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# Simultaneous Genotyping and Quantification of Hepatitis B Virus for Genotypes B and C by Real-Time PCR Assay▽

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**Hepatitis B virus (HBV) is an important cause of human chronic liver diseases and is a major public health problem. Viral load and HBV genotype play critical roles in determining clinical outcomes and response to antiviral treatment in hepatitis B patients. Viral genotype detection and quantification assays are currently in use with different levels of effectiveness. In this study, the performance of a real-time genotyping and quantitative PCR (GQ-PCR)-based assay was evaluated. Through the use of genotype-specific primers and probes, this assay provides simultaneous identification and quantification of genotypes B and C in a single reaction. Our GQ-PCR correctly identified all predefined genotypes B and C, and no cross-reaction between genotypes B and C were observed. The GQ-PCR identified more cases of HBV infections with mixed genotypes B and C than direct sequencing did. Samples from 127 HBV-infected Chinese patients were genotyped with GQ-PCR, revealing 56.7% HBV as genotype B, 13.4% as genotype C, and 29.8% as mixed genotypes B and C. This assay provides a reliable, efficient, and cost-effective means for quantification of the B and C genotypes of HBV in single or mixed infections. This assay is suitable for sequential monitoring of viral load levels and for determining the relationship between the genotype viral load and stage of disease in Asians.**

Hepatitis B virus (HBV) is one of the most serious and prevalent health problems, affecting more than 2 billion people worldwide. Chronic HBV infection greatly increases the risk for liver cirrhosis and hepatocellular carcinoma (HCC). HBV infection is associated with up to 80 to 90% of HCC patients in China, India, North and South Korea, Singapore, and Vietnam (17). Although highly effective vaccines against hepatitis B virus have been available since 1982, there are still more than 400 million chronic carriers, 75% of whom reside in the Asia-Pacific region (18).

HBV has been classified into eight genotypes (A to H) based on divergence over the entire HBV genomic sequence of greater than 8% (16). The clinical picture, the response to treatment, and the long-term prognosis, as well as the seroconversion profile, are influenced by the HBV genotypes (8). China is a country seriously affected by the burden of chronic HBV infection. The prevalence of chronic HBV infection in China is 5 to 20% of the general population (16, 29). The most prevalent genotypes in China are genotypes B and C (36). In mainland China, an area of HBV endemicity with one-third of the HBV carriers in the world and chronic carriers of hepatitis B surface antigen (HBsAg), patients are commonly infected during early childhood (31). Chronic HBV infection greatly increases the risk for liver cirrhosis and HCC. HBV infection is associated with up to 80 to 90% of HCC patients in China (17).

Previous studies indicated that HBV genotype C takes a more aggressive disease course than genotype B (2, 3, 4, 7, 9, 11, 14, 22, 34). Genotype C, in comparison to genotype B, is also associated with a lower response rate to antiviral therapy (10, 13, 15, 19, 27, 28, 29, 35) and precore mutations (30). Therefore, the role of HBV genotypes in predicting outcome should be evaluated further. Evidence has suggested that coinfection with different HBV genotypes is associated with higher viral replication and a more severe course of the disease (5, 6, 12, 26, 33). Several methods have been developed for HBV quantification and genotyping in a single reaction by real-time PCR (20, 23, 32). However, those assays include additional melting-curve analyses for HBV genotypes.

Reliable and easy methods to concurrently genotype and quantitate HBV genotypes B and C are prerequisites for molecular epidemiological tests, clinical studies, antiviral therapy, and detection of coinfection. Owing to its high sensitivity, specificity, and broad dynamic range, quantitative real-time PCR (QRT-PCR) has become increasingly important in the diagnostic laboratory and has been used for HBV detection, genotyping, and quantification (1). In this study, a novel real-time PCR-based assay was developed for simultaneous genotyping and quantification of HBV for individual B and C genotypes and for mixed genotypes.

## MATERIALS AND METHODS

**Samples.** Serum samples from 127 HBV-infected and hospitalized patients were collected from the Second Affiliated Hospital of Chongqing Medical University and stored at −20°C until use. All patients were HBsAg, HBeAg, or

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TABLE 1. Primers and probes used for TaqMan real-time PCR detection and absolute quantification of HBV genotypes B and C

| Primer or probe | Sequence 5′→ 3′ | Positions (nt)[a] | Labeling 5′→ 3′ |
|---|---|---|---|
| Forward primer, HBV-B | AGACTCGTGGTGGACTTC | 250–267 | HEX–BHQ-1 |
| Genotype B probe | TCGCAGTCCCAAATCTCCA | 311–329 | HEX–BHQ-1 |
| Reverse primer, HBV-B | ACAAGAAGATGAGGCATAGC | 418–437 | HEX–BHQ-1 |
| Forward primer, HBV-C | CTTGTTGGTTCTTCTGGACTAC | 430–451 | FAM–BHQ-1 |
| Genotype C probe | CCTCTACTTCCAGGAACATCAAC | 476–498 | FAM–BHQ-1 |
| Reverse primer, HBV-C | AGGATGATGGGATGGGAATAC | 600–620 | FAM–BHQ-1 |

[a] nt, nucleotides.

anti-HBe positive after serological testing. Informed written consent was obtained from all patients.

