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Genotyping Hepatitis C Virus Isolates from Spain, Brazil, China, and Macau by a Simplified PCR Method

PAUL V. HOLLAND,[1] JOSEP M. BARRERA,[2] M. GUADALUPE ERCILLA,[3] CLARA F. T. YOSHIDA,[4] YU WANG,[5] GABRIEL A. B. DE OLIM,[6] BERNIE BETLACH,[1] KEN KURAMOTO,[1] AND HIROAKI OKAMOTO[7]*

Sacramento Blood Center, Sacramento, California[1]; Liver Unit, Hospital Clinic I Provincial, University of Barcelona,[2] and Servicio de Immunologia, Hospital Clinic I Provincial de Barcelona,[3] Barcelona, Spain; National Reference Center for Viral Hepatitis, Virology Department, Oswaldo Cruz Institute, Rio de Janero, Brazil[4]; Institute of Hepatology, Beijing Medical University, Beijing, China[5]; Centro de Transfuoes de Sangue, Macau[6]; and Immunology Division, Jichi Medical School, Tochigi-Ken, Japan[7]

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An improved and simplified method of genotyping was developed for classifying hepatitis C virus (HCV) isolates into the five common genotypes, i.e., I/1a, II/1b, III/2a, IV/2b, and V/3a, by PCR with genotype-specific primers deduced from the core gene. Sense and antisense primers, specific for each of the five common genotypes, were designed by comparison of 319 core gene sequences from HCV isolates of various genotypes from genetic groups 1 to 9. In the first round of PCR, a sequence of 433 bp representing nucleotides 319 to 751 was amplified with universal primers. The second round of PCR was performed with respective sense and antisense primers in two separate reactions, one for the amplification of genotypes I/1a and II/1b and the other for the amplification of genotypes III/2a, IV/2b, and V/3a. The specificity of genotyping was confirmed with a panel of 191 serum samples containing HCV isolates whose core gene sequences were known: 110 serum samples infected with HCV of the five common genotypes and 81 serum samples infected with HCV of other genotypes. The use of sense and antisense primers for genotype II/1b (primers 389 and 492) abolished the cross-reaction of the antisense primer for genotype II/1b (primer 133) with some HCV isolates of genotype I/1a found by our original method. The new method was used for genotyping 130 HCV isolates from Spain, 53 from Brazil, 106 from China, and 30 from Macau. A total of 329 bp of the NS5b region (nucleotides 8279 to 8607) of five isolates from Spain and five isolates from Macau which could not be classified as any of the five common HCV genotypes or genotype 2c were sequenced, and the sequences were compared with those of HCV isolates of known genotypes; two isolates from Spain were deduced to be of genotype 4d and one was deduced to be of genotype 1d, while the remaining two isolates from Spain had novel genotypes in genetic group 2; however, all five isolates from Macau were of genotype 6a.

Hepatitis C virus (HCV) is unique among viruses infecting human beings in that, one, the detection of the viral genome remains the most sensitive and reliable means of establishing infection and, two, variations in nucleotide sequences are used for classifying them. Since the discovery of HCV by Choo et al. in 1989 (6), more than 80 genotypes have been identified, and the sequences of the subgenomic regions of these genotypes differ by >20%. How HCV genotypes should be classified in the context of clinical hepatology has not yet been settled.

The classification of HCV genotypes by Simmonds et al. (31) groups HCV isolates by a phylogenetic analysis of sequence variation in a part of a nonstructural region (NS5b) of 222 bp. They found two different tiers of sequence variation within this region and, on that basis, classified HCV into six types designated by Arabic numbers (1, 2, 3, etc.) and divided them further into subtypes labeled by letters (a, b, c, etc.).

We have proposed a different classification scheme, from the viewpoint that a full characterization of HCV cannot be accomplished without taking into account the entire genomic sequence of ~9,500 nucleotides (nt) (27, 28). The full-length sequence was first determined for the prototype HCV, HCV type 1 (HCV-1), prevalent in the United States (7), which was named genotype I (10). Thereafter, strains of the genotypes commonly found in Japan were sequenced in full for the designation of genotypes II, III, and IV (11, 24, 26). Later, a strain with a genotype frequently seen in the United States, England, Thailand, and New Zealand was sequenced in its entirety and was named genotype V (30). The five HCV genotypes are the most common around the world (3, 17, 18) and account for the vast majority of HCV isolates detected in blood donors and hepatitis patients in the United States, Canada, and Europe, as well as in most areas of Asia and Oceania. In the classification of Simmonds et al. (31) genotype I corresponds to 1a, genotype II corresponds to 1b, genotype III corresponds to 2a, genotype IV corresponds to 2b, and genotype V corresponds to 3a.

