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RAPID COMMUNICATION

Comparison of amplicon-sequencing, pyrosequencing and real-time PCR for detection of YMDD mutants in patients with chronic hepatitis B

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Abstract

AIM: To compare the sequencing of PCR products, pyrosequencing, and real-time PCR for detection of Tyrosine-methionine-aspartate-aspartate (YMDD) mutants in patients with chronic hepatitis B.

METHODS: Mixtures of plasmids and serum samples from 69 chronic hepatitis B patients treated with lamivudine were tested for YMDD mutations by sequencing of PCR products, pyrosequencing, and real-time PCR, respectively. Time required and reagent costs of the three assays were evaluated.

RESULTS: Real-time PCR detected 100%, 50%, 10%, 1% and 0.1% of YVDD plasmid in mixtures with 10^6 copies/mL of YMDD plasmid, whereas sequencing and pyrosequencing only detected 100% and 50% of YVDD plasmid in aliquots of the corresponding mixtures. Completely concordant results were obtained from 60 (87%) out of the 69 clinical serum samples by the three assays. Mutants were detected by real-time PCR in less than 20% of the total virus population, but no mutant was detected by sequencing and pyrosequencing. In addition, real-time PCR required less time and was more cost-effective than the other two assays. However, throughput of pyrosequencing was the highest.

CONCLUSION: Among the three assays compared, real-time PCR is the most sensitive, cost-effective, and time saving for monitoring YMDD mutants in patients with chronic hepatitis B on lamivudine therapy.

Key words: Tyrosine-methionine-aspartate-aspartate mutant; Hepatitis B virus; Real-time PCR; Sequencing; Pyrosequencing

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INTRODUCTION

It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) and that more than 1 million will die of liver cirrhosis and hepatocellular carcinoma (HCC) each year[1-3]. Lamivudine is an effective antiviral agent for patients with chronic hepatitis B and advanced liver diseases[4]. However, long-term lamivudine monotherapy induces emergence of lamivudine-resistant HBV mutants in some patients chronically infected with HBV[4,5]. 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 HBV polymerase[6]. Virological breakthrough and alanine transaminase (ALT) flare have been observed in 2 and 3 mo after the emergence of YMDD mutants, respectively[7]. Several technologies developed can detect lamivudine-resistant mutants[8], but the number of studies actually comparing these assays is limited.

Nucleotide sequencing of PCR products is routinely used to detect lamivudine resistance. However, this method is expensive and laborious and only can detect mutant virus when it comprises at least 25% of the total virus population[9]. Pyrosequencing has a high throughput and can detect minor sequence variants[10]. Real-time PCR is a rapid and highly sensitive method for detection of mutant HBV[11-14].

In the present study, we compared the sequencing of PCR products, pyrosequencing, and real-time PCR for detection of YMDD mutants in mixed plasmids and clinical samples from chronic hepatitis B patients treated with lamivudine. Time required and reagent costs were also analyzed to enable a comparison among these assays for their cost-effectiveness.

MATERIALS AND METHODS

Plasmids and controls

Plasmids and controls were prepared as previously described[14]. Briefly, three previously identified serum samples containing YMDD, YVDD and YIDD respectively were used as a template and amplified by PCR. PCR products were cloned using pGEM-T systems (Promega, Madison, WI, USA), and clones were sequenced using ABI 3100 sequencer (Applied Biosystems, Foster, CA, USA).

Patients, samples and extraction of HBV DNA

Serum samples were collected from 69 patients with chronic HBV infection. Real-time PCR showed that all the patients after treatment with lamivudine for three months to three years were HBV-DNA positive. HBV DNA was extracted from serum samples using the QIAamp blood kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. HBV DNA was measured on Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with quantitative real-time PCR reagents (Fosun Diagnostics, Shanghai, China) approved by the State Food and Drug Administration of China for *in vitro* diagnostic use.

Sequencing of PCR products

HBV DNA samples were prepared for sequencing by PCR amplification as described by Allen *et al*[6]. HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, CA, USA) and eluted from the column with 80 μL of distilled deionized water. The DNA quality and concentration of DNA were determined by absorbance measurements at 260 and 280 nm and by gel electrophoresis on 2.5% agarose gel. All sequencing reactions were performed on ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA).

Pyrosequencing

Pyrosequencing was carried out as described by Lindstrom *et al*[10]. In brief, the primer (5'-AGT GGG CCT CAG TCC GTT TC-3') was designed to anneal the adjacent YMDD motif. HBV DNA was extracted from serum samples and amplified by nested-PCR. One of the inner PCR primers was biotinylated to prepare single-stranded DNA with PCR products on the streptavidin-coated beads for pyrosequencing. Pyrosequencing was performed on the automated 96-well PSQ pyrosequencer (Pyrosequencing AB, Uppsala, Sweden) with the PSQ SNP 96 reagent kit (Pyrosequencing AB, Uppsala, Sweden). The nucleotide dispensation order was ATGTATGATG, which was designed on sequences of the interested YMDD motif of the wild-type and mutant HBV.

