

New universal primers for genotyping and resistance detection of low HBV DNA levels

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Abstract

HBV (hepatitis B virus) genotyping is important in determining the clinical manifestation of disease and treatment response, particularly, in patients with low viral loads. Also, sensitive detection of HBV antiviral drug resistance mutations is essential for monitoring therapy response.

A sensitive direct sequencing method for genotyping and the drug resistance mutation detection of low levels of HBV DNA in patients' plasma is developed by PCR amplification of the DNA with novel universal primers.

The novel, common, and universal primers were identified by alignment of RT region of all the HBV DNA sequences in databases. These primers could efficiently amplify the RT region of HBV virus at low DNA levels by directly sequencing the resulting PCR products, and mapping with the reference sequence made it possible to clearly obtain the HBV subtypes and identify the resistance mutations in the samples with HBV DNA level as low as 20 IU/mL. We examined the reliability of the method in clinical samples, and found it could detect the HBV subtypes and drug resistance mutations in 80 clinical HBV samples with low HBV DNA levels ranging from 20 to 200 IU/mL.

This method is a sensitive and reliable direct sequencing method for HBV genotyping and antiviral drug resistance mutation detection, and is helpful for efficiently monitoring the response to therapy in HBV patients.

Abbreviations: ETV = entecavir, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, IFN- α = interferon-alpha, LAM = lamivudine, LdT = telbivudine, NAs = nucleoside analogs, qPCR = quantitative real-time PCR.

Keywords: genotyping, HBV, low DNA, resistance mutation

1. Introduction

Hepatitis B virus (HBV) is one of the most serious and prevalent health problems affecting >2.4 billion people worldwide.^[1]

Editor: Ken Rosenthal.

YT, BL, and HL contributed equally to this work.

This work was financially supported by the Hubei Provincial Natural Science Foundation of China (2010CDB06903), National Natural Science Foundation of China (81000771), National Key Basic Research Program of China-973 Program (2012CB526706), and the National Natural Science Foundation of China (81271694).

All the authors in the manuscript have no conflict of interests to declare.

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Medicine (2016) 95:33(e4618)

Received: 6 July 2016 / Received in final form: 21 July 2016 / Accepted: 25 July 2016

<http://dx.doi.org/10.1097/MD.0000000000004618>

There are >350 million chronic HBV carriers, 75% of whom reside in the Asia-Pacific region, especially in China. People with hepatitis B are at an increased risk of developing hepatic decompensation, cirrhosis, and hepatocellular carcinoma (HCC). The patients with decompensated cirrhosis have a poor prognosis with a 14% to 35% probability of survival for 5 years.^[2,3] The estimated worldwide mortality caused by HBV infection is about 780,000 deaths a year.

Many drugs have been approved for the treatment of chronic hepatitis B. Antiviral therapy is an efficient way to prevent bad clinical outcomes of HBV infection. It has been shown to be effective in suppressing HBV replication, decreasing inflammation and fibrosis in the liver, and preventing progression of liver disease.^[4] Currently, there are 2 types of anti-HBV drugs: interferon-alpha (IFN- α) and nucleoside analogs (NAs),^[5,6] such as lamivudine (LAM),^[7-10] telbivudine (LdT),^[11] and entecavir (ETV).^[12] The clinical efficacy of the treatment depends on various factors, such as severity of liver disease, serum ALT levels, and serum HBV DNA levels. The AASLD and APASL guidelines recommend antiviral therapy for patients with compensated cirrhosis and serum HBV DNA level >2000 IU/mL regardless of ALT level. The EASL guideline recommends treatment of patients with any detectable level of serum HBV DNA.^[13] Low levels of serum HBV DNA occur in patients during drug treatment, which is considered to be one of the indicators of drug resistance in the HBV patients.^[14,15] The drug treatment not only reduces HBV loads to a low level, but also results in resistance mutations in the patients.^[14-16] Therefore, it is very important to develop a sensitive and clinically applicable method for genotyping and resistance detection of low HBV DNA in plasma of the patients.

