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Greater Diversity of Hepatitis C Virus Genotypes Found in Hong Kong than in Mainland China

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A hepatitis C virus (HCV) genotyping PCR assay based on type-specific primers was expanded to include genotype 6a as well as genotypes 1a, 1b, 2a, 2b, and 3a. The nucleotide sequences of a 194-bp fragment in the center of the HCV core gene showed that the homologies between genotype 6a and genotypes 1a, 1b, 2a, 2b, 3a, and 5 were 81.2, 82.1, 73.8, 77.3, 81.4, and 78.9%, respectively. A high degree of homology (99.6%) was seen in the amplified core region among eight clinically unrelated genotype 6a isolates. Although the Hong Kong Chinese patients had predominantly genotype 1b (70%), it was noteworthy that genotype 6a was the second most common genotype (14%). Four other HCV genotypes—1a, 1b, 2a, and 2b—were also present. In contrast, HCV infection in mainland China was confined to genotypes 1b and 2a. Thus, we found a greater diversity of HCV genotypes in Hong Kong than in mainland China.

Hepatitis C virus (HCV) is a single-stranded RNA virus containing a linear genome with a length of about 9,600 nucleotides with positive polarity (6). It is now recognized that HCV infection is the major etiological agent of posttransfusion hepatitis type non-A, non-B. HCV infection frequently progresses to chronic liver disease (7, 10). On the basis of phylogenetic analysis, HCV has been grouped into six to nine major genotypes, each of which contains one or more subtypes (1–4, 11, 12). The distribution of HCV genotypes varies in different geographical areas. In the United States and Northwestern Europe the predominant HCV genotype is 1a. The predominant genotype in Japan is 1b. Genotype 1b is also found in Central Europe, together with genotypes 1a, 2a, 2b, and 3a. Genotype 4 subtypes are found in the Middle East, being predominant in Egypt, and are also found in Central and South Africa (8). Genotype 5 has been found in South Africa (3, 4, 11, 16), whereas genotype 6a has so far been found only in Hong Kong, Macau, and Vietnam (2, 11, 12). The genetic heterogeneity and the high mutation rate of HCV pose difficulties for the development of vaccines and antiviral therapy (2). Therefore, significant efforts are being devoted to exploring the heterogeneity of HCV worldwide to obtain information on the prevalence of known genotypes and the identification of new HCV genotypes.

An earlier study of Chinese patients with a typing assay that can classify HCV genotypes into 1a, 1b, 2a, and 2b showed that HCV genotypes in mainland China were confined to 1b and 2a (13). The presence of new genotypes, including genotypes 3a and 6a, was not examined. There has been no report on the distribution of HCV genotypes in Hong Kong, which is geographically linked to mainland China. In this study, a typing assay based on type-specific primers directed against the HCV core region (8, 15) was expanded so that genotype 6a can be identified in addition to genotypes 1a, 1b, 2a, 2b, and 3a. Partial sequence determination of the core region was performed with the identified genotype 6a isolates to study the nucleotide homology within genotype 6a and to allow comparison with other HCV genotypes. The expanded typing assay was used to determine the prevalence of HCV genotypes in anti-HCV antibody-positive patients from Hong Kong and mainland China.

