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Genotyping of Canadian Hepatitis C Virus Isolates by PCR

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We used PCR for hepatitis C virus (HCV) genotyping with type-specific primers from the core and NS5 genes. Type I was predominant in the general population (58% in blood donors) as well as in different risk groups, such as intravenous drug abusers (58%), blood transfusion recipients (64%), hemophiliacs (62%), and patients with HCV chronic liver disease (76%). Types II, III, and IV were less prevalent in Canada, being found in 10.92, 6.72, and 5.88% of the population, respectively. The type II core primer was not type specific and reacted with the majority of our type I HCV samples, suggesting a false-positive dual infection with two different genotypes (I and II). Digestion of these amplified type I and type II products with restriction endonuclease *Acc*I proved to be very useful in the exclusion of false-positive dual type I and type II infections.

The identification of hepatitis C virus (HCV) as the major cause of parenterally transmitted non-A, non-B hepatitis has resulted in the subsequent cloning and sequencing of a number of HCV isolates from different areas of the world. On the basis of these detailed complete or partial sequence homology analyses, several distinct HCV genotypes have been described (2, 3, 11, 12, 15). At present, they are an essential tool for studying the worldwide distribution and epidemiology of HCV infections. The importance of HCV genotyping is further emphasized by reports linking a response to interferon treatment of HCV chronic liver disease with certain genotypes (7, 14) and data showing a correlation between serological reactivities and particular genotypes (16). Recently, a direct PCR for HCV genotyping was introduced (12); this method eliminates the need for time-consuming procedures, such as sequencing or identification by restriction fragment length polymorphisms (RFLPs) (9) of amplified HCV PCR products. To study the incidence of HCV genotypes in Canada, we have compared two PCR-based methods that use type-specific primers (Table 1) from the core and NS5 genes and have used the provisional designations of five HCV genotypes (I to V) from the typing scheme of Okamoto et al. (12, 13).

One hundred ninety-seven serum samples found positive for antibody to HCV by a second-generation enzyme immunoassay (EIA-2; Ortho) were included in this study. Of these, 174 were found reactive by a recombinant immunoblot assay (RIBA-2; Chiron, Emeryville, Calif.), and 23 were found indeterminate. The serum samples were obtained from blood donors, patients with HCV chronic liver disease, and groups at high risk for HCV, such as intravenous drug abusers (IVDA), hemophiliacs, and patients receiving blood transfusions. These specimens were collected from seven of the nine provinces in Canada, including the largest ones, such as Ontario, Quebec, and British Columbia.

HCV RNA was extracted from 50 μl of serum by the method of Chomczynski and Sacchi (6). Reverse transcription and nested PCR with primers from the conserved 5′-noncoding region (5′-NCR) were performed as previously described (4). One hundred nineteen specimens were found positive for HCV RNA and further subjected to PCR genotyping with primers from the core and NS5 genes by previously reported protocols (5, 12, 13).

Of these 119, 106 (89%) were found to be positive (Fig. 1A) with primers from the core gene. Genotypes II (10.92%), III (6.72%), and IV (5.88%) were each easily distinguishable by the PCR procedure reported previously (12). In three samples, the 123-bp band corresponding to type IV was detected together with bands corresponding to type I and/or type II when all four subtyping primers were used in the second-stage PCR. However, type IV positivity could not be confirmed in a separate PCR with only the type IV-specific primer. In the majority of cases (61.34%), PCR products indicative of the simultaneous presence of both genotypes I and II were found by use of core gene primers, while only 4.2% were genotype I positive. Nonspecific cross-annealing between a primer for type II and an HCV type I cDNA template was suspected. Attempts to increase the specificity of the reaction by raising the annealing temperature in the second-stage PCR from 60 to 65°C did not resolve the problem, and positive, although weaker, amplification of both type I and type II could still be detected.

Predicted cleavage patterns suggested that restriction enzyme *Acc*I would cleave type I (57-bp) but not type II (144-bp) PCR-amplified cDNA. However, if a type II PCR product were the result of nonspecific cross-annealing of a type II-primer with a cDNA template for type I, it would be then cleaved by *Acc*I (Fig. 2). We tested all 73 samples with suspected false-positive dual infections with HCV genotypes I and II, and in 53 samples (72.6%), *Acc*I cleaved both the 57-bp type I band and the 144-bp type II band. On the other hand, the 144-bp band from samples containing only type II was not cleaved. This finding suggests that *Acc*I could resolve most, although not all, dual type I and type II HCV infections, as true infections with only type I. We further confirmed this finding by separately sequencing type I and type II amplified PCR products from three samples positive for both genotype I and genotype II. We used sense (104) and antisense (132 and 133) primers to sequence both strands of the PCR-amplified cDNA. The nucleotide sequences of both type I and type II amplified PCR products were identical in their overlapping region, with a conserved *Acc*I restriction site. In addition, regions downstream of the *Acc*I site in type II cDNA were also found to be homologous to type I HCV and not to type II HCV, further proof that the original RNA template for reverse transcription-PCR is type I HCV. Others have also noted the nonspecific nature of the type II primer and proposed their own core primers (19). In a recent study, 22% of U.S. HCV isolates were

