Comparison of ligase detection reaction and real-time PCR for detection of low abundant YMDD mutants in patients with chronic hepatitis B

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Abstract

AIM: To compare the ligase detection reaction (LDR) and real-time PCR for detection of low abundant YMDD mutants in patients with chronic hepatitis B infection.

METHODS: Mixtures of plasmids and serum samples from 52 chronic hepatitis B patients with low abundant lamivudine-resistant mutations were tested with LDR and real-time PCR. Time required and reagent cost for both assays were evaluated.

RESULTS: Real-time PCR detected 100, 50, 10, 1 and 0.1% of YIDD plasmid, whereas LDR detected 100, 50, 10, 1, 0.1, and 0.01% of YIDD plasmid, in mixtures with YMDD plasmid of 10^6 copies/mL. Among the 52 clinical serum samples, completely concordant results were obtained for all samples by both assays, and 39 YIDD, 9 YVDD, and 4 YIDD/YVDD were detected. Cost and time required for LDR and real-time PCR are 60/80 CNY (8/10.7 US dollars) and 4.5/2.5 h, respectively.

CONCLUSION: LDR and real-time PCR are both sensitive and inexpensive methods for monitoring low abundant YMDD mutants during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

Key words: YMDD mutants; Hepatitis B virus; Real-time PCR; Ligase detection reaction

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INTRODUCTION

Lamivudine is an effective antiviral agent for treatment of patients with chronic hepatitis B and advanced liver diseases[1]. However, long-term lamivudine monotherapy leads to emergence of lamivudine-resistant hepatitis B virus (HBV) mutants in some patients chronically infected with HBV[2,3]. The incidence is 16%-32% in the first year and increases to 38%, 57%, and 67% after 2, 3, and 4 years, respectively[1,4]. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase, which is part of the catalytic site of the HBV polymerase[5]. Viological breakthrough and alanine transaminase (ALT) elevation have been shown to occur 2-28 wk and 12-31 wk after the emergence of YMDD mutants, respectively[3,4]. Initially, YMDD mutants consist of minor populations. They gradually replace the wild-type virus, reaching a 100% lamivudine-resistant variant population, and this replacement occurs in parallel with the increase in HBV DNA load[3]. Sensitive methods for early detection of lamivudine-resistant mutants will be helpful for physicians to make clinical decisions in treatment of patients with HBV infection.

Several technologies have been developed for the detection of lamivudine-resistant mutants[5,6]. Although nucleotide sequencing of PCR products is widely used to detect lamivudine resistance, it is expensive and laborious, and can detect only mutant viruses representing at least 50% of the total virus population[8]. Inno-LiPA, pyrosequencing, real-time PCR, and ligase detection reaction (LDR) are able to detect low abundant YMDD mutants in the wild-type HBV[9,12-15]. However, only few studies have compared these methods. We have previously compared real-time PCR and pyrosequencing for detection of YMDD mutants in patients with chronic hepatitis B[14]. In the present study, we compared LDR and real-time PCR for detection of low abundant YMDD mutants in mixed plasmids and clinical
samples from lamivudine treated patients with chronic hepatitis B.

**MATERIALS AND METHODS**

**Plasmids and controls**

Plasmids and controls were prepared as previously described\(^{14,16}\). In brief, three previously identified serum samples containing HBV with YMDD, YVDD and YIDD sequences were used as template and amplified by PCR. PCR products were cloned using pGEM-T systems (Promega, Madison, Wisconsin, USA), and clones were sequenced using ABI 3100 sequencer (Applied Biosystems, Foster, California, USA).

**Patients and samples**

Serum samples were collected from 196 patients with chronic HBV infection. All patients were treated with lamivudine for three months to three years and serum HBV-DNA levels were above 1.0 × 10\(^8\) copies/mL by real-time PCR. Among these samples, 52 samples with YMDD mutants below 50% of total HBV population (determined by real-time PCR\(^{14}\)) were selected for comparison of LDR and real-time PCR. All these 52 samples were found to contain only the YMDD variant by sequencing of PCR products, but found to contain YVDD or YIDD variants with real-time PCR or LDR.