Many quantitative real-time PCR (qPCR) methods have been developed to detect the HBV DNA concentration <200 IU/mL, even at 20 IU/mL, such as COBAS TaqMan HBV Test, v2.0

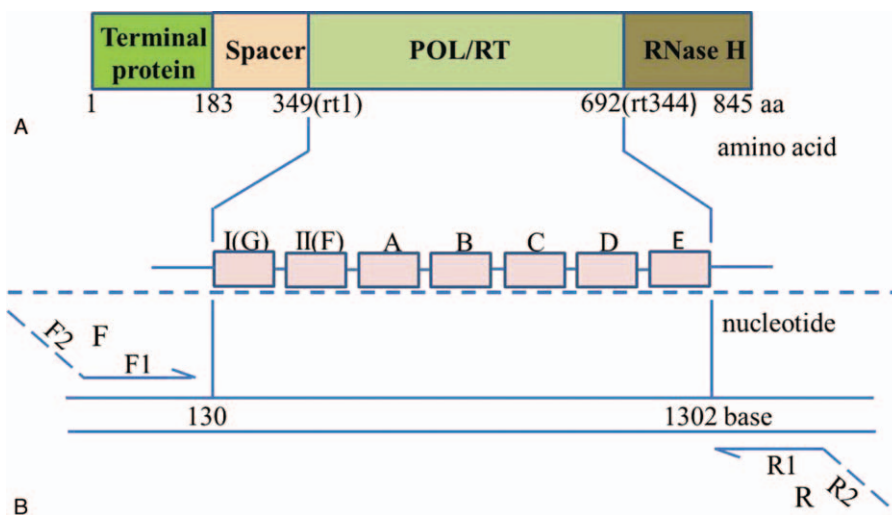


Figure 1. Primers' location for polymerase chain reaction amplification of the full-length of hepatitis B virus DNA POL/RT. (A) Schematic structure of the RT region of polymerase, which including 7 domain regions. (B) Location of the designed primers. The nucleotide sequence for 130 to 1302 base pair of the RT region is extracted from the NCBI Reference Sequence (Accession No. NC_003977).

(Roche Molecular Systems Inc.). However, the molecular methods for the analysis of the HBV subtype or resistance are currently based on common PCR, the sensitivity of which is only ≥ 200 IU/mL of HBV viral load.^[14,15] The detection by common PCR method often fails to detect HBV viral load between 100 and 200 IU/mL, and the repeatability of the method is poor. Here, we report a direct sequencing methodology with identified new common and universal primers to examine subtyping and resistance detection in a low virus load (from 20 to 200 IU/mL).

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of Renmin Hospital, Wuhan University School of Medicine. A written informed consent was obtained from each participant in accordance with the Ethics Committee of the Renmin Hospital of the Wuhan University.

2.2. Patients and plasma preparations

A total of 90 samples of 3 to 5 mL EDTA-anticoagulated peripheral blood were obtained from individuals for quantitative real-time PCR (qPCR) with COBAS TaqMan HBV Test kit (Roche, Swiss). HBV infection was confirmed in the Department of Infectious Diseases, Renmin Hospital of Wuhan University, and patients were treated with NAs, including lamivudine, adefovir, entecavir, tenofovir, or/and famciclovir, for >1 year. The HBV DNA titers for each patient were determined by qPCR as reported previously^[16] (data not shown). The HBV DNA titers of 80 of 90 patients were from 20 to 200 IU/mL, and 10 of these patients were from 200 to 10^8 IU/mL in the peripheral blood. The patients' age ranged from 22 to 67 years (median, 42.9 years), and 43 patients had the history of interferon therapy for more than a half year. All samples were centrifuged for 5 minutes at 3000g, and the supernatants were collected and stored at -70°C .

2.3. HBV DNA extraction

DNA was extracted from plasma samples with the UltraSens Virus Kit (QIAamp, German, Cat No: 53706) using the designation buffers and reagents in the kit and following the manufacturer's manual. Briefly, 0.8 mL Buffer AC and 5.6 μL of carrier RNA solution were pipetted onto the top of 1 mL plasma. After the solutions were mixed and incubated at room temperature for 10 minutes, they were centrifuged to have the supernatant discarded. Next, 300 μL Buffer AR and 20 μL proteinase K were added and vortexed. Buffer AB (300 μL) was added, mixed thoroughly by vortex, and transferred to QIAamp spin column. The solutions were centrifuged once again and the tube containing the filtrate was discarded. The silica pellet was washed with 500 μL Buffer AW1 and 500 μL Buffer AW2, respectively. The nucleic acids were eluted in 30 μL Buffer AVE and stored at -70°C .

