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Distribution of Viral Genotypes in Italy Determined by Hepatitis C Virus Typing by DNA Immunoassay

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The distribution of hepatitis C virus (HCV) genotypes in Italy was investigated by PCR amplification of the E1 region and hybridization with type I- and type II-specific nonisotopic probes. Positive PCR results were obtained for 65 of 72 patients (90.3%). Type I was detected in 13 of 72 patients (18%), type II was detected in 39 patients (54.2%), and a mixed type I-type II infection was detected in 7 patients (9.7%). Six amplification products not classified by this method shared a low level of homology with HCV types I and II. HCV type I was significantly associated with human immunodeficiency virus, whereas HCV type II was detected in older subjects who were negative for human immunodeficiency virus markers. These results indicate different epidemiological distributions of HCV types I and II in Italy.

Hepatitis C virus (HCV), a positive-stranded RNA virus (6) distantly related to the pestiviruses and flaviviruses (14), has been identified as the major etiologic agent of non-A, non-B hepatitis (NANBH) (6). Comparative sequence analysis of the viral genome has shown that the HCV 5′ untranslated region is very well conserved (7, 9, 10, 12, 17, 18, 27–29), whereas considerable sequence divergence has been reported for other genomic regions, including the E1 and E2 genes, which encode the putative viral surface proteins (10, 12, 27, 29–31).

On the basis of sequence divergences, different HCV genotypes have been identified. Several classifications have been proposed (2–4, 8, 10, 15, 17, 23, 24) (Table 1). Okamoto et al. (17) divided completely sequenced HCV genomes into four groups (HCV I through IV). Other proposed classifications are based on partial sequencing of the HCV 5′ untranslated region (1, 4), C, NS3, NS5 (4, 23), and E1 (2) regions.

The differential prevalence of HCV genotypes appears to be linked to the geographic area of origin (26), and the possible relationship between HCV genotypes and clinical features of infection (phenotypic expression of disease) has recently aroused considerable interest (19, 25, 26, 32). Several different approaches for HCV type determination have been proposed, including differential hybridization with type-specific probes (16, 25, 26), restriction fragment length polymorphism (16, 26), and PCR with type-specific primers (19). To investigate the distribution of HCV genotypes in Italy, we have developed an HCV typing system based on reverse transcription PCR of the E1 region, coupled with hybridization on microtiter plates with type-specific nonisotopic probes. This approach was chosen since extensive sequence variation has been described for the E1 region among all HCV types so far identified (2, 10, 12, 27, 29–31), including types I and II (classification of Okamoto et al. [17]), which are the most frequently detected in Europe (16, 25, 26) and are not discriminated by analysis of other genomic regions (2–4, 16, 23, 25).

HCV type determination by differential hybridization. Preliminary nucleotide sequence analysis of the HCV E1 region was performed by cloning in the Bluescript plasmid vector (Stratagene, San Diego, Calif.) the reverse transcription PCR products derived from serum samples of eight NANBH patients. Nucleotide sequence analysis was performed on denatured double-stranded DNA by the dideoxy-chain termination method (22) with the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). At least three independent clones were analyzed for each isolate in order to deduce a consensus sequence.

The alignment with HCV genomic fragments belonging to HCV types I and II (7, 27) revealed that both genotypes were represented among the analyzed isolates. In two cases (sequences a and a′ and e and e′ in Fig. 1), E1 sequences belonging to both type I and type II were isolated from the same patient. HCV type II sequences were found for a donor-recipient pair involved in posttransfusion hepatitis (sequences f and g).

Most of the substitutions occurring between the two HCV types at both the nucleotide and the amino acid levels clustered in segments displaying changes specific for the viral type. Comparative sequence alignment indicated the existence of a

TABLE 1. Proposed classifications of HCV genotypes

Type as classified by (reference):			
Okamoto et al. (17) or Mori et al. (15)[a]	Cha et al. (3)	Simmonds et al. (23)	Bukh et al. (2)
I	I	1a	I/1a
II	II	1b	II/1b
NC[b]	NC	1c	NC
III	III	2a	III/2a
IV	III	2b	IV/2b
NC	III	2c	2c
V	IV	3a	(V)/3a
VI	IV	3b	NC
NC	NC	4a	4a
NC	NC	NC	4b
NC	NC	NC	4c
NC	NC	NC	4d
NC	V	5a	5a
NC	NC	6a	6a

[a] Types V and VI are classified by Mori et al.; I through IV are by Okamoto et al.
[b] NC, not classified.