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MATERIALS AND METHODS

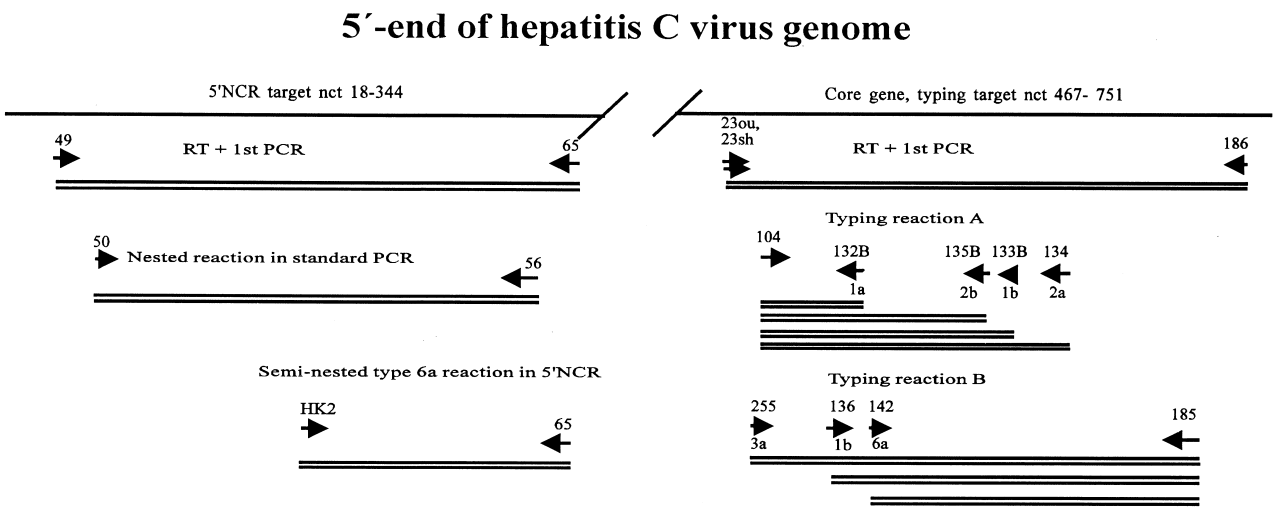
Subjects. Fifty-eight serum samples were collected from anti-HCV-positive Chinese patients in Hong Kong. Forty-nine of them had presumably acquired HCV infections in Hong Kong, 6 had acquired them in mainland China, 2 had acquired them in the United States, and 1 had acquired her infection in Australia. The sources of infection were as follows: blood transfusion, 51 patients, 27 of whom were also on renal dialysis; intravenous drug use, 4 patients; occupational exposure, 2 patients; and no known source, 1 patient.

Fifty-six serum samples were collected from anti-HCV-positive patients in mainland China. Twenty-nine were from Wuhan (middle part), 7 were from Kuming (Southwest), 10 were from Jilin (Northeast), and 8 were from Shangdong (East Coast). The sources of infection in the 39 patients who were HCV RNA positive were blood transfusion for 18 patients (including 12 renal dialysis patients) and unknown for 21 patients.

Diagnostic HCV reverse transcription-PCR. RNA was extracted from 50 μl of serum by the acid phenol-guanidinium thiocyanate method (5). Water was used as a negative control between every sample and was carried through all subsequent steps. Weak positive controls were used between every six samples. Reverse transcription and the first-round PCR assay were carried out in a single reaction mix with primers from the 5′ noncoding region (5′-NCR) as described previously (14). The first-round PCR was amplified for 35 cycles (94°C for 1.5 min, 50°C for 2 min, and 72°C for 3 min); the second-round PCR was amplified for 35 cycles, but with a two-temperature profile (94°C for 1 min, 60°C for 1 min) (Fig. 1, upper left).

General HCV typing assay. The general HCV typing assay was performed with a modification of the Okamoto PCR-based typing assay (9) as recently described (15). Briefly, consensus primers against the core region were used for the first-round PCR. Two sets of primers were used for the second-round PCRs (Fig. 1, right). Reaction A (genotypes 1a, 1b, 2a, and 2b) and reaction B (genotypes 1b, 3a, and 6a) were used to generate PCR products of different sizes that could be distinguished by agarose gel electrophoresis. The modified primer sequences and PCR stringency (annealing temperature of 63°C for 25 cycles) were as described previously (15). Two minor modifications were used: the redundant bases in primer 185 were replaced with inosine, and a mixture of two slightly different sense primers (23ou and 23sh, the latter two bases shorter at the 3′ end) was used in the first-round PCR.