**HBV DNA extraction.** Viral DNA was extracted from 200 μl of serum using a QIAamp MinElute virus spin kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Precipitated DNA was dissolved in 100 μl of elution buffer and stored frozen at −20°C until use.

**Clinical quantification of HBV-DNA.** Serum HBV-DNA was quantified at the laboratory of the Second Affiliated Hospital of Chongqing Medical University by real-time PCR using an ABI 7700 instrument (Applied Biosystems Inc., Foster City, CA) and HBV-DNA Q-PCR kit (Da An Gene Co., Sun Yat-Sen University, China) with a quantification range of $1 \times 10^3$ to $1 \times 10^7$ copies/ml. Samples with higher viral loads had been diluted before measurement. Although we still used number of copies per milliliter, the new recommendations that HBV viral levels be expressed in number of IU/ml and the conversion should be established for this new assay.

**Plasmids.** Two plasmids containing full-length genomic DNA of HBV genotype B were generated with the viral DNA isolated from patients who were hospitalized in the Second Affiliated Hospital of Chongqing University. We chose sequences that subtyped with NCBI genotyping tools (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and aligned with NCBI's recommended sequence strains for genotype B under accession number AF100309 and for genotype C under accession number AB014381. The two selected plasmids served as standard templates for the amplification. A serum sample containing HBV genotypes B and C, as confirmed by real-time PCR and DNA direct sequencing, was employed as an alternative construct.

**Generation of HBV genotypes B and C copy number standards for quantitative PCR.** We used HBV genotype B and C plasmids for generating the copy number standard curves for viral load quantification. The plasmid DNA was purified with a plasmid purification kit from Omega Bio-Tek (Norcross, GA); the corresponding concentration was determined by using NanoDrop ND-1000 spectrophotometers (Thermo Fisher Scientific, Inc.) and expressed as number of copies per milliliter. Serial dilutions of the plasmid ranging from $1 \times 10^2$ to $1 \times 10^9$ copies/ml were used for generating the standard curve.

**Real-time genotyping and quantitative PCR (GQ-PCR).** To locate the region of HBV genome suitable for differentiating the B and C genotypes, we analyzed the full-length sequences, particularly those of the B and C genotypes. For the primer selection, 1,213 complete sequences of HBV genotypes A to H were retrieved from GenBank, with 185 and 126 complete sequences for HBV genotypes B and C, respectively. A preference was given to HBV sequences submitted by laboratories from all over the world to account for geographic and demographic differences. The PCR primers were designed on the basis of sequence alignment of each genotype by MEGA 4.0 software (24) and Oligo 6.71 software (Molecular Biology Insights, Cascade, CO). When designing the primers, we used Geneious software version 4.6.5 (Biomatters Ltd., Auckland, New Zealand) and searched for nucleotide sequences that are genotype specific between genotypes and genotype conserved within the same genotype for genotypes B and C, respectively, which are located in part of the reverse transcriptase (RT) region. TaqMan probes were designed around type-specific motifs for genotype B and C using the Beacon Designer v.7.51 software (Premier Biosoft International, Palo Alto, CA). Each genotype-specific probe was labeled with a distinct fluorescent dye at the 5′ end (HEX [5-hexachlorofluorescein] dye for genotype B and FAM [6-carboxyfluorescein] dye for genotype C) and BHQ-1 (black hole quencher 1) quencher at the 3′ end (Table 1).

Simultaneous absolute quantification of two genotypes was done by generating two absolute standard curves. One standard curve was generated by using a HEX-labeled TaqMan probe for the quantification of genotype B, while the other was generated by using a FAM-labeled probe for the quantification of genotype C. The amplification profile was as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and 62°C for 30 s. The PCR was performed in a total volume of 10 μl containing 1 μl of DNA template, 5 μl of iQ Mutilplex Powermix (Bio-Rad Laboratories, Hercules, CA), 0.3 μM each probe, and 5 μM each primer. The mixtures were processed for PCR amplification with the CFX96 real-time PCR detection system (Bio-Rad Laboratories). Amplification data were collected and analyzed with CFX Manager software version 1.1 (Bio-Rad Laboratories).

**Direct sequencing analyses.** The direct sequencing analysis for PCR-amplified HBV viral genomic fragments was performed by using an ABI PRISM Big-Dye kit (Applied Biosystems, Foster City, CA) and then using an ABI 3100 genetics analyzer. Double peaks represent infections with mixed genotypes.

