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Original Article

Detection of Hepatitis B Virus M204V Mutation Quantitatively via Real-time PCR

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Abstract

Background and Aims: Drug-resistant DNA mutations of the hepatitis B virus (HBV) affect treatment response in chronic hepatitis B patients. We have established a new, sensitive, specific, accurate and convenient real-time PCR method to detect HBV mutations quantitatively. ***Methods:*** Blood samples were collected from patients showing viral breakthrough, primary nonresponse, or poor response during treatment, and mutations were detected via direct sequencing to assess our method. A plasmid containing the M204V mutation was synthesized and standard curves plotted. ***Results:*** The determination coefficient for linear correlation between Ct and log plasmid copy numbers was 0.996, where Ct value was −3.723log (DNA concentration) +48.647. Coefficients of variation indicated good reproducibility. Correctness was within tolerable bias. Limit of detection was 10^3 copies/mL. Specificity, accuracy, positive predictive value and negative predictive value were 92.86%, 100%, 96.88%, 100% and 94.74%, respectively. ***Conclusions:*** These results show that our method can be used to detect HBV M204V mutations with the advantages of sensitivity, specificity and efficiency, providing a new choice for monitoring drug resistance.

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Introduction

Hepatitis B virus (HBV) infection remains a global disease, with significant morbidity and mortality.[1,2] One of the main treatment options for patients infected with chronic hepatitis B (CHB) is the nucleotide analogs (NAs). Currently, NAs approved for clinical use in China include lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF).[3] However, antiviral resistance is the most important factor in the failure of hepatitis B treatment.

The occurrence of drug resistance may lead to recurrence of hepatitis, and even cause progression of the disease, including virological breakthroughs, biochemical rebounds, hepatitis condition aggravation, and even hepatic failure.[4,5] Long-term drug resistance monitoring of patients with CHB who are taking LAM showed the proportion of hepatitis recurrence increased significantly compared to patients without drug resistance; in addition, the incidence of compensation cirrhosis was increased in patients with long-term drug resistance.[6,7] Not only for its impact on hepatitis, the occurrence of drug resistance will also lead to an increase in the incidence of hepatitis B-related liver cancer. A meta-analysis showed that the incidence of liver cancer in patients with LAM resistance is significantly higher than in treatment-naive patients (42/594 vs. 126/3,287, p=0.001).[8] Thus, the occurrence of drug resistance mutations not only limits the choice of treatment options and increases the cost of treatment but is also closely related to the progression and prognosis of the disease.

Antiviral drug-resistant HBV variants occur spontaneously in CHB patients following exposure to NAs.[9,10] Genotypic antiviral resistance refers to the presence of unique nucleotide mutations in drug target genes, which are the HBV polymerase genes that have been shown to be associated with antiviral resistance during HBV treatment with NAs. There are two types of mutations associated with drug resistance: primary resistance mutations and compensatory mutations. Primary resistance mutations directly lead to a decrease in the sensitivity to the drug, while the compensatory mutations can restore or enhance the replication of virus. The mutations rtM204V/I represent one of the most common primary resistance mutations in hepatitis B patients, which directly decrease the susceptibility to NAs, especially to LAM and LdT.[11–15]

LAM is the most widely used and longest-serving antiviral drug, but it also has the highest resistance rate. Recent studies have indicated that the percentage of LAM-associated resistance mutations that appear after 1 year

Keywords: HBV DNA; Drug-resistance mutation; Real-time PCR; DNA sequencing.

Abbreviations: ADV, adefovir dipivoxil; CHB, chronic hepatitis B; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; LdT, telbivudine; NAs, nucleotideanalogs; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate.

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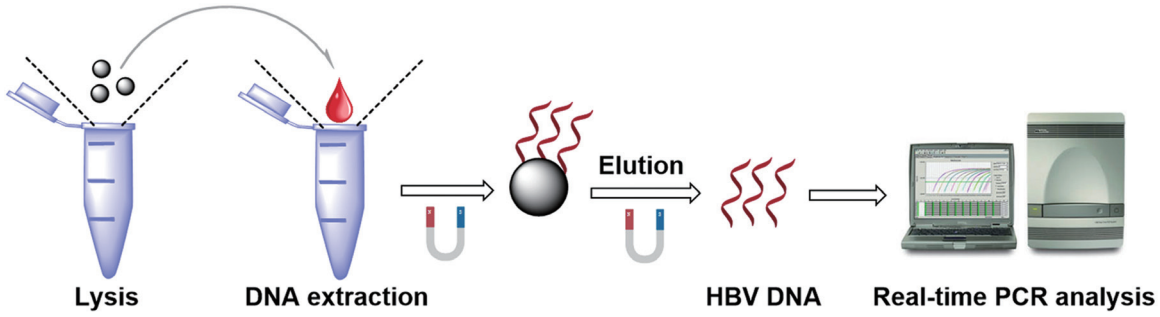