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TABLE 1. Oligonucleotide primers used for amplification of the HCV genome

Name	Region (nucleotide positions)	Polarity[a]	Sequence (5′ to 3′)	Reference
C1	5′-NCR (36–55)	+	CTGTGAGGAACTACTGTCTT	4
C2	5′-NCR (237–256)	−	AACACTACTCGGCTAGCAGT	4
C3	5′-NCR (55–74)	+	TTCACGCAGAAAGCGTCTAG	4
C4	5′-NCR (177–196)	−	GTTGATCCAAGAAAGGACCC	4
256	Core (480–499)	+	CGCGCGACTAGGAAGACTTC	12
186	Core (731–750)	−	ATGTACCCCATGAGGTCGGC	12
104	Core (488–517)	+	AGGAAGACTTCCGAGCGGTC	12
132	Core (525–544)	−	TGCCTTGGGGATAGGCTGAC	12
133	Core (612–631)	−	GAGCCATCCTGCCCACCCCA	12
134	Core (642–661)	−	CCAAGAGGGACGGGAACCTC	12
135	Core (591–610)	−	ACCCTCGTTTCCGTACAGAG	12
14	NS5 (8601–8621)	−	CCTGGTCATAGCCTCCGTGAA	5
111	NS5 (8266–8301)	+	CAGTCACTGAGAAGCGACATCCGTACG	5
57	NS5 (8460–8485)	+	ACCCTCACATGTTACTTGAAGGCCAC	5
121	NS5 (8479–8506)	+	AGGCCACTGCGGCCTGTCGAGCTGCGAA	5
65	NS5 (8315–8338)	+	TGATCTCGACCCCCAAGCCCGCGT	5
61	NS5 (8381–8403)	+	TATGTTCAACAGCAAGGGCCAGA	5
59	NS5 (8309–8334)	+	GGCTTGTTCCCTGCCTCAAGAGGCCA	5

[a] Orientation of the primer sequence (+, sense; −, antisense).

found to have both genotype I and genotype II, and sequencing of several of the amplified PCR products identified mutations giving rise to the detection of false-positive dual infections when the same core primers were used (8).

We have also sequenced two specimens showing mixed type I and type II PCR products; however, unlike the ones mentioned above, they were not cleaved by *Acc*I. According to the nucleotide sequence, they also belonged to type I; however, because of a point mutation in the second nucleotide of the *Acc*I site (an A-for-T substitution), the amplified PCR product could not be cleaved. The observed mutation was located in the third base of the codon and did not change the corresponding amino acid. These results suggest that at least some of the samples with dual positivity and whose type I and type II PCR products are not cleaved by *Acc*I also belong to just type I.

The results of PCR genotyping with NS5 gene primers are shown in Fig. 1B. Almost half of the 119 samples tested were positive for type I (48.73%). No samples were positive for both type I and type II. Of the 73 samples with dual positivity for type I and type II by the core gene primers, 48 (65.75%) were positive for type I when tested with both alternative NS5 type I-specific primers 111 and 65, 6 (8.21%) were positive when tested with primer 111 only, and 3 (4.1%) were positive when tested with primer 65 only.

