

Simultaneous detection of hepatitis B virus genotypes and mutations associated with resistance to lamivudine, adefovir, and telbivudine by the polymerase chain reaction-ligase detection reaction

Authors

Yong-Zhong Wang¹
Jun-Hua Xiao²
Long-Gen Liu³
Chun-Yan Ye⁴
Hong-Yu Shen⁵
Tian-Min Xu⁴
Ke-Zhuan Zhu⁴

¹Professor; Director, Institute for the Study of Liver Diseases, The Third People's Hospital of Changzhou, China

²PhD, Professor; Director, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, China

³Deputy Director, Third People's Hospital of Changzhou;

Professor, Department of Infectious Diseases, Institute for the Study of Liver Diseases, The Third People's Hospital of Changzhou, China

⁴Professors, Department of Infectious Diseases, Institute for the Study of Liver Diseases, The Third People's Hospital of Changzhou, China

⁵MS; Supervising Laboratorian, Institute for the Study of Liver Diseases, The Third People's Hospital of Changzhou, China

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Correspondence to:

Yong-Zhong Wang
300 North Lanling Road
Changzhou
213001 – Jiangsu Province
China
wyz213003@163.com

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ABSTRACT

Objectives: Detection of mutations associated to nucleos(t)ide analogs and hepatitis B virus (HBV) genotyping are essential for monitoring treatment of HBV infection. We developed a multiplex polymerase chain reaction-ligase detection reaction (PCR-LDR) assay for the rapid detection of HBV genotypes and mutations associated with lamivudine, adefovir, and telbivudine resistance in HBV-infected patients. **Methods:** HBV templates were amplified by PCR, followed by LDR and electrophoresis on a sequencer. The assay was evaluated using plasmids that contained wild-type or mutant HBV sequences and 216 clinical samples. **Results:** The PCR-LDR assay and sequencing gave comparable results for 158 of the 216 samples (73.1%) with respect to mutation detection and genotyping. Complete agreement between the two methods was observed for all the samples (100%) at codon 180 and codon 204. Concordant results were observed for 99.4% of the 158 samples at codon 181 and 98.7% at codon 236. The genotyping results were completely concordant between the PCR-LDR assay and sequencing. The PCR-LDR assay could detect a proportion of 1% mutant plasmid in a background of wild-type plasmid. **Conclusion:** The PCR-LDR assay is sensitive and specific for detection of HBV genotypes and drug resistance mutations, and could be helpful for decision making in the treatment of HBV infection.

Keywords: hepatitis B; drug resistance; mutation; genotype.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem worldwide. Antiviral therapies with interferon, pegylated interferon, or nucleos(t)ide analogs are widely used to treat HBV infection. Given that these therapies cannot eradicate HBV infection, the focus of the treatment is to prolong the suppression of viral replication, prevent complications of infection, and establish immune control by the host over the virus.¹ The effectiveness of interferon and pegylated interferon therapy depends on the specific genotype of HBV with which the patient is infected.^{2,3} In addition, the presence of mutations that confer drug resistance often lead to the failure of treatment with nucleos(t)ide analogs.⁴ HBV has been classified into eight major genotypes (designated from A to H), on the basis of an intergroup divergence of 8% or more in the full-length nucleotide sequence, and most of the genotypes show a distinct geographic distribution.^{5,6} There is evidence that genotypes A and B are associated with higher rates of hepatitis B e antigen

(HBeAg) seroconversion than genotypes C and D during treatment with interferon or pegylated interferon.^{2,3,7,8} Genotyping of HBV is essential to detect patients who are highly likely to respond to interferon or pegylated interferon.^{9,10}

The primary mutation that confers resistance to the nucleoside analog lamivudine is rtM204V/I, which is located in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV DNA polymerase, and is often accompanied by the mutation rtL180M. HBV that carries rtM204I or rtM204V + rtL180M is also resistant to telbivudine in cell culture.⁴ The primary mutations that confer resistance to the nucleotide analog adefovir (ADV) are rtN236T and/or rtA181T/V.⁴ Resistance to entecavir may be caused by a two-hit mechanism with initial selection of M204V/I mutation followed by the occurrence of rtT184S/A/I/L/F/G, rtS202G/I, or rtM250V.¹¹ The frequency of mutations for lamivudine resistance increases with the duration of lamivudine treatment, and reaches cumulative rates of 15%, 38%, 56%, and 67% in years 1, 2, 3, and 4 of treatment, respectively.¹²⁻¹⁵ In chronic hep-

atitis B patients who have not been exposed to nucleoside treatment previously, adefovir resistance mutations only emerge in the second year of treatment, and the frequency of these mutations reaches cumulative rates of 3%, 6%, 18%, and 29% at the end of 2, 3, 4, and 5 years of therapy, respectively.^{16,17} Adefovir resistance mutations are much more common in patients who have previously received lamivudine therapy.¹⁸

