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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 mutationsis 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/nucleotideanalogues 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

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

Primers	Sequences (5'-3')	Positions (nt)	Length (bp)	Amplicon size (bp)
KF181	GGGACTCAAGATGTTGTACAG	767~787	21	
181LO	CCTCAGTCCGTTTCTCYTGG+C	651~671	21	137
181LA	CCTCAGTCCGTTTCTCYTGG+T	651~671	21	137
KF236	CACYTGTATTCCCATCCCAT	595~614	20	
236LO	CAWCKTTTKGTTTTRTKAGGG+T	839~860	22	266
236LA	CAWCKTTTKGTTTTRTKAGGG+G	839~860	22	266

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.

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 inserum 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.

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 rtN236mutations (L236O, L236A) were also designed (**Table 1**). Blast analysis was performed to demonstrate the specificity of the primers.

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-

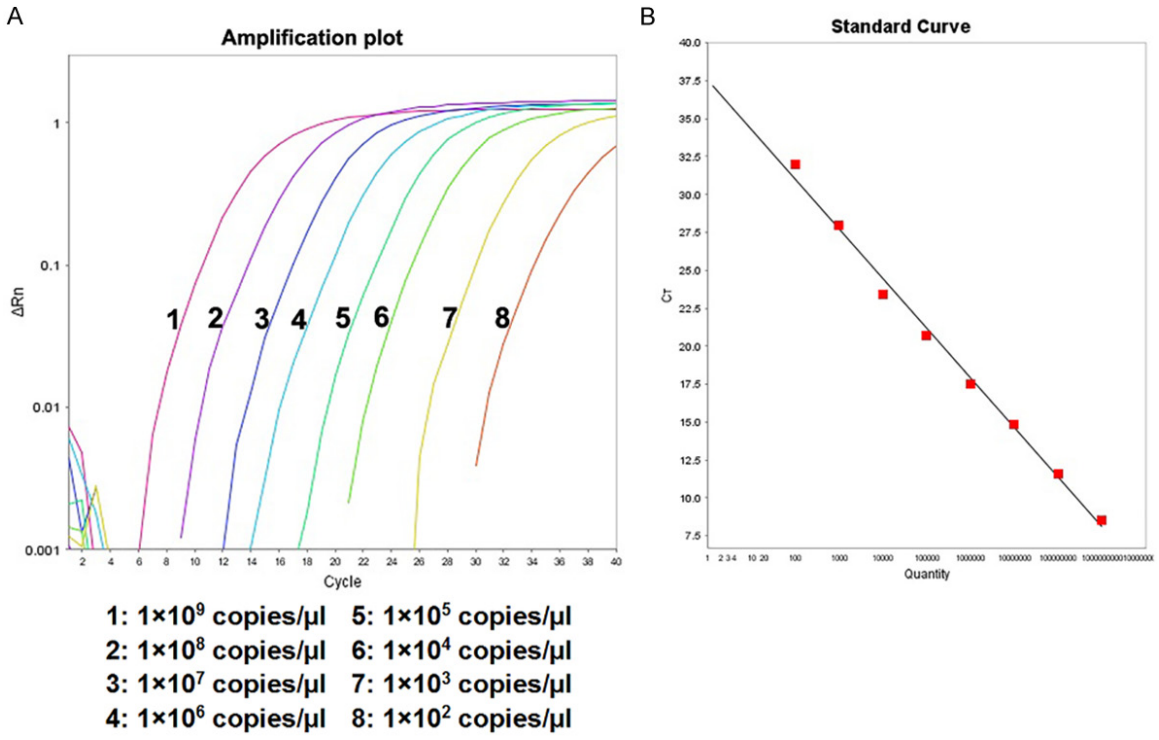

Figure 1. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtA181. A. The concentration from left to right were 1×10^9 copies/μl to 1×10^2 copies/μl, respectively. B. Standard curve of rtA181: Y=-3.651X+45.843 (R^2=0.987). The amplification efficiency was 94.904%.

