JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 161–164 Vol. 35, No. 1
0095-1137/97/$04.00+0

Subtype 2c of Hepatitis C Virus Is Highly Prevalent in Italy and Is Heterogeneous in the NS5A Region

F. MAGGI, M. L. VATTERONI, C. FORNAI, A. MORRICA, M. GIORGI, M. BENDINELLI,* AND M. PISTELLO

Virology Section and Retrovirus Center, Department of Biomedicine, University of Pisa, Pisa, Italy

Received 26 June 1996/Returned for modification 24 September 1996/Accepted 15 October 1996

Hepatitis C virus (HCV) isolates, obtained from 50 Italian patients with community-acquired infection, that had previously been classified as subtype 2a or 2b by current rapid genotyping methods were further characterized by partial sequence analysis. All the isolates were reclassified: 45 within subtype 2c and the other 5 as subtype 1b, 3a, or 4d. Thus, subtype 2c is much more prevalent than previously recognized, with about 30% of all HCV strains detected in Italy being subtype 2c. In contrast, isolates of subtypes 2a and 2b appear to be infrequent, if not absent. Further studies showed that subtype 2c isolates are heterogeneous in the NS5A region, in that they may or may not contain a 57-nucleotide (nt) segment spanning from nt 7533 to nt 7589 of the viral genome. Partial nucleotide sequencing of the NS5B region of four 2c subtypes excluded the possibility that the isolates possessing or not possessing the 57-nt segment in the NS5A region may have resulted from recombination phenomena.

On the basis of differences in nucleotide genome sequences, hepatitis C virus (HCV) isolates are currently grouped into at least six different genotypes, most of which can be further divided into several subtypes (16). Information on the geographical and epidemiological distributions of such genotypes is rapidly accumulating, and evidence suggests the interesting possibility that severity of infection and responsiveness to interferon treatment are subtype related (15, 17).

Several relatively rapid and simple methods for identifying the genotypes of HCV isolates have been described. These include analysis of subgenomic regions of the virus by genotype-specific PCR (10, 11), specific probe hybridization (18), and restriction fragment length polymorphism analysis (5). However, such methods are dependent on the existence of small nucleotide differences in subgenomic regions and must be constantly updated to permit the identification of newly recognized HCV types and subtypes.

In a previous report we partially sequenced six HCV isolates that had remained untyped by the genotype-specific PCR method described by Okamoto et al. (10) and found all of them to belong to subtype 2c, thus suggesting that this subtype is more prevalent in Italy than was previously realized (4). Concomitantly, Okamoto et al. (13) showed that 14 untypeable Italian HCV isolates also belonged to subtype 2c. Since subtype 2c is not recognized by current HCV genotyping assays, in the present study we examined the possibility that its prevalence in Italy is underestimated. For this purpose, we partially sequenced 50 HCV isolates that had originally been classified as subtype 2a or 2b on the basis of one of three rapid genotyping systems in current use. The results indicate that subtype 2c causes approximately 30% of all cases of community-acquired hepatitis occurring in Italy. In contrast, isolates of subtypes 2a and 2b appear to be infrequent if not absent. In addition, we found that subtype 2c isolates are heterogeneous in the NS5A region, in that they may or may not contain a 57-nucleotide (nt) segment spanning from nt 7533 to nt 7589 of the viral genome.

* Corresponding author. Mailing address: Dipartimento di Biomedicina, Università di Pisa, Via San Zeno 35, I-56127 Pisa, Italy. Phone: 39 50 559.411. Fax: 39 50 555.477. E-mail: bendinelli@biomed.unipi.it.

MATERIALS AND METHODS

Patients. The 50 patients selected for the study (23 males and 27 females with community-acquired infections) were ages 21 to 72 years and lived in various parts of Italy. They were all positive for anti-HCV antibody by third-generation immunoenzymatic and immunoblotting assays (Ortho, Milan, Italy) and were viremic by our in-house nested reverse transcriptase-PCR, performed as described previously (20) with primers covering the 5′ untranslated region of the viral genome (5′-UTR). As indicated in Table 1, all the patients studied had been classified in our laboratory as harboring isolates of subtype 2a or 2b by one of the following methods: an in-house subtype-specific PCR (15) by the method of Okamoto et al. (10, 11), restriction fragment length polymorphism analysis performed as described previously (5), and a commercially available line probe assay (Innogenetics, Zwijndrecht, Belgium) (18). The first method is based on the core region; the last two methods are based on the 5′-UTR of the HCV genome.

