

## Original Article

# Molecular Epidemiology of Hepatitis Viruses and Genotypic Distribution of Hepatitis B and C Viruses in Harbin, China

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**SUMMARY:** We carried out a molecular-based epidemiological survey of hepatitis viruses, including hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV), in Harbin, China. The study population of 358 subjects consisted of 132 healthy blood donors and 226 liver disease patients residing in Harbin City and surrounding suburbs. The infection rate of each virus among healthy subjects was 14.4% (19/132) for HBV and 2.3% (3/132) for HCV. In contrast, among liver disease patients, the infection rates were 72.6% (164/226) for HBV and 7.5% (17/226) for HCV, respectively ( $P < 0.01$  and  $P < 0.05$ , respectively). In particular, nearly 64% of hepatocellular carcinoma patients in Harbin was found to be infected with HBV. The most common viral genotypes were HBV type C (80%) and HCV type 1b (31.3%). Interestingly, a high prevalence of the HBV pre-S1/S2 deletion mutant was found in 13 of 58 (22.4%) subjects. Moreover, testing for HEV among 202 subjects resulted in the detection of anti-HEV IgG in 53 cases (26.2%). The prevalence of anti-HEV IgG has already reached 20% in tested cases aged less than 10 years. These results suggest that HBV infection is widespread in Harbin, China and has led to a high incidence of acute and chronic liver disease in this region.

## INTRODUCTION

Viral hepatitis exists throughout the world and is a major global public health problem. Particularly, hepatitis B virus (HBV) and hepatitis C virus (HCV) are the causative agents responsible for parenteral transmitted diseases. It is known that the prevalence of HBV and HCV infections varies according to geographical areas and that their infections appear to correlate with the severity of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma. In Heilongjiang Province of China, adequate levels of information regarding the molecular epidemiology of hepatitis viruses has not been available thus far, although many epidemiological studies have been reported from China (1-7).

In the present study, we conducted a molecular-based epidemiological study of HBV, HCV, and hepatitis E virus (HEV) among liver disease patients and healthy individuals residing in Harbin, China. Harbin is the capital of Heilongjiang Province and is located in the Northern part of China. We also identified the genotypic distribution of HBV and HCV in this area.

## MATERIALS AND METHODS

**Study population:** We tested 358 serum samples, including samples of 226 patients with liver diseases (27 acute hepatitis, 142 chronic hepatitis, 29 liver cirrhosis, and 28 hepatocellular carcinoma) and 132 healthy blood donors in Harbin, China.

All subjects were residents of Harbin City or surrounding suburbs. Clinical diagnosis was based on liver- function tests, hepatitis virus markers, autoantibodies, tumor markers, ultrasonography, and liver histopathology. The healthy group was made up of persons who had had health checkups in medical centers. They had shown neither clinical symptoms nor abnormalities in laboratory tests. Informed consent for participation in this study was obtained from each individual. These serum samples were collected from 2000 to 2001 and stored at  $-40^{\circ}\text{C}$  or below until use.

**Nucleic acid extraction and PCR for HBV DNA and HCV RNA detection:** Both DNA and RNA were extracted simultaneously from 100  $\mu\text{l}$  of serum using the SepaGene RV-R kit (Sanko Junyaku Co., Ltd., Tokyo). After extraction, the samples were precipitated with isopropanol and washed in ethanol. The resulting pellet was resuspended in 50  $\mu\text{l}$  of RNase-free water. The sequences of PCR primers were as follows. ① For HBV (X region): 5'-TGCCAACTGGATCCTTCGCGGGACGTCCTT-3' (MD24, sense primer, nucleotide [nt] 1392-1421) and 5'-GTTCACGGTGGTCTCCATG-3' (MD26, antisense primer, nt 1625-1607) for the outer primer pairs (233 bases), and 5'-GTCCCCTTCTTCATCTGCCGT-3' (HBx1, sense primer, nt 1487-1507) and 5'-ACGTGCAGAGGTGAAGCGAAG-3' (HBx2, antisense primer, nt 1604-1584) for the inner primer pairs (118 bases). ② For HCV (5'-untranslated region): 5'-GCGACACTCCACCATAGAT-3' (19, sense primer, nt 2 to 20) and 5'-GCTCATGGTGCACGGTCTA-3' (20, antisense primer, nt 312-330) for the outer primer pairs (329 bases), and 5'-CTGTGAGGAACTACTGTCT-3' (21, sense primer, nt 28-46), and 5'-ACTCGCAAGCACCTATCA-3' (22, antisense primer, nt 277-295) for the inner primer pairs (268 bases). The nucleotide positions were deduced from HBVadr4 (8) for HBV and HC-J1 (9) for HCV.