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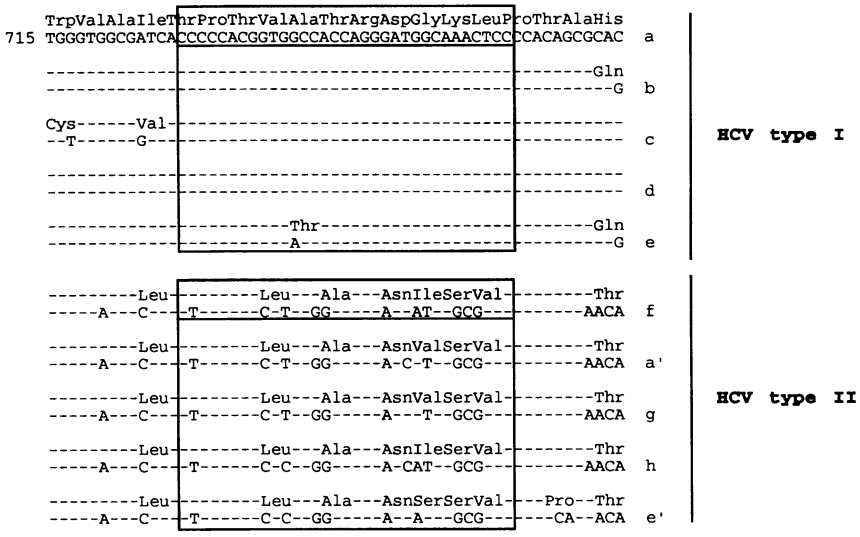

FIG. 1. Nucleotide and amino acid heterogeneity between HCV types I and II in the E1 region. The target sequences for differential hybridization (boxed) and the probes' sequences (underlined) are shown. Sequence pairs a and a′ and e and e′ were derived from a single patient each.

fragment (nucleotides 728 to 760) containing several type-specific variations, most of which were conserved within a single genotype. Only one point mutation was detected in this fragment among the five type I sequences, whereas type II sequences displayed one or two base changes in comparison with each other. In contrast, at least 10 nucleotide mutations between viral types I and II were present (Fig. 1).

Biotinylated type-specific oligonucleotide probes were synthesized according to sequences a (probe HC-I) and f (probe HC-II) (Fig. 1 and Table 2) and used to develop a differential hybridization assay for HCV typing. Hybridization was carried out with the DNA enzyme immunoassay (DEIA) (11, 13, 21). The DEIA system relies on a monoclonal antibody that selectively reacts with double-stranded and not with single-stranded DNA.

To evaluate the method, the PCR products corresponding to sequences a, b, d, e, f, and g were hybridized with both type-specific probes HC-I and HC-II for 1 h at 55°C. The DEIA results were consistent with the known nucleotide sequence (Fig. 2). Optical density (OD) values obtained by testing HCV type I sequences with HC-II probe and vice versa were used to derive the cutoff value of the method (mean + 3 standard deviations of 20 determinations = 200 OD at 450 nm [OD_{450}] units). Hybridization with the homologous probe led to OD values of at least 3.5-fold the cutoff value (range, 730 to 2,730 OD_{450} units). With the hybridization conditions used, the DEIA results were not influenced by the presence (in the amplified fragment) of up to two point mutations compared with the probe sequence.

Distribution of HCV types in Italy. The distribution of HCV types in 72 Italian patients hospitalized for acute (n = 21) or chronic (n = 51) NANBH was analyzed. The clinical and biochemical characteristics of patients are depicted in Table 3. All patients were found to have HCV RNA-positive serum as determined by using primers located in the conserved 5′ untranslated region of the viral genome (11, 21). The HCV E1 region was reverse transcribed and amplified from all samples by nested PCR. Two distinct sets of primers, whose sequences were conserved among different HCV genotypes, were used (Table 2). The first set, selected on the basis of HCV type I (7) and II (27, 29) sequences, included primers P, HE5, HC4, HE2, and HE3. The second set of primers, homologous to

TABLE 2. Oligonucleotide primers and probes used for HCV typing

Primer[a]	Sequence (5′→3′)[b]	Nucleotide positions	Use
P (a)	CCAGGTACAACCGAACCAATTGCC	1662–1639	cDNA synthesis
HE5 (a)	CCCGCCAGGACTCCCCAGTG	1073–1054	External PCR primer
HC4 (s)	GTAAGGTCATCGATACCCTT	359–378	External PCR primer
HE2 (a)	GCTTGTGGGATCCGGAGCAG	1028–1009	Internal PCR primer
HE3 (s)	GCCTACCAAGTGCGCAAC	571–588	Internal PCR primer
CLK4 (a)	TCATCCAGGTGCAGCCGAACC	1671–1652	cDNA synthesis; external PCR primer
CLK1 (s)	TTTGCCGACCTCATGGGGTAC	387–408	External PCR primer
CLK3 (a)	CAGCTGCCATTGGTGTTGAT	1259–1239	Internal PCR primer
CLK2 (s)	GGGAATTTACCCGGTTGCTC	498–518	Internal PCR primer
HC-I (s)	CCCCCACGGTGGCCACCAGGGATGGCAAACTCC	728–760	Type I-specific probe
HC-II (s)	CTCCCACGCTTGCGGCCAGGAATATCAGCGTCC	728–760	Type II-specific probe

[a] a, antisense; s, sense.
[b] The type-specific mutations are underlined.