Previously, we reported a method that can be used to define four genotypes, genotypes I/1a, II/1b, III/2a, and IV/2b, by selective amplification by PCR with genotype-specific primers deduced from a comparison of 44 core gene sequences (27). When genotype V/3a was described, our initial method was modified to detect this genotype too (28). Our method involved two rounds of PCR amplification, in the first round of which core gene sequences are amplified with universal primers. The products are then amplified in a second-round PCR with universal sense primers and a mixture of five antisense primers which are specific to the five common genotypes and which amplify fragments with length polymorphisms.

Recently, however, the specificity of our method has been questioned by research groups in Canada, the United States,

* Corresponding author. Mailing address: Immunology Division, Jichi Medical School, Minamikawachi-Machi, Tochigi-Ken 329-04, Japan. Phone: (81) 285-44-2111, extension 3334. Fax: (81) 285-44-1557.

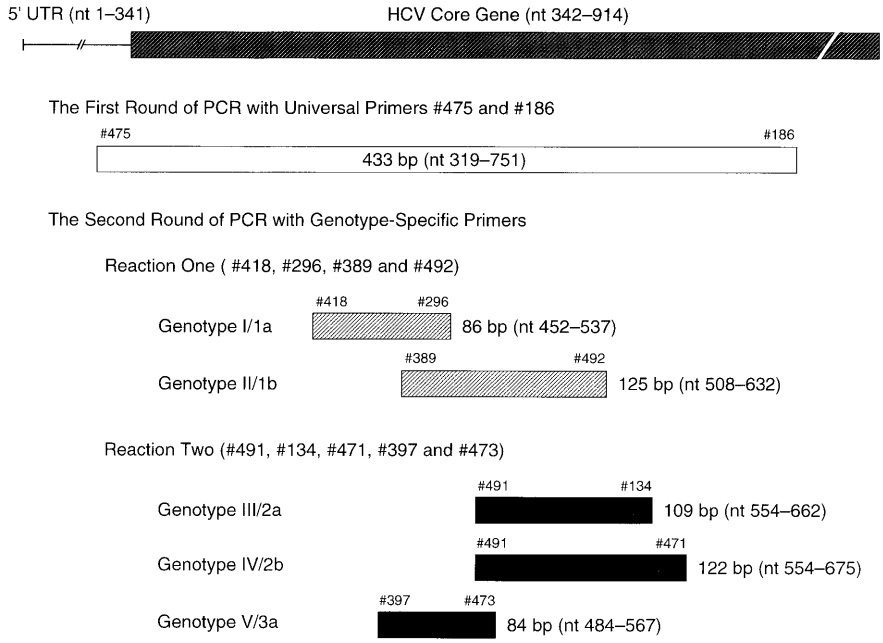

FIG. 1. Genotyping of HCV isolates by PCR with type-specific primers. The first round of PCR was performed with universal sense and antisense primers as described previously (21). The second round was performed with two separate reactions. In reaction one, two pairs of sense and antisense primers specific for genotype I/1a (primers 418 and 296) or II/1b (primers 389 and 492) were used for the detection of these genotypes. In reaction two, three pairs of sense and antisense primers specific for genotypes III/2a (primers 491 and 134), IV/2b (primers 491 and 471), and V/3a (primers 397 and 473) were used; the sense primer for III/2a and IV/2b (primer 491) was common and specific for genetic group 2.

and England, where genotype I/1a occurs frequently (2, 12, 13, 37). They reported that the primer for genotype II/1b (primer 133) in our original method (27) anneals to cDNA transcribed from HCV isolates of genotype I/1a, so that ~20% of viremic samples are misclassified as mixed infection with genotypes I/1a and II/1b. To address this issue, we developed a second-generation method for genotyping by PCR which can clearly distinguish genotype I/1a from genotype II/1b (21).

Although the second-generation method is specific, it involves five separate reactions in the second round of PCR, which makes its general application cumbersome. In the study described in this report we wished to modify further the second-generation method without affecting its specificity and to simplify it for easier application. The method was used to genotype HCV isolates from Spain, Brazil, China, and Macau. Furthermore, 329 bp of the NS5b region of 10 of these HCV isolates from Spain and Macau which could not be classified as any of the five common genotypes or genotype 2c (19) were sequenced, and two novel genotypes in genetic group 2 were identified.

