4, 2.5, and 8.0 pmol/50-μl reaction volume) and five different magnesium concentrations (1.0, 1.5, 2.0, 2.5, and 4.0 mM) were tested. It was found that in the reaction tube, the optimal primer and magnesium concentrations were 2.5 pM and 1.5 mM, respectively (data not shown). These conditions were used for both the first- and second-round PCRs.

Two rounds of amplification were carried out as described below. The first round utilized primers Sc2 and Ac2. Two second-round PCRs were performed for each sample, one with primer mix 1 and the other with mix 2 (Table 1). Eight microliters of the second-round PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and evaluated under UV light. The HCV genotype for each sample was determined by identifying the genotype-specific cDNA bands, as exemplified in Fig. 1. To evaluate the specificity of this genotyping method, samples with known genotypes (1a, 1b, 2a, 2b, and 3a, two samples each; 4, 5a, and 6a, one sample each) were also evaluated with this method, and in all cases the genotype-specific bands were detected.

For the reverse transcription-PCR in this HCV genotyping system, RNA was extracted from serum with RNAzol (Cinna/Biotecx Laboratories, Friendwood, Tex.) and reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase with random hexamer (GIBCO BRL, Gaithersburg, Md.) (26). Two microliters of this cDNA was amplified for 40 cycles with the following parameters: a preliminary 20 cycles of amplification at 94°C for 1 min (denaturing), 45°C for 1 min (annealing), and 72°C for 1 min (extension), followed by 20 additional cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For the second-round PCR, 0.5 μl of first-round PCR product was amplified for 30 cycles; each cycle consisted of 94°C for 1 min, 62°C for 45 s, and 72°C for 1 min.

Sequencing. To test the validity of this newly developed genotyping system, the nucleotide sequences of HCV from 23 of the 68 samples which gave discordant results with the typing system of Okamoto et al. and this new assay were determined (Table 3). Briefly, the second-round PCR was performed with primers S7 and A5 (Table 1). The PCR product was cloned into a TA cloning vector (Invitrogen, San Diego, Calif.), and the nucleotide sequence was determined bidirectionally by the dideoxy chain termination method.

Molecular evolutionary method. To clarify the relationship among different HCV isolates, a phylogenetic tree was constructed as described elsewhere (19). Briefly, the nucleotide sequences of multiple HCV isolates were aligned to maximize homology in the core region (9, 23). The numbers of nucleotide substitutions per site at all positions were estimated by the six-parameter method (36). Based on the numbers of nucleotide substitutions at all positions, a phylogenetic tree of the HCV core region was constructed by the neighbor-joining method. All analyses were performed with molecular evolutionary analysis software (ODEN version 1.1).

Nucleotide sequence accession numbers. Nucleotide sequences obtained for the first time in this study have been submitted to DDBJ at the National Institute of Genetics, Mishima, Japan, and are shown in Table 3.

RESULTS

Table 4 shows the genotype distribution of the 607 samples obtained from different geographic locations. The distribution of the HCV genotypes in this study population is in accord with the known geographic distribution of HCV genotypes.

Table 5 shows a comparison of results generated by the two genotyping systems. The genotype was determined in 562 of 607 samples (92.6%) by the typing system of Okamoto et al., whereas 592 of 607 (97.5%) could be genotyped by the newly developed assay. The genotypes of 15 samples were not determined by either method. All 15 of these samples were negative for core gene by PCR, and unfortunately we did not have enough serum or RNA for these samples for further PCR testing based on other genomic regions. When results obtained with both systems were compared, the genotypes of 539 of the 607 samples (88.8%) were concordant while those of 68 (11.2%) were discordant. It is worth noting that 30 samples which were judged to be indeterminate by the genotyping system of Okamoto et al. could be genotyped by the newly developed assay. Half of the 68 samples with discordant genotyping