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To obtain simultaneous detection of hepatitis B and C viral genomes, we used the multiplex PCR method as described previously (10). Detection was performed in one step that combined cDNA synthesis and PCR in a single tube. That is, for HCV, the first PCR was combined with the reverse transcriptase (RT) step in the same tube containing 50  $\mu$ l of a reaction buffer prepared as follows: 10 units of RNase inhibitor (Promega, Madison, Wis., USA), 100 units of Moloney murine leukemia virus RT (Promega), 40 ng of each outer primer for HBV and HCV, 300  $\mu$ M of each of the four deoxynucleotides, 2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA), and 1X reaction buffer containing 1.5 mM MgCl<sub>2</sub>. To obtain an automatic hot-start reaction, we used AmpliTaq Gold DNA polymerase instead of regular thermostable DNA polymerase. The thermocycler was programmed first to incubate the samples for 50 min at 37°C for the initial RT step and then to preheat at 95°C for 10 min to activate AmpliTaq Gold, followed by 40 cycles at of 94°C for 30 s, at 50°C for 45 s, and at 72°C for 1 min using a Perkin-Elmer 2400 or 9700 Thermal Cycler (Perkin-Elmer). For the second reaction, 2  $\mu$ l (1/25 volume) of the first PCR product was added to a tube containing the second set of each inner primer, deoxynucleotides, AmpliTaq Gold DNA polymerase, and PCR buffer as in the first reaction, but without RT and omitting the initial 50-min incubation at 37°C. Amplification was performed for 40 cycles with the following parameters: preheat at 95°C for 10 min, 20 cycles of amplification at 94°C for 30 s, annealing at 53°C for 45 s, and extension at 72°C for 1 min, followed by an additional 20 cycles at 94°C for 30 s, at 55°C for 45 s, and at 72°C for 1 min. The PCR products were run on 3% agarose gel, stained with ethidium bromide, and evaluated under UV light. The sizes of the PCR products were estimated according to the migration pattern of a 50-bp DNA ladder (Pharmacia Biotech, Piscataway, N.J., USA). To avoid the risk of false-positive results, PCR assays were carried out with strict precautions against cross-contaminations. Furthermore, all PCR assays were performed in duplicate to confirm reproducibility.

**Genotyping of HBV and HCV by PCR:** Genotyping of HBV was determined by a PCR method using type-specific primers as reported previously (11). In addition, HCV genotyping was assayed by PCR, as reported by Ohno et al. with slight modification (12).

**Nucleotide sequencing of HBV pre-S1/S2 gene:** Using 58 HBV DNA-positive samples, we amplified the HBV pre-S1/S2 gene by semi-nested PCR using the following primers: P1: 5'-TCACCATATTCTTGGGAACAAGA-3' (sense, nt 2817-2839) and S1-2: 5'-CGAACCCTGAACAAATGGC-3' (antisense, nt 704-685) for the outer primer pairs, and P1 and S2-2: 5'-GGCACTAGTAACTGAGCCA-3' (antisense, nt 687-668) for the inner primer pairs. PCR products were separated by 2% agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, Calif., USA). Recovered PCR products were subjected to direct sequencing from both directions using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequences of amplified cDNA were determined using a sequencer (ABI model 377 and 310; Applied Biosystems, Foster City, Calif., USA).