MATERIALS AND METHODS

Core gene primers. The nucleotide (nt) position was numbered starting with the 5′ end of an HCV genome of genotype II/1b (HC-J4/83) (22). Six genotype-specific primers, primers 389, 397, 471, 473, 491, and 492, were designed on the basis of sequence variation within 192 bp in the core gene spanning nt 484 to 675 of the HCV genome (corresponding to nt 143 to 334 of the core gene) among 319 isolates of various genotypes and previously used in a second-generation method of genotyping by PCR (21). Two primers, primers 134 and 296, are from the original method (27, 28). The remaining sense primer, primer 418, was designed anew. It had the sequence 5′-GCCGCGCAGAGGCCCTTGAT-3′, representing nt 452 to 471 of the core gene (the intentionally incorporated G-to-A as well as A-to-T mismatches are underlined).

Extraction of RNA and cDNA synthesis. RNAs were recovered from sera or plasma (50 to 100 μl) with an extraction reagent containing guanidinium isothiocyanate and phenol (ISOGEN-LS; Nippon Gene Co. Ltd., Tokyo, Japan) and were dissolved in distilled water (5.3 μl) treated with diethylpyrocarbonate. They were heated at 70°C for 1 min, chilled quickly on ice, and added to a reagent mixture which contained 20 pmol of primer 186 (21), 100 U of reverse transcriptase (Superscript II; GIBCO-BRL, Gaithersburg, Md.), 8 U of RNase inhibitor (rRNasin; Promega Corp., Madison, Wis.), and 5 nmol each of the deoxynucleoside triphosphates (dGTP, dATP, dTTP, and dCTP), as well as 2 μl of 5× buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$) and 1 μl of 0.1 M dithiothreitol, which were included in the reverse transcriptase package. The reactant (in a total volume of 10 μl) was covered with 20 μl of mineral oil (Sigma Chemical Co., St. Louis, Mo.) and was incubated at 42°C for 60 min to reverse transcribe the HCV RNA into cDNA by the method described previously (21).

PCR. Reverse-transcribed cDNA was heated at 95°C for 15 min, and a half portion (5 μl) was subjected to the first round of PCR for 35 cycles with primers 475 (sense) and 186 (antisense), both deduced from well-conserved areas in the 5′ untranslated region (5′ UTR) and the core gene (21), respectively, to obtain fragments of 433 bp, irrespective of different HCV genotypes (Fig. 1).

The second round of PCR was performed in two separate reactions with five sets of sense and antisense primers which were specific for each of the five common genotypes (Fig. 1); however, the sense primers for genotypes III/2a and IV/2b were identical (primer 491). A thin-walled microtube successively received mineral oil (30 μl), the reagent mixture (49 μl), and the product of the first-round PCR (1 μl corresponding to a 1/50 portion of the total) and was spun down immediately. The reagent mixture had the same composition as that used in the first-round PCR, except that it contained 15 pmol each of genotype-specific sense and antisense primers, two sets for reaction one and three sets for reaction two, in place of the universal primers (primers 475 and 186). PCR was performed for 25 cycles, with each cycle consisting of denaturation at 94°C for 30 s, primer annealing at 64°C (reaction one) or 62°C (reaction two) for 30 s, and primer extension at 72°C for 30 s. The extension in the last cycle was carried out for 7.5 min. The primers and products of the first- and second-round PCRs are illustrated along with their nucleotide positions in Fig. 1.

Electrophoresis on agarose gel. The products of the second-round PCR (10 μl) were applied to a 2-mm-thick slab gel spread onto a plate (7 by 10 cm). The gel (4%) was made up of three parts of NuSieve and one part of SeaKem LE agarose (NuSieve 3:1 agarose; FMC BioProducts, Rockland, Maine) in Tris-borate buffer (89 mM [each] Tris and boric acid [pH 8.0]) containing 2 mM EDTA. The current was run at 30 mA for 30 min in the same buffer. Then, the gel was stained with ethidium bromide and was observed under UV illumination.

Plasma and serum samples containing HCV RNA. Each of the five standard positive controls contained an HCV sample whose full-length sequence is known: HC-J1 (genotype I/1a), HC-J4/91 (II/1b), HC-J6 (III/2a), HC-J8 (IV/2b), or NZL1 (V/3a). They were diluted to an HCV RNA titer of 10^4/ml, HCV RNA was determined by reverse transcription PCR with nested primers from the 5′ UTR (25), and were tested for their genotypes. Their electrophoretic patterns are