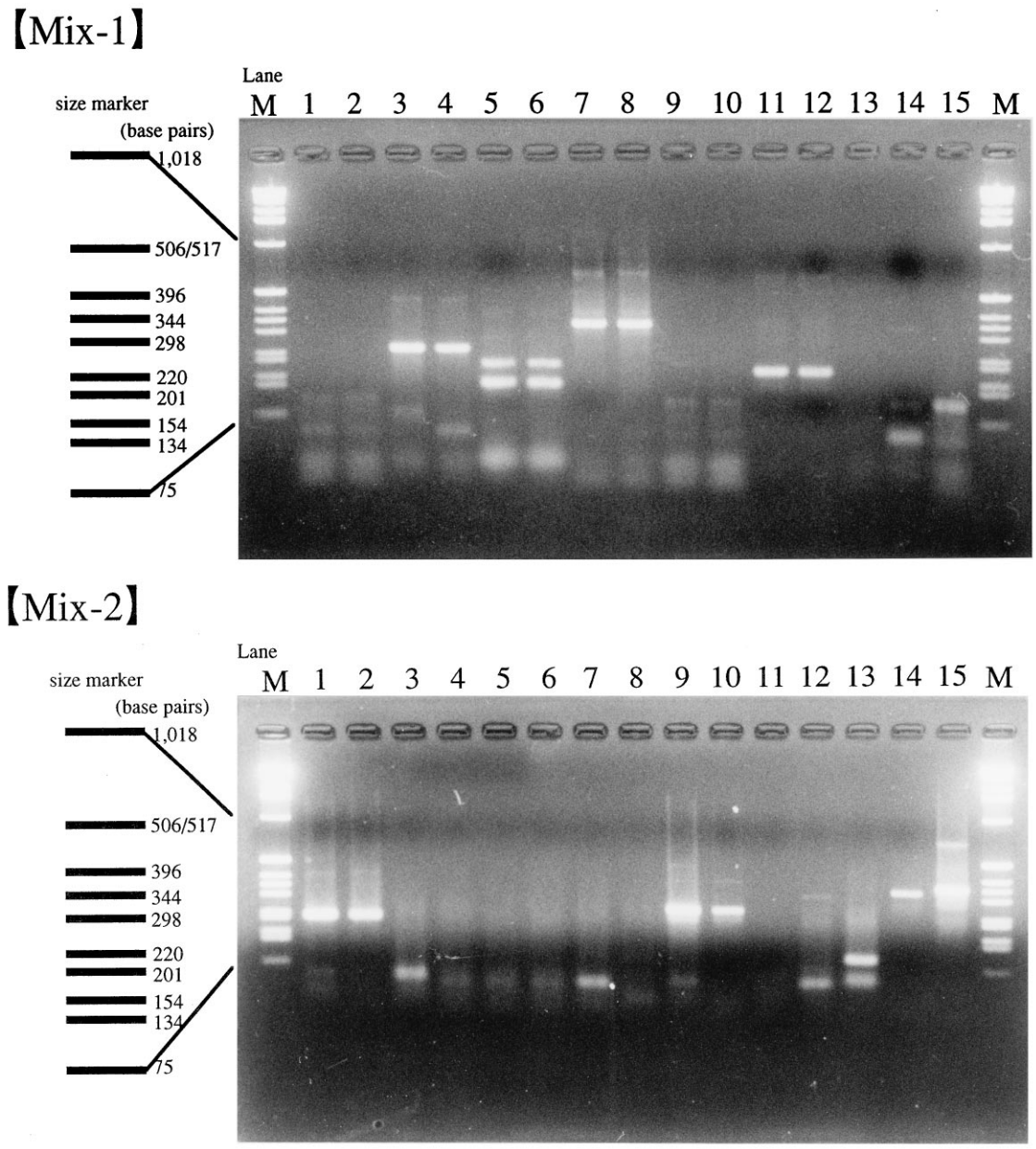

FIG. 1. Typical electrophoresis patterns of PCR products from different HCV genotypes determined by our new typing system. All samples were analyzed twice by PCR with either mix 1 or mix 2. Mix 1 will allow for the specific detection of PCR products for HCV genotypes 1b, 2a, 2b, and 3b. Mix 2 will allow for the specific detection of HCV genotypes 1a, 3a, 4, 5a, and 6a. The detection of genotype-specific products in mix 1 and mix 2 was designed so that the differences in the sizes of PCR products could be evaluated on gels easily. In the analysis of results, one should look for the strong specific bands seen on either gel. Faint nonspecific bands may be generated through weak priming, but they are usually very weak when the reaction conditions that we have optimized are used. The sizes of the HCV genotype-specific products are given in the footnotes of Table 1. For example, in lanes 1 and 2, no specific product is detected with mix 1 (upper panel), but a specific band of 209 bp is seen with mix 2 (note that the molecular marker scale is enlarged and that the PCR product band corresponds to a size of between 201 and 220 bp). Similarly, HCV genotype 1b samples showed only two bands of 234 bp with mix 1 (lanes 3 and 4); genotype 2a showed two bands (139 and 190 bp) in mix 1 (with two different sense primers) (lanes 5 and 6), while genotype 2b showed a specific band of 337 bp in mix 1 (lanes 7 and 8). A similar interpretation can be applied to genotype 3a (lanes 9 and 10) (232 bp in mix 2), genotype 3b (lanes 11 and 12) (176 bp in mix 1), genotype 4 (lane 13) (99 bp in mix 2; a faint nonspecific band was also seen in this case), genotype 5a (lane 14) (expected size of 320 bp in mix 2), and genotype 6a (lane 15) (336 bp in mix 2).

results were judged to be of mixed genotype by the typing system of Okamoto et al.