2.4. Primers design and PCR analysis

The PCR was designed to amplify the DNA fragment of the full-length sequences of RT (344 aa) (Fig. 1A), wherein the primer sequences were F1, 5'-CTGCTGGTGGCTCCAGTT-3'; R1, 5'-GCTAGGAGTTCCGCAGTATGG-3'; F2, 5'-TTCTTAGCGTATTGGAGCTC-3'; and R2, 5'-AAGTATCAGTATTGCGTTGTT-3', respectively. The F and R primer sequence was: 5'-TTCTTAGCGTATTGGAGCTC CTGCTGGTGGCTCCAGTT-3' (F=F2+F1) and 5'-AAGTATCAGTATTGCGTTGTTGCTAGGAGTTCCGCAGTATGG-3' (R=R2+R1), respectively (Fig. 1B). The PCR with the primer pair F1R1 reaction was done as follows: 94°C , 3 minutes; 94°C 30 seconds; 56°C , 50 seconds; 72°C , 50 seconds; for 35 cycles; 72°C 10 minutes and the amplification length was about 1230 bp. The PCR amplification with the primer pair FR reaction was done as follows: 94°C 3 minutes; 94°C 30 seconds, 64°C 90 seconds, 72°C 50 seconds, for 35 cycles; 72°C 10 minutes. The PCR amplification with the mixed primer pairs FR and F2R2 reaction was done as follows: 94°C 3 minutes; 94°C 30 seconds, 64°C 90 seconds, 72°C 50 seconds, 20 cycles; then 94°C 30 seconds, 56°C 30 seconds,

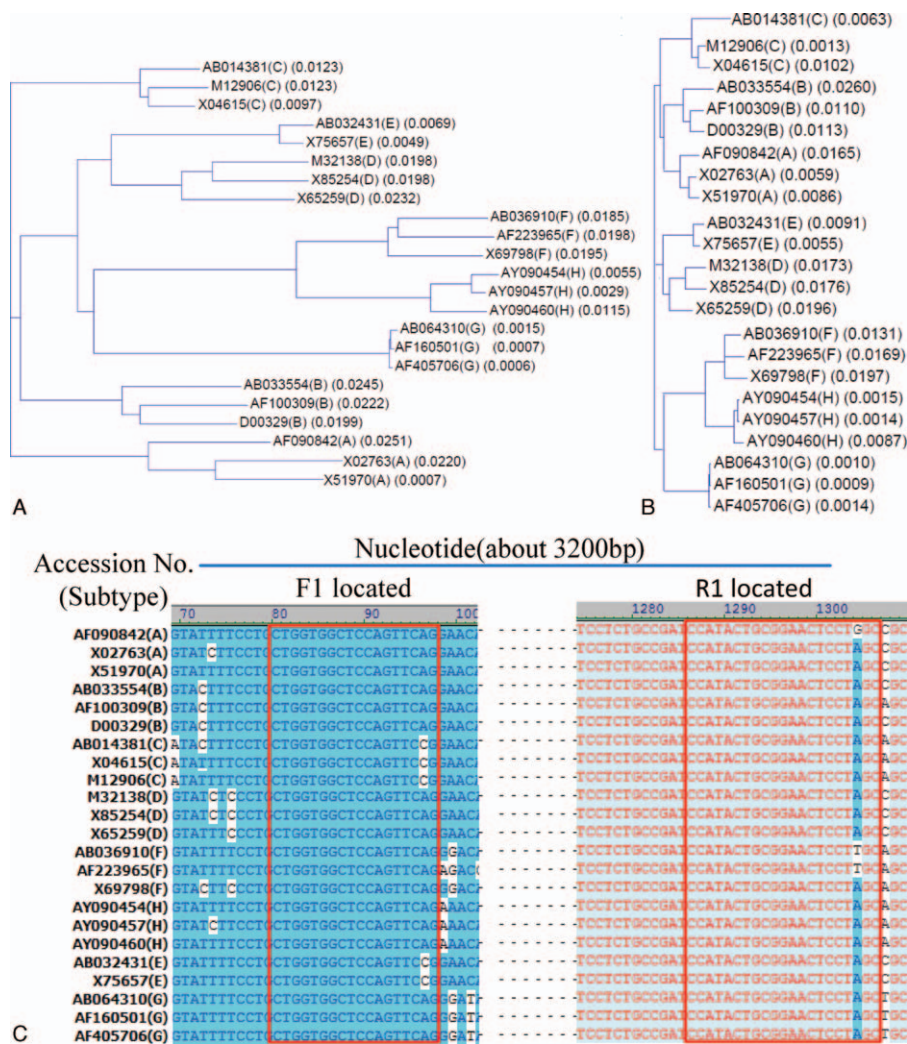


Figure 2. Primer screening by blast sequence comparison of the hepatitis B virus (HBV) DNA RT region in HBV genomic databases. (A) The result of phylogenetic analysis with 23 full-length HBV RT DNA sequences from HBV database of Stanford University. (B) The result of phylogenetic analysis with 23 full-length HBV genomic DNA sequences from NCBI database (<http://www.ncbi.nlm.nih.gov/projects/genotyping>). (C) The cluster result indicating the location of PCR primers in RT region of 23 HBV DNA sequences.

