Comparison of the Novel Real Time PCR Assay with Sequence Analysis, Reverse Hybridization and Multiplex PCR for Hepatitis B Virus B and C Genotyping

Yao Zhao,1,2† Xiuyu Zhang,2,5† Yuan Hu,2 Wen-Lu Zhang,2 Jie-Li Hu,2 Ai-Zhong Zeng,3 Jin-Jun Guo,4 Wen-Xiang Huang,3 Wei-Xian Chen5, You-Lan Shan,4 and Ai-Long Huang2* 

Pediatric Research Institute, Ministry of Education Key Laboratory of Child Development and Disorders, Key Laboratory of Pediatrics in Chongqing, Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, Children’s Hospital of Chongqing Medical University, Chongqing, China1; 
Institute for Viral Hepatitis, Key Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University, Chongqing, China2; 
Department of Infectious Diseases, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China3; 
Department of Gastroenterology and Hepatology, the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China4; 
Department of Clinical Laboratory, the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China5

†Y. Zhao and X.-Y. Zhang contributed equally to this work and are considered co-first authors.

Corresponding author:
Prof. Ai-Long Huang
Institute for Viral Hepatitis
Key Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education
Chongqing Medical University
Chongqing 400014, P.R. China
Tel: +86-23-6848-6135
Fax: +86-23-6848-5000
E-Mail: ahuang1964@yahoo.com.cn
ABSTRACT

By using a novel real time genotyping and quantitative PCR (GQ-PCR) assay, we compared with direct sequence analysis, reverse hybridization, and the multiplex PCR by detecting 127 HBV infected patients. We found that GQ-PCR had the highest concordance with sequence analysis and the highest detection rate for mixed genotype detecting.
Hepatitis B virus infection is highly prevalent worldwide, affecting more than 2 billion people. Approximately 400 million people are chronic carriers (7). Chronic HBV infection is associated with 1 million deaths annually and is a major risk factor of liver cirrhosis and hepatocellular carcinoma (10). The high HBV infection rate represents a considerable public health issue.

HBV has been classified into nine genotypes (A-I) based on >8% divergence in the entire HBV genome sequence or >4% in the S gene (5, 6). The distribution of HBV genotypes shows geographical variability. In China for example, genotypes B and C predominate and genotypes A and D are rarely detected (15).