To illustrate the genotyping efficacy, the typing system of Okamoto et al. was able to determine only 30 of 45 (66.7%) genotype 1a samples assigned by the newly developed genotyping system. Similarly, the genotyping system of Okamoto et al. was able to assign only 69 of 97 (71.1%) genotype 2a samples determined by the newly developed assay. Samples not assigned as genotype 1a or 2a were all designated mixed genotype by the genotyping system of Okamoto et al. In particular, of the 18 samples assigned as mixed genotype 1a + 1b by the typing system of Okamoto et al., 15 were found to be genotype 1a by the newly developed assay. In addition, most samples with a genotype other than 1a, 1b, 2a, or 2b as assigned

TABLE 2. The sequences of the 91 HCV isolates used for the design of genotype-specific primers in our genotyping system

Genotype	Isolate	Accession no.	Genotype	Isolate	Accession no.
1a	HCV1	M62321		F6-4	D16761
	GM1	M61718		F6-9	D16762
	GM2	M61719		US114	D14309
	HCVH	M67463		CR3N1	Z29461
	USA1	D16731		CR3N2	Z29462
	H77	M62381		CR3N3	Z29463
	H90	M62382		CR3N4	Z29464
	UK	D16699			
	HC J1	D00831	3b	HCV-TR	D11443
				648-1	D16720
1b	HCVJ	D00574		648-2	D16721
	HC J2	D10074		PAKIS	D82032
	HCV-BK	M58335		BANG1-1	D29647
	HC J4	D00832		BANG1-2	D29648
	HCV-JT	D11681		BANG1-3	D29649
	HCV-J	D90208		BANG2-1	D29650
	HCV JK1	X61596		BANG2-2	D29651
	JK2	X61592		BANG2-3	D29652
	JK3	X61593		BANG3-1	D29653
	JK4	X61594		BANG3-2	D29654
	JK5	X61595			
	HCVG3	M86779	4	EG21C	LO8162
	HCVT	M84754		EG29C	LO8163
	CHINA-1	D16694		EG33C	LO8164
	HC-CHINA	L02863		GB116C	D43675
	SAUDI	D16722		EGYPT	D16808
	HPC483	D13558		CR4N1	Z29465
	HPC491	D10750		CR4N2	Z29466
	W.INDI	D16697		CR4N3	Z29467
	ITALY	D16698		CR4N4	Z29468
				CR4N5	Z29469
1d	TD-6C	D14670			
	TD-34	D26383	5a	SA-K3	D16789
	TD-47	D26384		SAK13	D16791
				SA-K14	D16792
2a	HC J5	D10076		SA-K15	D16793
	HC J6	D00944		SA-K17	D16794
	J650-1	D16724		SA-K18	D16795
	MON	D16800		CR5N1	Z29470
	TD-17C	D14671		CR5N2	Z29471
	USA2	D16725		CR5N3	Z29472
				CR5N4	Z29473
2b	HC-J7	D10077		CR5N5	Z29474
	J663-2	D16728			
	USA3	D16734	6a	HK2	D43679
	CHINA-2	D16695			
	HC J8	D10988			
	SF1	D82033			
	SF2	D82034			
3a	E-b1	D10123			
	NZL1	D14305			
	TH85	D14307			
	F6-1	D16759			
	F6-3	D16760			