**Extraction and quantitation of HBV DNA**

HBV DNA was extracted from serum samples using the HBV DNA extraction reagents (Fosun Diagnostics, Shanghai, China) according to the manufacturer’s instructions. Serum HBV DNA levels were measured on ABI 7300 real-time PCR system (Applied Biosystems, Foster, CA, USA) by incubating the reaction mixture (50 μL) at 50 degree for two minutes, followed by 5 min at 95 degree, 40 cycles of PCR amplification (94 degree for 20 s and 53 degree for 30 s). The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The percentage of mutants in total virus was calculated by the following equations\(^{14}\):

(1) \(\Delta C_t = C_t \text{ of control} - C_t \text{ of mutants}\)

(2) Ratio of mutants to total virus = \(2^{\Delta C_t}\)

**Sequencing of PCR products**

HBV DNA samples were prepared for sequencing by amplification with PCR as described by Allen et al\(^3\). A Amplification of DNA extracted from serum samples was amplified by PCR. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, California, USA) and were eluted from the column with 80 μL of distilled deionized water. The DNA quality and concentration were determined by absorbance measurements at 260 and 280 nm and by gel electrophoresis on a 2.5% agarose gel. All sequencing reactions were performed on ABI 3130 DNA sequencer (Applied Biosystems, Foster, California, USA).

**Ligase detection reaction**

LDR was carried out as described by Xiao et al\(^8\). In brief, for one type of mutant (YMDD or YVDD), one common probe and two discriminating probes for mutant and wild-type YMDD were used in LDR, which was carried out in 20 μL of buffer, 1 pmol of each probe, and 5 μL of sample DNA. The reaction mixture was incubated at 94°C for 2 min, before adding 15 U of thermostable Taq DNA ligase (New England Biolabs, USA), followed by 20 cycles of 30 s at 94°C and 4 min at 65°C. Two PCR reactions were performed with the product of the LDR as template for 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

**Real-time PCR**

Real-time PCR for detection of YMDD mutants was performed as previously described\(^{14,16}\). In brief, parallel reaction C, V and I were used to detect total HBV, YVDD and YIDD variants, respectively. The amplification was performed on ABI 7300 PCR system (Applied Biosystems, Foster, CA, USA) by incubating the reaction mixture (50 μL) at 50 degree for two minutes, followed by 5 min at 95 degree, 40 cycles of PCR amplification (94 degree for 20 s and 53 degree for 30 s). The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The percentage of mutants in total virus was calculated by the following equations\(^{14}\):

(1) \(\Delta C_t = C_t \text{ of control} - C_t \text{ of mutants}\)

(2) Ratio of mutants to total virus = \(2^{\Delta C_t}\)

**Mixing experiments**

Mixing experiments were carried out as previously described\(^{16}\). In brief, mutant plasmid containing YIDD sequence and wild-type plasmid were mixed at a final concentration of 10\(^6\) copies/mL, and the percentage of the YIDD plasmid in the mixture was 100%, 50%, 10%, 1%, 0.1%, and 0.01%, respectively. The mixtures were analyzed by LDR and real-time PCR respectively. For real-time PCR, each mixture was analyzed five times, and the mean Ct value of the five runs was used to determine the ratio of mutant to total viruses. For LDR assay, each mixture was analyzed only once.

**Time study**

Two skilled technicians were selected to perform the assays. Time required for each assay was measured by direct observation during the procedures performed by the technicians, including the process of DNA extraction, amplification, detection, and analysis.

**Cost analysis**

Cost for each assay was estimated based on the prices of reagents in China. The cost of instruments and labors was not included.

**RESULTS**

**Detection of mixed plasmids**

Mixtures of plasmids contained YIDD and YMDD at different ratios were detected by LDR and real-time PCR, respectively. LDR detected YIDD, YIDD/YMDD in the mixtures containing 100%, 50%, 10%, 1%, 0.1% and 0.01% YIDD plasmid. Real-time PCR detected YIDD in the mixture containing 100% YIDD plasmid and YIDD/ YMDD in the mixtures containing 50%, 10%, 1% and 0.1% YIDD plasmids, but detected only YMDD in the mixture.
containing 0.01% YIDD plasmid (Table 1). It means that real-time PCR and LDR are able to detect 1000 and 100 copies/mL of mutant virus in the background of wild type viruses, respectively. The results of real-time PCR were consistent with our previous study with mixtures of YVDD and YMDD plasmids.[10]

**Comparison of LDR and real-time PCR for detection of clinical samples with low abundant YMDD mutants**

We tested clinical serum samples from 52 lamivudine treated patients with chronic hepatitis B who had low abundant YMDD mutants. All the samples were detected as YMDD virus by sequencing the PCR products. The results obtained by LDR and real-time PCR were consistent (Table 2). Both methods detected 39 YIDD, 9 YVDD, and 4 YIDD/YVDD variants in the virus population obtained by real-time PCR. The percentage of mutants in the virus population obtained by real-time PCR ranged from 4% to 40%. The percentage of the four YIDD/YVDD mixed mutants was 10%/20%, 30%/20%, 40%/10%, and 20%/30%, respectively.

**Time required**

In this study, we used 96-well PCR equipment and all the 52 samples were dealt with in a run. The total assay time for LDR and real-time PCR was 4.5 and 2.5 h, respectively.