HBV infection is a heavy health burden in China. National surveys found that the prevalence of HBV was 9.8% in 1992 and 7.2% in 2006 among the population aged 1–59 years. Due to the National Hepatitis B Vaccination to Infant Program, the prevalence of HBV has been dramatically reduced.¹⁹ However, because this program was only started in 1992, the prevalence rate of HBV is still high in those aged above 18 years, and the treatment of HBV-infected patients is still an important issue in China. The State Food and Drug Administration of China has approved interferon, pegylated interferon, lamivudine, adefovir, telbivudine, and entecavir for the treatment of HBV infection. All these drugs are widely used in China to treat HBV-infected patients. HBV genotyping and the detection of mutations that confer drug resistance help the selection of an appropriate treatment strategy and monitoring of the treatment. However, there are a limited number of methods that enable genotyping and mutation detection to be carried out simultaneously. Although nucleotide sequencing of PCR products is widely used for HBV genotyping and mutational analysis, the technique can only detect mutant virus when it comprises at least 25% of the total viral population.²⁰ The reverse hybridization-based line-probe assay LiPA DR is more sensitive and can detect resistance mutations when the mutant virus comprises at least 5% of the viral population;^{21,22} however, it is too expensive to be adopted widely in developing countries such as China. In the present study, we established a polymerase chain reaction-ligase detection reaction (PCR-LDR) assay for the simultaneous detection of the mutations rtL180M, rtM204V/I, rtA181T/V, and rtN236T and the HBV genotypes A, B, C, and D, and evaluated its performance using clinical samples.

MATERIALS AND METHODS

Plasmids

The plasmids that contained the mutant and wild-type sequences for rt204, rt181, and rt236 were constructed previously.^{23,24} The plasmids that contained the mutant and wild-type sequences for rt180 were constructed using the same method described previously.²⁴

Serum samples and extraction of HBV DNA

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave their informed consent. Serum samples were collected from 216 patients with

chronic hepatitis B. Of the 216 patients, 52 were treated with lamivudine alone, 82 were treated with adefovir alone, 51 were treated with lamivudine initially but became resistant to lamivudine and were then treated with adefovir, 19 were treated with telbivudine, and 12 were treated with entecavir. Given that drug resistance mutations often arise after one year of treatment, we selected patients who had been treated for more than 12 months but still had sufficient HBV DNA in their serum to be detected by real-time PCR, in order to include enough mutant samples to evaluate the assay. HBV DNA was extracted from 100 µL of serum using a QIAamp DNA Blood Kit (Qiagen, Chatsworth, CA) in accordance with the manufacturer's instructions.

Measurement of serum HBV DNA levels

Serum levels of HBV DNA were determined by using a real-time PCR kit (Fosun Diagnostics, Shanghai, China). The kit has been approved by the Chinese Food and Drug Administration for *in vitro* diagnosis and has a lower limit of detection of 84 IU/mL.²⁵

Primers for PCR and LDR

Primers and probes (synthesized by Invitrogen, Shanghai, China) to detect the HBV mutations and genotypes are listed in Tables 1 and 2.

Multiplex PCR and LDR

PCR amplifications were carried out in a volume of 50 µL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl₂, 200 µmol/L dNTPs, 300 mmol/L each primer, 1.5 units of Gold DNA polymerase (Qiagen), and 5 µmol/L HBV DNA template. The amplifications were performed in a PE 9600 thermal cycler (Applied Biosystems, Foster City, CA) by heating to 95°C for 10 min, cycling for 35 cycles at 95°C for 30 s, 54°C for 50 s, 72°C for 60 s, and then performing a final extension at 72°C for 10 min. LDR reactions were carried out in a volume of 20 µL containing 20 mmol/L Tris-HCl (pH 7.6), 10 mmol/L MgCl₂, 100 mmol/L KCl, 10 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L NAD⁺, 12.5 mmol/L each probe, 3 µL of PCR product, and 0.1 mmol/L DNA ligase. The LDR was performed in a PE 9600 thermal cycler by incubating at 95°C for 2 min and cycling for 30 cycles at 95°C for 30 s and 65°C for 4 min. The reaction was stopped by adding 0.5 µL of 0.5 mmol/L EDTA. Aliquots of 2.5 µL of the LDR products were mixed with an equal volume of loading buffer containing 80% formamide, 10 mmol/L EDTA, and 1.2% Blue Dextran. The mixture was denatured at 94°C for 2 min, chilled rapidly on ice before loading on a 10% polyacrylamide gel with 7 mol/L urea, and electrophoresed on an ABI 377 DNA Sequencer (Applied Biosystems) for 30 min. The results were analyzed by using the GeneMapper software (Applied Biosystems).