by our typing assay were determined to be of mixed genotype by the typing system of Okamoto et al.

To further elucidate the reliability of the newly developed assay, 23 of the 68 samples with discordant results by the genotyping system of Okamoto et al. were further analyzed by sequencing of the core region and phylogenetic-tree construction. Table 3 shows the relationship between the genotyping results and those elicited by sequencing. Figure 2 shows the phylogenetic tree for these 23 samples in relation to the known HCV genotype sequences. This tree shows that genotypes for these discordant samples were all assigned correctly by the newly developed assay.

DISCUSSION

HCV is known to have marked genetic heterogeneity, and it was estimated to have a nucleotide substitution rate of between 1.44×10^{-3} and 1.92×10^{-3} substitution per site per year (24, 28). Accumulation of nucleotide substitution in the HCV genome results in diversification and evolution into different genotypes (19). Presently, HCV can be classified into at least six major types and a series of subtypes (3, 25). There is increasing evidence that patients infected with different HCV genotypes may have different clinical profiles, severity of liver disease, and response to alpha interferon therapy (11, 18, 34, 35, 40). Hence, a convenient and reliable HCV genotyping system is essential for large-scale epidemiological and clinical studies.

In this report, a new genotyping method, based on genotype-

TABLE 3. Genotyping data from our system, the method of Okamoto et al., and sequencing and phylogenetic -tree construction for the 23 HCV isolates for which discordant results in genotype assignment were obtained by the method of Okamoto et al. and with our system[a]

Genotype as determined by:			Accession no.	Designation
Our system	Okamoto's system	Sequencing		
1a	1a + 1b	1a	D16775	USA1
	1a + 1b	1a	D16695	USA2
	1a + 1b	1a	D16781	USA3
	1a + 1b	1a	D49454	HG1
1b	NT[b]	1b	D49462	USA4
	NT	1b	D49455	HG2
	NT	1b	D16698	USA5
2a	1b + 2b	2a	D49458	SL1
	1b + 2a + 2b	2a	D49460	SL2
	NT	2a	D49461	SL3
	NT	2a	D49456	HG3
3a	2a	3a	D49450	EG-1
	1a + 2a	3a	D49451	EG-2
	1a + 1b	3a	D49491	USA9
	NT	3a	D49466	USA10
3b	1a + 1b	3b	D29654	BANG3-2
	1a + 1b + 2b	3b	D29649	BANG1-3
	1a + 1b + 2b	3b	D29652	BANG2-3
4	1b + 2a	4	D49452	EG-3
	1b + 2a	4	D49453	EG-4
5a	NT	5a	D16789	SA-K3
6a	1b	6a	D49457	HG-4
	1b	6a	D49458	HG-5

[a] Of the 68 samples which showed discordant results with the genotyping system of Okamoto et al., 23 were randomly selected for genotype determination by sequencing. All these sequences were registered with DDBJ. Of the 21 HCV type 2a samples not typed by the method of Okamoto et al., 18 were from Japanese patients, indicating that the observations by Giannini et al. (8) and Kleter et al. (14) were also potential problems in genotyping Japanese HCV type 2a isolates (the three SL HCV type 2a samples were from Japan).

[b] NT, not typed.