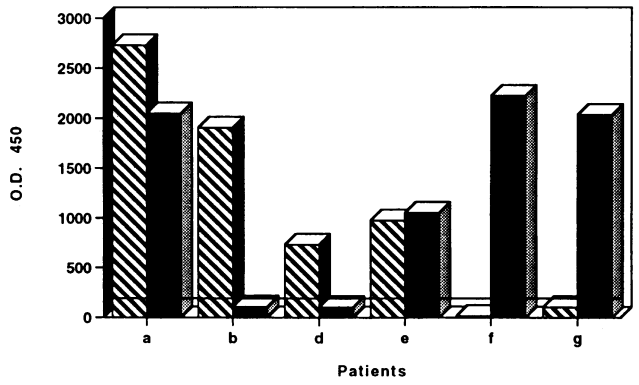

FIG. 2. HCV typing of PCR products for patients a through g. Amplified E1 gene fragments were hybridized with type-specific probes HC-I (▧) and HC-II (■). The horizontal line represents the cutoff value of the method (mean + 3 standard deviations of 20 determinations = 200 OD_{450} units).

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    TrpValAlaIleThrProThrValAlaThrArgAspGlyLysLeuProThrAlaHis
715 TGGGTGGCGATCACCCCCACGGTGGCCACCAGGGATGGCAAACTCCCCACAGCGCAC  a

    ---------Leu---------Leu---Ala---AsnIleSerVal---------Thr
    -----A---C----T------C-T--GG-----A--AT--GCG----------AACA  b

    ------LeuLeu--------------AlaProTyrProAsnAla---LeuGluSer
    ------CTCC----------C-----TG-GCCTT--CCG--CGC---GCTT-A-TCT  c

    ---------Leu---------------AlaProHisLeuSerAla---LeuGluSer
    --------TC----------C-----TG-GCCAC--CTG-GCGCT--GCTT-A-TCT  d

    ---ThrProVal---------------Val---TyrValGlyAla---------Ser
    ---ACTC-AG-G--A--T--A-----AGT----T-C-T-GG-GCA-----C--TTCG  e

    ---ThrProVal---------------Val---HisIleGlyAlaThr------Ser
    ---ACCC-AG-G--A--T--A-----AGT----C-CAT-GG-GCAA----C--TTCG  f

    ------ProVal------AsnMet---IleSerArgProGlyAlaLeu---LysGly
    ------C--G----T----ATA----T-TA--TCGACC-GGCGCT-T---CAA-GGG  g

    ------ProVal------AsnMet---ValSerGlnProGlyAlaPhe---GluGly
    ------C--G-T--C----ACA----TGTA--TC-ACCTGGCGCTTT---T-AAGGG  h
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FIG. 3. Alignment of E1 sequences derived from unclassified PCR products. Sequences a and b were used to derive type I- and type II-specific probes (underlined), which correspond to sequences a and f, respectively, in Fig. 1. Sequences c through h are for PCR products not classified by the DEIA.

HCV type II, III (18), and IV (17) sequences, included primers CLK 1 to 4. Total RNA was isolated from 200 μl of serum by the guanidinium thiocyanate-phenol-chloroform procedure (5). After denaturation (90°C for 2 min), 1/3 of the RNA preparation was reverse transcribed as previously described (21) and 1/10 of the cDNA was amplified by nested PCR with external primers for 35 cycles of denaturation for 1 min at 94°C, annealing of primers for 1 min at 45°C, and extension for 2 min at 72°C. In the second step, 3 μl of the first reaction mixture was amplified with internal primers, by the same protocol, for a further 25 cycles.

All 72 RNA samples were analyzed with the first set of primers, with positive results for 51 samples (70.8%). The negative samples were amplified with the second primer set, and 14 of 21 (66.6%) were found to be PCR positive. On the whole, amplification of the E1 region gave positive results for 65 of 72 patients (90.3%). Hybridization by the DEIA with type I- and II-specific probes detected HCV type I in 13 of 72 patients (18%) and HCV type II in 39 patients (54.2%). A mixed type I-type II infection was present in seven patients (9.7%), whereas six amplification products (all obtained with primer set 2) did not hybridize with either probe HC-I or probe HC-II.