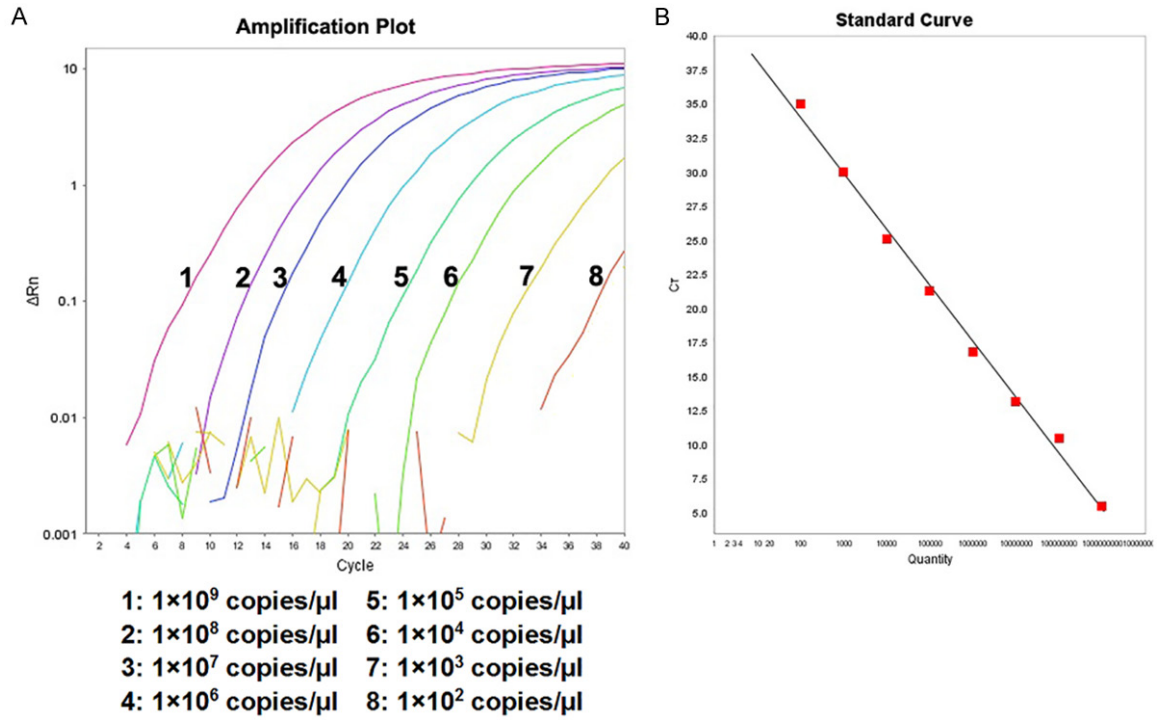

Figure 2. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtA181V. A. The concentration from left to right were 1×10^9 copies/μl to 1×10^2 copies/μl, respectively. B. Standard curve of rtA181V: Y=-4.093X+41.928 (R^2=0.995). The amplification efficiency was 95.510%.