Real-time PCR

Real-time PCR for detection of YMDD mutants was performed as previously described[14]. In brief, parallel reactions C, V and I were used to detect total HBV, YVDD, and YIDD, respectively. The reactions differed only in the reverse primer they contained. The amplification was performed on Mx3000P PCR system (Stratagene, La Jolla, CA, USA) by incubating the reaction mixture (50 μL) at 50℃ for 2 min, at 95℃ for 5 min, followed by 40 cycles of PCR amplification at 94℃ for 20 s and at 53℃ for 30 s. The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The threshold cycle (Ct) is the cycle at which a significant increase in fluorescence occurs. The percentage of mutants in total viruses was calculated by the equations of Bernard (ΔCt = Ct of control-Ct of mutants)[15] and Shi (ratio of mutants to total viruses = $2^{\Delta Ct}$)[14].

Mixing experiments

Mixing experiments were used to evaluate the abilities of the sequencing, pyrosequencing and real-time PCR to accurately detect and quantify minor sequence variants. Mutant plasmid containing YVDD sequence and wild-type plasmid were mixed at a final concentration of 10^6 copies/mL, and the percentage of YVDD plasmid in the mixture was 100%, 50%, 10%, 1%, 0.1% and 0.01% respectively. The mixtures were analyzed by the three methods, respectively. For real-time PCR, each mixture was analyzed five times, and the mean of the five runs was calculated.

Time study

Time analysis was carried out as described by Krafft and Lichy[16]. Three skilled technologists were selected to perform the assays. Time required for each assay was calculated by direct observation during the procedures.

Cost analysis

Costs for each assay were estimated based on the prices of reagents in China. The costs of instruments and labors were not included.

RESULTS

Detection of YVDD and YMDD mutants in plasmid mixture

YVDD and YMDD mutants in the plasmid mixture were detected at different ratios by sequencing, pyrosequencing and real-time PCR, respectively. YVDD, YVDD/YMDD mutants in the mixture containing 100% and 50% of YVDD plasmid and only YMDD in the mixture containing 10%, 1%, 0.1% and 0.01% YVDD plasmid were detected by sequencing and pyrosequencing. YVDD mutants in the mixture containing 100% YVDD plasmid, YVDD/YMDD mutants in the mixture containing 50%, 10%, 1% and 0.1% YVDD plasmid and only YMDD mutants in the mixture containing 0.01% YVDD plasmid were detected by real-time PCR. The ratio of YVDD to YMDD mutants in the real-time PCR assay was almost identical to the true ratio in the mixtures (Table 1).

Comparison of sequencing, pyrosequencing and real-time PCR for detection of YMDD and YVDD mutants in clinical samples

We detected YMDD and YVDD mutants in clinical serum samples from 69 chronic hepatitis B patients treated with lamivudine. The results obtained by sequencing, pyrosequencing and real-time PCR were compared (Table 2). Completely concordant results were obtained from 60

Table 1 Results of sequencing, pyrosequencing and real-time PCR for detection of mixed plasmids containing YVDD and YMDD at a final concentration of 10^6 copies/mL

YVDD plasmid in the mixture	Sequencing	Pyrosequencing	Real-time PCR	ΔCt (mean ± SD)	Calculated percentage (mean ± SD) (%)
100%	YVDD	YVDD	YVDD	-0.024 ± 0.06	98 ± 4.0
50%	YVDD	YVDD	YVDD	-0.98 ± 0.07	51 ± 2.5
10%	YMDD	YMDD	YVDD	-3.12 ± 0.09	11 ± 0.7
1%	YMDD	YMDD	YVDD	-6.60 ± 0.14	1 ± 0.1
0.1%	YMDD	YMDD	YVDD	-10.12 ± 0.20	0.09 ± 0.01
0.01%	YMDD	YMDD	YMDD	ND	0

ND: Not detected.

(87%) samples by the three assays. YMDD mutants in 24 samples and YVDD mutants in one sample were detected by pyrosequencing, while YMDD mutants in 22 samples and YVDD mutants in three samples were detected by real-time PCR. The percentage of mutants in the virus population obtained by real-time PCR in the three YVDD samples was 20%, 7% and 6% respectively. Twenty-four types of YIDD mutant and two mixed types of YIDD and YVDD mutants were detected by pyrosequencing, while 21 types of YIDD mutants and 5 mixed types of YIDD and YVDD mutants were detected by real-time PCR. The results were identical in 18 YVDD samples obtained by pyrosequencing, but YVDD mutants were detected in one sample by sequencing and pyrosequencing while YMDD mutants were detected in one sample by real-time PCR.

Among the five samples containing mixed types of YIDD and YVDD mutants by real-time PCR, mixed mutants were detected by pyrosequencing whereas only dominant mutants were detected by sequencing in two samples.