**Evaluation of sensitivity, specificity, precision, and efficiency of the quantitative PCR assay.** The sensitivity of the quantitative PCR assay was determined using a 10-fold dilution series of the clones of HBV genotypes B and C. The primer pairs and probes tested had similar sensitivities and high efficiencies. All samples with viral loads of at least $5.0 \times 10^2$ copies/ml could be detected successfully. The specificity of the assay for genotyping was evaluated by determining the cross-reactions between the primers/probes and target fragments of the two genotypes with a concentration gradient using a 10-fold dilution series from $1 \times 10^2$ to $1 \times 10^{13}$ copies/ml and a temperature gradient using a series from 50°C to 68°C. Assessment of the precision of the assay was performed with eight replicates of the real-time PCR run on six different days with DNA from the two genotypes.

**Phylogenetic analysis.** New sequences were accepted by GenBank under the accession numbers reported below. The genome sequences of the following eight different HBV genotypes (A to H) were obtained from GenBank under the indicated accession numbers and used as reference sequences for the phylogenetic tree: genotype A, AF090842; B, AF100309; C, AB014381; D, X65259; E, AB032431; F, AB036910; G, AF160501; and H, AY090454. For the mixed-genotype sequence, we used the higher peak for mapping. Nucleotide sequences were multiply aligned using the CLUSTAL X program (version 2.0). TREEVIEW (version 1.6) was run for phylogenetic tree construction. Those sample sequences appearing on the same node with reference sequences were interpreted to be from the same genotype.

**Statistics.** The distribution of the variable was determined by the Shapiro-Wilk test. Nonnormal distributions were analyzed with the Mann-Whitney U test for comparison of viral loads between groups. Statistical analyses were performed using the Statistical Package for Social Sciences software version 16.0 (IBM Company, Chicago, IL), and significance was set at a $P$ value of less than 0.05. The figure showing the results of the phylogenetic analysis (see Fig. 3) was made by GraphPad InStat software version 5.0 (San Diego, CA).

**Nucleotide sequence accession numbers.** New sequences were submitted to GenBank under accession numbers GU202958 to GU203084 (for samples ZY1-1 to ZY1-17 and ZY1 to ZY110).

## RESULTS

**Design primers and probes for distinguishing genotypes B and C.** To locate the region of the HBV genome suitable for differentiation of the B and C genotypes, we analyzed 1,213 complete sequences of HBV genotypes A to H retrieved from GenBank, with 185 and 126 complete sequences for HBV genotypes B and C, respectively. The most suitable region containing nucleotide sequences that are genotype specific among genotypes and genotype conserved within the same genotype for genotypes B and C, respectively, is the reverse transcriptase (RT) region (Fig. 1). Two pairs of PCR primers