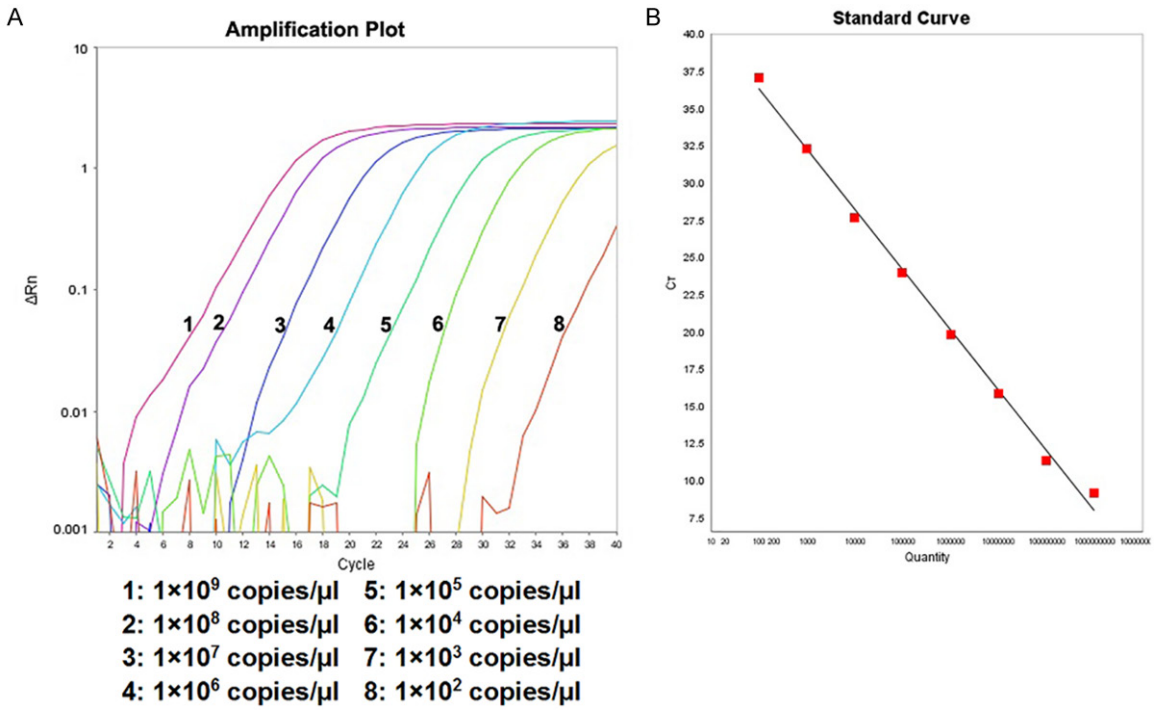

Figure 3. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtN236. A. The concentration from left to right were 1×10^9 copies/μl to 1×10^2 copies/μl, respectively. B. Standard curve of rtN236: Y=-4.048X+44.374 (R^2=0.996). The amplification efficiency was 96.807%.

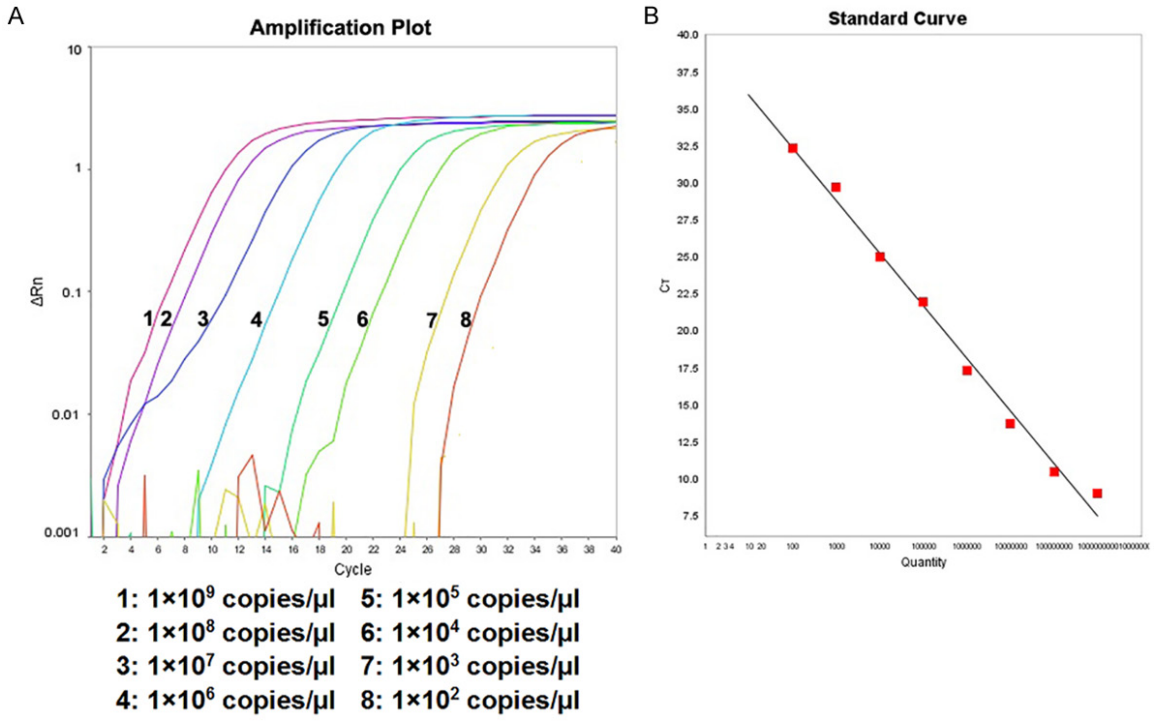

Figure 4. Amplification plot and standard curve of RT-AS-LNA-qPCR for rtN236T. A. The concentration from left to right were 1×10^9 copies/μl to 1×10^2 copies/μl, respectively. B. Standard curve of rtN236T: Y=-3.548X+39.439 (R^2=0.99). The amplification efficiency was 95.349%.