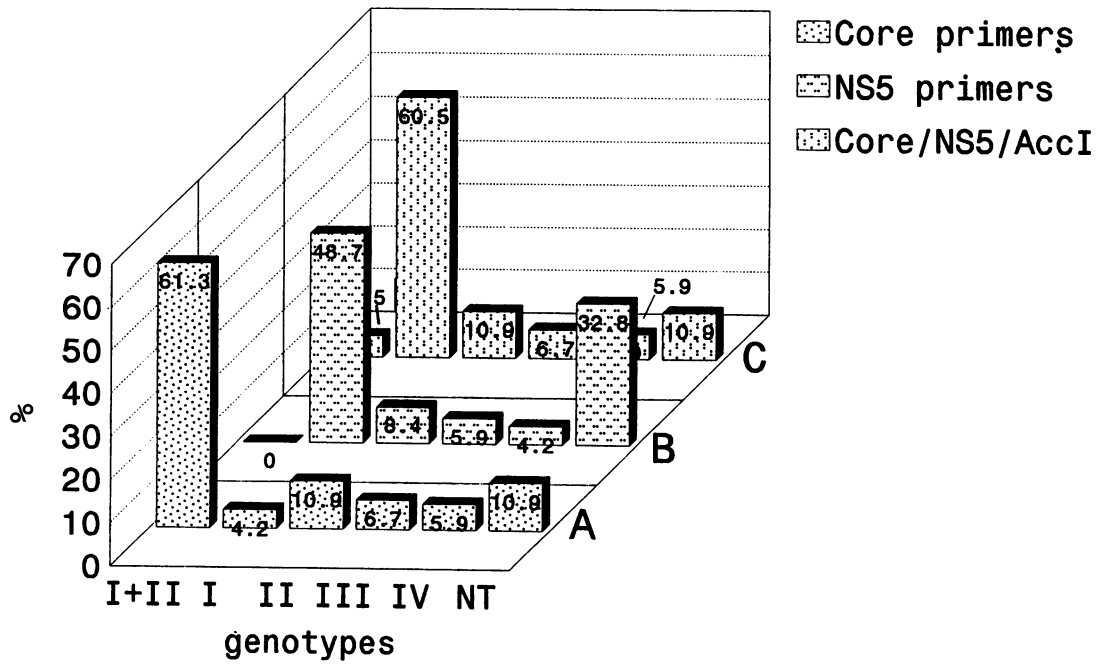

FIG. 1. Distribution of HCV genotypes in Canada. The percentage of each genotype is shown within the columns. NT, not typed.

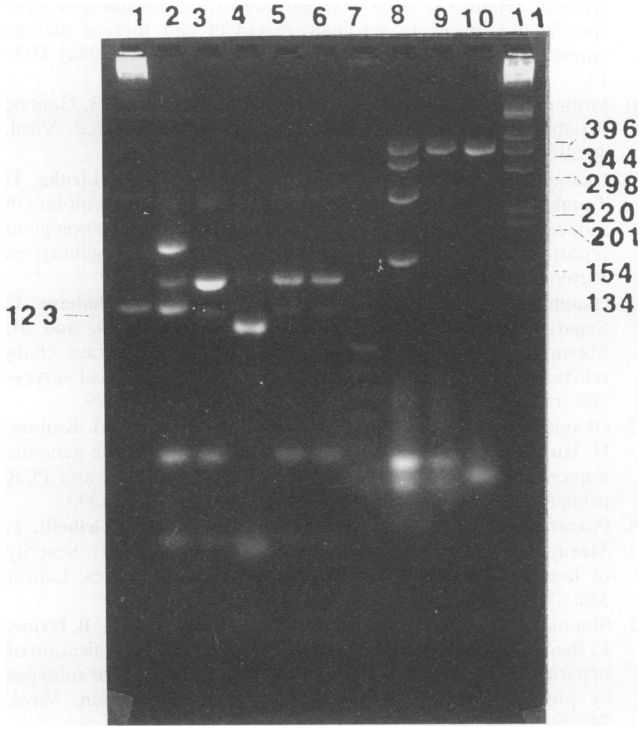

FIG. 2. Restriction endonuclease digestion with *Acc*I of HCV specimens showing both type I (57-bp) and type II (144-bp) PCR bands. Lanes: 1, 123-bp ladder; 2, PCR products obtained for HCV types I (57 bp), II (144 bp), III (174 bp), and IV (123 bp) with core primers; 3, sample 7a (HCV types I and II; core primers); 4, sample 7a plus *Acc*I; 5, sample 7b (types I and II; core primers); 6, sample 7b plus *Acc*I; 7, 10-bp ladder (the strongest band in this lane equals 100 bp); 8, PCR products obtained for HCV types I (356 bp), II (162 bp), III (241 bp), and IV (313 bp) with NS5 primers; 9, sample 7a, NS5 primers; 10, sample 7b, NS5 primers; 11, 1-kbp ladder. A metaphor agarose gel (4%) was stained with ethidium bromide and photographed on a UV light illuminator. Both band I and band II in sample 7a were cleaved with *Acc*I (144-bp band II into 33 and 111 bp; 57-bp band I into 33 and 24 bp) to yield a three-band digest pattern (111, 33, and 24 bp). Bands in sample 7b were not cleaved with *Acc*I; however, both samples 7a and 7b (lanes 9 and 10) were found to contain only type I with NS5 primers. A primer-dimer-trimer PCR product can be seen at the bottom of lanes 2, 3, 8, 9, and 10.