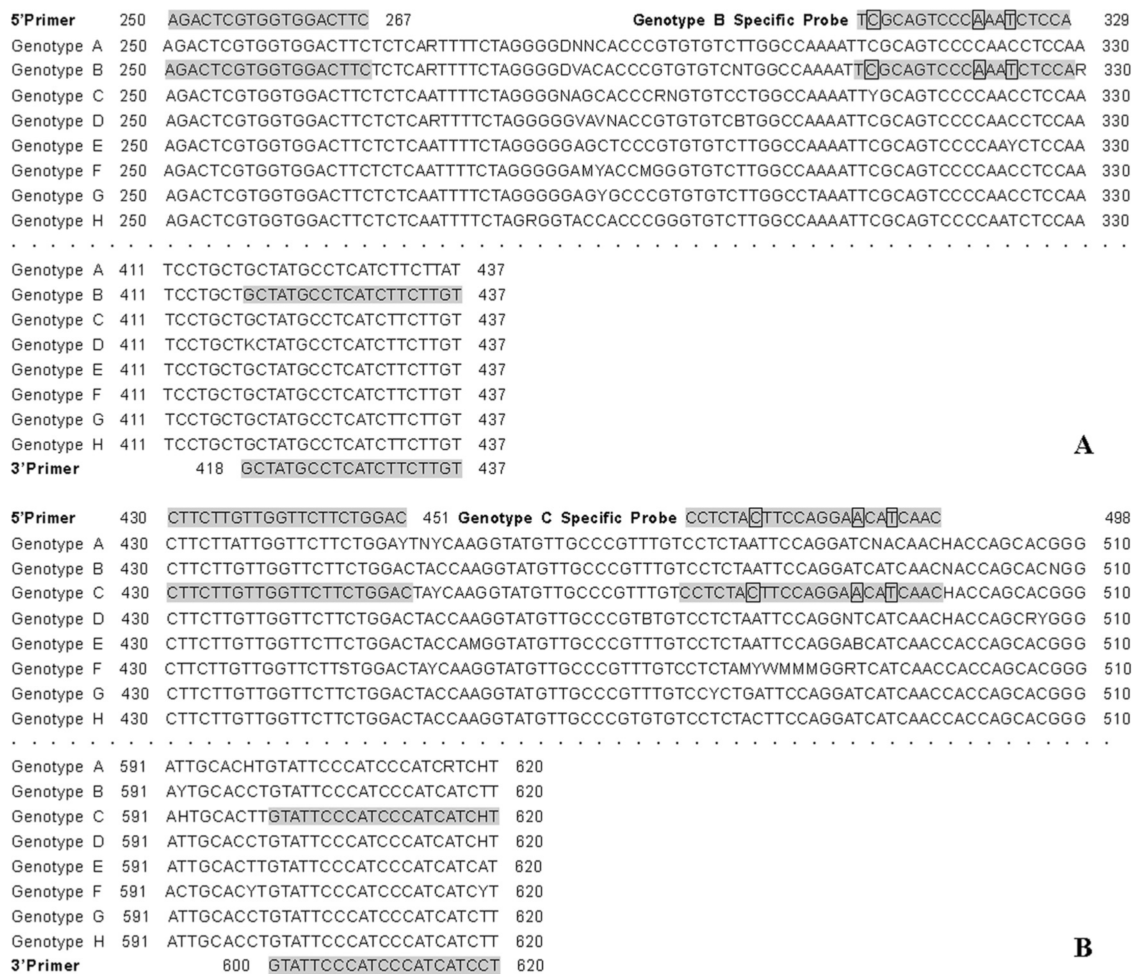

FIG. 1. Multiple alignment of nucleotide sequences in the HBV genome for genotypes A to H, using the MEGA v.4.0 and Geneious v.4.6.5 software. Primers (5′ and 3′) show homology with consensus sequences of all HBV genotypes. Probes are designed to distinguish genotypes B and C from other genotypes, and critical positions are boxed. A genotype-specific probe shows absolute homology only with genotypes that are conserved within the same genotype for genotypes B or C. (A) The shaded sequences indicate the locations of genotype B primers and probes. The sequences of genotypes A and C to H are shown below each genotype alignment. (B) The shaded sequences indicate the locations of genotype C primers and probes. The sequences of genotypes A, B, and D to H are shown below each genotype alignment.

were designed in the conserved part of this region with genotype B- or C-specific nucleotides in between. The first pair of primers produces a 187-bp-long amplicon, and the other produces a 190-bp-long amplicon. TaqMan probes were designed around genotype B-specific motifs within the first amplicon for genotype B and around genotype C-specific motifs within the second amplicon for genotype C. Both of the probe sequences contain three divergent nucleotides, with the single-nucleotide polymorphisms (SNPs) several nucleotides apart (8 and 2 nucleotides, respectively) to ensure specificity for the two genotypes. Furthermore, neither of the genotype-specific probes cross-targets the consensus sequences of other genotypes.

**Specificity and efficiency of the GQ-PCR assay.** To determine the sensitivity of the quantitative PCR assay, a 10-fold dilution series (9.0 to 1.0 $\log_{10}$ genomic copies) of the clones of HBV genotypes B and C were used to perform the GQ-PCR assay. The primer pairs and probes tested had similar sensitivities and efficiencies. All samples with a minimum of 100 copies/μl of the genotype B or C plasmid could be constantly detected (Table 2).

TABLE 2. Identification of HBV genotype of HBV DNA with the standard dilutions by GQ-PCR

| Genotype | No. (%) of samples at HBV DNA concn (copies/ml) of[a]: | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1,000 | 500 | 150 | 100 | 50 | 10 | 1 |
| B | 20 (100.0) | 20 (100.0) | 20 (100.0) | 20 (100.0) | 16 (80.0) | 10 (41.7) | 7 (29.2) |
| C | 20 (100.0) | 20 (100.0) | 20 (100.0) | 20 (100.0) | 14 (70.0) | 9 (37.5) | 8 (33.3) |

[a] The total number of samples tested was 20 for all concentrations except for 10 and 1 copies/ml, for which the total number of samples tested was 24 each.

