Original Article
An allele specific locked nucleic acid real time quantitative PCR for detection of HBV rtA181V and rtN236T mutations

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Abstract: Background: Drug-resistant mutations of hepatitis B virus (HBV) are the major causes for unsuccessful therapy for chronic hepatitis B infection (CHB). Adefovir dipivoxil (ADV) has been widely used in patients who failed to lamivudine (LMV) treatment. Early detection of ADV-resistant mutations is of great clinical significance. In this study, we established an easy-to-use approach, real time allele specific locked nucleic acid quantitative PCR (RT-AS-LNA-qPCR) for early quantification of the rtA181V and rtN236T mutations associated with resistance to ADV and focused on its performance evaluation. Methods: Four recombinant plasmids for rtA181 and rtN236 mutations were constructed. The assay was established and evaluated with standard recombinant plasmids and 102 serum samples from patients who experienced with ADV. Results: The linear range of the assay for the detection of rtA181V and rtN236T was between 1×10^9 copies/μl and 1×10^2 copies/μl. Sensitivity of the assay was 10^-5 in the wild-type background of 1×10^7 copies/μl. The detection sensitivity of the assay was 0.03% in the detection of clinical samples. RT-AS-LNA-qPCR had a high concordance with direct sequencing in detecting mutations associated with resistance to ADV. RT-AS-LNA-qPCR was more sensitive than direct sequencing in detecting minor variants which could detect these mutations earlier. Conclusions: RT-AS-LNA-qPCR assay was able to sensitively and specifically detect the rtA181V and rtN236T mutations associated with resistance to ADV. This easy-to-use approach may be a useful tool for monitoring ADV resistance mutations in patients with chronic HBV infection and for optimization of ADV therapy.

Keywords: Hepatitis B virus, mutation, adefovir dipivoxil, locked nucleic acid, polymerase chain reaction

Introduction
Approximately 240 million people worldwide are chronically infected with hepatitis B virus (HBV) and more than 75% of those reside in Asia-Pacific area, especially in China [1]. Chronic HBV carriers may lead to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1, 2]. Oral Nucleoside/nucleotide analogues such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir (TDF) are widely used for the treatment of CHB [3]. Although recent treatment guidelines recommend that entecavir and tenofovir are the first line treatment for CHB, ADV is still widely prescribed in some developing countries, such as China, due to the effect on lamivudine-resistant mutations occurring upon prolonged treatment and the economic considerations in rural areas in China. Unfortunately, HBV resistance to ADV develops and increases overtime. The most important mutations associated with ADV resistance are rtA181V and rtN236T, respectively found within the Band D functional domains of the HBV reverse transcriptase [4]. Researches have indicated that the cumulative annual incidence of genotypic resistance to ADV was estimated to be 3, 9, 18 and 28% after 2, 3, 4 and 5 years of treatment, respectively [5]. Consequently, early and periodic detection of HBV drug-resistant mutants has received much attention due to its importance for the strategic treatment of chronic hepatitis B virus-infected patients. Many available assays such as line probe assay (LiPA), next-generation sequencing (NGS), selective real-time PCR (sPCR), mass spectrometric analysis (MS), coamplification at lower denaturation temperature (COLD)-PCR etc. for detecting ADV-resistant minority variants have their respective advantages and disadvantages [6-8]. Conventional sequencing fails to detect
minority populations in less than 20% of a total viral population [9]. INNO-LiPA and MS are capable of detecting minor variants, but more strict experiment conditions and instruments are required [7].

Herein our aim was to establish an easy-to-use approach, real time allele specific locked nucleic acid quantitative PCR (RT-AS-LNA-qPCR) for early quantification of the rtA181V and rtN236T mutations associated with resistance to ADV and focused on its performance evaluation.

Materials and methods

Subjects

A total of 102 chronic HBV patients who were undergoing ADV mono-therapy were recruited in the Center of Liver diseases of the First Affiliated Hospital of Fujian Medical University from October 2014 to May 2016. Of these patients, 71 (69.6%) were males and 31 (30.4%) were females with an average age of 46.59 ± 12.95 years old. Serum samples were collected at baseline and every 12 weeks. All the recruited patients met the following criteria: I) presence of HBV DNA in serum and an elevation of serum ALT at least 2 times higher than the normal level during the year prior to the initiation of the study; II) hepatitis B surface antigen (HBsAg) positive inserum for at least six months. All investigations were conducted with the approval of the ethics committee of the First Affiliated Hospital of Fujian Medical University and according to the tenets of the Declaration of Helsinki. Written informed consent was also obtained before collecting serum samples and studying the clinical feature of each patient.