72°C 50 seconds, for 35 cycles; 72°C 10 minutes. The PCR was conducted with HotStar Taq Master Mix Kit (Qiagen, German) according to the recommendations of the manufacturer. The PCR products amplified with primer pairs were electrophoresed on a 1.2% agarose gel for gel purification, and stored at -70°C.

2.5. Sanger sequencing

The purified amplification was sequenced with an ABI PRISM BigDye 3.1 terminator cycle sequencing kit (Applied Biosystems). The upstream sequencing primer was the same as F1, and the downstream primer was the same as R1. The sequencing reaction mixture contained 2 µL of Terminator Ready Reaction Mix, 6 µL of 2.5 Sequencing Buffer, 3 µL of template, 8 µL of deionized water, and 1 µL of either of the 2 PCR primers, primers F1 and R1. The cycle sequencing profile was 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, followed by incubation at 4°C. The sequencing fragments were purified with 70% ethanol, 95% ethanol, and 3mol/L sodium acetate. Sequencing was performed on an ABI Prism 3130 Genetic Analyzer with ABI

Prism 3130 Collection and Sequencing Analysis software. The sequences generated by the forward and reverse sequencing primers were assembled and analyzed with the software program Sequencher 5.2.3 (Gene Codes Corporation, Ann Arbor, MI). The resulting complete sequences were translated into amino acid sequences to analyze the HBV DNA mutation.

2.6. HBV genotype analysis

The genotypes were analyzed using the HBV sequences available in the NCBI database (<http://www.ncbi.nlm.nih.gov/projects/genotyping>), which contains 23 HBV genomic DNA reference sequence for 8 HBV subtypes, including Subtype A (Accession No. X02763, X51970, AF090842), Subtype B (Accession No. D00329, AF100309, AB033554), Subtype C (Accession No. X04615, M12906, AB014381), Subtype D (Accession No. X65259, M32138, X85254), Subtype E (Accession No. X75657, AB032431), Subtype F (Accession No. X69798, AB036910, AF223965), Subtype G (Accession No. AF160501, AB064310, AF405706), and Subtype H (Accession No. AY090454, AY090457, AY090460).