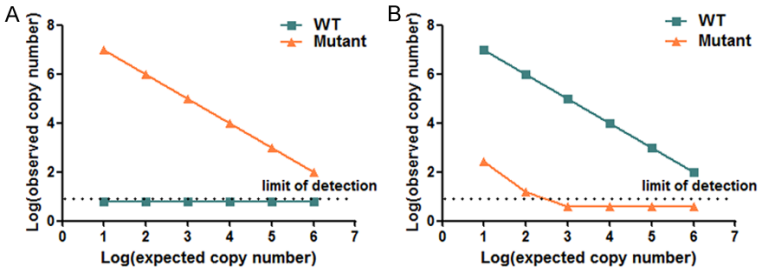

Figure 5. Cross-reactivity test of RT-AS-LNA-qPCR for rtA181. A. Specificity of mutant primer rtA181V (KF181/181LA). No nonspecific amplification was observed. B. Specificity of wild-type primerrtA181 (KF181/181LO). Nonspecific amplification was detected when the concentration of mutant template was 1×10^6 copies/μl and higher, but the corresponding mismatch products decrease significantly.

ies/μl to 1×10^1 copies/μl using Easy Dilution Buffer (Takara, Japan) to generate standard concentrations.

RT-AS-LNA-qPCR

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 ul 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 ddH_2O 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).

Statistical analysis

Data are expressed as the mean ± SD. Data was analyzed by the SPSS version 21.0 software (SPSS Inc, USA) and GraphPad Prismsoftware version 5.0 (GraphPad Software, 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 resultsor (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 templa-

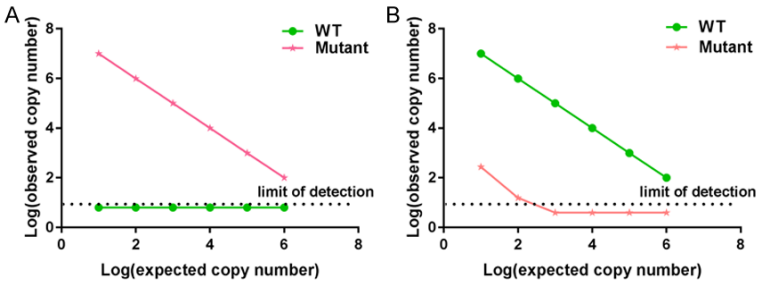

Figure 6. Cross-reactivity test of RT-AS-LNA-qPCR for rtN236. A. Specificity of mutant primer rtN236T (KF236/236LA). No nonspecific amplification was observed. B. Specificity of wild-type primer rtN236 (KF236/236LO). Nonspecific amplification was detected when the concentration of mutant template was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

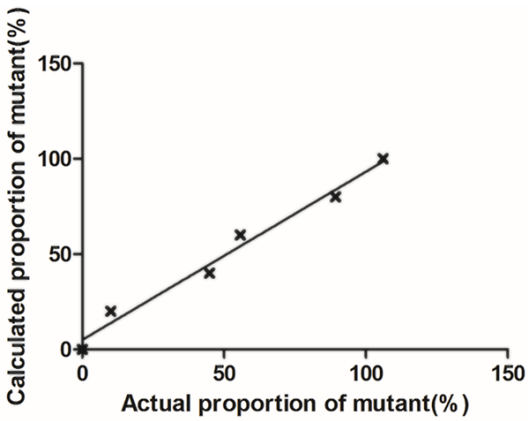

Figure 7. The correlation between actual and calculated proportion of mutant plasmid DNA at concentration of 1×10^7 copies/μl for rtA181. There was a significant linear correlation (R^2=0.9809, P=0.0001) between actual proportion of mutant and calculated proportion of correlation for HBV standard plasmid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

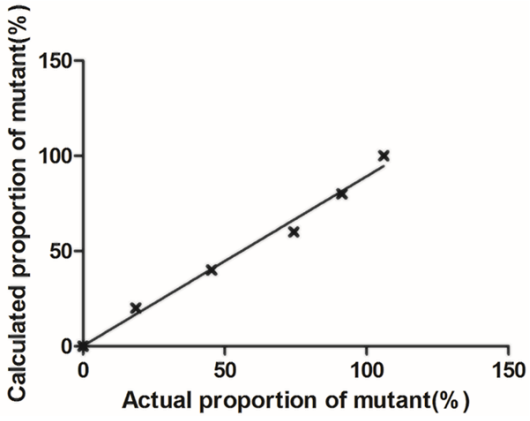

Figure 8. The correlation between actual and calculated proportion of mutant plasmid DNA at concentration of 1×10^7 copies/μl for rtN236. There was a significant linear correlation (R^2=0.9882, P<0.0001) between actual proportion of mutant and calculated proportion of correlation for HBV standard plasmid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