To further assess the reliability of the DEIA, five PCR samples typed with the DEIA were subjected to cloning and nucleotide sequence analysis, leading to confirmation of type assignment. The occurrence of double infection (type I plus type II) was confirmed in five of seven cases by nucleotide sequencing or by hybridization of single clones derived from each PCR with either HC-I or HC-II probe. Partial nucleotide sequence analysis of the E1 region indicated that the six PCR products not hybridizing with probe HC-I or HC-II shared a low level of homology with HCV sequences belonging to types I and II (Fig. 3). Comparison with published sequences of the E1 region indicated homology between sequence c and type 4b (classification of Bukh et al. [2]), sequence d and type 4c, sequences e and f and type (V)/3a, and sequence g and type 2c (first described for an isolate from Sardinia, Italy [2]). Sequence h showed features intermediate between those of type III/2a and type 2c (2).

TABLE 3. Clinical and biochemical characteristics of NANBH patients

Characteristic	Value for group		Total
	Acute NANBH	Chronic NANBH	
No. of patients			
Total	21	51	72
Female	8	20	28
Male	13	31	44
Mean age ± SD (yr)	33 ± 16	42 ± 15	39 ± 15
Parenteral risk factors			
Yes	17	32	49
No	4	19	23
Other viral markers[a]			
HBsAg	0	1	1
Anti-HBc	2	26	28
Anti-HIV	1	18	19

[a] HBsAg, hepatitis B surface antigen; HBc, hepatitis B core.

The prevalence of types I and II, which are distributed worldwide, is characteristic of different countries: type I is very infrequent in Japan (where almost all such patients had been transfused with imported coagulation factors) (16, 25) but is the major genotype in the United States (26). The present study indicated that HCV type II is prevalent in northern Italy and that type I accounts for only 13% of the infections. These results are partially consistent with previous studies indicating prevalence of HCV type II, followed by types IV, III, and I, in patients from northern and central Italy with community-acquired HCV infection (20). Coinfection with types I and II was observed in 10% of our subjects. The occurrence of mixed infections has already been described elsewhere (8, 16, 19, 20), and the apparently high prevalence observed in this study may be explained by the fact that six of the seven patients positive for both genotypes had parenteral risks of infection.

The distribution of viral types among the studied patients was statistically analyzed for any significant correlation with age, sex, selected risk factors, clinical presentation (stage) of the disease (acute or chronic), and presence of other viral markers (anti-hepatitis B core and anti-human immunodeficiency virus [anti-HIV] antibodies). In patients with chronic hepatitis, duration of infection, liver histology, and anti-HCV reactivity (4 Antigen RIBA; Ortho Diagnostic Systems, Raritan, N.J.) were also considered. Student's *t* test and the chi-square test were used to compare differences between groups of patients infected with different HCV genotypes. We

TABLE 4. Logistic regression models of HCV types I and II based on epidemiological variables found to be significant in bivariate models

Patient variable	No. (%) of patients positive for variable with HCV genotype		Statistical analysis			
			Bivariate		Multivariate	
			OR[a]	*P*	OR	*P*
	Type I	Non-type I				
Total positive	13	52				
Anti-HIV positive	8 (61.5)	11 (21)	6	0.01	7.28	0.017
Age >40	1 (7.7)	26 (50)	0.09	0.02	0.15	0.158
Drug addiction	9 (69)	15 (29)	5.53	0.01	1.7	0.57
	Type II	Non-type II				
Total positive	39	26				
Anti-HIV positive	4 (10)	15 (57.7)	0.08	0.0001	0.14	0.005
Age >40	23 (59)	4 (15)	7.9	0.001	3.8	0.05

[a] OR, odds ratio.

then used logistic regression to investigate which clinical characteristics were associated with the infecting genotype, by a multivariate model that incorporated characteristics found to be significant in bivariate models (Table 4). Use of logistic regression models showed that infection with HCV type I was significantly associated with HIV coinfection, whereas HCV type II was significantly associated with patients who were over age 40 who had no HIV markers.

These results are consistent with different epidemiological distributions of HCV types I and II in Italy. This evidence may indicate a recent spread of type I HCV, in association with HIV, in the geographic area under study and might be suggestive of a different efficacy in the blood-borne transmission of HCV type I. This hypothesis is supported by a previous report indicating an increased prevalence of HCV type I in Italian hemophiliacs, who are treated with mainly imported concentrates, but not in patients with community-acquired hepatitis (20).

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