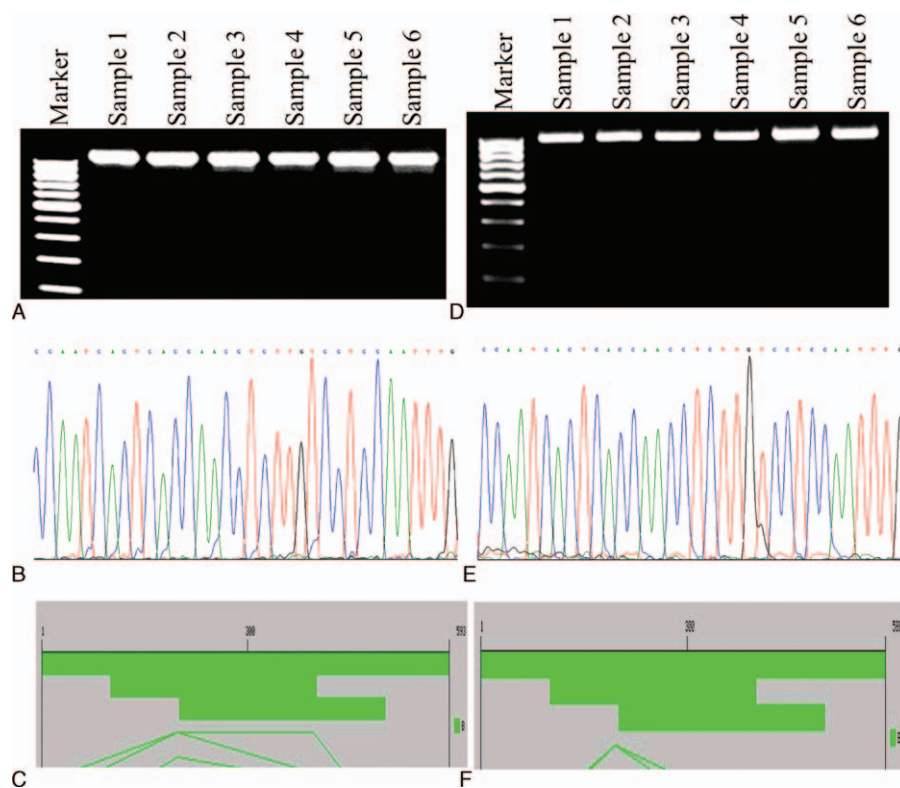


Figure 3. High specificity of the primers for amplification of the RT region of hepatitis B virus (HBV) DNA. (A) The bands of PCR products from the patients' plasma samples with Primer F1R1 by gel electrophoresis. (B) The result of Sanger sequence for the PCR products of F1R1 and sequenced with primer F1 or R1. (C) The HBV subtypes of the patients obtained by the blast sequence comparison of the HBV sequences (B) with the online HBV DNA database. (D) The bands of PCR products from same plasma samples with Primer FR by gel electrophoresis. (E) The result of Sanger sequence for the PCR products of FR and sequenced with primer F2 or R2. (F) The HBV subtypes of the patients obtained by blast sequence comparison of the HBV sequences (E) with the online HBV DNA database. ALL of the samples' HBV DNA copy ≥ 200 IU/mL.

2.7. Phylogenetic analysis

All nucleotide sequences from HBV strains were aligned using the ClustalW 2.0 software with a reference panel of reported 23 HBV genomic DNA sequences available in the HBV sequence database (<http://www.ncbi.nlm.nih.gov/projects/genotyping>) and full-length RT sequences provided by Stanford University (<http://hivdb.stanford.edu/HBV/releaseNotes/>), which contains 23 HBV DNA reference sequences for 8 HBV subtypes as above. Pairwise evolutionary distance matrices for the RT nucleotide sequences were computed using the p-distance algorithm of the MEGA software package (version 5.2, 2012; Pennsylvania State University, University Park, PA). Sequence distance matrices were analyzed with conventional statistical software (SYSTAT, v13.0; Systat Software, Inc., Point Richmond, CA) using files derived from Mega distance tables. For RT sequences, a p-distance of 0.12 was found to delineate strains of the same subtype and strains of a different subtype. Phylogenetic analysis was performed with the MEGA software package using the p-distance algorithm for distance determination and the neighbor-joining method for tree drawing. The reliability of phylogenetic classification was evaluated by a 1000-replication bootstrap test using the same software package.

3. Results

3.1. The common primers designed for amplification of almost all HBV subtypes

The RT region of HBV genomic DNA sequences was extracted from NCBI HBV database (<http://www.ncbi.nlm.nih.gov/proj>)

and HBV RT region database of Stanford University (<http://hivdb.stanford.edu/HBV/releaseNotes/>), respectively (Fig. 1). Phylogenetic analysis was used to identify the HBV subtypes. It could distinguish the subtype for each of the RT region sequence of the Stanford HBV database (Fig. 2A), which is similar to that in NCBI HBV genomic database (Fig. 2B). The data indicated RT region sequence could be used for clinical HBV genotyping. Therefore, we aligned the sequences of the RT region from all the HBV subtypes to design the primers, which could cover almost all HBV subtypes by Vector NT 11.0 software (Invitrogen, Carlsbad, CA). The identified common primers (F1 and R1) were shown in the red frame, which could be used to amplify the RT region of the 23 HBV DNA subtypes from the HBV DNA database (Fig. 2C).

We designed the primer F2 and R2, as a universal primer, which could not amplify any HBV DNA sequence, human genomic DNA sequence, or other nucleotide sequence. The F2 and R2 primers are further linked at the end of F1 and R1 to construct another universal primer F and R, respectively (Fig. 1B).

3.2. The specificity of the designed primers

The HBV DNA was extracted from 10 HBV patients' plasma with HBV DNA levels from 200 to 10^8 IU/mL. First, the 2 pairs of primers F1R1 were used to amplify the RT region in the 10 samples. The correct size of the PCR products with F1R1 primers was about 1200 bp length as shown in Figure 3A. The resulting