te i.e., different concentrations of wild-type and mutant-type recombinant plasmid (1×10^7 copies/μl~1×10^2 copies/μl) were amplified with mutant specific and wild-type specific primers, respectively. Nonspecific amplification phenomenon was not observed by mutant specific primers set (KF181/181LA) (**Figure 5**). However, nonspecific amplification with the wild-type specific primers set (KF181/181LO) was detected at the concentration higher than or equal to 1×10^6 copies/μl. But, the observed copy number was not in accordance with the excepted, representing at least 4 logs less than the expected copy number. Interestingly, no nonspecific amplification was observed when the mismatch template was below 10^6 copies/μl. Similar results were also observed while performed for rtN236 (**Figure 6**).

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 1×10^7 copies/μl (**Figures 7** and **8**).

Reproducibility test: The High (1×10^7 copies/μl), medium (1×10^5 copies/μl) and low (1×10^3 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-

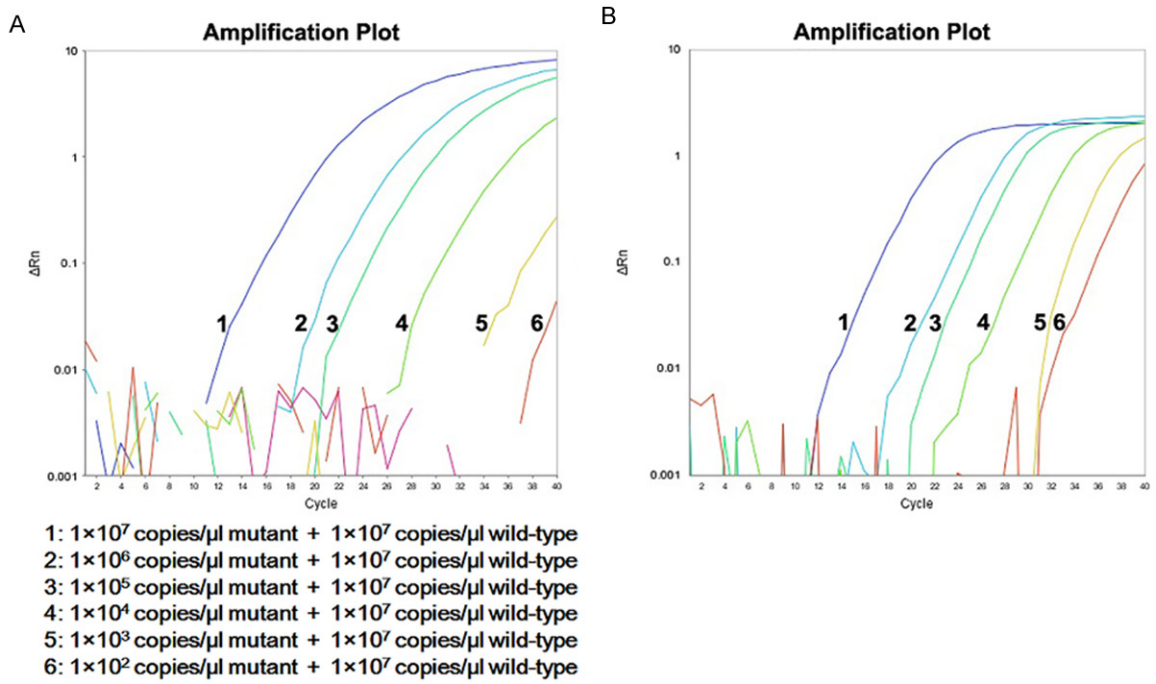

Figure 9. Sensitivity of RT-AS-LNA-qPCR in 1×10^7 copies/μl wild-type DNA background. From left to right were 1×10^7 copies/μl to 1×10^2 copies/μl mutant plasmids balanced mixing with 1×10^7 copies/μl wild-type DNA respectively, 100% pure wild-type DNA and no-template control. A. rtA181; B. rtN236.

Table 2. Sensitivity comparison of RT-AS-LNA-qPCR and sequencing on different proportions of clinical samples

Proportions of mutations (rtA181V/rtN236T)	RT-AS-LNA-qPCR	Sequencing
50%	WT/M	WT/M
25%	WT/M	WT/M
20%	WT/M	WT/M
10%	WT/M	WT/M
5%	WT/M	WT
1%	WT/M	-
0.5%	WT/M	-
0.05%	WT/M	-
0.04%	WT/M	-
0.03%	WT/M	-
0.02%	WT/M	-

WT: Wild type; M: mutant; -: not performed.

