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Identification of Numerous Hepatitis C Virus Genotypes in Montreal, Canada

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Hepatitis C virus genotypes were determined for 358 viremic individuals in Montreal, Canada, by restriction endonuclease analysis of PCR products and phylogenetic analysis of core gene sequences. Types 1, 2, and 3 occurred in 62.8, 14.2, and 13.7%, respectively; types 4 and 5 were found in 3.9 and 4.5%, respectively; and genotypes 6a and 7c and a novel genotype each occurred in 0.3%. Types 4, 6, and 7 and the novel genotype were mostly from persons who had immigrated to Canada.

Hepatitis C viruses are the major cause of parenterally transmitted non-A, non-B hepatitis. Their genomes display significant sequence heterogeneity and have been classified into types and subtypes (12). Six types (types 1 to 6) have so far been recognized, and each type has a variable number of subtypes (subtypes a, b, c, etc.). Recently, new variants have been identified and assigned into proposed types 7 to 11 (14, 15). Hepatitis C virus (HCV) genotypes appear to vary in their degree of pathogenicity and in their response to antiviral therapy (2). In addition, genotypes have been shown to be associated with age and mode of transmission (11) and may influence HCV RNA quantification (3).

HCV genotypes show different prevalences and geographic distributions (2). In North America, type 1 is reported to be predominant; types 2 and 3 are less prevalent (1, 5). Only a few variants of other types have been encountered (5, 7). In the present study, we analyzed the genotype distributions in 157 prospectively recruited volunteer blood donors and 201 patients infected with HCV. Most of the patients resided in Montreal, Canada.

HCV RNA was extracted either from 50 μl of serum by treatment with sodium dodecyl sulfate and proteinase K or from 100 μl of serum by treatment with RNAzol B (Biotecx Laboratories, Houston, Tex.) (8, 16). Reverse transcription was performed with primer DM50, while PCR was performed with primer pair DM50 (nucleotides [nt] −50 to −31) and DM51 (nt −271 to −247). Amplifications were carried out with either the DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.) or the GeneAmp PCR System 9600 (Perkin-Elmer) as described previously (8). For the System 9600 amplifications, an initial 1 min of denaturation at 94°C was followed by 40 cycles of 10 s at 94°C, 25 s at 55°C, and 75 s at 72°C. The last cycle was followed by a 5-min extension at 72°C. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The specificity of the 241-bp product was confirmed by hybridization with either probe Alx89 or probe DM53 end-labelled with [γ-^{32}P]ATP (9).

Types were determined by cleavage of the PCR products with *Bst*NI, *Bsr*I, *Hin*fI, *Mae*III, and *Hae*III as described previously (7). DNA compatible with type 5 was confirmed by cleavage with *Taq*I (9). Type 3 variants lacking the G residue at position −160 (*Mae*III site) have been reported previously (13) and would be misclassified as type 4 by our typing scheme. Therefore, the authenticity of type 4 variants was confirmed by cleavage with *Rsa*I (4). In addition, types 1 and 2 were subtyped as described by Davidson et al. (4) by cleavage of the amplified DNA with *Bst*UI and *Scr*FI, respectively. Genotypes 2a and 2c were grouped together since most 2c variants share with 2a variants common *Scr*FI cleavage sites (4). Digests were resolved by gel electrophoresis and were visualized by ethidium bromide staining.

For sequence analysis, a segment encompassing the complete core region was obtained by nested PCR. Amplified products for variants QC29 and QC30 were directly sequenced. Products for variants QC21, QC26, and QC27 were cloned prior to sequencing. Three clones were isolated for each variant, and their consensus sequence is reported. In addition, for each of these five variants, single-round PCR products of the 5′ noncoding region (5′ NCR) (nt −302 to −31) were directly sequenced. The phylogenetic relationships between core nucleotide sequences were inferred by the maximum-likelihood method (10). Different input orders were tested, and trees were further improved by global rearrangements.

The electropherotype profiles (EPs) obtained upon analysis of the 358 samples from both blood donors and patients are presented in Fig. 1B. Only two samples showed unexpected EPs. The virus (QC30) assigned to profile S had a T residue at position −221, creating a *Hin*fI site (pattern c, Fig. 1A). The virus (QC29) assigned profile T possessed an *Rsa*I cleavage pattern expected for type 3 (pattern e, Fig. 1A). However, its *Bst*NI cleavage pattern was not compatible with that type.