Fig. 1. Process of real-time PCR based on magnetic nanoparticles for detecting HBV DNA.

may vary from 7% to 30%.[15–17] The accumulation rate of LAM-associated drug resistance following 5 years of therapy is 70%.[7] As a recommended first-line drug, ETV is also widely used in clinical practice. In addition to a background of the mutations rtM204V/I, the other mutations, such as rtI169T, rtS184G, rtS202I and rtM250V, are associated with the emergence of ETV resistance.[18–21] A retrospective study conducted in China from 2009 to 2016 demonstrated that 73.7% of male CHB patients developed HBV genotypic resistance mutations to NAs, and that ETV-associated resistance increased to 17.1% in 2016.[22] Moreover, rtM204V combined with some other mutations may lead to a resistance to TDF.[23] Due to the widespread use of the above-mentioned drugs, drug-resistant specimens are the most readily available. Therefore, we chose mutations rtM204V/I to be detected.

Real-time PCR, a relatively new quantitative testing technique, includes a new platform that allows the initial concentration of the sample template to be estimated. In contrast to conventional PCR, it uses fluorescent dyes or specific fluorescent labeled probes to monitor the reaction process and illustrate the amount of DNA present, in real time, at each cycle of amplification. Two methods are used to calculate the initial DNA concentration: absolute quantitation and relative quantitation. Relative quantitation is used to determine the relative changes in expression in a similar target nucleic acid sequence and the correction sample. Absolute quantitative analysis determines the absolute value of a nucleic acid sequence in a sample.[24–28] Real-time PCR is widely used in molecular diagnostics to detect and identify bacteria and viruses. In addition, because real-time PCR is quantifiable, it is used to evaluate disease progression and efficacy of antiviral/antibiotic therapies.

The current study developed a real-time PCR assay aimed at quantitatively detecting HBV mutations. The schematic diagram of this work is shown in Fig. 1.

Methods

Sample preparation

We collected blood samples from patients treated with NAs, especially LAM and ETV, showing viral breakthrough (defined as a confirmed increase in HBV DNA levels of more than 1 log10 copies/mL compared to the lowest HBV DNA level on-therapy), primary nonresponse (defined as less than 1 log10 copies/mL decrease in the HBV DNA level from baseline following 12 weeks of therapy), or poor response (defined as more than 1 log10 copies/mL decrease in the HBV DNA level from baseline but detectable following at least 12 months of therapy) in response to NAs at the Beijing Friendship Hospital between 2015 and 2019. The exclusion criteria were as follows: co-infection with other hepatitis viruses or/and human immunodeficiency virus; poor treatment compliance; and patients who refused to participate in the experiment.

The study was approved by the Medical Ethics Committee of Beijing Friendship Hospital, affiliated with Capital Medical University.

Process for the detection system

DNA extraction: HBV DNA was extracted using nano-magnetic beads according to the following steps: nano-beads and heated lysis buffer were mixed in a 70 °C water bath to dissolve insoluble substances, and then mixed evenly before use. A 100 μL serum sample was added to a new centrifuge tube and mixed with 400 μL of cracking buffer solution, and was heated for 10 m at 70°C and mixed every 2–3 min. Subsequently, 300 μL of binding buffer and 20 μL of magnetic beads were added to the above centrifuge tube in turn, and mixed. The mixture was left to stand at room temperature for 5 m, with mixing once every 2 m. Next, 200 μL each of cleaning buffers I, II and III were added to the centrifuge tube, respectively, a magnet was used to adsorb the nano-beads for 30 s, and the supernatant was discarded. Centrifugation at high speed was used to separate the nano-beads from the mixture, followed by extraction. Next, 50 μL eluting buffer was added to the centrifuge tube, heated to 70°C and left for 5 m at 70°C. Nano-beads adsorbed on the magnets and supernatant fluid containing DNA were transferred to an RNA-free centrifuge tube and stored at −20°C for further use.