Strategy for including genotype 6a in the typing assay. To identify potential genotype 6a isolates, a sense primer [5′GA(TC)GACCGGGTCCTTTCCATT-3′; nucleotides 181 to 199) in the 5′-NCR was designed with a 3′ end including the two-base insertion (underlined) described in the HK2 isolate by Bukh et al. (1). With this primer in a seminested PCR under stringent conditions (annealing temperature of 60°C for 35 cycles) together with our standard outer anti-sense 5′-NCR primer (Fig. 1, lower left), 8 of 56 Hong Kong samples and none of the



Primer	Primer sequence (5′to 3′)	Product size
49	GGCGACACTCCACCATAGATC	
65	CATGGTGCACGGTCTACGAGAC	327-(330) bp
50	GGAACTACTGTCTTCACGCAGA	
56	TCGCAAGCACCCTATCAGGCAG	260 -(263) bp
HK2	GA(TC)GACCGGGTCCTTTCCATT	
65	CATGGTGCACGGTCTACGAGAC	167 bp

Primer	Primer sequence (5′to 3′)	Product size
23ou	TAGATTGGGTGTGCGCGCGA	
23sh	CAGGTTGGGTGTGCGCGC	
186	ATGTACCCCATGAGGTCGGC	285 bp
104	AGGAAGACTTCCGAGCGGTC	
132B	TGGGGATAGGCTGACGTCTACCT	52 bp
133B	CCATCCTGCCCACCCCA	141 bp
134	CCAAGAGGGACGGGAACCTC	173 bp
135B	GCAACCCTCGTTTCC(GA)TACAGAG	126 bp
185	GCAIGTIAGGGTATCGATGACIT	
255	GTGTGCGCGACGCGTAAA	248 bp
136	AACCTCGTGGAAGGCGACAA	214 bp
142	AGCCCCAGGGCAGGCA	187 bp

FIG. 1. Schematic drawing of PCRs used to diagnose HCV viremia (upper left) and identify genotype 6a by primers in the 5′-NCR (lower left) and typing reactions in the core (right). Primer sequences are included. I, inosine; nct, nucleotides.

mainland China samples that were positive in the standard diagnostic reverse transcription-PCR assay were found to be positive.

To include genotype 6a in the expanded typing assay, the first step involved building up a sequence library of genotype 6a in the core region and constructing a specific primer for genotype 6a. By using consensus core primers (104 and 185) on the eight genotype 6a isolates, a segment of the core gene was amplified and sequenced. The aligned sequences were used for primer design. Several primers were tested before sense primer 142 (5′ AGCCCCAGGGGCAGGCA 3′; nucleotides 549 to 565) was decided on. This primer has a melting temperature (54.9°C) slightly lower than that of the other primers used in our system (57°C). However, attempts to prolong the 5′ end of this primer resulted in a thermostable loop structure according to the nearest-neighbor algorithm and equal performance in vitro. Together with our antisense consensus primer, a 187-bp product was generated that could be easily differentiated from the 1b and 3a products in reaction B (Fig. 1, right). PCR with 67 other samples of known genotypes 1a, 1b, 2a, 2b, 3a, and 4a to 4d from mainland China, Sweden, Saudi Arabia, and Ethiopia showed that there was no cross-reactivity with the type 6a-specific core primer. However, five of eight genotype 6a isolates yielded a faint 2b band in PCR A. All primers used in PCR and sequencing are shown in Fig. 1.

Sequencing. The PCR products from the 5′-NCR as well as from the core consensus amplification were purified by gel electrophoresis; both strands were sequenced by the dideoxy chain termination method as described previously (17). All inner consensus primers in PCR amplification were also employed as sequencing primers. To eliminate gel artifacts caused by secondary structure due to GC-rich contents in the HCV genome, dITP was used instead of dGTP. Sequence analysis and alignment were performed with the Pileup (Genetics Computer Group, Madison, Wis.) and MacMolly (Tetra, version 2.0; Soft Gene Gmbh, Bocholt, Germany) programs.

RESULTS

Diagnostic PCR. Fifty-six (97%) of 58 patients from Hong Kong and 39 (70%) of 56 patients from mainland China were HCV RNA positive with primers in the 5′-NCR. The lower rate of detectable viremia in samples from mainland China may be due to poor storage conditions during shipment to Sweden, where the nucleic acid analysis assays were performed.