Time required

The time required for each assay of a certain number of samples was dependent on the throughput of the instruments used for detection. We measured the time required for each assay based on a 4-sample run because the highest throughput of ABI 3130 sequencer used in this study was 4 samples per run. The total assay time for sequencing, porysequencing and real-time PCR was 5, 4.5 and 2 h, respectively.

Costs

The cost for each assay was calculated based on the prices of the reagents in China. The sequencing reagents were from Applied Biosystems (Applied System, Foster, CA, USA). Pyrosequencing reagents were from Pyrosequencing AB (Pyrosequencing AB, Uppsala, Sweden). Primers and probes were synthesized in TaKaRa Biotech (TaKaRa, Dalian, China). Real-time PCR mixtures were from Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The total reagent cost for each sequencing, pyrosequencing and real-time PCR was 120, 150 and 80 Yuan RMB, respectively.

Table 2 Comparison of results obtained by sequencing, pyrosequencing and real-time PCR *n* (%)

Type	Sequencing	Pyrosequencing	Real-time PCR
rtM204	25 (36)	24 (35)	23 (33)
rtM204I	26 (38)	24 (35)	21 (30)
rtM204V	18 (26)	19 (27)	20 (29)
rtM204I + rtM204V	0 (0)	2 (3)	5 (8)

DISCUSSION

Lamivudine is one of the first-line medicines for chronic hepatitis B. Mutations in the YMDD motif of polymerase gene have been detected in patients with chronic hepatitis B treated or untreated with lamivudine[14,17-19]. Clinical breakthrough can be observed 2 wk to 7 mo after the emergence of YMDD mutations[7,9,19-20]. Acute and severe exacerbation due to YMDD mutations can cause considerable morbidity and mortality[21-24]. Lamivudine therapy after the emergence of YMDD mutations has no effect on chronic hepatitis B patients[25]. Antiviral agents, such as adefovir and entecavir, are effective against lamivudine-resistant HBV[26-28]. Pegylated interferon is also one of the first-line therapeutic options for hepatitis B virus infection and can induce sustained responses in some of lamivudine-resistant patients[29-31]. Monitoring YMDD mutation during lamivudine therapy contributes to the clinical decision of treatment of chronic hepatitis B patients.

Many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B[8]. However, they are different in sensitivity, specificity, cost, and time required. Sequencing of PCR products is a conventional assay and pyrosequencing is a high throughput method that can detect minor sequence variants. In the present study, we compared sequencing of PCR products, pyrosequencing, and real-time PCR for detection of YMDD mutations in chronic hepatitis B patients treated with lamivudine. The results obtained by sequencing, pyrosequencing and real-time PCR were completely concordant in 60 (87%) patients. YVDD mutants were detected by real-time PCR in three samples. The percentage of the mutants in total viruses in the three samples was 20%, 7%, and 6%, respectively, all of which were below the detection limit of sequencing[9]. YVDD mutants were detected by pyrosequencing in the sample with 20% of mutants. These results suggest that real-time PCR is more sensitive, cost-effective and time saving than sequencing and pyrosequencing. In one sample, YVDD mutants were detected by sequencing and pyrosequencing and YMDD mutants were detected by real-time PCR. Further study of the clinical data of the sample revealed that it contained 620 copies/mL of HBV DNA, which was below the limit of real-time PCR assay[14].

Pyrosequencing is a high throughput, non-gel-based DNA sequencing technique based on real-time detection of the released pyroghosphate during DNA synthesis[10]. Pyrosequencing is widely used in detection of single nucleotide polymorphisms (SNP) in the human genome[32].

However, pyrosequencing only can detect DNA sequence with a length of no more than 40 base-pair, while sequencing of PCR products can detect much longer DNA sequences. Real-time PCR detects specific point mutations recognized by the primers or probes.

The time required for each assay is dependent on the throughput of instruments used. In the present study, ABI 3130 was used for sequencing of PCR products. Its highest throughput was 4 samples per run. The instruments used for pyrosequencing and real-time PCR were 96-well plates and had the highest throughput of 96 samples and 32 samples, respectively. Although pyrosequencing has the highest throughput, relatively few laboratories have used pyrosequencing equipments[10]. Real-time PCR assay is well suited for routine screening of YMDD mutants because real-time PCR systems are routinely used in many laboratories.

For detection of mutants in virus population, real-time PCR can calculate the ratio of mutants to total viruses[14], while sequencing and pyrosequencing only can estimate the ratio by the signal intensity. The background noises affect the results. When serum HBV-DNA is below 10^8 copies/mL, no false positive results have been observed[14]. However, the accuracy of the ratios calculated in real-time PCR can be affected by variations in the ΔCt value, especially in cases where mutants are above 50% in the virus population.

In conclusion, among the three assays studied, real-time PCR is the most sensitive, cost-effective, and time saving method for monitoring YMDD mutations in patients with chronic hepatitis B on lamivudine therapy.

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