Table 1. Primers for RT-AS-LNA-qPCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
<th>Positions (nt)</th>
<th>Length (bp)</th>
<th>Amplicon size (bp)</th>
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<td>21</td>
<td></td>
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<tr>
<td>181LO</td>
<td>CCTCAGTCGTTTCTCTGTGG+C</td>
<td>651~671</td>
<td>21</td>
<td>137</td>
</tr>
<tr>
<td>181LA</td>
<td>CCTCAGTCGTTTCTCTGTGG+T</td>
<td>651~671</td>
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<tr>
<td>KF236</td>
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<td>595~614</td>
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<td>236LO</td>
<td>CAWCKTTTGTGTTTTRKAGGG+T</td>
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<td>CAWCKTTTGTGTTTTRKAGGG+G</td>
<td>839~860</td>
<td>22</td>
<td>266</td>
</tr>
</tbody>
</table>

KF181/KF236 indicate the same common forward primers to each reaction; 181LO/181LA/236LO/236LA indicate specific RT-AS-LNA-qPCR reverse primers. Y indicates C/T; K indicates G/T; W indicates A/T; R indicates A/G; +C/+T+G indicate LNA nucleosides.

HBV DNA extraction from serum samples

HBV DNA was extracted from 200 μl of serum using QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and stored at -20°C until used.

Primers

PCR primers containing LNA were designed using PrimerPremier 5.0 software (Premier Biosoft International, USA) along with the principle of allele-specific PCR (AS-PCR), general principles for LNA primer design rules provided by Exiqon (http://www.exiqon.com/oligo-tools) and the HBV reverse transcriptase polymorphism analysis of the most common genotypes (B and C) in China obtained from GenBank. Briefly, the forward primers, KF181 and KF236, at nucleotide positions 767~787 and 595~614, were for the amplification of both wild-type and mutant DNA of rtA181 and rtN236, respectively. The reverse primers modified with a LNA (LNA primer) at the 3’-end terminal position (L181O, L181A) were designed to detect wild type and the mutant type of rtA181 mutations, respectively. Similarly, LNA primers for wild type and rtN236 mutations (L236O, L236A) were also designed (Table 1). Blast analysis was performed to demonstrate the specificity of the primers.

Table 1. Primers for RT-AS-LNA-qPCR

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KF181/KF236 indicate the same common forward primers to each reaction; 181LO/181LA/236LO/236LA indicate specific RT-AS-LNA-qPCR reverse primers. Y indicates C/T; K indicates G/T; W indicates A/T; R indicates A/G; +C/+T+G indicate LNA nucleosides.

Standard plasmid DNA construction

Four clinical samples sequenced harboring wild-type and mutant DNA sequences of rtA181 and rtN236 were used as templates for plasmids construction. Target DNA was amplified with primers (KF, L) listed in Table 1. Then PCR products were cloned into pMD 18-T vector (Takara, Japan) and transformed into E. coli DH5α competent cell (Takara, Japan). The positive plasmids were sequenced to confirm the presence of the expected sequences and then isolated using TIANprepMini Plasmid kit (Tiangen Biotech Company, Beijing, China). Plasmids were quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The corresponding copy number was calculated and 10-fold serially diluted from 1×10^{10} cop-
Figure 1. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtA181. A. The concentration from left to right were 1×10^9 copies/μl to 1×10^2 copies/μl, respectively. B. Standard curve of rtA181: Y=-3.651X+45.843 ($R^2=0.987$). The amplification efficiency was 94.904%.

Figure 2. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtA181V. A. The concentration from left to right were 1×10^9 copies/μl to 1×10^2 copies/μl, respectively. B. Standard curve of rtA181V: Y=-4.093X+41.928 ($R^2=0.995$). The amplification efficiency was 95.510%.
Figure 3. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtN236. A. The concentration from left to right were $1 \times 10^9$ copies/μl to $1 \times 10^2$ copies/μl, respectively. B. Standard curve of rtN236: $Y = -4.048X + 44.374$ ($R^2 = 0.996$). The amplification efficiency was 96.807%.