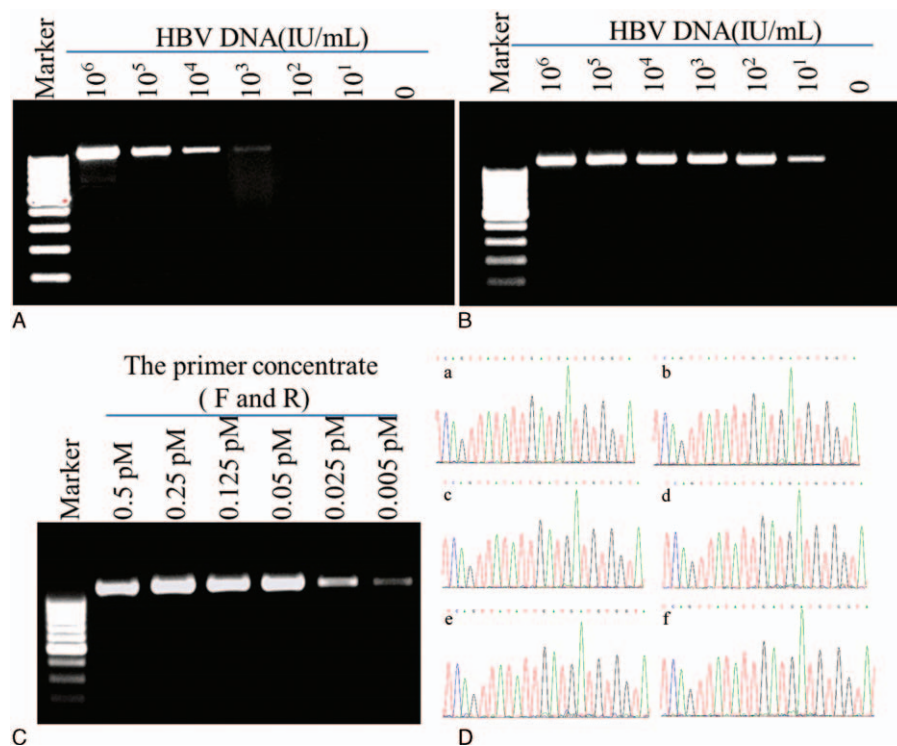


Figure 4. High sensitivity of the mixed primer to amplify the hepatitis B virus (HBV) DNA. (A) The bands of PCR products with Primer FR in the samples with different HBV DNA level: 0, 10 IU/mL, 20 IU/mL, 200 IU/mL, 10^3 IU/mL, 10^4 IU/mL, and 10^5 IU/mL. (B) The bands of PCR products with Primer FR and F2R2 in the samples with different HBV DNA level: 0, 10 IU/mL, 20 IU/mL, 200 IU/mL, 10^3 IU/mL, 10^4 IU/mL, and 10^5 IU/mL. (C) The bands of PCR products with the final concentration of primer FR ranged from 0.5 to 0.005 pmol/L. (D) The sequence of resulting PCR products in C, a to f indicated the sequence result from the PCR products amplified with different concentration of primer F and R ranged from 0.5 to 0.005 pmol/L, respectively.

gel-purified PCR products were sequenced with the primer F1 or R1. The representative sequence is shown in Figure 3B. The resulting sequence was used for the HBV subtyping by blast sequence comparison with the online NCBI HBV database, and the representative data are shown in Figure 3C. We used the pair of FR primers to amplify the RT region, and the correct size of the PCR products for this pair of primer was about 1300 bp as shown in Fig. 3D. The PCR products were also sequenced with the primer of F2 or R2 but not F1 or R1 as shown in Figure 3E; and the resulting sequence was blasted online to identify the subtypes as representatively shown in Figure 3F. These data showed that the 2 pairs of primers could efficiently amplify the RT region of HBV. Also, the HBV DNA sequence amplified by the pair of primer F1R1 (Fig. 3B) was consistent with that of primer FR (Fig. 3E), and the resulting HBV subtypes with the 2 pairs of primers (F1R1 and FR) were also identical (Fig. 3C and Fig. 3F).

3.3. Sensitivity of the identified primers for PCR amplification

To examine the sensitivity of the identified primers, HBV DNA was serially diluted from 10^5 to 10 IU/mL; and the primer pair was also serially diluted from 10 to 0.1 pmol/L. We tested the sensitivity of the PCR amplification for the pair of primer FR (FR only) and 2 pairs of primers FR plus F2R2 (FR+F2R2), respectively. The result showed that FR could only amplify the HBV DNA >200 IU/mL with the primer of final concentration of 0.5 pmol/L F and 0.5 pmol/L R (Fig. 4A). The FR + F2R2 primers could amplify the HBV DNA at 20 IU/mL with each of the primers (F, R, F2, and R2) of the final concentration no <0.5

pmol/L (Fig. 4B). With FR + F2R2 primers, HBV DNA could be detected as low as 20 IU/mL, even for the concentration of F or R primer diluted to 0.005 pmol/L with 0.5 pmol/L F2 and 0.5 pmol/L R2 (Fig. 4C). The PCR products on Fig. 4C were purified and sequenced, and all of the sequences from a to f were identical (Fig. 4D). These data indicated that the designed primers, particularly the mixed primer (FR + F2R2), could efficiently amplify the RT region of HBV DNA with an extremely high sensitivity.