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 1×10^7 copies/μl concentration of wild-type DNA (**Figure 9**). The results showed RT-AS-LNA-qPCR could accurately and steadily detect 1×10^2 copies/μl of mutant DNAin the 1×10^7 copies/μl concentration of wild-type DNA, indicating that the sensitivity of the assay was 10^{-5} in the wild-type background of 1×10^7 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

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

RT-AS-LNA-qPCR	Direct sequencing			Total
	WT	WT+Mut	Mut	
WT	**83**	0	0	83
WT+Mut	*2*	**2**	*10*	14
Mut	0	0	**5**	5
Total	85	2	15	102

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)

RT-AS-LNA-qPCR	Direct sequencing			Total
	WT	WT+Mut	Mut	
WT	**83**	0	0	83
WT+Mut	*3*	**4**	*6*	13
Mut	0	0	**6**	6
Total	86	4	12	102

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

both RT-AS-LNA-qPCR assay and sequencing. 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 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. While 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, and the established method is consistent with the subclone sequencing finding (data not shown).

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 1×10^6 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 1×10^6 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.

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Disclosure of conflict of interest

None.

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References

[1] Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS and Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int 2016; 10: 1-98.

[2] Zeng Y, Yang B, Wu Y, Chen J, Shang H, Chen X, Su M, Wu S, Lin J and Ou Q. Clinical significance of periodic detection of hepatitis B virus YVDD mutation by ultrasensitive real-time amplification refractory mutation system quantitative PCR during lamivudine treatment in patients with chronic hepatitis B. J Med Microbiol 2015; 64: 237-242.

[3] European Association For The Study Of The Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 2012; 57: 167-185.

[4] Zhao WF, Shao YL, Chen LY, Wu JH, Zhu YL, Gan JH and Xiong H. Establishment of a new quantitative detection approach to adefovir-resistant HBV and its clinical application. World J Gastroenterol 2010; 16: 1267-1273.

[5] Niesters HG, Zoulim F, Pichoud C, Buti M, Shapiro F, D'Heuvaert N, Celis L, Doutreloigne J and Sablon E. Validation of the INNO-LiPA HBV DR assay (version 2) in monitoring hepatitis B virus-infected patients receiving nucleoside analog treatment. Antimicrob Agents Chemother 2010; 54: 1283-1289.

[6] Lupo J, Larrat S, Hilleret MN, Germi R, Boyer V, Nicod S, Bargues G, Leroy V, Seigneurin JM, Zarski JP and Morand P. Assessment of selective real-time PCR for quantitation of lamivudine and adefovir hepatitis B virus-resistant strains and comparison with direct sequencing and line probe assays. J Virol Methods 2009; 156: 52-58.

[7] Lowe CF, Merrick L, Harrigan PR, Mazzulli T, Sherlock CH and Ritchie G. Implementation of next-generation sequencing for hepatitis B virus resistance testing and genotyping in a clinical microbiology laboratory. J Clin Microbiol 2016; 54: 127-133.

[8] Liu C, Lin J, Chen H, Shang H, Jiang L, Chen J, Ye Y, Yang B and Ou Q. Detection of hepatitis B virus genotypic resistance mutations by coamplification at lower denaturation temperature-PCR coupled with sanger sequencing. J Clin Microbiol 2014; 52: 2933-2939.

[9] Zeng Y, Li D, Wang W, Su M, Lin J, Chen H, Jiang L, Chen J, Yang B and Ou Q. Establishment of real time allele specific locked nucleic acid quantitative PCR for detection of HBV YIDD (ATT) mutation and evaluation of its application. PLoS One 2014; 9: e90029.

[10] Pallier C, Castera L, Soulier A, Hezode C, Nordmann P, Dhumeaux D and Pawlotsky JM. Dynamics of hepatitis B virus resistance to lamivudine. J Virol 2006; 80: 643-653.

[11] Rupp J, Solbach W and Gieffers J. Single-nucleotide-polymorphism-specific PCR for quantification and discrimination of Chlamydia pneumoniae genotypes by use of a “locked” nucleic acid. Appl Environ Microbiol 2006; 72: 3785-3787.

[12] Morandi L, de Biase D, Visani M, Cesari V, De Maglio G, Pizzolitto S, Pession A and Tallini G. Allele specific locked nucleic acid quantitative PCR (ASLNAqPCR): an accurate and cost-effective assay to diagnose and quantify KRAS and BRAF mutation. PLoS One 2012; 7: e36084.