Table 1. Primers and probes used in the PCR-LDR protocol for detection of HBV drug-resistant mutations

Primers and probes	Sequence	Length of product (bp)
PCR primers for mutation detection		
Primer 1-F	5'-CTACCAGCACGGGACCATGC-3'	575
Primer 2-R	5'-TACATGCATATAAAGGCATTGAGG-3'	
LDR probes for mutation detection		
L180	P-GAGAAACGGACTGAGACCTACCTACCTACCTACCT-FAM	
L180-M	ACCTACCACCTACCTACCTAGTAACTAGACCAT	70
L180-W	ACCTACCTACCTACCTACCTAAAGTAAACTGAGCCA	72
M204-1	P-ATATAACTGAAAGCCACCTACCTACCTACCTACCTAA-FAM	
M204-2	P-ATAACTGAAAGCCAAACCTACCTACCTACCTACCT-FAM	
M204-W	ACCTACCTACCTACCTACCTAAAATACCACATCATCC	74
M204-I	CCACCTACCTACCTACCTACCTACCTAAATACCACATCATCA	78
M204-V	ACCTACCTACCTACCTAACCTACCTATAACCACATCACCCAC	80
A181-1	P-CCAAGAGAAACGGACTGAGGCCCACTCCCATAGGAATCTTGCG-FAM	
A181-2	P-TCAAGAGAAACGGACTGAGGCCCACTCCCATAGGAATCTTG-FAM	
A181-M	AGCCCTACGAACCACTGAACAAATGGCACTAGTAAACTGAA	84
A181-W	AAAGCCCTACGAACCACTGAACAAATGGCACTAGTAAACTGAG	86
N236	P-TCAAATGTATACCCAAAGACAAAAGAAAATTGGTAATAGAGGTAA-FAM	
N236-M	CCATGAAGTTAAGGGAGTAGCCCCAACGTTTGGTTTTATTAGGGG	90
N236-W	TCCCATGAAGTTAAGGGAGTAGCCCCAACGTTTGGTTTTATTAGGGT	92

Table 2. Primers and probes used in the PCR-LDR protocol for HBV genotyping

Primers and probes	Sequence	Length of product (bp)
Primers for HBV genotyping		
Primer-F	5'-ACTGCCTCTCCCATATCGTC-3'	376
Primer-R	5'-GACAAACGGGCAACATACCT-3'	
LDR probes for HBV genotyping		
Genotype B	P-TCGAGGAATATGATAAAAACGCCGAGCCTACCTACCTACCTACCTACCT-FAM CCTACCTACCTACCTACTCCTACCTACCTTAAGATGAGGCATAGCAGCAGGATGT	108
Genotype C	P-TACCAAGGTATGTTGCCCGTTTGTCCACCTACC TACCTACCTAGCTACCTACCTA-FAM CCTACCTACCTACCTACCTACCTGACCCTGCACCGCCAACATGGAGAAGAC	110
Genotype A	P-TACCAAGGTATGTTGCCCGTTTGTCCACCTACC TACCTACCTAGCTACCTACCTA-FAM CCTACCTACCTACCTACCTACCTGACCCTTGCTTTGTTGGTTCTTCTGGAT	112
Genotype D	P-CGAAAGCCCAGGATGATGGGATGGGCTACCTACTAGTAGCCTACCTACTACT-FAM CCTACCTACCTACCTACCTAGTAGCTACCTACTGGGCCCACTCCCATAGGAATTTTC	118

Sequencing and subcloning

To validate the mutation detection and HBV genotyping results, the 575- and 376-bp PCR products, respectively, were purified and sequenced by Invitrogen. Discrepancies between the PCR-LDR and sequencing results were resolved by sequencing subcloned PCR products. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and 20 clones for each sample were selected for sequencing.