At present, several HBV genotyping methods are in widespread use, such as direct sequencing, restriction fragment length polymorphism, reverse hybridization assay and enzyme-linked immunosorbent assay (3). Direct sequencing and reverse hybridization (INNO-LiPA HBV Genotyping) assays are accepted generally, but are complex to perform and also costly. Our laboratory developed a real time PCR assay for simultaneous genotyping and quantitation of HBV genotype B and C (14). In the present study, we evaluated the real time genotyping and quantitative PCR (GQ-PCR) method by determining the genotypes of 127 HBV infected patients. The genotype profiles were compared with results obtained by direct sequence analysis, multiplex PCR method and the HBV INNO-LiPA genotyping kit.
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 anti-HBe positive after serological testing. Informed written consent was obtained from all patients. Viral DNA was extracted from 200 µl of serum using the QIAamp MinElute Virus Spin Kit (QIAGEN GmbH, Germany) according to manufacturer’s instructions. Precipitated DNA was dissolved in 100 µl of elution buffer and stored frozen at -20°C until use. The samples were analyzed by a method developed previously in our laboratory (14), using iQ™ Mutilplex Powermix (Bio-Rad Laboratories, Hercules, USA). The amplification step was performed using CFX Manager™ Software version 1.6 (Bio-Rad Laboratories). A portion of the S gene was amplified with the primers PS1: 5’TCTAGACTCGTGGTGGACT-3’ and PS2: 5’-GATGATGGGATGGGAATACA-3’. PCR reaction in 50 µl volume was performed at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds with the final step at 72°C for 10 minutes. PCR products were handled by ABI PRISM Big-dye kit (Applied Biosystems, Foster City, CA), separated by ABI 3100 Genetics Analyzer (Applied Biosystems, Foster City, CA), and finally analyzed by Sequence Navigator software sequencer (ABI, version 1.01). Sequences of these 127 samples were genotyped using NCBI genotyping tools (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). An in vitro reverse hybridization assay INNO-LiPA (Innogenetics, Gent, Belgium) was performed according to the manufacturer’s operating manual. HBV DNA was amplified by nested PCR, the amplicons were hybridized to genotype-specific probes impregnated on membrane strips and the hybrids were detected with chromogenic substrates. The multiplex PCR for HBV genotyping was carried out as described by Chen et al. (4). Because HBV genotypes E, F, G and H are not found in China, we added only the specific primer pairs for HBV genotypes B and C into the PCR reaction. Other
reaction conditions were not changed. For the samples where genotyping results of two methods were discordant, four representative samples were selected for TA cloning. The PCR purified products were cloned using the pMD18-T Vector Kit (TaKaRa, DaLian, China). Twenty to forty positive colonies from each sample were picked, and then sequenced and genotyped as the S gene sequence analysis data became available. The agreement between the real time genotyping and quantitative PCR and the other three methods was analyzed by Cohen’s Kappa test. Chi-square test was used to compare the variance of the results. P values of less than 0.05 were considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Sciences software version 16.0 (IBM Company, Chicago, USA).
All of the 127 samples were genotyped successfully by GQ-PCR, reverse hybridization, multiplex PCR and direct sequence analysis (Table 1). As assessed by these four methods, genotype B was the major genotype. The reverse hybridization assay did not detect the genotype D alone, but seven individuals co-infected with genotype B, one with C and two with mixed genotypes (B and C) were detected. Since GQ-PCR has been designed to detect genotypes B and C, it cannot detect genotype D. The phylogenetic tree construction showed that the genotype agreed well with the sequence analysis, and the amplicons of the expected genotypes grouped together in a distinct cluster (14). All samples demonstrated very good agreement with the GQ-PCR method based on direct sequence analysis (Kappa = 0.768, P = < 0.001). All samples detected by the GQ-PCR method as a single genotype B were also classified as genotype B by sequence analysis. Likewise, good agreement was observed for the single genotype C sample. Discrepancies were mainly due to differences in the ability of the two methods to detect mixed genotypes. GQ-PCR assay had the highest detection rate for mixed genotype identification and was 1.73 (38/22) times higher than the sequence analysis method. The genotyping concordance between the GQ-PCR and the reverse hybridization assays was also good (Kappa = 0.733, P = <0.001). 22 and 38 samples were detected as mixed genotype B and C by the reverse hybridization assay and GQ-PCR, respectively. The agreement between the reverse hybridization assay and the sequence analysis was lower than the GQ-PCR and sequence analysis also since the Kappa value was 0.704 (P = <0.001) and 0.768 (P = <0.001). Between the GQ-PCR method and the multiplex PCR method, the Kappa value is 0.645 (P = <0.001). The multiplex PCR method could not identify the genotypes of six samples, because the viral loads of those samples were below 10^4 copies/ml. 14 single-genotype samples determined by the multiplex PCR method were identified as mixed-genotype samples by the GQ-PCR assay. From the 16 samples for which genotyping results were discordant when assessed
by sequence analysis and the GQ-PCR method, four samples were selected at random for TA cloning. The same region as the S gene sequence analysis was amplified. 4 samples with a single genotype infection as detected by sequence analysis were all identified as mixed genotype infections by clonal analysis, and these results were consistent with GQ-PCR (Table 2). Our data therefore demonstrate that GQ-PCR is suitable for detecting mixed genotype samples.
A variety of investigations demonstrate that the HBV genotype may influence the severity and prognosis of disease. Several methods have now been developed for HBV genotyping (8, 11, 13). Direct sequence analysis is used as the gold standard of HBV genotyping, but it is considered to be technically challenging, time consuming and costly. Furthermore, sequence analysis is not suitable for detecting HBV infections of mixed genotypes (12). Although the INNO-LiPA HBV genotyping assay is a sensitive method that detects mixed genotypes, it is too expensive to be used for clinical applications and large-scale epidemiological investigations, especially in developing countries like China. The low-cost multiplex-PCR method and the PCR-RFLP-based method for HBV genotyping is reported to be superior for the detection of double infections and can be performed rapidly, but we cannot ignore the possibility that a single nucleotide exchange may make this form of analysis unreliable (1, 3, 4). This is because it not only increases false negatives, but also reduces the accuracy of the genotyping results. There are also several methods for HBV quantification and genotyping using a single-step PCR reaction, but these assays rely on the identification of HBV genotype based on melting-curve analysis (2, 9). Our laboratory has developed a real time genotyping and quantitative PCR (GQ-PCR) procedure for simultaneously genotyping and quantitating HBV for the individual B, C and mixed genotypes. Compared to previously published methods, the GQ-PCR is more convenient in operation, more efficient, faster, and cheaper owing to the advantages of real-time fluorescence quantitative PCR.