**Assay for HEV antibody by enzyme-linked immunosorbent assay (ELISA):** Immunoglobulin G (IgG) and IgM antibodies to HEV were measured by ELISA. The ELISA to detect anti-HEV using virus-like particles expressed by a recombinant baculovirus was performed as reported previ-

ously (13).

**Statistical analysis:** Statistical analyses were performed using the Student's *t* test or Fisher's exact test. A difference with a *P* value of <0.05 was considered significant.

## RESULTS

**HBV and HCV infections and their genotyping:** HBV DNA and HCV RNA were detected in 19 (14.4%) and 3 (2.3%), respectively, of a population of 132 healthy individuals (Table 1). In contrast, among 226 liver disease patients, HBV DNA and HCV RNA were detected in 164 (72.6%) and 17 (7.5%), respectively (*P* < 0.01 and *P* < 0.05, respectively). HBV DNA was present in 70.4% of acute hepatitis, 76.1% of chronic hepatitis, 65.5% of liver cirrhosis, and 64.3% of hepatocellular carcinoma patients, respectively. As shown in Table 2, genotype C (80%) of HBV was the most prevalent in the 183 patients tested. For HCV, among 16 samples, genotype 1b (31.3%) was the most common. Finally, 3.3% of HBV and 18.8% of HCV cases examined were unclassifiable in these populations by PCR genotyping.

**Prevalence of anti-HEV antibody:** The prevalence of anti-HEV antibodies was 26.2% (53/202) for the IgG class and 1.5% (3/202) for the IgM class. The age-specific prevalence has already reached 20% in 0 to 10 year-olds and 16.7% in 11 to 20 year-olds, respectively (Fig. 1).

**Pre-S1/S2 deletion mutant of HBV:** We analyzed the nucleotide sequences of the HBV pre-S1/S2 region obtained from 58 Chinese residents of Harbin. The results revealed that 13 of 58 (22.4%) HBV isolates had a deletion mutant in this region. The number of nucleotides deleted varied from 18 to 258 bases, but was not associated with a frame shift of

Table 1. Prevalence of HBV and HCV in Harbin, China

Category	<i>n</i>	HBV DNA	HCV RNA	Coinfection
Acute hepatitis	27	19 (70.4)	2 (7.4)	2 (7.4)
Chronic hepatitis	142	108 (76.1)	13 (9.2)	8 (5.6)
Liver cirrhosis	29	19 (65.5)	1 (3.4)	2 (6.9)
HCC <sup>1)</sup>	28	18 (64.3)	1 (3.6)	1 (3.6)
Subtotal	226	164 (72.6)	17 (7.5)	13 (5.8)
Blood donor	132	19 (14.4)	3 (2.3)	0
Total	358	183 (51.1)	20 (5.6)	13 (3.6)

<sup>1)</sup>hepatocellular carcinoma.

Numbers in parentheses indicate percentages.

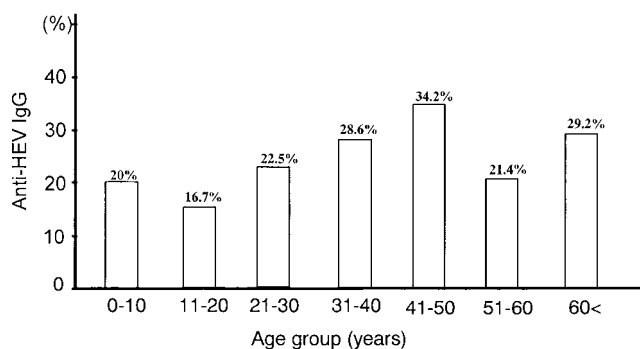


Fig. 1. Age-specific prevalence of anti-HEV IgG among 202 subjects residing in Harbin, China.