Sequencing. The regions selected for sequencing were the 197-bp fragment of the core region, nt 160 to nt 356, which had proved to be valuable for subtype 2c isolate identification in a previous study (4), and the 222-bp fragment of the NS5B gene from nt 7975 to nt 8196. Sequencing was performed as described previously (4). Briefly, amplicons were obtained by nested reverse transcriptase-PCR and were sequenced on an automatic DNA sequencer (Pharmacia, Uppsala, Sweden).

Phylogenetic analysis. Sequence data were edited by using the PC-GENE software package (IntelliGenetics, Geel, Belgium) and were aligned with the consensus sequences of known subtypes, as retrieved by Bukh et al. (2), with the CLUSTAL V program (9). The consensus sequence of the Italian subtype 2c isolates was obtained by multiple alignment with the PILEUP and PRETTY programs of the GCG package (version 7.0). Phylogenetic analysis was carried out with the PHYLIP package (8). Briefly, multiple sequence alignments were resampled by bootstrap resampling (100 data sets) with SEQBOOT, and distances were estimated by analyzing 100 data sets with the Kimura two-parameter model of DNADIST. Phylogenetic trees were constructed by using the neighbor-joining algorithm on the previous set of pairwise distances with NEIGHBOR and 100 bootstrap replicates. Consensus trees were built by using CONSENSE, and unrooted trees were drawn by using DRAWTREE.

NS5A gene amplification. A partial nucleotide sequence of the NS5A region spanning from nt 7500 to nt 7612 was chosen for PCR amplification by using degenerated primers external to a type-specific 57-nt sequence and subsequent analysis of the size of the amplicons produced by the procedure described by Tanaka et al. (19). Briefly, the cDNAs resulting from reverse transcription of the viral RNAs were subjected to nested PCR by using the following conditions: denaturation at 94°C for 45 s, primer annealing at 50°C for 30 s, and product extension at 72°C for 1 min for 35 cycles for the first step with sense primer C (5′-CCTCTCGAGGG[A/G]GA[A/G]CC[G/A/T]GG-3′; positions 7500 to 7519) and antisense primer D (5′-CCTGTCCAGG[A/T][A/G]TA[G/A/T]GACAT-3′; positions 7593 to 7612) and for 25 cycles for the second step with sense primer A (5′-AGGGAGAGCCTGG[A/G]GA[T/C]CC-3′; positions 7507 to 7526) and

TABLE 1. Characterization by sequence analysis of 50 Italian HCV isolates originally classified as subtype 2a or 2b by current genotyping methods

Original genotype	Method used for original genotyping[a]	No. of isolates examined	No. of isolates with the following classification based on sequence homology:			
			1b	2c	3a	4d
2a	PCR	23	0	21	0	2
	LiPA	12	2	9	1	0
	RFLP	3	0	3	0	0
2b	PCR	12	0	12	0	0

[a] PCR, genotype-specific PCR; LiPA, line probe assay; RFLP, restriction fragment length polymorphism analysis.

antisense primer B (5′-GAGTATGACAT[G/A/T]GAGCAGCA-3′; positions 7584 to 7603). This was followed by amplicon electrophoresis in a 4% agarose gel and ethidium bromide staining.

RESULTS

Table 1 presents the results of characterization by sequence analysis of the 197-bp fragment of the core region. Surprisingly, none of the 50 HCV isolates was of subtype 2a or 2b. Five isolates were classified in a genotype outside of genotype 2. All the other isolates were classified as genotype 2c since their sequences were 93 to 95% homologous to the consensus sequence of this subtype and only 85 to 89% and 82 to 89% similar to subtype 2a and 2b consensus sequences, respectively. Phylogenetic analysis and bootstrap resampling methods indicated that the latter isolates as well as the corresponding consensus sequence (2cIT) clustered together with the consensus sequence of subtype 2c, thus confirming that all 45 isolates belonged to this subtype (Fig. 1A and B).