RESULTS

Specificity and sensitivity

The strategy of the PCR-LDR assay has been described in detail previously.²⁴ Its specificity and sensitivity were determined by using mutant and wild-type plasmids and clinical samples. All the plasmids produced the expected results. No nonspecific signals were observed at template concentrations of 2×10^3 , 2×10^5 , or 2×10^7 copies/mL. Serum samples from 10 blood donors, and 20 samples from patients with hepatitis A and C were tested, and gave no signal. Serial dilutions of the plasmids and serum samples were used to determine the lower limit of detection of the assay. Each plasmid was assayed three times. The PCR-LDR assay could detect mutant and wild-type plasmids at concentrations of 5×10^7 , 5×10^6 , 5×10^5 , 5×10^4 , 5×10^3 , and 500 copies/mL. Thus, the lower limit for the PCR-LDR assay was 500 copies/ml of mutant or wild-type plasmid. Three clinical serum samples with 1×10^7 IU/mL wild-type HBV, 6×10^6 IU/mL rtA181T, and 2×10^6 IU/mL rtN236T were serially diluted 10-fold and each dilution was tested in triplicate in the PCR-LDR assay. The PCR-LDR assay could repeatedly detect HBV in dilutions containing 600 IU/mL of HBV or above, but could not detect HBV in dilutions containing an HBV load lower than 600 IU/mL. Therefore, the lower limit of detection for serum samples was 600 IU/mL.

Mixed plasmid experiments

Mixed plasmid experiments were performed to determine the ability of the PCR-LDR assay to distinguish mutant plasmids in a background of wild-type plasmids. Mutant and wild-type plasmids were mixed to give final total concentrations of 1×10^5 and 1×10^6 copies/mL. Each mixture contained 100, 10, 1, or 0.1% mutant plasmid. The mutant plasmids were detected in all the mixtures except for those that contained 0.1% mutant plasmid.

Comparison of the LDR assay and sequencing

The PCR-LDR assay was also evaluated using serum samples from 216 patients who had been treated with lamivudine, adefovir, telbivudine, or entecavir. All samples were screened by both the PCR-LDR assay and sequencing. Discrepancies between the results obtained with the two methods were

resolved by sequencing subcloned PCR products. The results of both assays are summarized in Table 3. The PCR-LDR assay and sequencing gave comparable results for 158 (73.1%) of the 216 samples with respect to mutation detection and genotyping. In the remaining 58 samples (26.9%), the mutations and genotypes could not be detected by either method because insufficient product was amplified in the PCR step. Complete agreement between the two methods was observed for all the samples (100%) at codon 180 and codon 204. Concordant results were observed for 157 out of 158 analyzable samples (99.4%) at codon 181 and 156 out of 158 samples (98.7%) at codon 236. Among the discrepancies, PCR-LDR detected rtA181V/T and rtN236T double mutations in two samples and a rtM204V and rtA181V/T double mutation in one sample, whereas the sequencing only detected a rtA181V/T single mutation in the two samples and a rtM204V single mutation in the one sample, respectively.

Table 3. Comparison of PCR-LDR and sequencing results

	Results of PCR-LDR (n)	Results of sequencing (n)
Mutation types		
Single rtM204I	14 (6.5%)	14 (6.5%)
Single rtM204V	3 (1.4%)	4 (1.9%)
Single rtA181V/T	14 (6.5%)	16 (7.4%)
Single rtN236T	6 (2.8%)	6 (2.8%)
rtL180M+M204I	4 (1.9%)	4 (1.9%)
rtL180M+M204V	15 (6.9%)	15 (6.9%)
rtM204I+rtM204V	2 (0.9%)	2 (0.9%)
rtM204I+rtA181V/T	2 (0.9%)	1 (0.5%)
rtM204I+rtN236T	1 (0.5%)	1 (0.5%)
rtA181V/T+rtN236T	4 (1.9%)	2 (0.9%)
rtL180M+rtM204V+rtA181V/T	2 (0.9%)	2 (0.9%)
rtM204I+rtM204V+rtA181V/T	1 (0.5%)	1 (0.5%)
Wild-type	90 (41.7%)	90 (41.7%)
Not detected	58 (26.9%)	58 (26.9%)
Total	216 (100%)	216 (100%)
Genotypes		
Genotype B	32 (14.8%)	32 (14.8%)
Genotype C	116 (53.7%)	116 (53.7%)
Genotype B+C	3 (1.4%)	3 (1.4%)
Genotype D	7 (3.2%)	7 (3.2%)
Not detected	58 (26.9%)	58 (26.9%)
Total	216 (100%)	216 (100%)