Figure 4. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtN236T. A. The concentration from left to right were $1 \times 10^9$ copies/μl to $1 \times 10^2$ copies/μl, respectively. B. Standard curve of rtN236T: $Y = -3.548X + 39.439$ ($R^2 = 0.99$). The amplification efficiency was 95.349%.
RT-AS-LNA-qPCR for HBV mutation detection

RT-AS-LNA-qPCR was performed on the ABI 7500 Instrument (Applied Biosystems) using the SYBR® Premix Ex Taq™ (Takara, Japan). Amplification was carried out in a final volume of 25 μl containing 12.5 μl of SYBR Green Mix (Takara, Japan), 0.7 μl of each primer (10 μM), 0.5 μl of 50×ROX II reference dye (Takara, Japan), 8.6 μl of ddH₂O and 2.0 μl of DNA template. Real-time PCR conditions were set as follow: 30 s at 94°C, followed by 40 cycles of denaturation for 5 s at 94°C and extension for 30 s at 60°C. All samples were amplified in duplicate. In each run, negative and positive controls and a standard curve were included.

Direct sequencing

All clinical samples were subjected to direct sequencing. The HBV DNA fragments containing the polymerase RT (reverse-transcriptase) domain were amplified using HBV sequencing kit (Shenyou, Shanghai) according to the manufacturer’s protocol. The PCR products used for direct sequencing were purified by the High PCR Product Wizard kit (Qiagen GmbH, Hilden, Germany) and sequenced with the ABI Prism 3130 Genetic Analyzer (Life Technologies, USA). Concordance was assessed by Cohen’s Kappa test. Results were considered complete concordance if RT-AS-LNA-qPCR and direct sequencing showed the identical results. Results were considered partial concordance if (I) RT-AS-LNA-qPCR provided additional information compared to that provided by sequencing, meaning that RT-AS-LNA-qPCR showed a mixture of wild-type and mutant sequences, whereas sequencing showed only one of the two results or (II) sequencing showed a mixture of wild-type and mutant sequences but RT-AS-LNA-qPCR showed only a wild-type or a mutant sequence. Results were considered complete discordance if one test showed a wild type and the other showed a mutant. All statistical tests were two-sided and the level of statistical significance was set at \( P<0.05 \).

Results

Identification of recombinant plasmids by sequencing

Recombinant wild-type and mutant plasmids of rtA181 and rtN236 containing inserts spanning the target region and specific single-base mutations were successfully constructed. The recombinant clones were picked out and sequenced. Then, the obtained DNA sequences were subjected to BLAST alignment against HBV genome database. The BLAST results showed the DNA sequence of the recombinant plasmids were consistent with the HBV reference sequence.

Performance of RT-AS-LNA-qPCR

Linear range: The 1×10^10 copies/μl - 1×10^1 copies/μl of each recombinant plasmid was used to test the linear range and detection limit of RT-AS-LNA-qPCR. As can be seen from Figures 1-4 (Figure 1 was taken as the typical representative, and similarly for the other figures), there was an excellent linear correlation between the cycle number and the HBV DNA copy number from the concentration of 1×10^9 copies/μl to 1×10^2 copies/μl.

Specificity test: The cross-reactivity test was performed using a dilution series of template DNA.
RT-AS-LNA-qPCR for HBV mutation detection

Figure 6. Cross-reactivity test of RT-AS-LNA-qPCR for rtN236. A. Specificity of mutant primer rtN236T (KF236/236LA). No nonspecific amplification was observed. B. Specificity of wild-type primer rtN236 (KF236/236LO). Nonspecific amplification was detected when the concentration of mutant template was $1 \times 10^6$ copies/μl and higher, but the corresponding mismatch products decrease significantly.

Figure 7. The correlation between actual and calculated proportion of mutant plasmid DNA at concentration of $1 \times 10^7$ copies/μl for rtA181. There was a significant linear correlation ($R^2=0.9809$, $P=0.0001$) between actual proportion of mutant and calculated proportion of correlation for HBV standard plasmid DNA at concentration of $1 \times 10^7$ copies/μl.