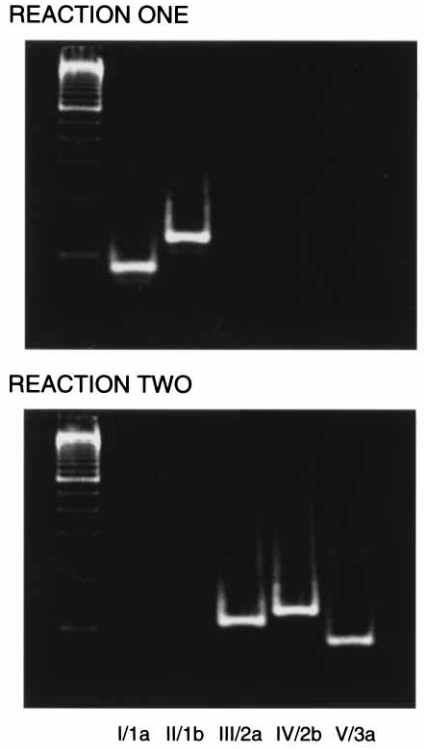

FIG. 2. Electrophoretogram of PCR products. Patterns of reaction one and reaction two in the second-round PCR are shown for five serum samples containing HCV genotypes I/1a, II/1b, III/2a, IV/2b, or V/3a whose full-length sequences are known. The migration positions of molecular size markers (100-bp ladder; GIBCO-BRL) are indicated on the left. The lengths of PCR products were 86 bp for genotype I/1a, 125 bp for II/1b, 109 bp for III/2a, 122 bp for IV/2b, and 84 bp for V/3a.

shown in Fig. 2. The five controls were serially diluted twofold to evaluate the sensitivity of genotyping.

A panel of 191 serum or plasma samples containing HCV isolates of various genotypes were used to evaluate the specificity of genotyping. There were 20 samples of genotype I/1a, 42 of II/1b, 3 of 1c, 18 of III/2a, 15 of IV/2b, 14 of 2c, 4 of 2e, 2 of 2f, 15 of V/3a, 2 of 3b, one each of 3c to 3f, 19 of genetic group 4, 1 of genetic group 5, 5 of genetic group 6, 10 of genetic group 7, 4 of genetic group 8, 5 of genetic group 9, 5 of genetic group 10 and 3 of genetic group 11. Genotypes had been determined by sequence analysis of (i) the core gene or its parts (486 bp [nt 342 to 827] or 390 bp [nt 342 to 731]), (ii) the E1 gene, or (iii) a part of the NS5b region (1,093 bp [nt 8279 to 9371] or 329 bp [nt 8279 to 8607]), or the combination of the E1 gene and a part of the NS5b region.

Serum or plasma samples positive for HCV RNA were obtained from 130 blood donors in Spain, 53 donors in Brazil, 106 donors in China, and 30 patients with chronic hepatitis in Macau. Genotyping was performed with 100 μl of each sample.

Amplification by PCR and sequence analysis. HCV RNAs were converted to cDNA with the antisense primer 81, with a sequence of 5′-CTAGTCATAGCC TCCGTGAA-3′, and were amplified by the first round of PCR with primer 80 (sense; 5′-GACACCCGCTGTTTTGACTC-3′) and primer 81 for 35 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 90 s [8.5 min in the last cycle]) to obtain a fragment of 377 bp (nt 8256 to 8632). The product was subjected to a second round of PCR with nested primers, primer 317 (sense; 5′-ACCCGCTGTTTTG ACTCNAC-3′ [N = G, A, T, or C]) and primer 316 (antisense; 5′-CATAGGC TGCGTGAAGGCTC-3′), for 30 cycles under the same conditions used for the first round, and a fragment of 369 bp (nt 8259 to 8627) was obtained.

The amplification product was ligated with the M13 phage vector, and clones carrying the target sequence were selected. Three clones were propagated from each sample, and their nucleotide sequences were determined with the Auto-Read DNA sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Then, the consensus sequence of three clones was adopted for each sample.

Nucleotide sequence accession numbers. The nucleotide sequences reported here are deposited in the DDBJ/GenBank/EMBL nucleotide sequence databases under accession numbers D85929 to D85938.

RESULTS

Specificity and sensitivity of HCV genotyping by PCR. The electrophoretic patterns from the two reactions of the second-round PCR are shown in Fig. 2 for representative HCV isolates of the five common genotypes whose entire sequences are known. A panel of 191 HCV isolates, genotyped by sequence analysis of the core gene, was tested by this simplified PCR genotyping method (Table 1). The method correctly determined the genotypes of all 110 HCV isolates of the five common genotypes: I/1a in 20 isolates, II/1b in 42 isolates, III/2a in 18 isolates, IV/2b in 15 isolates, and V/3a in 15 isolates. For isolates of the other genotypes, 71 (88%) of 81 samples were determined not to be the five common genotypes. Cross-reactions with noncorresponding genotypes were observed in 3 (21%) of 14 isolates of genotype 2c in the detection of genotype IV/2b; furthermore, all 4 isolates in genetic group 8 cross-reacted with I/1a and all 3 isolates in genetic group 11 cross-reacted with I/1a.