It has been reported that subtypes 2a and 2b contain a 57-nt sequence (positions 7533 to 7589) in the NS5A region which is not present in subtypes 1a, 1b, 1c, and 3a (12, 19). Although partial nucleotide sequences have been reported for the 5′-UTR, core, E1, E2/NS1, NS5B, and 3′-UTR regions of subtype 2c (13), no information is available on the NS5A region of this HCV subtype (3). To investigate whether subtype 2c isolates contain the 57-nt sequence that would appear to be specific for other type 2 genomes (Fig. 2A), we examined the 45 subtype 2c isolates identified in the present study for the presence of this sequence.

Four of the 45 isolates examined yielded no specific amplification bands. The others gave clearly visible bands, but the amplicons produced were not uniform in size (Fig. 2B); 14

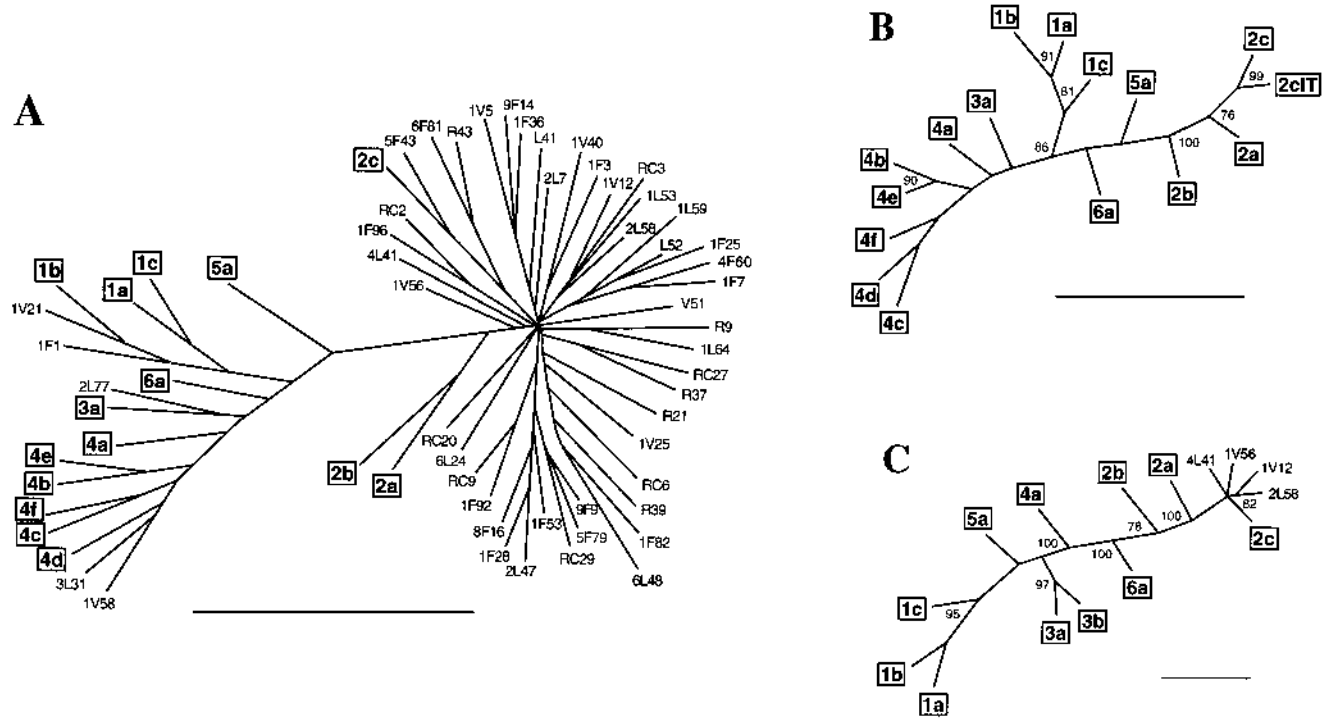

FIG. 1. Phylogenetic trees of HCV isolates constructed on the basis of a core sequence of 197 bp (nt 160 to nt 356) and an NS5B sequence of 222 bp (nt 7975 to nt 8196). Individual core sequences (A) and consensus core sequences (2cIT) (B) of 50 Italian isolates originally classified as belonging to subtypes 2a or 2b are compared with the consensus core sequences of the major HCV