TABLE 3. HBV genotypes and their absolute quantities determined by GQ-PCR

| Sample[a] | Predicted genotype | Genotype determined by the GQ-PCR assay | DNA level (copies/ml) determined by the GQ-PCR assay | | | | Mean DNA level (copies/ml) | SD | CV[b] (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Run 1 | Run 2 | Run 3 | Run 4 | | | |
| 1 | B | B | $6.981 \times 10^7$ | $6.759 \times 10^7$ | $7.547 \times 10^7$ | $4.087 \times 10^7$ | $6.344 \times 10^7$ | $1.540 \times 10^7$ | 24.284 |
| 2 | C | C | $5.477 \times 10^5$ | $4.958 \times 10^5$ | $9.260 \times 10^5$ | $3.463 \times 10^5$ | $5.790 \times 10^5$ | $2.466 \times 10^5$ | 42.597 |
| 3 | B | B | $2.145 \times 10^5$ | $9.260 \times 10^5$ | $1.255 \times 10^5$ | $3.508 \times 10^5$ | $4.042 \times 10^5$ | $3.560 \times 10^5$ | 89.063 |
| 4 | B | B | $3.021 \times 10^8$ | $6.314 \times 10^8$ | $3.854 \times 10^8$ | $2.541 \times 10^8$ | $3.933 \times 10^8$ | $1.678 \times 10^8$ | 42.664 |
| 5 | C | C | $2.186 \times 10^6$ | $1.193 \times 10^6$ | $9.548 \times 10^5$ | $1.113 \times 10^6$ | $1.362 \times 10^6$ | $0.558 \times 10^6$ | 41.006 |
| 6 | B | B | $1.594 \times 10^9$ | $1.667 \times 10^9$ | $1.906 \times 10^9$ | $1.803 \times 10^9$ | $1.743 \times 10^9$ | $0.139 \times 10^9$ | 7.990 |
| 7 | B | B | $8.282 \times 10^4$ | $6.179 \times 10^4$ | $6.314 \times 10^4$ | $7.954 \times 10^4$ | $7.182 \times 10^4$ | $1.090 \times 10^4$ | 15.179 |
| 8 | B | B | $1.561 \times 10^7$ | $1.479 \times 10^7$ | $1.673 \times 10^7$ | $1.676 \times 10^7$ | $1.597 \times 10^7$ | $0.095 \times 10^7$ | 5.965 |
| 9 | C | C | $2.519 \times 10^6$ | $2.558 \times 10^6$ | $3.580 \times 10^6$ | $4.512 \times 10^6$ | $3.292 \times 10^6$ | $0.950 \times 10^6$ | 28.856 |
| 10 | B | B | $2.222 \times 10^7$ | $1.914 \times 10^7$ | $2.530 \times 10^7$ | $2.720 \times 10^7$ | $2.347 \times 10^7$ | $0.354 \times 10^7$ | 15.082 |
| CS with $6 \times 10^9$ copies/ml | B | B | $4.636 \times 10^9$ | $6.294 \times 10^9$ | $4.728 \times 10^9$ | $5.107 \times 10^9$ | $5.191 \times 10^9$ | $0.763 \times 10^9$ | 14.696 |
| CS with $3 \times 10^9$ copies/ml | C | C | $2.595 \times 10^9$ | $2.069 \times 10^9$ | $2.651 \times 10^9$ | $2.844 \times 10^9$ | $2.540 \times 10^9$ | $0.331 \times 10^9$ | 13.051 |
| CS with $6 \times 10^7$ copies/ml for genotype B | B and C | B and C | $7.362 \times 10^7$ | $7.792 \times 10^7$ | $6.921 \times 10^7$ | $6.660 \times 10^7$ | $7.184 \times 10^7$ | $0.498 \times 10^7$ | 6.937 |
| CS with $3 \times 10^7$ copies/ml for genotype C | B and C | B and C | $1.351 \times 10^7$ | $1.287 \times 10^7$ | $2.005 \times 10^7$ | $2.290 \times 10^7$ | $1.733 \times 10^7$ | $0.493 \times 10^7$ | 28.442 |

[a] CS, control sample of the known concentration.
[b] CV, coefficient of variation.

Next, the genotyping capacity of the GQ-PCR assay to differentiate between HBV genotypes B and C was examined using a 10-fold dilution series ($1 \times 10^2$ to $1 \times 10^{13}$ copies/ml) of the HBV plasmid DNA samples of known genotype B or C. As shown in Table 3 (the last three samples), both genotypes B and C were identified correctly and no cross-reactions were observed. We examined plasmids of other HBV genotypes, i.e., A, D, E, F, G, and H, and sera of HCV- and HIV-infected patents, and no fluorescent signals were detected. Assessment of the precision of the assay was performed four times with the real-time PCR, which was run on six different days for the two genotypes, and coefficients of variation for genotypes B and C were 0.577% and 0.859%, respectively. Taken together, these results demonstrate that this novel GQ-PCR assay is able to distinguish between HBV genotypes B and C.

**Simultaneous genotyping and quantification by the GQ-PCR assay.** The GQ-PCR assay was designed for simultaneous quantification and genotyping of HBV in a single-tube reaction. To determine the quantification capacity of our GQ-PCR assay, HBV DNA samples of known genotype and viral load were used for the validation (Table 3). To ensure the accuracy of the experiment, every reaction plate in each experiment contained HBV DNA standard dilutions for generating two standard curves and three to eight no-template controls. All sample determinations were done four times (Table 3) to calculate the mean quantity and standard deviation. As shown in Table 3, the results of the quantification by the GQ-PCR assay were almost in the same order of magnitude as the predicted concentration. The samples numbered 1 to 10 in Table 3 were the genotyped clinical specimens. These results demonstrate that the GQ-PCR assay is efficient and reliable for simultaneous genotyping and quantification of HBV genotypes B and C.

**Concurrent HBV genotyping and quantification of clinical samples.** To evaluate the accuracy of GQ-PCR for clinical use, we performed parallel amplification and quantification of 127 samples from unknown HBV-infected patients by our assay and a commercial assay (HBV-DNA FQ-PCR kit; Da An Gene Co., Sun Yat-Sen University, China). The results of the quantification were of the same order of magnitude for all samples tested with only a few discrepancies, and the statistical correlation of the two assays was significant ($r = 0.700$, $P = 0.003$). HBV DNA was not detected in any of the serum samples obtained from healthy individuals, again confirming the high specificity of this novel assay.