Primers and probes of M204V

Primers and probes were designed by the Wawasye Nanotech Company (Wuhan, China) and synthesized by Sangon Biotech (Shanghai, China). The probes were quenched using BHQ1 or MGB at the 3′-end (Table 1). Better probes were selected by subjecting the positive serum, containing the mutant gene of HBV polymerase M204V previously confirmed by Sanger sequencing, to a series of tests.

Preparation of the reaction system

We blended 12.5 μL of SYBR Premix (2x) with 0.5 μL of forward primer, 0.5 μL of reverse primer, 0.1 μL of specific probe and 9.4 μL of deionized water as a reaction system. Subsequently, we added 2 μL of template DNA to the experimental group and 2 μL of deionized water to the control

Table 1. Primer and probe sequences with their respective dye and quencher

	Target gene	Name	Sequences
Primer		primer forward	5′-GCACTTGTATTCCCATCCCATCAT-3′
		primer reverse	5′-AGCAAAGCCCAAAAGACCCACAAT-3′
Probe	M204	204-2-P	5′-FAM-TCTGTACAACATCTTGAGTCCCTT-BHQ1-3′
	M204	204-2-CP	5′-FAM-TRAACCCTAATAAAACCAAACGTTGG-BHQ1-3′
	M204V	204-2-VP	5′-FAM-CATCATCCACATARC-BHQ1-3′
	M204I	204-2-IP	5′-FAM-CCACATCATCAATATA-BHQ1-3′
	M204V	204-2-VP2	5′-FAM-CATCATCCACATARC-MGB-3′
	M204I	204-2-IP2	5′-FAM-CCACATCATCAATATA-MGB-3′

group in order to obtain a final reaction volume of 25 μL each.

PCR amplification detection of mutations in 204 HBV nucleotides

Amplifications were processed using the FAMTM/SYBR® Green channel of the SLAN®-96P Real-Time PCR System (Shanghai Hongshi Medical Technology, Shanghai, China). Cycling conditions were as follows: 5 m at 95°C, followed by a two-step cycling stage of 40 cycles of 15 s at 95°C and 60 s at 59°C. Samples were considered positive when presenting a typical amplification curve with a Ct value of ≤35. Analyses of samples with Ct values of 35 and 38 were repeated.

Plasmid design and standard curve protraction

In order to obtain standard curves for the purposes of quantification and estimation of efficacy of the new real-time PCR protocol, a specific plasmid containing the M204V mutation was designed and synthesized by Sinogene Biotech (Beijing, China). Standard curves based on a 10-fold dilution of the plasmid were plotted. The formula [DNA (copies/μL)=6.02×10^{23} (copies/mol)×DNA concentration (μg/μL)/[DNA length (bp)×660(daltons/bp)] was used to calculate the DNA load. Next, we analyzed linear relationships based on amplification results. Log DNA concentration (horizontal axis; X) was plotted against Ct values (vertical axis; Y).

Results and discussion

We enrolled 32 CHB patients with drug-resistant gene mutations. The patients' information is shown in Table 2.

We designed two different probes for 204 nucleotide mutations, to ensure the presence of a mutation, and subsequently designed two specific probes each for M204V or M204I. The specificity and sensitivity of the probes were confirmed via the amplification of positive serum samples, which had been verified as containing the M204V/I mutation via Sanger sequencing. We repeated a portion of the experiments on the same specimen in order to verify its accuracy and repeatability. An acceptance criterion of Ct <38 was used. According to the results of 16 positive serum samples, the 204-2-P probe was more specific and sensitive. The true positive rates were 100% and 62.4%, respectively. Both 204-2-VP and 204-2-IP were abandoned due to the absence of a fluorescent signal. The specificity and sensitivity of probes 204-2-VP2 and 204-2-IP2 were confirmed. The positive rate of detection was 100% and the false positive rate was 0%.

Amplification curves of the 10-fold dilution series of the plasmid are shown in Fig. 2. The efficiency of quantitative real-time PCR was confirmed via plasmid-based curves. The plasmid DNA concentration was 2,000 ng/μL and DNA length was 2,980 bp. We introduced relevant parameters to the formula, stated above, to obtain the initial DNA load. The serial dilution curves indicated that the relationship between Ct value and log DNA concentration was linear (Fig. 3). The linear coefficient of determination (R^2) was 0.996, indicating a significant linear relationship for the quantitative real-time PCR. The slope of the standard curve was −3.723.