HCV genotypes in Hong Kong Chinese patients. As shown in Table 1, the predominant HCV genotype was 1b (70%). 6a was the second most common genotype (14%). All eight patients with genotype 6a had contracted their infections in Hong Kong. Three infections were associated with transfusion and hemodialysis, one was associated with transfusion and perito-

TABLE 1. HCV genotypes in 56 Chinese patients from Hong Kong

Source of infection	No. PCR positive	No. with genotype						
		1a	1b	2a	2b	3a	6a	Mixed
Blood transfusion hepatitis	21	0	14	2	1	1	3	0
Renal dialysis and blood transfusion	27	0	21	0	0	0	4	2[a]
Intravenous drug use	4	0	3	1	0	0	0	0
Occupational exposure	3	0	1	0	0	1	1	0
Unknown	1	0	1	0	0	0	0	0
Total	56	0	40	3	1	2	8	2

[a] One sample contained mixed 1a plus 2a infection; the second one contained 1b plus 2b.

TABLE 2. HCV genotypes in 39 Chinese patients from mainland China

Source or type of infection	No. PCR positive	No. with genotype						
		1a	1b	2a	2b	3a	6a	Mixed
Blood transfusion hepatitis	6	0	1	1	0	0	0	4[a]
Renal dialysis and blood transfusion	12	0	12	0	0	0	0	0
Sporadic hepatitis	21	0	14	4	0	0	0	3[a]
Total	39	0	27	5	0	0	0	7

[a] All with mixed 1b plus 2a infections.

neal dialysis, three were associated with blood transfusion only, and one was associated with occupational exposure. Of the six patients presumably infected in mainland China, four were found with genotype 1b, one was found with genotypes 1a and 2a, and one was found with genotypes 1b and 2b; two patients infected in the United States had genotype 1b, and one patient infected in Australia had genotype 2a. There was no clinical connection among the eight patients with HCV genotype 6a. Genotypes 1a, 2a, 2b, and 3a were also present but at lower frequencies. Genotype 1a was only detected in a single patient who had a mixed infection of genotypes 1a and 2a. The only other patient with a mixed infection had genotypes 1b and 2b. All 56 patients who were HCV RNA positive could be classified by our current typing assay.

HCV genotypes in mainland Chinese patients. As shown in Table 2, HCV genotypes in mainland Chinese were restricted to 1b and 2a. Genotype 1b was predominant, while genotype 2a was the second most common, being found in 69 and 31%, respectively, of the patients who were HCV RNA positive. Interestingly, a high proportion of patients (18%) showed a pattern of mixed infection, all of whom had a combination of genotypes 1b and 2a. Four patients could not be classified with the current core typing assay, probably because of the lower level of RNA in their samples. This was supported by failure of amplification by core consensus primers. However when the 5′-NCRs of those four samples were sequenced, their sequences revealed that two of them were identical to genotype 1b and the other two were identical to genotype 2a compared with sequences published by Bukh et al. (1). Those four samples were therefore classified as genotypes 1b and 2a, respectively.

Sequence comparisons among different HCV genotypes. The sequences of the amplified core regions between primers 104 and 185 (194 bp in length) from the eight genotype 6a isolates were aligned and compared with published sequences from all other known genotypes. The nucleotide homologies compared with genotypes 1a, 1b, 2a, 2b, 3a, and 5a were 81.2, 82.1, 73.8, 77.3, 81.4, and 78.9%, respectively. In contrast, there was 99.6% homology among the sequences of the eight genotype 6a isolates (Fig. 2).