3.4. Reliability of HBV subtyping and resistance mutation detection in clinical samples

The HBV DNA was extracted from 80 patients' plasma with low load HBV DNA infection. The RT region of HBV was PCR amplified with FR + F2R2 primers at the final concentration of 0.025 pmol/L for F and R and 0.5 pmol/L for F2 and R2. The primers successfully amplified the RT with the correct size in all the samples, and the representative 8 PCR products are shown on Figure 5A. After sequencing the purified PCR products for all the samples with low level of HBV DNA, the accurate subtype for each sample was identified by mapping to NCBI reference sequences through blast sequence comparison with the online HBV database. Figure 5B shows the representative mapped genotypes of B, C, D, and mix B/C. The HBV mutations in the samples were also analyzed by comparing their sequences with NCBI reference sequences. The common resistance mutations such as rtI169, rtV173, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtN236, and rtM250 were found in the samples; also some rare mutations, such as rtL80, rtV84, rtV214 and rtQ215,

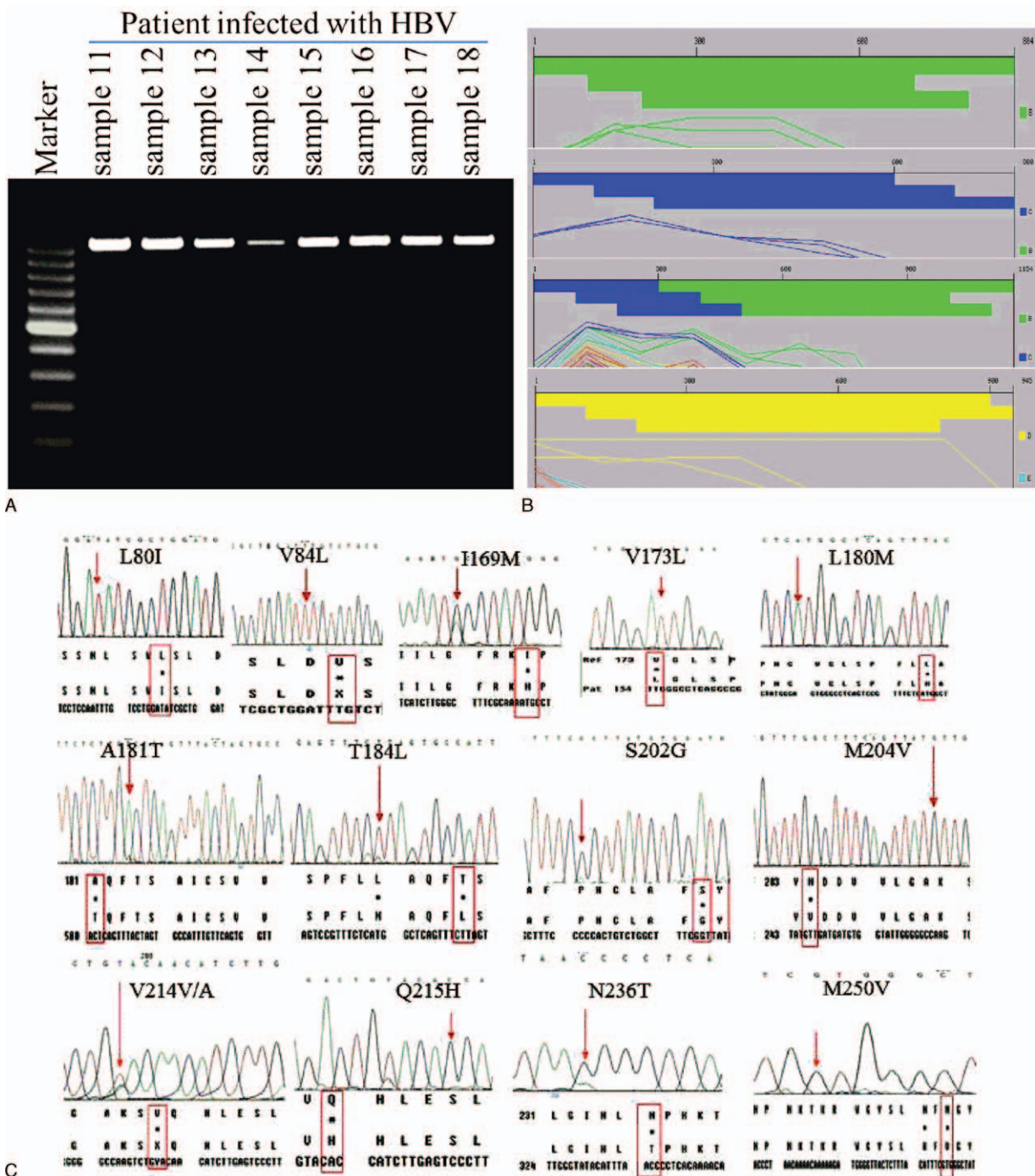


Figure 5. The results of subtypes and drug resistance mutation detections in clinical samples. (A) The bands of polymerase chain reaction products from the patients' samples with the mixed primer FR and F2R2 by gel electrophoresis. (B) The result of HBV subtypes of the patients obtained by blast sequence comparison of their hepatitis B virus (HBV) sequence with online NCBI HBV database. The HBV subtypes in the patients include B, C, D, and mix B/C subtype. (C) The antiviral drug resistance mutations detected in 80 clinical HBV samples (HBV DNA from 20 to 200IU/mL).