Next, the 127 clinical samples were analyzed and their genotypes were determined by the GQ-PCR assay. Genotyping of the 127 patient samples with the GQ-PCR assay revealed 56.7% (72 samples) of HBV as genotype B, 13.4% (17 samples) as genotype C, and 29.9% (38 samples) as mixed genotypes B and C (Table 4). To confirm the reliability of our genotyping, we amplified and sequenced the RT region of HBV DNA from all 127 samples. After genotyping with NCBI genotyping tools (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi), we

TABLE 4. Comparison of HBV genotypes determined by GQ-PCR and direct sequencing

| Genotype(s) determined by GQ-PCR | No. of samples genotyped by direct sequencing | | | |
|---|---|---|---|---|
| | B | C | B and C | Neg[a] |
| B | 72 | | | |
| C | | 17 | | |
| B and C | 15 | 2 | 21 | |
| Neg | | | | 3 |

[a] Neg, negative.

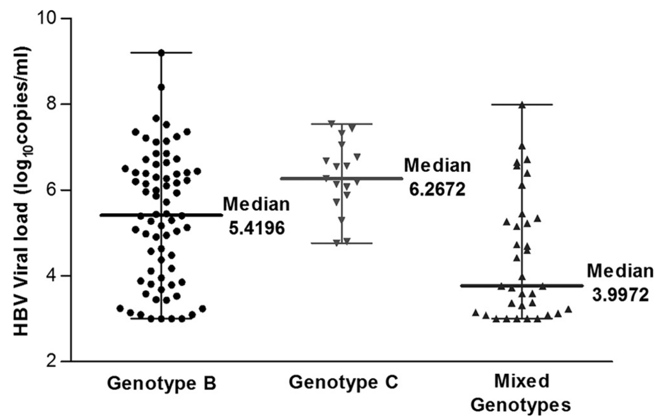


FIG. 2. HBV viral loads in single-genotype infections (genotype B or genotype C) and mixed-genotype infections in 127 plasma samples. Horizontal bars indicate the medians for the two groups. The differences between the single-genotype group and the mixed-genotype group, and between genotypes B and C, were statistically significant ($P < 0.05$). The median HBV viral load in the single-genotype group was higher than that in the mixed-genotype group, and that in the genotype C group was higher than that in the genotype B group.

identified genotype B in 87 samples, genotype C in 19 samples, and mixed genotypes B and C (double summits) in 21 samples (Table 4). The genotyping concordance between GQ-PCR and direct sequencing was 86.6% for the 127 samples. The discrepancies may be due to the inability of direct sequencing to detect mixed populations representing less than 20% of the total quasispecies pool. Mixed infections are very difficult to identify by sequencing. In fact, our GQ-PCR assay identified more cases of HBV infections with mixed genotypes than direct sequencing did. Of 127 samples, 17 (13.4%) single-genotype samples determined by direct sequencing were identified as mixed-genotype samples by the GQ-PCR assay. A comparison of the genotyping results of the GQ-PCR assay and direct sequencing is shown in Table 4.

The medians ± standard deviations of HBV viral load as measured by the GQ-PCR assay (expressed as $\log_{10}$ copies per milliliter of serum) in the 127 specimens were 5.4196 ± 2.54 and 6.2672 ± 1.11 $\log_{10}$ copies/ml for the infection with single genotypes B and C, respectively, and 3.9952 ± 2.08 $\log_{10}$ copies/ml for the infection with mixed genotypes. These data demonstrate that the viral load was higher in patients with a single-genotype infection than that in patients with mixed-genotype infections ($P < 0.05$) and was higher with genotype C infection than with genotype B ($P < 0.05$) (Fig. 2).

Final verification was done by a phylogenetic analysis. A phylogenetic tree was constructed with the sequences from the amplicons and standard genotype B and C strains (GenBank accession numbers AF100309 and AB014381). All of our sequences from the 127 amplicons were accepted by GenBank under GenBank accession numbers GU202958 to GU203084. In the phylogenetic tree, we used our numbers instead of the GenBank accession numbers. The tree revealed that the amplicons and strains of the expected genotype were grouped together in a well-supported distinct cluster (Fig. 3).