DISCUSSION

Our paper is the first to describe a genotyping assay that included genotype 6a based on type-specific primers in the core region of HCV. When our studies were initiated, no sequence information for genotype 6a in the core region was available for the design of a 6a-specific primer. The strategies we used to detect genotype 6a involved (i) using a special 5′-NCR primer which contained two insertions in its 3′ end to identify genotype 6a isolates that carried those base insertions on the basis of the data of Bukh et al. (1), (ii) amplifying a core segment in those genotype 6a isolates by core consensus primers and then sequencing those core segments to obtain the core sequence motif of genotype 6a for specific primer construction, and (iii) detecting genotype 6a by including a 6a-specific primer in an expanded typing assay with the same high stringency. The validity of this assay in differentiating genotype 6a from other genotypes was verified by testing 67 samples of genotypes 1a, 1b, 2a, 2b, 3a, and 4a to 4d collected from mainland China, Sweden, Saudi Arabia, and Ethiopia (data not shown). This is further supported by the sequence data and the finding of a high degree (99.6%) of homology in the eight genotype 6a isolates and lower degrees of homology of 81.2, 82.1, 73.8, 77.3, 81.4, and 78.9% from genotypes 1a, 1b, 2a, 2b, 3, and 5, respectively. The high degree of homology among the eight genotype 6a isolates from clinically unrelated patients is surprising, since homologies among isolates of other genotypes in this region are about 97.8, 95.1, 94.2, 97.1, and 95.7% for genotypes 1a, 1b, 2a, 2b, and 3a, respectively (15). Studies are ongoing to determine if such a high degree of homology can be found in other regions of the HCV genome.

The distributions of the HCV genotypes in mainland China and Hong Kong were different. Although the predominant HCV genotype was the same (1b), genotype 6a, the second most common (14%) genotype in Hong Kong, was not detected in patients from mainland China. The second most common genotype in mainland China, 2a, was found in 31% of the patients alone or in combination with 1b but was found in only 9% of the Hong Kong patients. Mixed infection was detected in 18% of the patients in mainland China but in only 4% of the patients in Hong Kong. All of the patients in Hong Kong could be classified by the current typing assay, but 10% of the samples from mainland China could not classified on the basis of primers in the core region; these samples were provisionally classified according to the 5′-NCR sequence motif. The difficulty in classifying these samples may be related to low-level viremia or the presence of variations in the core region that prevent annealing of the primers.

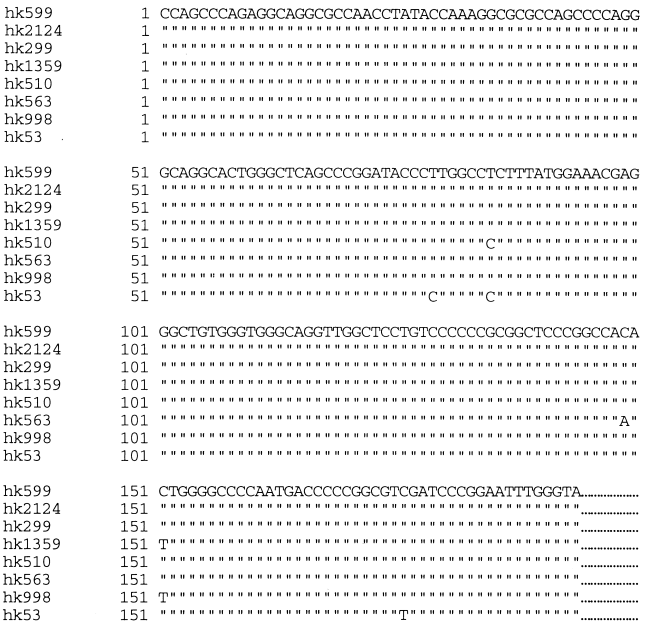


FIG. 2. Alignment of a 194-bp segment in the core region in eight genotype 6a isolates.

In summary, we found a greater diversity in HCV genotypes in patients from Hong Kong than in mainland China despite their geographical proximity. This may be related to the fact that Hong Kong is an international metropolis that gives its inhabitants more contact with the outside world. The lack of genotype 6a in mainland China is surprising, since there is frequent travel between the two places. Further studies including a larger population from mainland China, especially the southern cities that are adjacent to Hong Kong, are needed to conclude if genotype 6a is indeed absent in mainland China.

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