When serial twofold dilutions of serum samples containing each of the five common genotypes were tested, they were genotyped at a sensitivity of one-fourth to one-eighth that for the detection of HCV RNA by nested PCR with 5′ UTR primers. In other words, amplification of HCV RNA is more sensitive for the 5′ UTR than for the core region.

HCV isolates of genotype I/1a which were mistyped as II/1b by a cross-reaction with antisense primer 133 by the original method. HCV isolates of genotype I/1a were amplified by the second-round PCR with the pair of primer 104 (universal sense primer) and primer 133 (antisense primer specific for genotype II/1b) when the annealing was performed at 60°C. However, the cross-reaction of I/1a sequences with primer 133 decreased substantially compared with that of the prior mixture of four or five antisense primers used in the original method (27, 28).

The misannealing of genotype I/1a sequences with antisense primer 133 was rarely observed when the annealing temperature was increased to 64°C (Fig. 3). Sense and antisense primers (primers 389 and 492, respectively), newly designed for genotype II/1b, also did not amplify I/1a sequences at either 60 or 64°C. Nonspecific amplification did not occur in the PCR with the combination of the universal sense primer (primer 104) from the previous method and antisense primer (primer 492) from the improved method or the combination of the sense primer (primer 389) from the improved method and antisense primer (primer 133) from the previous method.

HCV genotypes in blood donors and hepatitis patients in Spain, Brazil, China, and Macau. Table 2 presents the HCV genotypes in the blood of 289 donors from Spain, Brazil, and China plus 30 patients with chronic hepatitis from Macau. HCV isolates from Brazil and China were classified into the five common genotypes. Three donors from China had mixed infections with HCV of genotypes II/1b and III/2a. Also, a mixed infection with genotype I/1a and II/1b was observed in one donor from Spain. Mixed infections in these sera were confirmed by the second-generation method of genotyping by PCR (21).

HCV isolates with unclassifiable genotypes. The genotypes of eight HCV isolates from Spain and five from Macau could not be classified into the five common genotypes by the simplified method described herein. Three of the eight HCV isolates from Spain were genotyped as type 2c by PCR with primers specific for this genotype (19). HCV isolates from the remaining five samples from Spain, as well as all five samples from Macau, were sequenced; Table 3 compares their sequences with the corresponding sequences of the HCV isolates

TABLE 1. Specificity of a simplified genotyping method by PCR with HCV core primers[a]

Group and genotype	No. of isolates	No. (%) of isolates detected by:				
		Reaction one (64°C)		Reaction two (62°C)		
		I/1a (primers 418 and 296; 86 bp)	II/1b (primers 389 and 492; 125 bp)	III/2a (primers 491 and 134; 108 bp)	IV/2b (primers 491 and 471; 121 bp)	V/3a (primers 397 and 473; 84 bp)
Group 1						
I/1a	20	20 (100)	0	0	0	0
II/1b	42	0	42 (100)	0	0	0
1c	3	0	0	0	0	0
Group 2						
III/2a	18	0	0	18 (100)	0	0
IV/2b	15	0	0	0	15 (100)	0
2c	14	0	0	0	3 (21)[b]	0
2e	4	0	0	0	0	0
2f	2	0	0	0	0	0
Group 3						
V/3a	15	0	0	0	0	15 (100)
3b	2	0	0	0	0	0
3c-3f	4	0	0	0	0	0
Group 4	19	0	0	0	0	0
Group 5	1	0	0	0	0	0
Group 6	5	0	0	0	0	0
Group 7	10	0	0	0	0	0
Group 8	4	4 (100)[b]	0	0	0	0
Group 9	5	0	0	0	0	0
Group 10	5	0	0	0	0	0
Group 11	3	3 (100)[b]	0	0	0	0

[a] The HCV isolates in a panel of 191 serum samples were genotyped by the method described in the text. The HCV isolates were classified into genetic groups and genotypes by sequence analyses. The results of the second round of PCR, which was performed in two separate reactions with different sets of sense and antisense primers, are indicated.

[b] Nonspecific reaction for HCV isolates of genotypes different from those expected by the genotype-specific primer pairs used in the current method.

whose genotypes are known and have been reported previously (4, 16, 23, 31, 33, 34).