## DISCUSSION

Several studies indicate that HBV genotypes may affect the disease profiles and clinical outcome in chronic HBV infection. Here we developed a rapid and sensitive method for concurrent quantification and identification of HBV genotypes B and C in a single-step reaction. Genotypes B and C are the two most common HBV genotypes in China, accounting for approximately 95% of HBV-infected patients (29). Current data demonstrate that HBV genotype C takes a more aggressive disease course and has a more aggressive clinical phenotype than genotype B (2, 3, 4, 7, 9, 11, 14, 22, 34). A recent study by Chan et al. (4) indicated that genotype C HBV was associated with more severe liver fibrosis than genotype B HBV, probably because of delayed HBeAg seroconversion and prolonged active disease. The findings of Choi et al. (7) suggested that pre-S mutations, especially pre-S2 deletions and pre-S2 start codon mutations, were common in patients with genotype C HBV infection and were associated with advanced liver disease and active viral replication. A longitudinal study done by Chu and Liaw (9) showed that genotype C hepatitis B virus infection is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than that with genotype B HBV infection. The findings of Chan et al. (3) suggested that genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. HBV genotype C is also associated with a lower response rate to antiviral therapy (10, 15, 19, 27, 28, 29, 35). Wai et al. (27) showed that HBV genotype B is associated with a higher rate of interferon (IFN)-induced HBeAg clearance than HBV genotype C. Our previous findings (35) showed that genotype B had a better virological response to adefovir dipivoxil (ADV) therapy than genotype C did. Wang et al. (28) found that there was a reduction in IFN-α production in patients with genotype C infections, compared to that in patients with genotype B infections, and thus may correlate with the outcome of antiviral treatment in chronic hepatitis B patients and the progression of liver inflammation. In addition, patients with simultaneous infections with different genotypes are more frequently associated with higher HBV DNA levels, resistance to antiviral treatment, and severe progression of liver disease (4, 5, 12, 13, 26, 33). Therefore, in the Asia-Pacific region, the detection and discrimination of HBV genotypes B and C are very important to disease prognosis and clinical treatment.

The aims of this study were to develop a method for simultaneous genotyping and quantification of the two most prevalent HBV genotypes in the Asia-Pacific region and to improve the detection rate of mixed genotypes. Although many assays were developed for the quantification of HBV DNA and used in the laboratory diagnosis, when genotyping is requested, additional assays, such as specific PCRs, restriction fragment length polymorphism analysis (RFLP), and DNA sequencing, must be applied. In recent years, with the development of the real-time PCR, several methods have been developed for HBV quantification and genotyping in a single reaction by real-time PCR with additional melting-curve analyses (20, 23, 32). In those assays, the first step involves the use of the real-time PCR for quantification and the second step involves melting-curve analyses for differentiating between HBV genotypes B and C. One of the limitations of those assays is that they can