Figure 8. The correlation between actual and calculated proportion of mutant plasmid DNA at concentration of $1 \times 10^7$ copies/μl for rtN236. There was a significant linear correlation ($R^2=0.9882$, $P<0.0001$) between actual proportion of mutant and calculated proportion of correlation for HBV standard plasmid DNA at concentration of $1 \times 10^7$ copies/μl.

Accuracy test: A mixture of known quantity of wild-type and mutant standard plasmids were used to generate different proportions of mutant DNA, and then quantitative analysis of both wild-type and mutant DNA by RT-AS-LNA-qPCR was performed. Actual proportion of mutant had a significant linear correlation with calculated proportion of correlation for HBV standard plasmid DNA at concentration of $1 \times 10^7$ copies/μl (Figures 7 and 8).

Reproducibility test: The High ($1 \times 10^7$ copies/μl), medium ($1 \times 10^5$ copies/μl) and low ($1 \times 10^3$ copies/μl) concentrations of YMDD and YIDD recombinant plasmids were used as templates for 20 separate and simultaneous measurements by RT-AS-LNA-qPCR. For the rtA181, the intra-assay coefficient of variation (CV) was 2.47%, 2.51% and 8.94% for wild-type plasmids and 3.61%, 1.61% and 5.82% for mutant-type plasmids. For the rtN236, the intra-assay CV was 3.95%, 3.35% and 7.39% for wild-type plasmids and 3.22%, 5.26% and 8.12% for mutant-type plasmids.

The quantitative assay was also performed for 20 days consecutively. For rtA181, the inter-
assay CV was 4.13%, 7.35% and 12.8% for wild-type plasmids and 5.9%, 7.87% and 8.61% for mutant-type plasmids. For rtN236, the inter-assay CV was 7.04%, 5.63% and 9.12% for wild-type plasmids and 4.53%, 8.79% and 13.85% for mutant-type plasmids.

Sensitivity test: We tested the quantitative sensitivity of RT-AS-LNA-qPCR with serial dilutions of mutant plasmids DNA mixed with $1 \times 10^7$ copies/μl concentration of wild-type DNA (Figure 9). The results showed RT-AS-LNA-qPCR could accurately and steadily detect $1 \times 10^2$ copies/μl of mutant DNA in the $1 \times 10^7$ copies/μl concentration of wild-type DNA, indicating that the sensitivity of the assay was $10^{-5}$ in the wild-type background of $1 \times 10^7$ copies/μl.

Comparison of RT-AS-LNA-qPCR and direct sequencing

Sensitivity comparison using standard recombinant plasmids: Plasmids containing different proportions (50%, 25%, 20%, 15%, 10%, 5%, 1%, 0.5%, 0.05%, 0.04%, 0.03%) of mutations (rtA181V and rtN236T) were used to compare the sensitivity of RT-AS-LNA-qPCR and sequencing. RT-AS-LNA-qPCR could measure mutations at a proportion as low as 0.02% (detection limit) of the total population, while sequencing analysis can only measure at a proportion of 10% (Table 2).

Sensitivity comparison using serum samples

The RT-AS-LNA-qPCR assay was evaluated using serum samples from 102 patients treated with ADV. All samples were parallel tested by
RT-AS-LNA-qPCR for HBV mutation detection

Table 3. Concordance between RT-AS-LNA-qPCR and direct sequencing (rtA181)

<table>
<thead>
<tr>
<th>RT-AS-LNA-qPCR</th>
<th>Direct sequencing</th>
<th>WT</th>
<th>WT+Mut</th>
<th>Mut</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>83</td>
<td>0</td>
<td>0</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>WT+Mut</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Mut</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td>2</td>
<td>15</td>
<td>102</td>
<td></td>
</tr>
</tbody>
</table>

Complete concordant results are shown in bold. Partial concordant results are shown in italics. The two assays showed a high concordance (Kappa=0.623, P<0.001).

Table 4. Concordance between RT-AS-LNA-qPCR and direct sequencing (rtN236)

<table>
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<th>RT-AS-LNA-qPCR</th>
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<th>WT+Mut</th>
<th>Mut</th>
<th>Total</th>
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</thead>
<tbody>
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<td>WT</td>
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<td>Mut</td>
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<tr>
<td>Total</td>
<td>86</td>
<td>4</td>
<td>12</td>
<td>102</td>
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</table>

Complete concordant results are shown in bold. Partial concordant results are shown in italics. The two assays showed a high concordance (Kappa=0.708, P<0.001).