In this paper, we compared our GQ-PCR method with direct sequence analysis, reverse hybridization assay and the multiplex PCR method to evaluate the accuracy and sensitivity of the novel GQ-PCR assay. In conclusion, the GQ-PCR is a reliable, efficient, convenient and rapid method for HBV genotyping, that is especially sensitive for detecting mixed genotypes. This method will be more suitable for rapid clinical diagnosis and large-scale epidemiological
investigations compared to sequence analysis, multiplex PCR and reverse hybridization. It offers robust methodological support for research into the influence of HBV genotype on liver disease progression, selection of mutants and response to antiviral therapy.

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REFERENCES


Table 1. HBV genotyping result determined by GQ-PCR, multiplex-PCR, reverse hybridization assay and direct sequence analysis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Direct sequence analysis</th>
<th>GQ-PCR</th>
<th>Reverse Hybridization</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>86 (67.72%)</td>
<td>72 (56.69%)</td>
<td>75 (59.06%)</td>
<td>69 (54.33%)</td>
</tr>
<tr>
<td>C</td>
<td>19 (14.96%)</td>
<td>17 (13.39%)</td>
<td>20 (15.87%)</td>
<td>28 (22.05%)</td>
</tr>
<tr>
<td>B &amp; C</td>
<td>22 (17.32%)</td>
<td>38 (29.92%)</td>
<td>22(17.32%)</td>
<td>24 (18.90%)</td>
</tr>
<tr>
<td>B &amp; D</td>
<td>0</td>
<td>—</td>
<td>7(5.51%)</td>
<td>—</td>
</tr>
<tr>
<td>C &amp; D</td>
<td>0</td>
<td>—</td>
<td>1(0.79%)</td>
<td>—</td>
</tr>
<tr>
<td>B &amp; C &amp; D</td>
<td>0</td>
<td>—</td>
<td>2(1.57%)</td>
<td>—</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6(4.7%) a</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>127</td>
<td>127</td>
<td>127</td>
</tr>
</tbody>
</table>

* Multiplex PCR assay produced six negative result. Those samples were 3.13±0.18 log10 copies/ml; we were unable to amplify the viral DNA with the conventional PCR assay.

Table 2. Clones of HBV genotype propagated from four HBV/B and HBV/C co-infected patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sequence analysis</th>
<th>GQ-PCR</th>
<th>Reverse Hybridization</th>
<th>Multiplex PCR</th>
<th>Clonal analysis</th>
</tr>
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<tbody>
<tr>
<td>58</td>
<td>B</td>
<td>BC</td>
<td>B</td>
<td>B</td>
<td>B/C (24/1)</td>
</tr>
<tr>
<td>66</td>
<td>B</td>
<td>BC</td>
<td>BC</td>
<td>BC</td>
<td>B/C (37/3)</td>
</tr>
<tr>
<td>79</td>
<td>C</td>
<td>BC</td>
<td>BC</td>
<td>C</td>
<td>B/C (2/18)</td>
</tr>
<tr>
<td>86</td>
<td>C</td>
<td>BC</td>
<td>C</td>
<td>C</td>
<td>B/C (2/37)</td>
</tr>
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