subtypes. (C) Individual NS5B sequences of four subtype 2c isolates are compared with the consensus NS5B sequences of the major HCV subtypes. Consensus sequences are in boldface type and are boxed. The numbers at each branch point indicate percent probabilities (>75%) obtained with 100 replications of bootstrap resampling. Bars indicate 10% divergence.

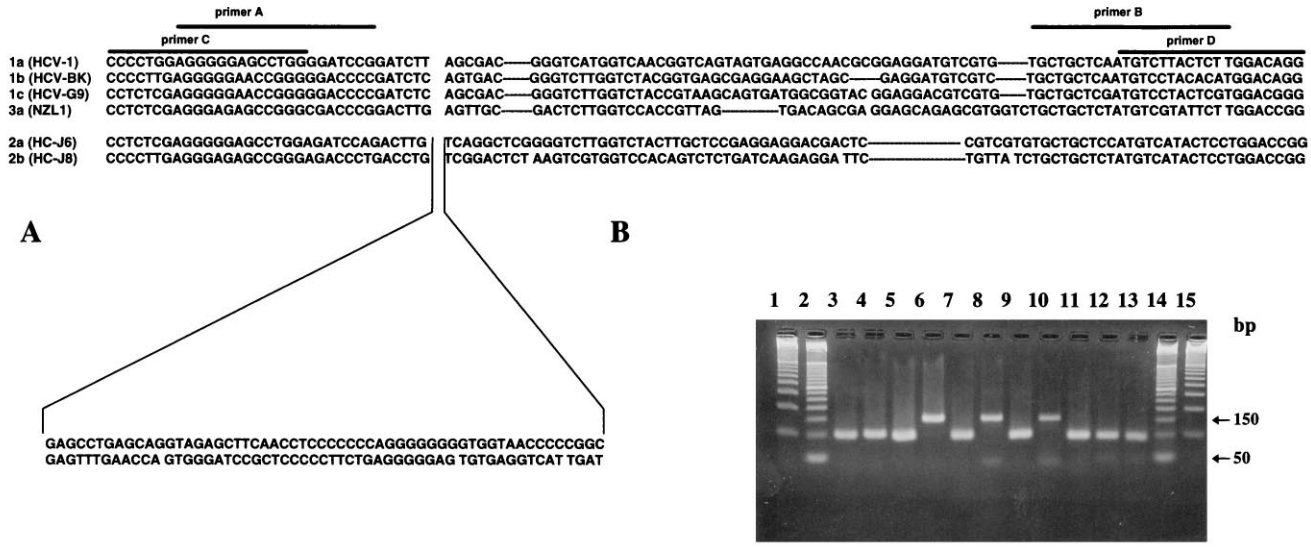

FIG. 2. (A) Nucleotide sequence alignment in the NS5A region (nt positions 7500 to 7612) of different HCV subtypes. The sequences of HCV-1 (subtype 1a), HCV-BK (subtype 1b), HC-G9 (subtype 1c), HCV-J6 (subtype 2a), HCV-J8 (subtype 2b), and NZL1 (subtype 3a) were deduced from the GSDB, DDBJ, EMBL, and NCBI DNA databases. The 57-nt insertion present in certain HCV subtypes is shown. Dashes indicate deletions. PCR primer positions (overlined) are reported. (B) Representative PCR results within the NS5A region of individual subtype 2c isolates. Lanes 3 to 5, 7, 9, and 11 to 13, amplicons approximately 97 bp long (absence of the 57-nt sequence); lanes 6, 8, and 10, amplicons approximately 154 bp long (presence of the 57-nt sequence); lanes 1 and 15 and lanes 2 and 14, DNA molecular size markers (100- and 50-bp ladders, respectively).