TABLE 4. HCV genotype distribution, as determined by our genotyping method, of serum samples from patients originating from different geographic regions[a]

Patients' country of origin	No. of samples with genotype													Total no. of samples
	1a	1b	2a	2b	3a	3b	4	5a	6a	1a + 2b	1b + 2a	1b + 2b	I[b]	
Japan	10	244	64	47	5					1		1	7	379
United States	25	20	4	9	2								3	63
Korea	1	35	9	4								1	3	53
Taiwan		19	10	3							2		1	35
China (Nanjing)		21	8	2							1			32
Hong Kong	2	15	1						2					20
Australia	7	5		1	1								1	15
Egypt		1	1		2		2							6
Bangladesh						3								3
South Africa								1						1
Total	45	360	97	66	10	3	2	1	2	1	3	2	15	607

[a] Note that the distribution of the number of samples from each geographic area was not similar. With this database, HCV type 1b is the most common genotype.
[b] I, indeterminate by our genotyping system.

specific primers for PCR of the core gene, by which HCV isolates can be classified into genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a is described. So far, the genotyping system described by Okamoto et al. is the most popular system used by HCV investigators in Japan (27). This system is also used by a number of researchers worldwide. However, for HCV isolates with HCV genotypes other than type 1 or 2, the typing system of Okamoto et al. may not suffice. In Western patients with HCV type 2a infection, the system of Okamoto et al. has also been reported to be less useful in genotyping (8, 14). Recently, it was also shown that the system designed by Okamoto et al. had a higher number of mixed-infection designations, and these samples were found to have non-type 1–non-type 2 isolates, due to nonspecific priming (16). This observation is confirmed in this study, in which a significant proportion of samples assigned to the mixed-infection category were in fact non-type 1–non-type 2 HCV. This is likely to result from nonspecific annealing of primers to the sequences.

It is well established that the matching of the two to three nucleotides at the 3′ end is one of the important parameters for specific priming. Therefore, the lower the number of HCV isolates employed in designing primers is, the lower the specificity of the primers would be. The typing system of Okamoto et al. was based on sequences of HCV isolates of genotype 1a, 1b, 2a, or 2b (32). Since a number of new HCV genotypes were identified only recently, the system designed by Okamoto et al. would be insufficient to differentiate these newly identified genotypes. In fact, Okamoto et al. have revised their assay to include HCV type 3a. Our study was embarked upon before the publication of their paper, and we did not have a chance to test their modified system versus our system. However, we point out that the region used by Okamoto et al. for their type 3a primer might not be a suitable region for the design of primers if all the common subtypes were to be detected. As our system is based on the nucleotide sequences of genotype 1a, 1b, 1d, 2a, 2b, 3a, 3b, 4, 5a, and 6a HCV isolates, we believe that this system may have a much broader application. However, the number of samples of HCV types 3 to 6 tested was still not large enough for definitive conclusions to be drawn. Certainly, this system should be further tested in areas in which HCV types 3 to 6 are common to further validate this genotyping method. If the accuracy of our system is confirmed in these areas, we anticipate that this convenient method will assist research workers in conducting large-scale epidemiological studies.

TABLE 5. Comparison of the genotyping results obtained by the method of Okamoto et al. with those obtained by our system

Genotype by Okamoto's genotyping system	No. of samples with genotype by our genotyping system of[a]:													Total no. typed
	1a	1b	2a	2b	3a	3b	4	5a	6a	1a + 2b	1b + 2a	1b + 2b	NT[b]	
1a	30													30
1b		357	1	1					2					361
2a			69		2									71
2b				62										62
1a + 1b	15				2	1								18
1a + 2b										1				1
1a + 1b + 2b						2								2
1b + 2a			3		3		2				3			11
1b + 2b			1	1								2		4
1b + 2a + 2b			1											1
2a + 2b			1											1
NT		3	21	2	3			1					15	45
Total	45	360	97	66	10	3	2	1	2	1	3	2	15	607

[a] The underlined numbers represent HCV samples with discordant genotyping results.
[b] NT, not genotypeable (indeterminate results).