One of the five HCV isolates from Spain (isolate BA107) which were not classifiable into any of the five common genotypes had an NS5b sequence (nt 8279 to 8607) showing 92.7% similarity with the NS5b sequence of isolate NL29 of genotype 1d (33). Likewise, two other isolates from Spain (isolates BA037 and BA083) were 91.2 to 92.1% similar to TK2 isolates of genotype 4d (4, 21) for the sequence compared. The sequences of the remaining two Spanish isolates (isolates BA045 and BA047) resembled the reported sequences of isolates of genetic group 2. However, BA045 was at most 85.4% similar to isolate NL50 of genotype 2e (33), and BA047 showed a maximal similarity of 85.1% to isolate BEBE1 of genotype 2c (19). These sequences of isolates BA045 and BA047 were only 81.8% similar to each other. On the basis of these results, they would belong to novel genotypes in genetic group 2.

The NS5b sequences (nt 8279 to 8607) of all five HCV isolates from Macau that were unclassifiable into the five common genotypes were 94.8 to 96.0% similar to the NS5b sequence of isolate VN506 of genotype 6a. Thus, the similarities of these five isolates to the various sequenced HCV genotypes are compared in Table 3.

DISCUSSION

Among the different parts of the HCV genome, consisting of three structural, six nonstructural, and two untranslated regions (8, 10), the core gene appears to be most suitable for genotyping by PCR. Genotyping by selective amplification of other parts of the HCV genome, such as the NS5b region, has been undertaken (5); however, genotype-specific primers with sequences deduced from the sequence of this region are less efficient than core gene primers in amplifying cDNA fragments transcribed from HCV RNA (2). Furthermore, because of sequence variations in the NS5b region which are much greater than those in the core gene (18), it would be difficult to design NS5b primers which can anneal with HCV cDNA of specified genotypes with high degrees of sensitivity and specificity.

Our original method for genotyping by PCR used genotype-specific, antisense primers which were deduced from a comparison of only 44 core gene sequences available at that time (27). Although the method distinguished four genotypes, genotypes I/1a, II/1b, III/2a, and IV/2b, in our hands, others found that the primer specific to II/1b (primer 133) may amplify cDNA of genotype I/1a erroneously. As a consequence, ~20% of HCV RNA samples were misclassified as mixed genotypes (13). We have also found that some HCV sequences of genotype I/1a could be amplified by primer 133 when annealing was performed at 58°C, a temperature lower than that used in the original method (unpublished data). Nonspecific amplification occurred even at an annealing temperature of 60°C when the second round of PCR was performed with the universal sense primer (primer 104) and only the antisense primer specific for genotype II/1b (primer 133), as indicated in Fig. 3. Amplification was less frequent, however, when the mixture of four or five antisense primers was used, as in the original method (27, 28), possibly because the authentic and more closely matched antisense primer for genotype I/1a

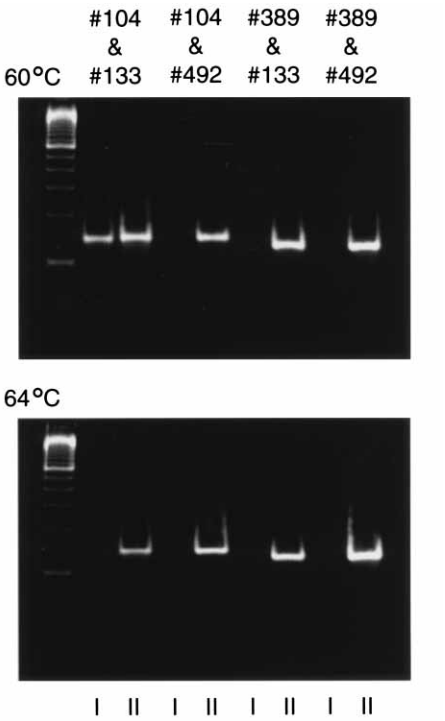

FIG. 3. Amplification of genotypes I/1a and II/1b by PCR with primers specific for genotype II/1b. The four lanes labeled I are for genotype I/1a, and the other four lanes labeled II are for genotype II/1b. Amplification in the second-round PCR was performed with four combinations of two sense and two antisense primers, with sense primer 104 and antisense primer 133 giving rise to a PCR product of 144 bp as in the original method (27), primers 389 and 492 giving rise to a PCR product of 125 bp in the improved method, as well as hybrids thereof: primers 104 and 492 gave rise to a PCR product of 144 bp, and primers 389 and 133 gave rise to a PCR product of 125 bp. Annealing was carried out at either 60 or 64°C. The migration positions of the molecular size markers (100-bp ladder; GIBCO-BRL) are indicated on the left.

(primer 296) overwhelmed primer 133 in the annealing with I/1a sequences (unpublished data). Although such a nonspecific annealing could be avoided when the temperature was increased to 64°C (Fig. 3), primer 133 is not specific for genotype II/1b.