isolates (34%) yielded amplicons approximately 154 nt long, a size compatible with the presence of the 57-nt sequence under scrutiny, while the remaining 27 isolates (66%) gave amplicons approximately 97 nt long, indicative of the absence of the 57-nt sequence. In contrast, 16 HCV isolates belonging to genotypes other than type 2, whose genotypes were determined by core sequence analysis and which were examined for comparison, were all found to be negative for this sequence, as expected (Table 2).

Recently, investigators have identified rare HCV isolates that could be classified within one subtype on the basis of analysis of specific regions of the viral genome but that contained sequences not corresponding to the same subtype in other parts of the genome (14). Thus, we reasoned that the existence of isolates classified as subtype 2c on the basis of the core sequence but heterogeneous with regard to the NS5A gene might result from recombination phenomena. To verify this possibility, we compared a 222-bp stretch of the NS5B gene from nt 7975 to nt 8196 from four subtype 2c isolates, two of which were positive and two of which were negative for the 57-nt sequence. Isolates showing low nucleotide homology in the core region were chosen for this study. Analysis of the sequences, obtained as described previously (4), showed that the four isolates were highly homogeneous among themselves (86 to 91%) and confirmed their taxonomic inclusion within subtype 2c (Fig. 1C). These findings confirmed the reliability of sequencing of the core region for typing HCV isolates and, considering the vicinity of the NS5B region to the NS5A region, made it extremely unlikely that subtype 2c isolates possessing or not possessing the 57-nt segment in the NS5A region may have resulted from recombinational events between different HCV genotypes.

TABLE 2. Molecular characterization by PCR analysis of the NS5A region of 41 subtype 2c and 16 nonsubtype 2c HCV isolates

HCV genotype	No. of isolates examined	No. of isolates with the 57-nt insertion
1a	2	0
1b	11	0
2c	41	14
3a	1	0
4d	2	0

DISCUSSION

The present results based on partial sequence analysis of the genomes of 50 viral isolates originally classified as subtype 2a or 2b show that in Italy, HCV subtype 2c is much more prevalent than was previously believed. In conjunction with our previous findings and those of other groups (4, 13), we estimate that the prevalence of this genotype in isolates involved in community-acquired HCV infections is about 30%. In contrast, subtypes 2a and 2b, which previous genotyping studies based on nonsequencing methods had indicated were responsible for about one-third of sporadic HCV infections, appear to be very uncommon, if not absent, in Italy. This observation suggests that other countries also should be monitored for the prevalence of this subtype and demands that genotyping methods be redesigned in such a way as to recognize this subtype as well as other subtypes.

The present data also indicate that widely used nonsequencing methods for HCV typing provide rather imprecise results. In this limited study they not only misidentified subtype 2c isolates as subtype 2a or 2b, which may be justified by the fact that the methods used were not designed to identify subtype 2c, but they also identified as subtype 2a five isolates (isolates from 10% of the patients studied) that sequence analysis has instead conclusively classified as belonging to type 1, 3, or 4. This poor performance of current rapid HCV genotyping methods is in keeping with the results that emerged from a more systematic evaluation of five such methods that we have recently conducted (1). Thus, there is little doubt that rapid HCV typing methods require careful redesign.

Finally, our results indicate that only one-fourth of subtype 2c isolates possess a 57-nt sequence that hitherto had been detected in the NS5A region of all the type 2 HCV isolates studied and that this variation is unlikely to stem from recombinational events between different HCV subtypes. At present, the significance of the presence or absence of this 57-nt segment in the HCV genome is not clear. In the present study, subtype 2c was associated with clinical conditions ranging from persistent chronic hepatitis to cirrhosis, and no correlation between NS5A pattern and infection severity was apparent (data not shown). However, since recent studies have correlated mutations in the NS5A region with susceptibility to interferon therapy (6, 7) and subtype 2c HCV isolates reportedly are poorly responsive to interferon treatment (13), it may be worthwhile to examine whether the presence or absence of the 57-nt segment correlates with interferon susceptibility.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministero della Sanità and Ministero della Università e Ricerca Scientifica, Rome, Italy.