We diluted plasmid concentrations to 6.12×10^6, 6.12×10^5, and 6.12×10^4 copies/mL to determine the reproducibility of real-time PCR. Six repetitions of the dilution series were analyzed. The coefficients of variation of log DNA concentration were 0.43%, 0.69%, and 0.58%, respectively, which indicate good reproducibility.

The accuracy of real-time PCR was determined via a 10-fold dilution series ranging from 6.12×10^6 to 6.12×10^2 copies/mL. The plasmids were diluted using a mixture of serum samples from patients with CHB. The average log DNA concentration of five replicates tested simultaneously under similar operating conditions was estimated. The biases were −0.174, −0.085, 0.005, and 0.06, respectively, which indicated good correctness.

In order to determine the limit of detection, the plasmids were diluted to 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies/mL. A Ct value <38 and a standard deviation of 0.5 were considered as the reference standard. The coefficients of variation of log DNA concentration were 0.43%, 0.69%, 0.58%, 2.79%, 5.54%, and 4.89%, respectively. The standard deviations of log DNA concentration were 0.03, 0.04, 0.03, 0.11, 0.16, and 0.12, respectively. The positive rates of detection were 100%, 100%, 100%, 100%, 83.3%, and 50%, respectively. Thus, the limit of detection for real-time PCR was determined to be 10^3 copies/mL.

A comparative analysis of 32 serum samples from CHB patients suspected of showing signs of drug-resistance was conducted. Both real-time PCR and direct sequencing protocols indicated that 13 out of the 32 samples (40.625%) were M204V-positive. One sample (3.125%) was identified as M204V-positive by direct sequencing, whereas the real-time PCR results could not be evaluated. Eighteen samples (56.25%) were identified as M204V-negative by both real-time PCR and direct sequencing. None of the samples were found to be M204V false positives. Direct sequencing is considered as the gold standard. Overall, the sensitivity of real-time PCR for detecting the M204V mutation was 92.86% (13/14), with a specificity of 100%.

Table 2. Detailed information of the 32 patients

No.	Sex	Genotype	HBV viral load, copies/mL	Mutations
1	Male	C	2.03×10^5	M204V, L180M
2	Male	B	1.317×10^5	M204V, L180M
3	Male	C	1.2×10^3	M204V, L180M
4	Female	C	1.4×10^4	M204I, L180M
5	Male	B	2.5×10^4	M204I, L180M
6	Male	B	3.03×10^2	M204I
7	Female	B	1.99×10^2	M204V, L180M, T184A
8	Female	C	4.33×10^3	M204V, M204I
9	Male	C	4.33×10^3	M204V, M204I, L180M
10	Male	C	2.3×10^7	M204V, L180M
11	Female	C	1.8×10^5	M204I
12	Female	C	10^7	M204I
13	Male	C	3.03×10^3	M204V, L180M, S202G, T184A
14	Female	B	4.79×10^3	M204V, L180M, S202G
15	Male	C	3.6×10^7	M204V, L180M, T184L
16	Female	C	2.2×10^7	M204V, L180M, T184A
17	Male	B	6.4×10^3	A181T, N236T
18	Female	B	$4.\ 3\times10^5$	A181V
19	Female	B	3.07×10^5	A181T
20	Male	C	1.68×10^4	A181T, A181V, N236T
21	Male	C	8.5×10^4	A181V
22	Male	C	2.2×10^5	M204V, L180M
23	Male	C	1.07×10^3	M204V, L180M
24	Male	C	1.8×10^3	M204V, L180M
25	Male	C	4.25×10^7	M204I, L180M
26	Female	B	3.51×10^3	M204I, L180M
27	Male	C	1.45×10^3	M204I
28	Male	C	4.2×10^4	A181T, N236T
29	Male	C	2.09×10^3	A181V
30	Male	B	5.2×10^2	A181T
31	Female	B	2×10^3	A181T, A181V, N236T
32	Female	B	1.29×10^3	A181V

Conclusions

The current study established a practical quantitative real-time PCR that monitors HBV DNA polymerase gene mutations in patients undergoing treatment with NAs, with particular reference to the M204V mutation. Compared with direct sequencing, quantitative real-time PCR is effective, low-cost, and convenient. It does not involve complex and expensive instruments. Furthermore, our quantitative detection system, which gathers a fluorescence signal from each cycle, is rapid, as each DNA test requires only 6–7 m. Moreover, the limit of detection is similar to that of direct sequencing. This new method is able to monitor HBV DNA variations quantitatively, thus providing a new method that monitors drug resistance during the early stages of therapy and assesses the relationship between genetic mutations and phenotypic resistance.