As shown in Table 3, for rtA181, among the 102 samples analyzed, 83 wild-type, 5 mutant-type and 14 mixtures of wild types and mutants were detected by RT-AS-LNA-qPCR. Compared with the results obtained from RT-AS-LNA-qPCR with that from sequencing, the complete coincidence rate was 88.2% (90/102), partial coincidence rate was 11.8% (12/102), and no complete discordance was observed. For rtN236, 83 wild-type, 6 mutant-type and 13 mixtures of wild types and mutants were detected by RT-AS-LNA-qPCR (Table 4). The complete coincidence rate was 91.2% (93/102), partial coincidence rate was 8.8% (9/102), and no complete discordance was observed. All the partial coincidence in results between the two methods were confirmed by subclone sequencing.

Discussion

Adefovir dipivoxil, a nucleotide analogue, has become a treatment option for CHB due to its effect on lamivudine-resistant mutations occurring upon prolonged treatment and the economic considerations in China. However, long-term therapy with ADV may lead to treatment failure because of the development of ADV resistance. Two mutations in the HBV polymerase gene, rtA181V and rtN236T, have been reported to confer resistance to ADV [4]. HBV drug resistance does not happen overnight. Instead, the emergence of a resistant strain is a stepwise process [2]. Previous research documented that minor pre-existing mutants could be gradually selected to become the dominant species and finally precede the occurrence of virological or biochemical breakthrough [10]. Therefore, developing a rapid, simple and reliable method to detect the ADV-resistant mutations as early as possible is of great clinical importance in clinical management of CHB.

Many assays recently have been described with their respective advantages and disadvantages. Locked nucleic acid (LNA) is an RNA homologue with a methylene bridge between the ribose 2-oxygen and 4-carbon atoms. LNA increases the melting temperature of LNA-DNA hetero duplexes by up to 3°C per LNA base, and thus, enhances the discriminatory power between match and mismatch under stringent annealing conditions [11]. Because of the increased Tm to improve the mismatch discrimination ability of PCR, LNA-modified nucleotides have been widely used for a variety of applications, including SNP genotyping [12]. In this study, LNA primers for quantitative amplification of HBV DNA, rtA181 and rtN236 mutations were designed, and the results showed that they could detect more than 10^2 copies/μl of variants. This study appears, to our knowledge, to be the first application of LNA PCR to quantify rtA181V and rtN236T mutations associated with ADV resistance. By using this method, it was possible to detect 0.001% of mutated strains in a wild type plasmid population. While the sensitivity was 0.03% when the assay was performed in the clinical sample. To assess the feasibility of this assay, wild type and mutant type of HBV rtA181 and rtN236 were tested in 102 patients with unsatisfactory response to ADV by direct sequencing. The results showed that the two assays had a high concordance. Moreover, RT-AS-LNA-qPCR assay could detect rtA181 and rtN236 mutations as well as some ADV mutations which were not identified by direct sequencing, thus supporting the new approach with a high concordance and a higher sensitivity. Beyond that, RT-AS-LNA-qPCR pro-
vides a low-cost, user-friendly method that can be easily implemented in clinical laboratories.

A limitation also exists for the RT-AS-LNA-qPCR assay though advantages of the assay over existing assays. We acknowledge that the specificity of wild-type primer is a little poor, the amplification of the mutant template with the wild-type primer occurred at a mutant viral concentration above $1 \times 10^6$ copies/μl. However, comparison of the perfect match primer, the nonspecific products amplified with mismatch primer declined 4 orders of magnitude. No nonspecific products occurred when mutant DNA level was below $1 \times 10^6$ copies/μl. To some extent, the error can be neglected because we focus mainly on minor mutants in high wild-type background and two separate PCR including wild-type and mutant reaction tubes were run in parallel.

In conclusion, the RT-AS-LNA-qPCR assay developed in this study was able to sensitively and specifically detect the rtA181V and rtN236T mutations associated with resistance to ADV. We believe that this easy-to-use approach may be a useful tool for monitoring ADV resistance mutations in patients with chronic HBV infections and for optimization of ADV therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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