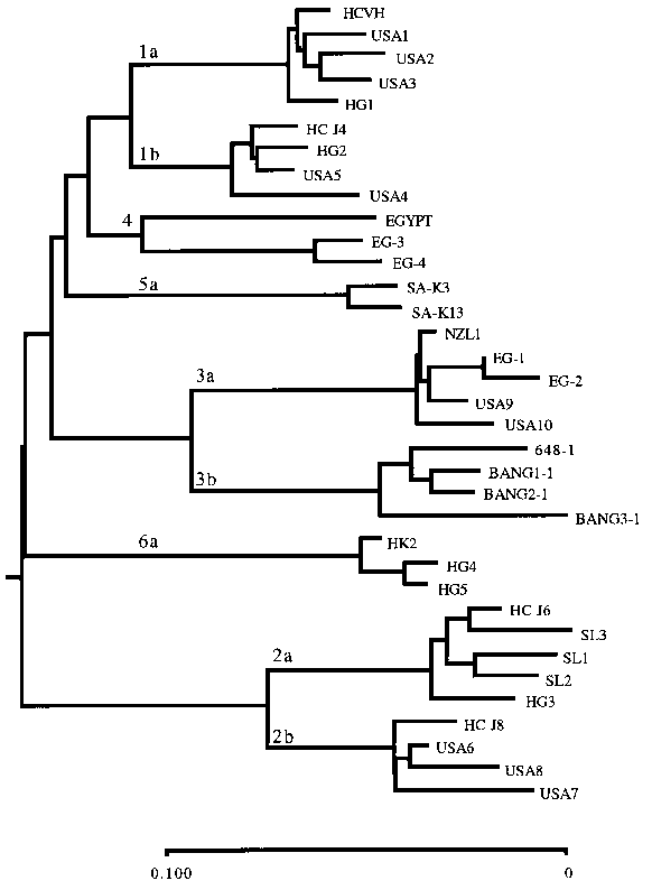

FIG. 2. Phylogenetic tree of the HCV core sequences of 23 HCV isolates for which results obtained by the method of Okamoto et al. and with our system were discordant. One known HCV isolate from each genotype is included as a reference (1a, HCVH; 1b, HC J4; 2a, HC J6; 2b, HC J8; 3a, NZL1; 3b, 648-1; 4, EGYPT; 5a, SA-K3; 6a, HK2). The tree was constructed by the neighbor-joining method. The branch corresponding to each genotype is labelled with the genotype assignment (from 1a to 6a) on the branch. The horizontal bar at the bottom represents the reference. Note that all samples with discordant results were assigned to the genotype which was assigned by our genotyping system, confirming the specificity of our genotyping system.

ACKNOWLEDGMENTS

M.M. was supported in part by a grant-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture (06670571), by Japanese Ministry of Health and Welfare health science research grants (non-A, non-B hepatitis research grants), and by the Viral Hepatitis Research Foundation of Japan. J.Y.N.L. was supported in part by grant DSR-RDA-1-15 from the Division of Sponsored Research of the University of Florida, by an American Liver Foundation Hans Popper Liver Scholar Award, by a Glaxo Institute of Digestive Health Clinical Investigator Award, and by Public Health Service grant AI41219.