REFERENCES

1. **Bendinelli, M., et al.** Submitted for publication.
2. **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.
3. **Bukh, J., R. H. Miller, and R. H. Purcell.** 1995. Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin. Liver Dis. **15:**41–63.
4. **Cammarota, G., F. Maggi, M. L. Vatteroni, L. Da Prato, L. Barsanti, M. Bendinelli, and M. Pistello.** 1995. Partial nucleotide sequencing of six subtype 2c hepatitis C viruses detected in Italy. J. Clin. Microbiol. **33:**2781–2784.
5. **Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, G. A. Medgyesi, H. Kiyokawa, G. Olim, G. Duraisamy, T. Cuypers, A. A. Saeed, D. Teo, J. Conradie, M. C. Kew, M. Lin, C. Nuchaprayoon, O. K. Ndimbe, and P. L. Yap.** 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. J. Gen. Virol. **76:**1197–1204.
6. **Enomoto, N., I. Sakuma, Y. Asahina, M. Kurosaki, T. Murakami, C. Yamamoto, N. Izumi, F. Marumo, and C. Sato.** 1995. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J. Clin. Invest. **96:**224–230.
7. **Enomoto, N., I. Sakuma, Y. Asahina, M. Kurosaki, T. Murakami, C. Yamamoto, Y. Ogura, N. Izumi, F. Marumo, and C. Sato.** 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. **334:**77–81.
8. **Felsestein, J.** 1991. PHYLIP manual, version 3.5c. University Herbarium, University of California, Berkeley.
9. **Higgins, D. G., A. J. Bleasby, and R. Fuchs.** 1992. Clustal V: improved software for multiple sequence alignments. Comput. Appl. Biosci. **8:**189–191.
10. **Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and infectious sources. J. Gen. Virol. **73:**673–679.
11. **Okamoto, H., H. Tokita, M. Sakamoto, M. Horikita, M. Kojjma, 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.
12. **Okamoto, H., and S. Mishiro.** 1994. Genetic heterogeneity of hepatitis C virus. Intervirology **37:**68–76.
13. **Okamoto, H., M. Crovatto, G. Pozzato, C. Feray, C. Brechot, and S. Mishiro.** 1995. Molecular and clinical characteristics of the hepatitis C virus genotype ’2c’ found in Italians in Italy and France. Int. Hepatol. Commun. **3:**161–165.
14. **Panigrahi, A. K., J. Roca, S. K. Acharya, S. Jameel, and K. Panda.** 1996. Genotype determination of hepatitis C virus from Northern India: identification of a new subtype. J. Med. Virol. **48:**191–198.
15. **Pistello, M., F. Maggi, L. Vatteroni, N. Cecconi, F. Panicucci, G. P. Bresci, L. Gambardella, M. Taddei, A. Bionda, M. Tuoni, and M. Bendinelli.** 1994. Prevalence of hepatitis C virus genotypes in Italy. J. Clin. Microbiol. **32:**232–234.
16. **Simmonds, P., E. C. Holmes, 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 analysis of the NS5 region. J. Gen. Virol. **74:**2391–2399.
17. **Simmonds, P.** 1995. Variability of hepatitis C virus. Hepatology **21:**570–583.
18. **Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Heuverswyn, and G. Maertens.** 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. **74:**1093–1102.
19. **Tanaka, T., K. Tsukiyama-Kohara, K. Yamaguchi, S. Yagi, S. Tanaka, A. Hasegawa, Y. Ohta, N. Hattori, and M. Kohara.** 1994. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology **19:**1347–1353.
20. **Vatteroni, M. L., M. Pistello, F. Maggi, N. Cecconi, F. Panicucci, and M. Bendinelli.** 1994. Hepatitis C virus serological and polymerase chain reactions in human immunodeficiency virus-positive and negative patients. Clin. Diagn. Virol. **2:**7–16.