The introduction of an antisense primer specific to genotype II/1b (primer 492) in combination with a sense primer specific to II/1b (primer 389), in place of the universal sense primer and primer 133 in the original method (27, 28), increased the specificity for detecting genotype II/1b. Increasing the annealing temperature from 60 to 64°C and curtailing the PCR procedure from 30 to 25 cycles were additional factors for improving the specificity of the current PCR-based genotyping technique.

To circumvent possible nonspecific reactions in the original method, we reported a second-generation method of genotyping with sense and antisense primers designed from a comparison of 319 core gene sequences. The second-generation method can classify samples into the five common genotypes, I/1a, II/1b, III/2a, IV/2b, and V/3a, for example, without misinterpreting any of 20 HCV isolates of genotype I/1a for genotype II/1b. Although that second-generation method is specific, it involves five different reactions with type-specific sense and antisense primers in the second round of PCR. For the purpose of simplifying that method, without losing specificity, we developed the current technique. In this third-generation PCR method, the products of the five different genotypes are not sufficiently different in length to allow their easy identification. Hence, the second-round PCR had to be performed in two separate steps, differentiating genotypes I/1a and II/1b in one reaction and distinguishing genotypes III/2a, IV/2b, and V/3a in the other (Fig. 1).

For some HCV isolates genotyping was not feasible by the current method. When they were amplified with core gene primers specific for genotype 2c, three HCV isolates from Spain were found to be of this genotype. An NS5b stretch of 329 bp (nt 8279 to 8607) of 10 HCV isolates with unclassified genotypes was sequenced for comparison with the reported sequences of HCV isolates with known genotypes. All five isolates from Macau belonged to genotype 6a, which was originally reported to be from Hong Kong (4, 31). Three of the five unclassifiable isolates from Spain were found to be of genotype 1d (33) or 4d (4); the remaining two HCV isolates from Spain, however, had sequences that classified them in genetic group 2 but that were different from those of any reported genotypes and, therefore, are considered to belong to novel genotypes in genetic group 2.

The differentiation of HCV into genotypes is at a critical point. The present count exceeds 80 genotypes, and new genotypes are being added to the list on the basis of variations in short stretches of the sequence of the HCV genome. It will probably not be in the interest of clinicians to deal with so many HCV genotypes, because their practical significance is still not clear.

In Japan, genotype II/1b has been associated with more severe disease and a poorer response to interferon than genotypes III/2a and IV/2b (9, 14, 35, 38). Because genotype I/1a rarely occurs in Japan, we have not been able to determine if there are any clinical differences between genotypes I/1a and II/1b, even though they belong to the same genetic group, group 1 (type 1 by the classification of Simmonds et al. [31]). Such differences, should they exist, may be important in areas where both genotypes I/1a and II/1b prevail, such as in the

TABLE 2. Prevalence of HCV genotypes in blood donors or patients from Spain, Brazil, China, and Macau

Country	N[a]	No. (%) of isolates of the following genotype:						
		I/1a	II/1b	III/2a	IV/2b	V/3a	Mixed[b]	Unclassified
Spain	130	19 (15)	87 (67)	1 (1)	0	14 (11)	1 (1)	8 (6)
Brazil	53	23 (43)	25 (47)	0	0	5 (9)	0	0
China	106	0	77 (73)	26 (25)	0	0	3 (3)	0
Macau	30	2 (7)	23 (77)	0	0	0	0	5 (17)
Total	319	44 (14)	212 (66)	27 (8)	0	19 (6)	4 (1)	13 (4)

[a] *N*, number of HCV reactive samples.
[b] Two different genotypes were detected. They were genotypes I/1a and II/1b in a donor from Spain, but II/1b and III/2a in three donors from China.

TABLE 3. Similarity of 10 HCV isolates of genotypes unclassifiable by PCR from Spain and Macau with those of known genotypes within a sequence of 329 bp in the NS5b region (nt 8279 to 8607)