The putative core sequence for the two variants (QC29 and QC30) displaying unexpected EPs was determined in order to establish their genotype status. The core sequences of variants assigned to type 4 (QC27), type 5 (QC21), and type 6 (QC26) were also determined. Phylogenetic analysis revealed that QC30 clustered with type 7 sequences and belonged to the same genotype as VN4 (7c* in Fig. 2). On the other hand, QC29 was closest to variants of types 3 and 10, while it remained distinct from them. Thus, QC29 likely constitutes a new genotype of HCV. QC21 and QC26 clustered with genotypes 5a and 6a, respectively (Fig. 2). QC27 clustered closest to genotype 4c, but it could be a novel subtype.

The distribution of types between patients and blood donors was not significantly different ($P > 0.05$; Mann-Whitney test) (Table 1). In both groups, type 1 was predominant. Types 2 and

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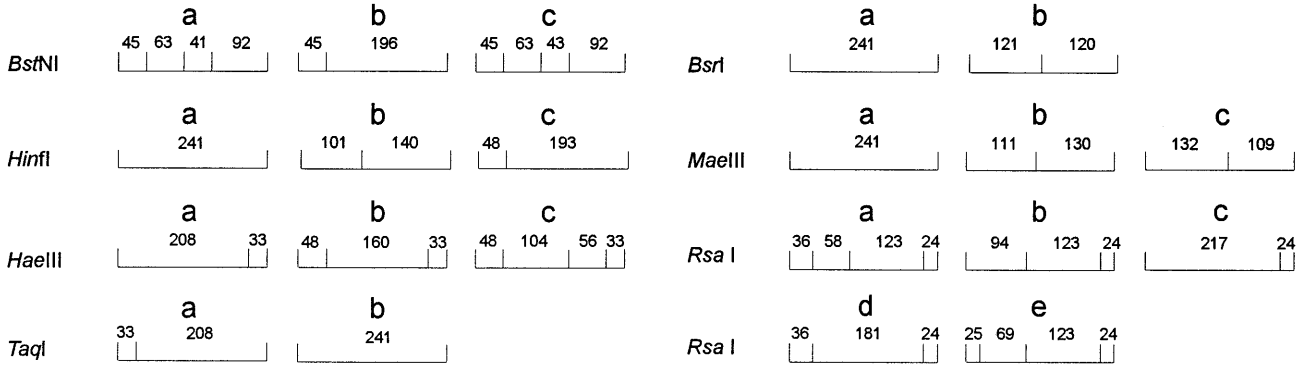
A



B

Electropherotype profile	*Bst*NI	*Bsr*I	*Hin*fI	*Mae*III	*Hae*III	Type	No. observed	Prevalence (%)	Electropherotype profile	*Bst*NI	*Bsr*I	*Hin*fI	*Mae*III	*Hae*III	*Taq*I	*Rsa*I	Type	No. observed	Prevalence (%)
A	a	a	a	a	a	1	225	62.8	K	b	a	b	c	b		e	3	-	-
B	b	b	a	a	c	2	25	7.0	L	b	a	b	a	a		a	4	6	1.7
C	b	b	a	a	b	2	22	6.1	M	b	a	b	a	a		b	4	5	1.4
D	b	b	a	a	b/c	2	1	0.3	N	a	a	b	a	a		c	4	2	0.6
E	b	b	a/b	a	b	2	1	0.3	O	a	a	b	a	a		d	4	1	0.3
F	b	b	a/c	a	b	2	1	0.3	P	b	a	a	a	a	a		5	15	4.2
G	b	b	a/b	a	c	2	1	0.3	Q	b	a	a	a	a	b		5	1	0.3
H	b	a	b	b	a	3	44	12.3	R	c	a	a	a	b			6	1	0.3
I	b	a	b	b	b	3	3	0.8	S	a	a	c	a	a			7	1	0.3
J	b	a	b	b	a/b	3	2	0.6	T	a	a	b	a	b		e	?	1	0.3

FIG. 1. Identification of HCV types by restriction endonuclease analysis of amplified 5′ NCR sequences with the primer pair DM50-DM51. (A) Cleavage patterns obtained with each restriction endonuclease and number of residues per segment. Some variants may contain a DNA segment 1 bp longer than indicated because of the insertion of an A residue near position −136. (B) Prevalence of each EP and corresponding HCV type for 358 clinical specimens.