REFERENCES

1. **Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q. L. Choo, and G. Kuo.** 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med **321:**1494–1500.
2. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1993. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc. Natl. Acad. Sci. USA **90:**8234–8238.
3. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.
4. **Chayama, K., A. Tsubota, Y. Arase, A. Saitoh, I. Koida, K. Ikeda, T. Matsumoto, M. Kobayashi, S. Iwasaki, S. Koyama, T. Morinaga, and H. Kumada.** 1993. Genotypic subtyping of hepatitis C virus. J. Gastroenterol. Hepatol. **8:**150–156.
5. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359–362.
6. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
7. **Enomoto, N., A. Takada, T. Nakao, and T. Date.** 1990. There are two major types of hepatitis C virus in Japan. Biochem. Biophys. Res. Commun. **170:** 1021–1025.
8. **Giannini, C., V. Thiers, J. Nousbaum, L. Stuyver, G. Maertens, and C. Brechot.** 1995. Comparative analysis of two assays for genotyping hepatitis C virus based on genotype-specific primers or probes. J. Hepatol. **23:**246–253.
9. **Gojobori, T., K. Ishii, and M. Nei.** 1982. Estimation of the average number of nucleotide substitutions when the rate of substitution varies with nucleotide. J. Mol. Evol. **28:**414–423.
10. **Inchauspe, G., S. I. Zebedee, D. H. H. Lee, M. Sugitani, M. Nasoff, and A. M. Prince.** 1991. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. Proc. Natl. Acad. Sci. USA **88:**10292–10296.
11. **Kanai, K., M. Kako, and H. Okamoto.** 1992. HCV genotypes in chronic hepatitis C virus and response to interferon. Lancet **339:**1543.
12. **Kaneko, S., M. Unoura, K. Kobayashi, K. Kuno, S. Murakami, and N. Hattori.** 1990. Detection of serum hepatitis C virus RNA. Lancet **335:**976.
13. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugiura, and K. Shimotohono.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87:**9524–9528.
14. **Kleter, G. E. M., L. J. van Doorn, L. Stuyver, G. Maertens, J. T. Brouwer, S. W. Schalm, R. A. Heijtink, and W. G. V. Quint.** 1995. Rapid genotyping of hepatitis C virus RNA isolates obtained from patients residing in Western Europe. J. Med. Virol. **47:**35–42.
15. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevence, G. E. Tegtmeier, F. Bonino, M. Colombo, W. S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244:**362–364.
16. **Lau, J. Y. N., M. Mizokami, J. A. Kolberg, G. L. Davis, L. E. Prescott, T. Ohno, R. P. Perrillo, K. L. Lindsay, R. G. Gish, K.-P. Qian, M. Kohara, P. Simmonds, and M. S. Urdea.** 1995. Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. J. Infect. Dis. **171:**281–289.
17. **McOmish, F., S. W. Cha, B. C. Dow, J. Gillon, W. D. Frame, R. J. Crawford, P. L. Yap, E. A. C. Follett, and P. Simmonds.** 1993. Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. Transfusion **33:**7–13.
18. **Mita, E., N. Hayashi, Y. Kanazawa, H. Hagiwara,, K. Ueda, A. Kasahara, H. Fusamoto, and T. Kamata.** 1994. Hepatitis C virus genotype and RNA titer in the progression of type C chronic liver disease. J. Hepatol. **21:**468–473.
19. **Mizokami, M., T. Gojobori, and J. Y. N. Lau.** 1994. Molecular evolutionary virology: its application to hepatitis C virus. Gastroenterology **107:**1181–1182.
20. **Mori, S., N. Kato, A. Yagyu, T. Tanaka, Y. Ikeda, B. Petchclai, and P. Chiewslip.** 1992. A new type of hepatitis C virus in patients in Thailand. Biochem. Biophys. Res. Commun. **183:**334–342.
21. **Mosley, J. W., R. D. Aach, B. Hollinger, C. E. Stevens, L. H. Barbosa, G. J. Nemo, P. V. Holland, W. H. Bancroft, and G. Kuo.** 1990. Non-A, non-B hepatitis and antibody to hepatitis C virus. JAMA **263:**77–78.
22. **Nakao, T., N. Enomoto, N. Takada, and T. Date.** 1991. Typing of hepatitis C virus genomes by restriction fragment length polymorphism. J. Gen. Virol. **72:**2105–2112.
23. **Nei, M., and T. Gojobori.** 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:**418–426.
24. **Ogata, N., H. J. Alter, R. H. Miller, and R. H. Purcell.** 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**3391–3396.
25. **Ohba, K., M. Mizokami, T. Ohno, K. Suzuki, E. Orito, Y. Ina, J. Y. N. Lau, and T. Gojobori.** 1995. Classification of hepatitis C virus into major types and subtypes based on molecular phylogenetic tree analysis. Virus Res. **36:**201–214.
26. **Ohno, T., M. Mizokami, C. J. Tibbs, K. Ohba, K. Suzuki, R. R. Wu, K. T. Nouri-Aria, and R. William.** 1994. New genotype of hepatitis C virus in South Africa. J. Med. Virol. **42:**409–413.
27. **Ohno, T., M. Mizokami, M. Yamauchi, K. Ohba, E. Orito, R. R. Wu, M. Mizuno, K. Sugihara, T. Wakita, and S. Kakumu.** 1995. Genotype distribution in Nagoya and new genotype (genotype 3a) from Japanese patient with hepatitis C virus. J. Gastroenterol. **30:**209–214.