were identified in the samples with this method (Fig. 5C). We identified 11.25% (9/80) HBV subtype B, 85.0% (68/80) HBV subtype C, 1.25% (1/80) HBV subtype D, and 2.5% (2/80) HBV subtype mix B/C in the 80 clinical samples (Fig. 6A). The detective mutation frequencies for the most common mutation rtM204 and rtL180 were 35.5% and 29.71%, respectively (Fig. 6B), whereas the rare mutation rtV214 and rtQ215 was 2.90% and 2.17%, respectively (Fig. 6B).

4. Discussion

HBV genotypes are important for the clinical manifestation of disease and treatment response of the patients, particularly those with low HBV loads. Also, sensitive detection of HBV-DNA resistance is essential for monitoring response to therapy. So far there have no common PCR methods available for HBV subtyping or resistance detection for low levels of HBV DNA.

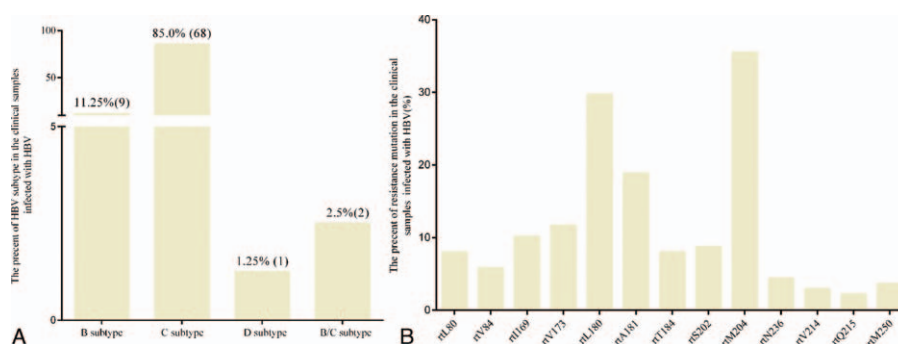


Figure 6. Frequency of hepatitis B virus (HBV) subtypes and drug resistance mutations in the patients' samples. (A) The ratio of HBV subtypes in the clinical HBV samples. (B) The ratio of the drug resistance mutations in the clinical HBV samples.

We developed a sensitive method in this study for efficiently genotyping and detecting resistance mutations in patients with low levels of HBV DNA (20 to 200 IU/mL). Our result showed that this method is a precise and reliable method for clinical application.

This method is characterized by the designed common and universal primers. We identified the common primer F1R1 that could cover all HBV subtypes in the databases. We also designed the universal F2R2 primers, and another universal primers FR by linking F2 and R2 to F1 and R1 primers, respectively. The new designed common primers F1R1 and FR could amplify all 23 HBV subtypes from databases and clinical samples. The F2R2 universal primer could be used as sequence primer for all amplified subtypes in the patients' samples. This pair of primers was also able to amplify all HBV subtypes if mixed with FR together, which increases the sensitivity of PCR amplification. Therefore, FR + F2R2 have the highest sensitivity for amplification, and we found that they can detect plasma HBV DNA as low as 20 IU/mL.

Antiviral treatment is effective to suppress HBV replication, decreasing the level of serum HBV DNA in the patients. However, drug treatment also induces resistance mutations in the patients.^[14–16] Therefore, sensitive detection of HBV-DNA resistance is essential for monitoring response to therapy. With this method, we could efficiently perform the subtyping and detection of resistance mutations in 90 patient samples including 80 samples with low levels of plasma HBV DNA (from 20 to 200 IU/mL). The ratio of HBV genotypes and resistance mutations is consistent with previous reports. These results indicated this method is a sensitive and reliable direct sequencing method for genotyping and resistance mutation detection in low levels of serum HBV DNA samples.

In summary, we developed a new direct sequencing method for genotyping and detecting resistance mutations in low levels of HBV DNA serum samples with the identified new common and universal primers. This method facilitates early identification of resistance mutation and monitoring therapy response in HBV patients.

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