Single genotypes II, III, and IV correlated much better with core gene PCR genotyping and accounted for 8.4, 5.9, and 4.2% of positive samples, respectively. Overall, one-third of all samples (32.77%) could not be subtyped with NS5 gene primers. It has been suggested that this result is due to very low virus concentrations in the samples tested (5). Indeed, the 40 cycles used in the NS5 PCR were not as sensitive as the two 30 cycles used in the nested PCR. As we used a universal hexamer for reverse transcription, the efficiency of this step should be the same for all three PCRs that we used (5′-NCR, core, and NS5). This fact and the fact that PCR genotyping for types II, III, and IV was much more effective than that for type I suggest that there may be another explanation; perhaps some of our HCV genotype I isolates were divergent from the proposed nucleotide sequence for type I-specific NS5 primers 111 and 65.

This study shows that the combination of the two PCR genotyping methods, i.e., those using core and NS5 primers, and *Acc*I for samples showing dual positivity proved to be quite useful and complementary. Each approach has its own shortcomings. Core PCR genotyping seemed to be more sensitive; however, a large number of our type I HCV isolates also reacted with a type II primer (133). This result required further clarification with *Acc*I RFLPs and NS5 PCR genotyping. NS5 primers for type I were more specific but failed to amplify 14 (19.4%) of our type I HCV isolates. However, combining PCR genotyping with the core and NS5 primers with *Acc*I RFLPs solved the problem of the false-positive dual HCV type I and type II infections and revealed that the majority of the samples tested were genotype I (60.5%) (Fig. 1C). In this group, 5 samples were found to be only type I positive by use of core primers, 57 samples were found to be type I and type II positive by use of core primers but only type I positive by use of NS5 primers, and 10 samples were found to be negative by use of NS5 primers and type I and type II positive by use of core primers but were cleaved by *Acc*I. Six more samples found to be positive for type I and type II by use of core primers (5%) could not be further characterized, as they were found to be negative by use of NS5 primers and were not cleaved by *Acc*I. Twenty-eight (23.5%) samples belonged to genotypes II, III, and IV; these included 6 samples that were reactive with core but not NS5 primers. Thirteen samples (11%) could not be subtyped.

The initial view that different genotypes are strictly confined to certain geographical areas, i.e., type I for the United States and type II for Japan, is being challenged by new reports, including this one. Indeed, three studies from Japan showed type II to be the major genotype (70%) and type III prevalence to range from 14 to 18% (5, 12, 17). Type I was of low prevalence, i.e., 0, 0.8, and 4%, in the same studies. Similar findings were reported for China: type II HCV was found in 69% of cases, and type III was found in 23% (18). While the presence of these two HCV types in Canada is relatively limited, i.e., 10.92% for type II and 6.72% for type III, type IV, which is rare in Japan (4.8%), was detected in seven samples (5.88%). Other than Japan and Canada, HCV genotype IV has been reported in Sweden, Denmark, and the United States; with the increasing number of studies on genotypes, it may be found in other parts of the world as well (2, 8). In Europe, the mix of different genotypes seems to be even more pronounced, with type II being the predominant one in Germany (10), types I, II, and V predominating in Great Britain (9), and types I and II predominating in France, Italy, and Spain (1).

Among different risk groups, HCV genotype I is the most prevalent, ranging from 58% in IVDA to 76% in patients with chronic hepatitis (Table 2). We found a significantly higher percentage ($P < 0.1$) of nontypeable samples (27%) among IVDA than among other risk groups or blood donors.

The majority of our HCV isolates (89%) were genotyped. A further reduction in the number of nontypeable HCV isolates

TABLE 2. HCV RNA genotypes in different risk groups

Risk group (no. of samples)	% of the following HCV RNA genotype				% Nontypeable
	I	II	III	IV	
IVDA (26)	58	0	8	8	27
Hemophiliacs (16)	62	25	0	6	6
Blood transfusion recipients (14)	64	14	7	0	14
Chronic hepatitis patients (17)	76	6	6	0	12
Blood donors[a] (31)	58	13	10	10	10

[a] Included for comparison only; blood donors do not belong to any risk category.

was achieved with the newly proposed type-specific primers for type V from the core region (11). After the submission of this paper, we tested all 13 previously nontypeable samples with type V core primers and found 2 to be positive. The same ones were found to be negative with type V NS5 primers.

Comparative sequence data from different parts of the HCV genome are most informative for typing and especially subtyping. However, PCR genotyping is more practical and easier to perform, and most HCV isolates can be typed, despite some difficulties with nonspecific amplification with type II core primer.

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