28. **Okamoto, H., M. Kojima, S. Okada, H. Yoshizawa, H. Iizuka, T. Tanaka, E. E. Muchmore, D. A. Peterson, Y. Ito, and S. Mishiro.** 1992. Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology **190:**894–899.
29. **Okamoto, H., K. Kurai, S. Okada, K. Yamamoto, H. Iizuka, T. Tanaka, S. Fukuda, F. Tsuda, and S. Mishiro.** 1992. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology **188:**331–341.
30. **Okamoto, H., S. Okada, Y. Sugiyama, K. Kurai, H. Iizuka, A. Machida, Y. Miyamura, and M. Mayumi.** 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J. Gen. Virol. **72:** 2697–2704.
31. **Okamoto, H., S. Okada, Y. Sugiyama, S. Yotsumoto, T. Tanaka, H. Yoshizawa, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1990. The 5′-terminal sequence of the hepatitis C virus genome. Jpn. J. Exp. Med. **60:**167–177.
32. **Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyamura, and M. Mayumi.** 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. **73:**673–679.
33. **Okamoto, H., H. Tokita, M. Sakamoto, M. Horikita, M. Kojina, H. Iizuka, and S. Mishiro.** 1993. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J. Gen. Virol. **74:**2385–2390.
34. **Pozzato, G., M. Moretti, F. Franzin, L. S. Croce, C. Tiribelli, T. Masayu, S. Kaneko, M. Onoura, and K. Kobayashi.** 1991. Severity of liver disease with different hepatitis C viral clones. Lancet **338:**509.
35. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q. L. Choo, M. Houghton, and G. Kuo.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **87:**6547–6549.
36. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing a phylogenetic tree. Mol. Biol. Evol. **4:**406–425.
37. **Simmonds, P., A. Alberti, H. J. Alter, F. Bonino, D. W. Bradley, T. C. Brecho, J. T. Brouwer, S. W. Chan, K. Chayama, D. S. Chen, Q. L. Choo, M. Colombo, T. M. Cuypers, T. Date, G. M. Dusheiko, J. I. Esteban, O. Fay, S. J. Hadziyannis, J. Han, A. Hatzakis, E. C. Holmes, H. Hotta, M. Houghton, B. Irvine, M. Kohara, J. A. Kolberg, G. Kuo, J. Y. N. Lau, P. N. Lelie, G. Maertens, F. McOmish, T. Miyamura, M. Mizokami, A. Nomoto, A. M. Prince, H. M. Reesink, C. Rice, M. Roggendorf, S. W. Schalm, T. Shikata, K. Shimotohno, L. Stuyver, C. Trepo, A. Weiner, P. L. Yap, and M. Urdea.** 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology **19:**1321–1324.
38. **Simmonds, P., E. C. Holmels, T. A. Cha, S. W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea.** 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic tree analysis. J. Gen. Virol. **74:**2391–2399.
39. **Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. V. Heuverswyn, and G. Maertens.** 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. **244:**1093–1102.
40. **Yoshioka, K., S. Kakumu, T. Wakita, T. Ishikawa, Y. Itoh, M. Takayanagi, Y. Higashi, M. Shibata, and T. Morishita.** 1992. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-α therapy: relationship to genotypes of hepatitis C virus. Hepatology **16:**293–299.