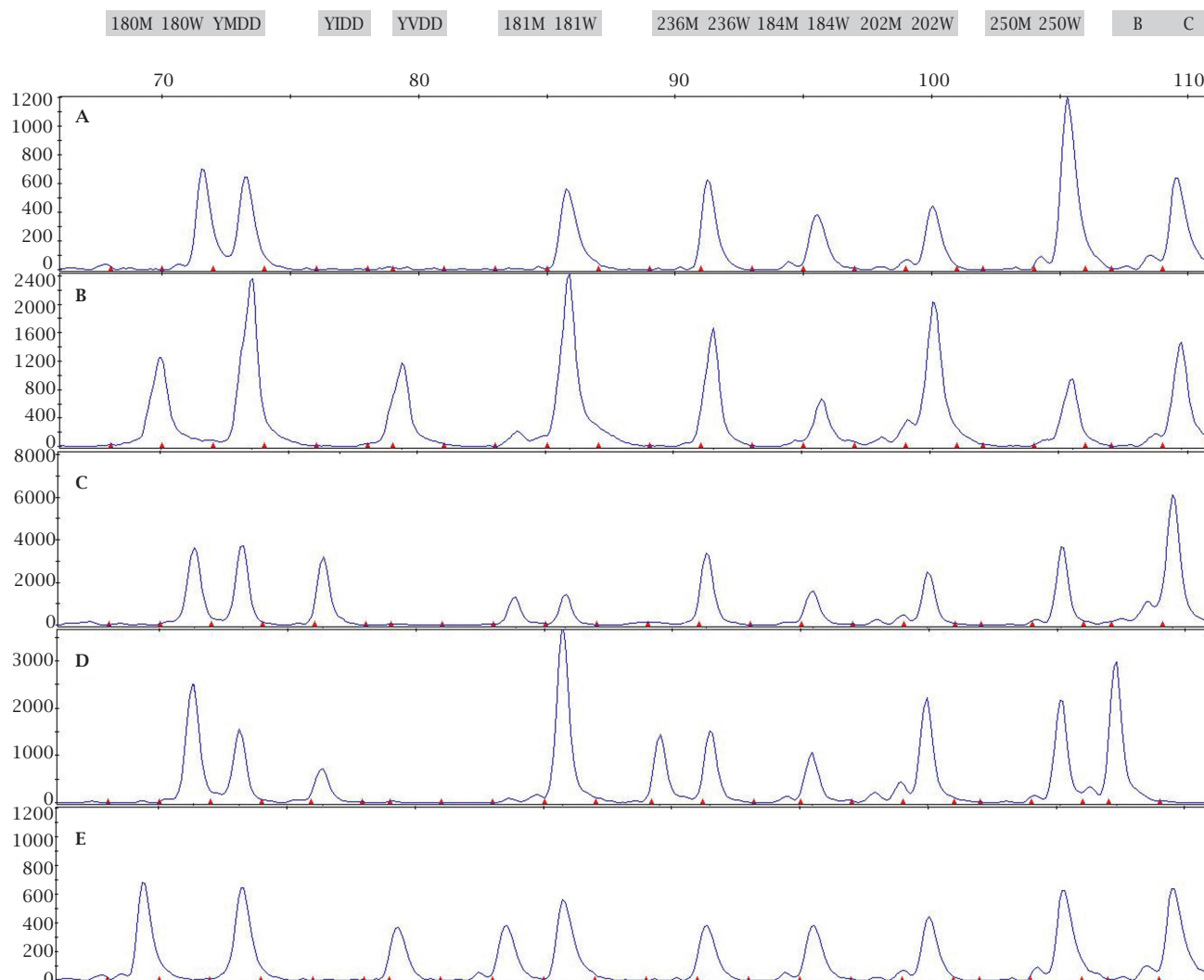


Figure 1: Some results of the PCR-LDR assay. The length of the LDR product is determined by the lengths of the LDR probes. Each mutant and/or wild sequences and genotypes produces a peak at the corresponding position when analyzed using GeneMapper software. (A) Wild-type HBV, genotype C. (B) rtM204V, genotype C. (C) rtM204I+rtA181V/T, genotype C. (D) rtM204I+rtN236T, genotype B. (E) rtL180M+rtM204V+rtA181V/T, genotype C.