3 were less prevalent and occurred at similar frequencies. Types 1 to 3 accounted for 92 and 90% of the types in blood donors and patients, respectively, who were mostly of Canadian origin. All except one of the people infected with type 4 were immigrants from Africa or the Middle East; the exception was a patient of Canadian origin. This patient reported intravenous drug use as a risk factor and had no history of travel to Africa or the Middle East. QC27 was from a patient of Egyptian origin. Type 5 was found to occur at a rate of 4 to 5% in both groups (9). One person infected with type 5 was of European origin, while the others, including the patient infected with QC21, were all of Canadian origin. It is unknown whether the former acquired the infection outside of Canada. The persons infected with the genotypes 6a (QC26) and 7c (QC30) were of Vietnamese origin. The novel genotype (QC29) was found in a Somali patient who had immigrated to Canada. Infection with more than one major type was not detected.

In blood donors, genotypes 1a and 1b occurred at similar frequencies, as did genotypes 2a/c and 2b (Table 1). In patients, genotype 1a also occurred at a frequency similar to that of genotype 1b, but genotype 2a/c was more prevalent than genotype 2b. The type 2 sample that could not be subtyped possessed *Scr*FI sites of genotypes 2a/c except for the cleavage site at position −163 (4). Interestingly, in both donors and patients, persons infected with genotype 2b were significantly younger than those infected with genotype 2a/c. This may be correlated to the mode of transmission, since the main risk factor identified was intravenous drug use for persons infected with genotype 2b but previous medical procedures (transfusions, injections, or surgeries) for persons infected with genotype 2a/c. It must be noted that these results should be interpreted with prudence, since some variants may be misclassified by this method of subtyping (13).

Newly discovered variants from Indonesia, Thailand, and Vietnam assigned to types 7, 8, 9, and 11 have 5′ NCR sequences that may not be differentiable from type 1 (15). However, these variants have rarely been encountered outside of southeast Asia and are expected to occur rarely in North America. Thus, the value obtained for type 1 in the present study most likely represents its true prevalence. Furthermore, there has been some confusion in the literature as to whether the variants assigned to types 7, 8, 9, and 11 should be classified into distinct types (14, 15) or within type 6 (6). Our phylogenetic analysis of core gene sequences revealed a close relation-