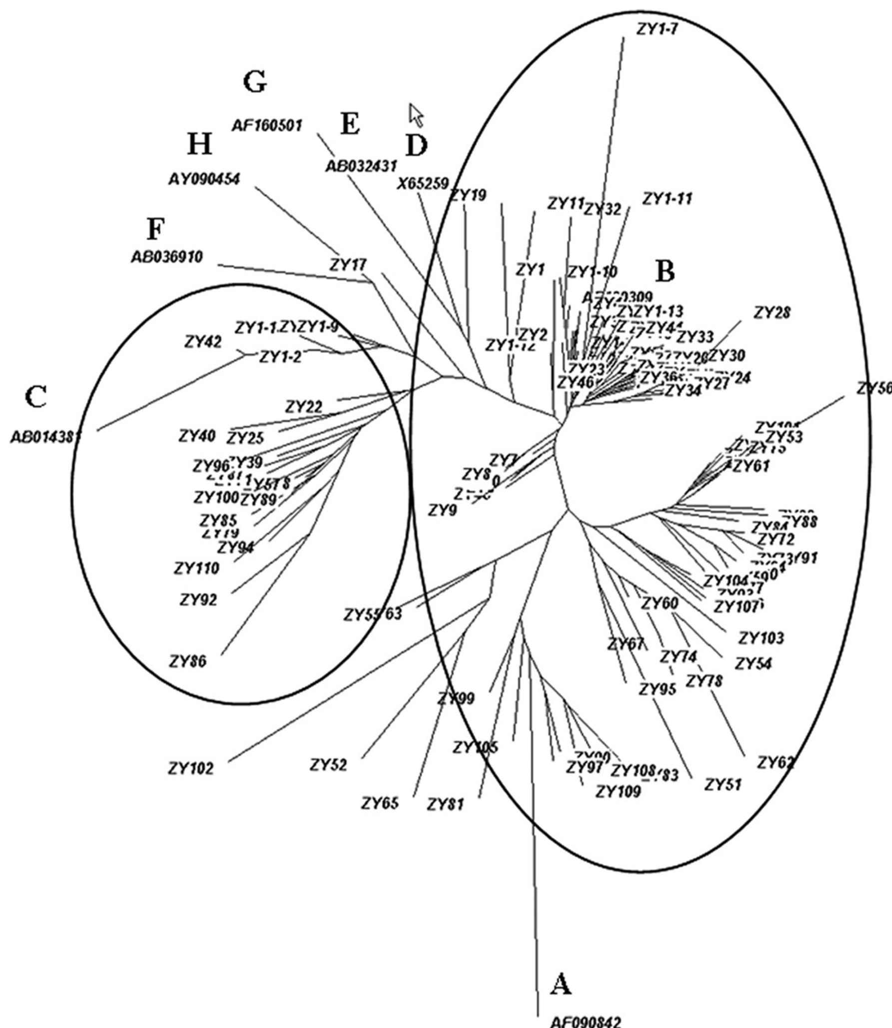


FIG. 3. Phylogenetic analysis of the RT region of HBV genomic DNA identified by the GQ-PCR assay. AF090842, AF100309, AB014381, X65259, AB032431, AB036910, AF160501, and AY090454 represent the standard strains for genotypes A through H, respectively. The neighbor-joining method was used for the phylogenetic analysis of 127 amplicons (GenBank accession numbers GU202958 to GU203084) and 8 representative sequences. Bootstrap analysis with 1,000 replicates was used to determine the robustness of the tree and the evolutionary relationship of HBV.

detect only the copy number of the total HBV DNA. Thus, they cannot be used to determine the precise quantity of each HBV genotype in the samples coinfected with genotypes B and C. Tanic and his colleagues (25) have developed a multiplex real-time PCR for concurrent quantification and genotyping of the HBV A and D genotypes. Our method also utilizes the advantages of multiplex real-time PCR, which can take advantage of the different primers and probes for quantification and genotyping at the same time. However, the assay of Tanic et al. can be used only to quantify and genotype DNA samples for genotypes A and D. Recently, a new genotyping method was developed on the basis of the TaqMan real-time PCR, which could identify all HBV genotypes (21). Using this method, each sample is processed in four multiplex real-time PCRs, each targeting two or three genotype-specific segments of HBV. However, when the need for simultaneous detection of genotypes B and C arose, more than one PCR was carried out. Moreover, our method could improve the detection rate of the mixed genotypes B and C. Therefore, the method described here allows, for the first time, simultaneous genotyping and quantification of HBV genotypes B and C for Asian populations in which genotypes B and C are predominant.

It has been reported that patients with simultaneous infections with different genotypes are more frequently associated with higher HBV DNA levels (4, 5, 12, 13, 26, 33). In our study, infection with mixed HBV genotypes was found in 29.9% (38 of 127) of patients, as detected by GQ-PCR. However, our data indicated that the viral load was higher in patients with a single-genotype infection than in those with mixed-genotype infections (Fig. 2). Although our results seem somewhat contradictory to previous reports, they are consistent with those of Toan et al. (26), who found that the HBV DNA level in patients infected with a single genotype was higher than that of patients infected with a mixture of genotypes in the chronic hepatitis B group. In fact, most of our patients were infected with chronic hepatitis B (data not shown). We also found that

patients with genotype C infections had a higher viral load than those infected with genotype B.

The accuracy of this new method for quantification was compared with that of a commercial assay with good statistical correlation. There were some discrepancies between HBV genotyping obtained by the GQ-PCR assay and that obtained by direct sequencing (Table 4). Through the screening of 127 clinical samples, we found that 17 (13.4%) single-genotype samples detected by direct sequencing were mixed genotypes using the GQ-PCR assay. The accuracy of this new method for genotyping was compared with direct sequencing and a genotype-specific PCR assay. Because coinfection of genotypes B and C was found in approximately 30% of the patients, but only half of them could be confirmed by sequencing, we used a genotype-specific PCR assay to verify the genotyping results. Coinfection with genotypes B and C was found in 18.9% (24 of 127) of the patients, which was a higher percentage than that found by direct sequencing but lower than that found by our method (other data not shown). These differences could be explained by the fact that the quantitative real-time PCR assay is highly sensitive and precise, whereas the mixed-genotype infection is very difficult to detect by direct sequencing. Since direct sequencing or Sanger sequencing can pick up mixed populations only at ratios above 20:80 and probably works only by reading maps to determine whether there are mixed genotypes, when the ratio of the mixed populations is lower, fewer of the mixed genotypes will be revealed. Moreover, the genotype-specific PCR assay had higher sensitivity than direct sequencing. Comparing the viral loads of 17 mixed-genotype HBV infections detected by our assay and direct sequencing, we found that the viral load of genotype B was higher than that of genotype C except for one specimen. Chen et al. (6) compared direct sequencing and clonal analysis and found that the genotype determined by direct sequencing is equivalent to the dominant genotype of clonal analyses, probably due to its presence being masked by a predominant strain. Therefore, the use of an absolute quantitative real-time PCR assay to detect mixed infections of different genotypes offers an unparalleled advantage, and it would be useful to know what the relative concentrations are for genotypes B and C in the mixed infections. The new method we developed in this study provides an efficient way for detecting mixed genotypes.

In conclusion, very good precision, sensitivity, and specificity of HBV genotyping and viral load quantification were achieved with our GQ-PCR assay. This assay provides a reliable, efficient, and cost-effective method for precise differentiation and quantification of genotypes B and C in single or mixed infections.

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