All the discrepancies corresponded to mutations that were detected by the PCR-LDR assay but not by sequencing of the same sample. The discrepancies were resolved by the sequencing of 20 randomly chosen subclones for each sample. In each case, at least three clones for the same sample had mutations that were consistent with the PCR-LDR results. To confirm the performance of this assay with respect to the detection of mutations at codons 181 and 236, we retested the 70 samples that had been shown to have rtA181V/T and/or rtN236T mutations in the previous study and 20 samples shown to be wild type,²⁴ and obtained consistent results (Figure 1). The genotyping results were completely concordant between the PCR-LDR assay and sequencing.

Fifty-eight samples could not be analyzed by either method because they could not be amplified with the PCR

primers. Real-time PCR quantification analysis showed that the concentration of HBV DNA in all these samples was below 600 IU/mL.

Time required

For a panel of 64 samples, serum DNA extraction takes one hour. PCR-LDR takes about four hours on an ABI 9600 thermal cycler. Electrophoresis takes about 1.5 hours on an ABI 377 sequencer. The total time for the PCR-LDR assay is about six hours. In contrast, the sequencing analysis takes about 13 hours, including one hour for serum DNA extraction, 2.5 hours for PCR on a ABI 9600 thermal cycler, one hour for purification of PCR products, 1.5 hours for sequencing reaction and purification, and seven hours for electrophoresis on a ABI 377 sequencer.

DISCUSSION

Although HBV genotyping and the detection of drug resistance mutations are important for monitoring the treatment of chronic hepatitis B, there are a limited number of methods for the simultaneous detection of HBV genotypes and mutations that confer resistance to lamivudine, adefovir, and telbivudine.²⁶ We have established a PCR-LDR assay to detect the major HBV genotypes and the rtL180M, rtM204I, rtM204V, rtA181V/T, and rtN236T mutations, which are associated with lamivudine, adefovir, and telbivudine resistance, in patients with chronic HBV infections. The assay could specifically detect mutant and wild-type HBV, and its lower limit of detection was 600 IU/mL for clinical serum samples. In a mixture of mutant and wild-type plasmids, the assay could detect mutant plasmids at a proportion of 1%. Therefore, the PCR-LDR assay was more sensitive than sequencing for the detection of a low level of mutant mixed with wild-type virus. In addition, the cost of the PCR-LDR assay is below 20 USD dollars per test, which is affordable for patients in China and other developing countries. Compared to sequencing, PCR-LDR is less time-consuming.

Fifty-eight samples could not be analyzed by either PCR-LDR or sequencing because the level of HBV DNA in the samples was too low to be amplified in the PCR step. Although the clinical significance of low serum levels of HBV DNA in patients with chronic HBV infection is unclear, patients who have a detectable serum level of HBV DNA have a much higher risk of relapse after the withdrawal of antiviral agents.²⁷ Mutations that occur at a low level may lead to HBV breakthrough and treatment failure during long-term antiviral therapy. The improvement of HBV DNA extraction protocols and nested PCR should be considered in future studies, to increase the sensitivity of detection of low levels of HBV DNA.

Theoretically, new mutations that cause resistance to antiviral agents used to treat HBV could be detected simultaneously by multiplex LDR with specific probes. We have designed specific probes to detect the mutations rtT184G, rtS202I, and rtM250V, which are associated with entecavir resistance. However, we did not detect these mutations in the clinical samples analyzed due to the low rates of these mutations and the relatively small number of patients treated with entecavir in our study. The performance of the PCR-LDR assay with respect to the detection of entecavir-resistance mutations needs to be evaluated in future studies.

Although at least eight HBV genotypes have been reported, the major HBV genotypes in China are B and C.²⁸ Genotypes A and D are found in a very small proportion of Chinese patients, and genotypes E, F, G, and H have not been reported in China. As a consequence, we could only evaluate the performance of the PCR-LDR assay for genotypes A, B, C, and D, and, in fact, we did not detect genotype A in this study. This might be due to the very small number of patients infected with genotype A in eastern China.²⁸

Separate mutations associated with lamivudine and adefovir resistance were detected simultaneously in samples from six patients. All these patients failed to respond to lamivudine treatment initially and were then treated with adefovir for more than 12 months. This result implies that sequential treatment with lamivudine and adefovir can lead to multi-drug resistance in HBV-infected patients, as reported in other studies.^{29,30}

In conclusion, the PCR-LDR assay developed in this study was able to detect HBV genotypes and the mutations associated with resistance to lamivudine, adefovir, and telbivudine sensitively and specifically. It might be a useful tool for decision making with respect to the treatment of HBV-infected patients.

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