Supplementary Material

Distribution and Hepatocellular Carcinoma-Related Viral Properties of Hepatitis B Virus Genotypes in Mainland China: A Community-Based Study

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Sample size estimation. Since 1992, Mainland China has recommended a routine infant hepatitis B immunization with a schedule of 0, 1, 6 months to prevent perinatal transmission of hepatitis B virus (HBV) using domestically produced hepatitis B vaccines. A national survey conducted in 1999 showed that the HBV vaccine coverage in infants under 12 months of age was 88.5% for urban areas and 62.7 % for rural areas, respectively (1, 2). The ultimate integration of HBV vaccine into the Expanded Programme Immunization (EPI) has been performed since 2002 in Mainland China. The prevalence of HBV in the children at the age between 1 and 14 years have decreased dramatically, especially in the children at the age between 1 and 4 years (2). According to the previous prevalence rates of hepatitis B surface antigen (HBsAg), the estimated prevalence rates (allowable errors) of HBsAg at the age groups of
1-4 years, 5-14 years and 15-59 years were set as 1.0% (0.5%), 4.0% (1.0%), and 9.0% (1.8%), respectively. According to the economic, geographic, and demographic situations, 160 national disease surveillance points (DSPs) were stratified into 6 strata, composed of country population and urban population in the developed area, the developing area, and the under-developed area, respectively. The sample sizes at the age groups of 1-4 years, 5-14 years and 15-59 years in each stratum were calculated based on following formula, respectively.

\[ n = \frac{u^2 \pi (1 - \pi)}{\delta^2} \]

- \( n \) = sample size
- \( \pi \) = the previous estimated prevalence of HBsAg
- \( \delta \) = allowable error
- \( u_\alpha \) = \( u \) value at the significant level \( \alpha \). \( \alpha = 0.05 \), \( u = 1.96 \)

For the age group between 1 and 4 years, the sample size was 1,522 as calculated with the above formula. By adding the 10.0% of possible dropout and 1.5 fold increase of the sample size for cluster sampling, estimated sample size in this age group was 1,522 \( \div 0.9 \times 1.5 \times 6 = 15,213 \). Estimated sample size in this age group between 5 and 14 years was 23,416 as calculated above. Estimated sample size in the age group between 15 and 59 years was 40,500. A total of the estimated sample size was 79,129 (15,213 + 23,416 + 40,500).

The actual number of the participants included in this study was 81,775 from all DSPs covering the 31 provincial administrative regions in Mainland China.
Extraction of HBV DNA from blood clots. About 400 microliters (μl) blood clots sample was added into a 1.5 milliliter (ml) eppendorf vial. After being diluted by 400μl distilled water, it was mixed by vortex for 5min. After the centrifugation at 2,000×g/min for 1min, 20 μl of the supernatant was transferred into a new vial. The same volume of the reagent I containing 3.5 mol per liter NaCl and 200g per liter PEG6000 (Bio Basic Inc, Markham Ontario, Canada) was added into the vial, mixed and centrifuged at 16,200×g/min for 15min. The supernatant was discharged, 80μl of the reagent II containing 50mmol per liter Tris-HCl and 1.0% NP-40 (Bio Basic Inc, Markham Ontario, Canada) was added. After being mixed adequately, the mixture was boiled for 8min, and then centrifuged at 16,200×g/min for 15min. HBV DNA in the supernatant was precipitated by 75% ethanol, and then dissolved with 50 μl 1×Tris-EDTA (TE) buffer. HBV DNA concentration of the extracts was measured in the LightCycler™480 (Roche, Basel, Switzerland) using quantitative HBV PCR fluorescence diagnostic kits (Fuxun Diagnostics, Shanghai, China). The kit has a certified lower limit of detection of 500 copies/ml. We used 3-5 μl of each extract as templates of multiplex PCR to identify HBV genotype and subgenotype.

Multiplex PCR and nested-multiplex PCR for the identification of HBV genotype and subgenotypes. The primers were designed by using Primer Primier 5.0 and Oligio 6.0 software packages. The primers used in the study were listed in Supplementary Table 1. For the samples with low level of HBV DNA, the nested multiplex PCR was used for the identification of HBV genotype. The nested-PCR was firstly carried out to amplify the samples with a pair of the outer primers which covered all the primers sites used for the
multiplex PCR. The multiplex-PCR was then used for the identification of HBV genotypes and subgenotypes as previously described (3, 4). All PCR procedures including the preparation of the templates, mixture of PCR components, and electrophoresis were carried out in separate laboratory rooms with specific facilities to avoid cross contamination.