Genotype[a]	Isolate	Accession no.	% Similarity				
			Spain				Macau (five isolates)[c]
			BA107	BA045[b]	BA047[b]	BA037 and BA083	
I/1a	HCV-1	M62321	81.8	64.4	65.0	69.9	66.9–67.5
II/1b	HC-J4/83	D01217	85.7	62.9	64.4	64.4–64.7	66.0–66.9
1c (Okamoto et al. [23])	HC-G9	D14853	80.2	64.1	66.3	66.6–67.2	68.4–69.3
1c (Simmonds et al. [31])[d]	2TY4	L23446	79.7	62.6	63.1	66.2–66.7	61.7–63.1
1d	NL29	L38377	92.7[e]	65.9	67.4	66.8–67.1	67.7–68.6
1e	CAM1078	L38361	77.8	64.4	65.7	63.2–63.8	66.0–66.9
1f	FR2	L38371	82.2	63.2	67.3	65.4–67.6	66.7–67.3
III/2a	HC-J6	D00944	67.8	83.3	85.1	67.8	63.5–64.7
IV/2b	HC-J8	D01221	67.5	78.4	78.7	65.7–66.0	63.8–65.0
2c	BEBE1	D50409	67.8	79.3	85.1	64.7–65.3	62.9–64.4
2d	NE92	L23632	66.3	82.1	84.8	63.8–65.0	64.7–65.7
2e (Tokita et al. [34])	JK020	D49760	65.0	79.6	83.9	64.1–64.4	61.7–62.6
2e (Stuyver et al. [33])	NL50	L44602	66.5	85.4	82.3	63.7–64.9	63.7–65.5
2f (Tokita et al. [34])	JK081	D49769	63.8	79.0	80.5	63.2–63.5	63.2–64.7
2f (Stuyver et al. [33])	NL33	L44601	65.0	84.8	84.2	64.4–65.3	62.9–64.4
2(I) (Mellor et al. [16])[d]	EUGAM29	U31268	66.0	73.8	75.2	63.1–64.1	62.6–64.1
V/3a	NZL1	D17763	64.7	59.6	61.4	67.8–68.4	64.4–66.0
4a (Simmonds et al. [31])[d]	EG-13	L23469	67.6	60.4	61.7	79.7–82.0	66.2–67.6
4d (Bukh et al. [4])	TK2	D85928	66.6	63.5	62.0	91.2–92.1	64.1–65.3
5a	FR741	D50467	69.3	63.8	64.7	63.8–64.1	64.1–65.3
6a	VN506	D21319	68.4	64.7	66.0	66.0–66.6	94.8–96.0
6b	Th580	D37855	65.0	60.8	61.1	62.9	78.4–79.3
7a	VN540	D21324	69.0	65.3	64.4	64.4–65.7	72.3–73.3
8a	VN507	D21320	63.8	62.3	67.8	67.8–68.1	70.1–71.1
9a	VN004	D21315	64.7	63.5	64.4	64.1–64.4	69.9–71.1
10a	JK049	D63821	63.8	62.0	67.2	68.4–69.0	65.7–66.9
11a	JK046	D63822	71.1	65.3	66.9	66.6–67.5	71.1–71.7

[a] Reporters of genotypes of unclear identity are indicated in parentheses, along with reference numbers.
[b] BA045 and BA047 had 81.8% similarity.
[c] The five isolates are MA007, MA025, MA026, MA054, and MA115.
[d] A sequence of 222 bp in the NS5b region (nt 8313 to 8534) was compared.
[e] Homology of >90% is indicated by underscoring.

United States, Canada, Europe, and Oceania. In a report from the United States, patients infected with HCV of genotype I/1a responded to interferon better than those infected with HCV of genotype II/1b during a short-term follow-up (13); nonetheless, they did not differ appreciably in their long-term responses. By contrast, reports from France document more favorable responses to interferon in patients infected with HCV genotype I/1a than in those infected with genotype II/1b (14, 20, 29). Whether genotypes I/1a and II/1b have different responses to interferon may influence the methods used for genotyping.

Three genotyping methods based on different principles are popular. They are restriction fragment length polymorphism analysis of the 5′ UTR and amplified core sequences by PCR (15, 17); line probe assay of the 5′ UTR and core amplicons for reverse hybridization with type-specific, synthetic oligonucleotides (32, 33); and selective amplification of genomic sequences by PCR with genotype-specific primers, as described here. If HCV isolates of genetic group 1 do not have a different response to interferon than isolates of other groups, clinicians would need to know whether their patients were infected with HCV of genetic group 1; it would not matter whether they were infected with genotype I/1a or II/1b. If, however, isolates of these genotypes do respond differently to therapy, physicians may wish to distinguish between genotypes I/1a and II/1b, especially in countries where these two genotypes occur frequently, such as in the United States, Canada, Europe, and Oceania (17, 18). At present, II/1b appears to be the only HCV genotype that is consistently associated with any clinical significance.

HCV genotypes may keep growing in number, but only a few appear to occur locally. Hence, genotyping of HCV isolates into >80 genotypes or even into 11 genetic groups is not required everywhere. The five common genotypes, genotypes I/1a, II/1b, III/a, IV/2b, and V/3a, account for practically all HCV isolates prevalent in industrialized countries but not in Africa, the Middle East, the Indian subcontinent, or Southeast Asia (3, 16, 18, 30, 33, 36). It is hoped that the simplified genotyping method described here may be used in epidemiological studies, for example, to determine the route of infection (27), which is not established for almost one-half of community-acquired HCV infections (1).

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