Table 2. Genotypic distribution of HBV and HCV in Harbin, China

HBV	Number Tested	Genotype							Unclassified
		A	B	C	D	E	F	B+C	
	183	11 (3)	10 (5.5)	147 (80)	1 (0.5)	0	0	8 (4.4)	6 (3.3)

HCV	Number Tested	Genotype					Unclassified
		1a	1b	2a	3a	3b	
	16	2 (12.5)	5 (31.3)	3 (18.8)	2 (12.5)	1 (6.3)	3 (18.8)

Numbers in parentheses indicate percentages.

the amino acid sequences. All isolates with such mutations were identified from liver disease patients: including 5 asymptomatic carriers, 1 patient with acute hepatitis, 3 with chronic hepatitis, 2 with liver cirrhosis, and 2 with hepatocellular carcinoma.

## DISCUSSION

Hepatitis virus infection is now a major problem both in developed and developing countries. In particular, hepatitis viruses are responsible for one of the most widespread infectious diseases in Asian countries. Although many studies have been carried out in China (1-7), an epidemiological investigation based on the molecular characterization of hepatitis viruses in Harbin has not been available thus far. Results of the present study show that Harbin City of Heilongjiang Province, located in the Northern part of China, is a very high endemic area of HBV infection, although a national expanded program of immunization, including vaccination against HBV, has already been started. In particular, nearly 70% of acute hepatitis cases and 64% of hepatocellular carcinoma cases in Harbin have been found to be related to HBV infection. A strong relationship between HBV and the occurrence of hepatocellular carcinoma in Harbin has also been confirmed by *in situ* detection of hepatitis virus genomes using carcinoma tissues (14).

In general, the route of viral infection in developing countries is not clear. The routes and risk factors involved were not identified in the present study. Currently, however, we are conducting an investigation to clarify the transmission route of these viruses in this area. Genotyping of HBV and HCV are important tools in clarifying the route and pathogenesis of these viruses (15-17). In particular, examination of sequence diversity among different isolates of the virus is important because variants may differ in their patterns of serologic reactivity, pathogenicity, virulence, and responses to therapy. On the other hand, HBV and HCV have genetic variations, corresponding to the geographic distribution (11, 15-17). It is known that types C and B of HBV and type 1b of HCV are primarily distributed in Asian countries (11). In China, two major genotypes of HBV have been reported thus far. That is, genotype B exist mainly in Southern parts (Shanghai area) and genotype C in Northern parts of China. In this study, we found that the major genotype distributed among Chinese peoples residing in Harbin, Heilongjiang Province, is type C for HBV and type 1b for HCV. It has been suggested that the HBV genotype C and HCV genotype 1b are associated with the presence of more serious liver disease and with a lower rate of response to interferon (18-24). In this study, we could not compare the relationship among genotypes of HBV and HCV, and the clinical outcome of liver disease, because the

most individuals were infected with genotype C of HBV, with very few being infected with HCV. Knowledge of the relation between the genotypes of HBV and HCV and their pathogenicity in chronic liver diseases, including hepatocellular carcinoma occurrence is awaited with great interest.

Surprisingly, we found in the present study a very high prevalence of HBV pre-S2 mutant (22.4%) that was partially truncated to various sizes. It has been reported that the pre-S2 deletion mutant appears to prevail at low or nonreplicative phases (25-27). The emergence of pre-S mutants may affect viral replication and evade immune surveillance. More investigations are needed to clarify the biology and clinical significance of the spread of HBV with the pre-S1/S2 mutant in this area.

HEV, previously referred to as enterically transmitted non-A, non-B hepatitis, is a major cause of epidemic hepatitis and of acute, sporadic hepatitis in developing countries (28). Many outbreaks of HEV-induced hepatitis have been reported in India, Southeast and Central Asia, Africa, and Mexico (29). Our results indicate that a high prevalence of anti-HEV antibody has already been reached in those less than 10 years of age in Harbin, suggesting that the enterically transmitted infectious disease is prevailing in this area.

In conclusion, HBV infection was found to be the primary cause of liver diseases in Harbin, China. In particular, more than half of hepatocellular carcinoma patients in Harbin were found to be infected with HBV, with the major HBV genotype being type C. Establishment of prevention, serological diagnosis, and treatment of hepatitis viruses is an important area of concern in this area.

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