The first round amplification with PCR Master Mix-Premix Ex Taq enzymes (TaKaRa, Dalian, China) was carried out as followed: 1) P1-S and P1-AS primers were used for the amplification of HBV genotypes B, C and D. P2-S and P2-AS primers were used for the amplification of the first round of HBV B1 and HBV B2. An Autorisierter thermocycler (Eppendorf AG, Humburg, Germany) was programmed to initially denature the samples for 3min at 95°C, followed by 35 cycles consisting of 94°C for 60 second (s), 58°C for 60s, 72°C for 60s, followed by a final elongation step at 72°C for 10min. 2) HBV genotypes A, E, and F and subgenotypes C1and C2 were amplified by using P3-S and P3-AS primers. The thermocycler was programmed to initially denature the samples for 3min at 95°C, followed by 35 cycles consisting of 94°C for 60s, 52°C for 60s, 72°C for 3min, followed by a final elongation step at 72°C for 10min.

The product (2μl) of the first round PCR served as a template for routine multiplex PCR, as previous described (3, 4). The thermocycler was programmed to initially denature the samples for 3min at 95°C, followed by 25 cycles consisting of 94°C for 60s, 62°C for 60s, 72°C for 60s, followed by a final elongation step at 72°C for 10min. About 60% of the samples with HBV DNA at the concentration of 500 copies/ml were able to be genotyped by using the nested-multiplex PCR, while about 40% of the samples with HBV DNA at
the concentration of 500 copies/ml were able to be genotyped by using routine multiplex PCR.

**HBV DNA sequencing and phylogensis.** The PreS-S region of HBV DNA was amplified for the direct sequencing and subsequent phylogensis to confirm HBV genotype and subgenotype identified by the multiplex PCR for the samples that were randomly selected from those with HBV genotyped (5). We used the nested-PCR to amplify HBV DNA if routine PCR failed to amplify HBV DNA. The detection limits of routine PCR and the nested PCR were $10^4$ copies/ml and $10^3$ copies/ml, respectively. Direct DNA sequence analysis of the PreS-S region, the enhancer II (EnhII)/basic core promoter (BCP)/precore (EnhII/BCP/Precore) region, and whole genome of HBV was carried out by using ABI PRISM BigDye sequencing kits and an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). The complete genome and the PreS-S region of HBV were sequenced as described previously (6-8). All sequences were analyzed in both forward and backward directions. Phylogensis was performed as previously described (5).

**Recombination analysis.** The whole HBV genomes of HBV subgenotype C9 isolates were randomly selected for recombination analysis by using Bootscanning program of SimPlot version 2.5 software package (9).

**References**


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Legends of Supplementary Figures

Supplementary Figure 1. Phylogenetic tree of complete HBV genome of HBV C9 with other HBV genotypes and subgenotypes. An alignment of complete sequences was performed with using the Alignment program of the MEGA 4.0 software. The alignment was further analyzed by boot-strapping with 1000 replicates using the Neighbourhood-Joining method in MEGA version 4.0.

Supplementary Figure 2. Bootscanning analysis over the complete genome of HBV subgenotype C9 isolate S3 using SimPlot. S3 was compared with representative HBV genotypes and subgenotypes: A1, AB076678; A2, AF536524; A3, AB194951; B1, AB010290; B2, AB073834; B3, AB033554; B4, AB031267; B5, AB219426; C1, AB031262; C2, AB014360; C3, X75656; C4, AB048704; C5, AB241109; C8, Ph105; D1, AB721605; D2, AB090268; D3, X85254; D4, AB048702; E, AB091255; F, AB036909; G, AB064311; H, AY090457.

Supplementary Figure 3. Phylogenetic tree of PreS-S region of HBV subgenotype D1. The HBV subgenotype D1 isolates from Mainland China are S16, S19, S20, S21, S22, S23, and D85 from the Uygur ethnic; D33, D613, C11, and C29 from the Han ethnic; S24 and S26 from the Kazak ethnic; S5 from the Zhuang ethnic; and S9 from the Yi ethnic. An alignment of complete sequences was performed with using the Alignment program of the MEGA 4.0
software. The alignment was further analyzed by boot-strapping with 1000 replicates using the Neighbourhood-Joining method in MEGA version 4.0.