Tests that determine the limit of detection are insufficient, requiring many further tests to confirm the limitation. Further assays are required to develop systems designed to monitor other mutations, and also to establish a system for multiplex detection purposes.

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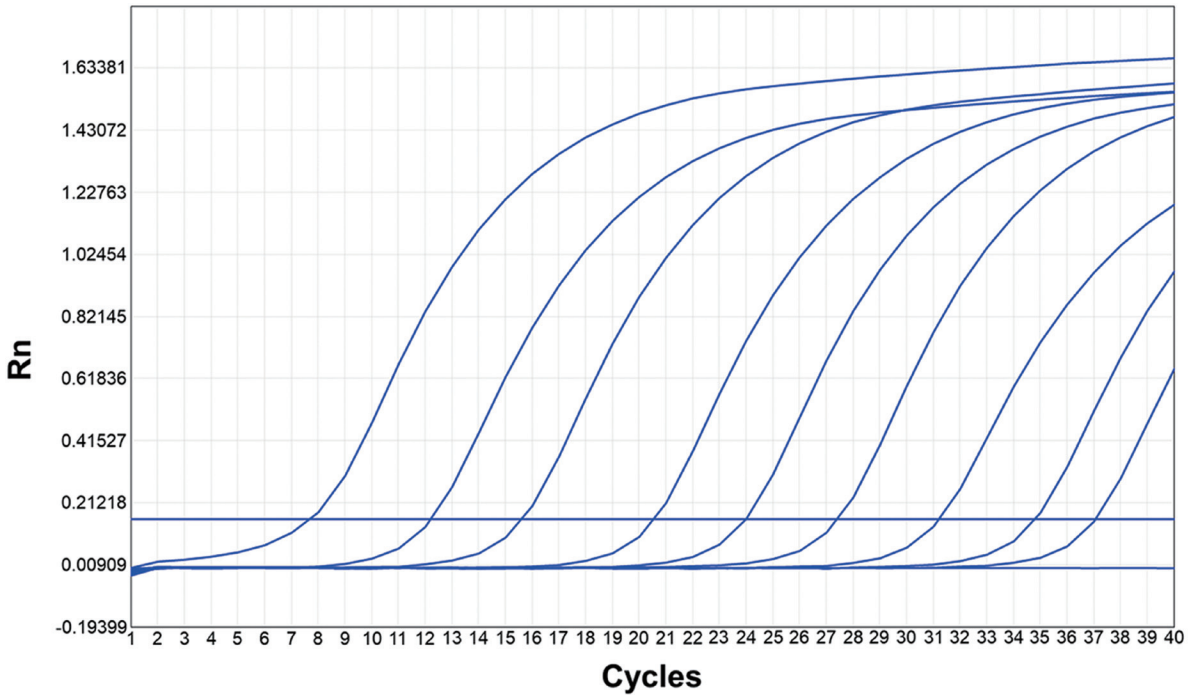

Fig. 2. Amplification curves of plasmids.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design (JJL, HM, YW), performance of experiments (JJL, LN), analysis and interpretation of data (JJL, GC), manuscript writing (JJL, GC), statistical analysis (JJL, GC, XML), critical revision of the manuscript (HM, YW), critical funding (HM, YW), and technical or material support (HM, LN, YW).

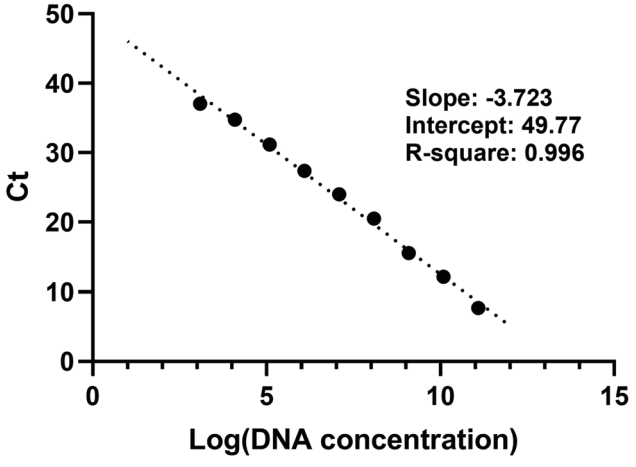

Fig. 3. Linear relationship between Ct values and log DNA concentrations.

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