**Cost**

The cost per test for each assay was calculated based on the prices of the reagents in China. Primers and probe were synthesized in TaKaRa Biotech (TaKaRa, Dalian, China). Real-time PCR mixtures were from Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The total reagent cost per test for LDR and real-time PCR was 60 and 80 CNY (8 and 10.7 US dollars), respectively. Although the cost of labor is similar in the same region, the cost of equipment used for LDR assay is much lower than that for PCR assay (5000 US dollars vs 60,000 US dollars).

**DISCUSSION**

Lamivudine has revolutionized the treatment of chronic hepatitis B. Lamivudine-resistant mutations in the YMDD motif of polymerase gene were detected in lamivudine treated and untreated patients with chronic hepatitis B.[14,17-19] Clinical breakthrough was observed 2 wk-7 mo after the emergence of YMDD mutations[8-10], causing considerable morbidity and mortality in those patients.[20-24]. Lamivudine-resistant mutants are frequently preexisting variants in HBV-infected patients and are selected during lamivudine therapy. These resistant variants initially represent a minority of the quasispecies and gradually replace the wild-type YMDD variants[10]. Detection of low abundant lamivudine-resistant mutants in the background of wild-type HBV as early as possible is helpful for virological follow-up and diagnosis of resistance in the clinical setting.

To date, many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B.[11] The differences in sensitivity, specificity, cost, and time required do exist in these methods. Real-time PCR is able to quantitatively detect a small portion of resistant mutants in HBV populations and LDR is a newly developed method for detection of low abundant mutants in the background of wild-type HBV. In the present study, we compared LDR and real-time PCR for detection of low abundant YMDD mutations in lamivudine treated patients. The results obtained by the two methods were completely concordant in all samples, and 39 YIDD, 9 YVDD, and 4 YIDD/YVDD variants were detected. The percentages of mutants in the virus population obtained by real-time PCR ranged from 4% to 40%. In the mixing experiment, LDR was able to detect as low as 0.01% (100 copies/mL) of YIDD plasmid, while real-time PCR only detected 0.1% (1000 copies/mL) of YIDD plasmid in the background of YMDD plasmid. This may be due to LDR employing two kinds of amplification cycles, 20 cycles of LDR and 30 cycles of PCR, in the testing process. These results suggest that LDR is more sensitive than real-time PCR. In addition, the cost of LDR is slightly lower than that of real-time PCR. However, real-time PCR is much more rapid and requires less manual work than LDR. Both methods are sensitive and inexpensive compared to other methods for detection of YMDD mutation[12]. Another advantage of the real-time PCR method is that it is able to calculate the ratio of mutants to total virus in samples.[13] This will be useful in the clinical studies on the dynamics of resistant mutants during lamivudine therapy.

Several antiviral agents, such as adefovir and entecavir, can provide effective therapies in patients with lamivudine-resistant HBV.[25-27] Pegylated interferon also induces sustained responses in a portion of lamivudine-resistant patients.[28-30] Monitoring low abundant YMDD mutation during lamivudine therapy by sensitive and inexpensive methods will be helpful for physicians to make better clinical decisions as early as possible in management of chronic hepatitis B.

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**Table 1  Results of LDR and real-time PCR for detection of mixed plasmids containing YIDD and YMDD at a final concentration of 10^6 copies/mL**

| YIDD plasmid in the mixture | LDR | Real-time PCR |
|-----------------------------|-----|---------------|
| 100% (10^6 copies/mL)       | YIDD| YIDD          |
| 50% (5 × 10^5 copies/mL)    | YIDD/YMDD | YIDD/YMDD |
| 10% (10^5 copies/mL)        | YIDD/YMDD | YIDD/YMDD |
| 1% (10^4 copies/mL)         | YIDD/YMDD | YIDD/YMDD |
| 0.1% (1000 copies/mL)       | YIDD/YMDD | YIDD/YMDD |
| 0.01% (100 copies/mL)       | YIDD/YMDD | YMDD         |

**Table 2  Comparison of results obtained by LDR and real-time PCR for 52 clinical samples**

| Types          | No. of samples (%) | LDR | Real-time PCR |
|----------------|--------------------|-----|---------------|
| YMDD           | 0 (0)              | 0 (0) |
| YIDD           | 39 (75)            | 39 (75) |
| YVDD           | 9 (17)             | 9 (17) |
| YIDD + YVDD    | 4 (8)              | 4 (8) |
In conclusion, both LDR and real-time PCR are sensitive and inexpensive methods for monitoring low abundant YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. LDR is more sensitive and less expensive, while real-time PCR is more rapid.

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