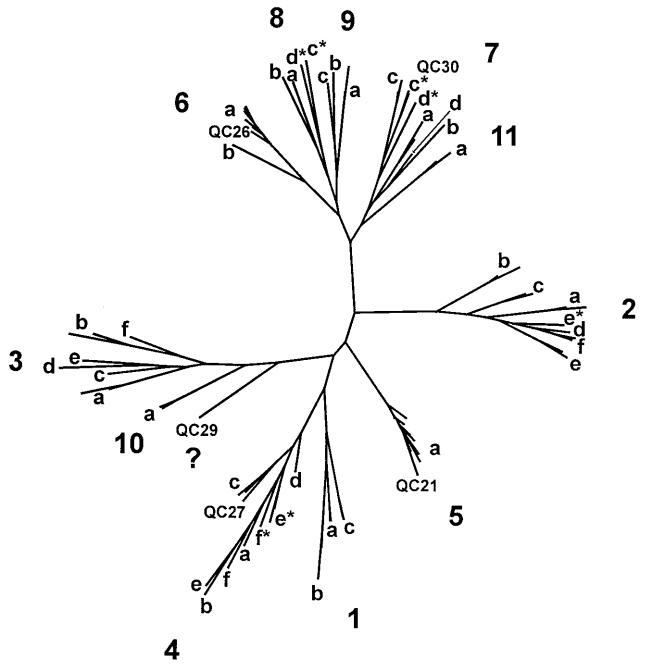


FIG. 2. Unrooted maximum-likelihood tree inferred from nucleotide sequences of the complete core region. All branch lengths are drawn to scale. The variants characterized in the present study are identified. Types and subtypes are also shown. Asterisks indicate the genotypes assigned by Stuyver et al. (14). Variants included in the phylogenetic analysis as representatives of various genotypes are listed below, with their GenBank accession numbers given in parentheses. Distinct genotypes bearing the same designation (2e, 4e, 4f, 7c, and 7d) are listed separately. 1a, HCV-1 (M62321) and HCV-H (M67463); 1b, HCV-J (D90208) and HCV-T (M84754); 1c, HC-G9 (D14853) and YS117 (D16189); 2a, HC-J5 (D10075) and HC-J6 (D00944); 2b, HC-J7 (D10077) and HC-J8 (D10988); 2c, FR8 (L38337) and S83 (U10211); 2d, NE92 (L29631); 2e, JK020 (D49745) and JK128 (D49756); 2e, FR4 (L38333); 2f, JK081 (D49754) and JK139 (D49757); 3a, HCV-K3a/650 (D28917) and NZL1 (D17763); 3b, HCV-Tr (D26556) and Th527 (D37839); 3c, NE048 (D16612); 3d, NE274 (D16620); 3e, NE145 (D16618); 3f, NE125 (D16614); 4a, Z4 (U10236); 4b, Z1 (U10235); 4c, Z6 (U10238) and Z7 (U10239); 4d, DK13 (U10192); 4e, Z5 (U10237); 4e, CAM600 (L29587) and GB809 (L29624); 4f, Z8 (U10240); 4f, FR12 (L38332); 5a, BE95 (L29577), SA1 (U10216), SA3 (U10217), SA4 (U10218), SA5 (U10219), SA6 (U10220), SA7 (U10221), SA11 (U10214), and SA13 (U10215); 6a, HK2 (U10198), VN11 (L38339), VN506 (D17500), VN538 (D17504), VN569 (D17506), and VN571 (D17507); 6b, Th580 (D37841); 7a, VN540 (D17505) and VN998 (D30796); 7b, VN235 (D17498); 7c, Th271 (D37844) and Th571 (D38078); 7c, VN4 (L38341); 7d, Th846 (D37843); 7d, VN12 (L38340); 8a, VN507 (D17501) and VN530 (D17502); 8b, VN405 (D17499); 8c, D97/93 (D63946); 8d, B4/92 (D63943); 9a, VN004 (D17496) and VN085 (D17497); 9b, Th555 (D37849) and Th602 (D37850); 9c, Th553 (D37848); 10a, JK049 (D63821) and JK072 (D49753); 11a, JK046 (D63822) and JK065 (D49751).

ship between variants of types 6, 7, 8, 9, and 11 and supports their classification into a single genetic group. The similar relationship observed between our novel variant (QC29) and those of types 3 and 10 also suggests that these should be classified into a single group.

HCV types 4 to 11 occur preferentially in certain geographical areas and have rarely been reported or not reported in North America (1, 5, 7). We have identified types 4, 5, 6, and 7 and a novel genotype in infected persons from Montreal. Furthermore, genotype 3b has been identified in an immigrant from Pakistan in an ongoing study on the response to interferon therapy (unpublished data). This variant possessed electropherotype profile K (Fig. 1B). Our report confirms the diversity of genotypes in Montreal, Canada, and the fact that types other than types 1 to 3 account for 8 to 10% of hepatitis C viruses.

TABLE 1. Prevalence of HCV genotypes in blood donors and patients and age distributions

HCV genotype	Blood donors (n = 157)		Patients (n = 201)	
	% Occurrence (no. of subjects)	Mean ± SD age (yr)	% Occurrence (no. of patients)	Mean ± SD age (yr)
1	61.1 (96)	36.7 ± 7.1	64.2 (129)	42.7 ± 14.6
1a	29.9 (47)	36.3 ± 7.1	30.3 (61)	40.3 ± 12.9
1b	30.6 (48)	37.1 ± 7.2	29.9 (60)	45.1 ± 16.5
1a + 1b	0.6 (1)	39	4.0 (8)	42.4 ± 9.1
2	15.9 (25)	40.2 ± 10.3	12.9 (26)	48.5 ± 16.8
2a/c	8.3 (13)	47.2 ± 8.4	9.0 (18)	52.3 ± 14.4
2b	7.6 (12)	32.6 ± 5.9[a]	3.5 (7)	38.6 ± 20.4[b]
2?	0		0.5 (1)	57
3	15.3 (24)	33.6 ± 6.4	12.4 (25)	40.1 ± 14.7
4	1.9 (3)	40.7 ± 13.6	5.5 (11)	53.6 ± 12.4
5	5.1 (8)	46.3 ± 7.9	4.0 (8)	44.4 ± 18.5
6	0.6 (1)	39	0	
7	0		0.5 (1)	67
?	0		0.5 (1)	44

[a] $P < 0.001$ versus genotype 2a/c (chi-square test).
[b] $P = 0.025$ versus genotype 2a/c (chi-square test).

Nucleotide sequence accession numbers. The newly described sequences reported here have been submitted to GenBank and can be retrieved under accession numbers U52810, U52811, and U33430 to U33437.

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