Detection of Hepatitis B Virus Genotypic Resistance Mutations by Coamplification at Lower Denaturation Temperature-PCR Coupled with Sanger Sequencing

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Mutations in the reverse transcriptase (rt) region of the DNA polymerase gene are the primary cause of hepatitis B virus (HBV) drug resistance. In this study, we established a novel method that couples coamplification at lower denaturation temperature (COLD)-PCR and Sanger sequencing, and we applied it to the detection of known and unknown HBV mutations. Primers were designed based on the common mutations in the HBV rt sequence at positions 180 to 215. The critical denaturation temperature (T_c) was established as a denaturing temperature for both fast and full COLD-PCR procedures. For single mutations, when a melting temperature (T_m)-reducing mutation occurred (e.g., C-G→T-A), the sensitivities of fast and full COLD-PCR for mutant detection were 1% and 2%, respectively; when the mutation caused no change in T_m (e.g., C-G→G-C) or raised T_m (e.g., T-A→C-G), only full COLD-PCR improved the sensitivity for mutant detection (2%). For combination mutations, the sensitivities of both full and fast COLD-PCR were increased to 0.5%. The limits of detection for fast and full COLD-PCR were 50 IU/ml and 100 IU/ml, respectively. In 30 chronic hepatitis B (CHB) cases, no mutations were detected by conventional PCR, whereas 18 mutations were successfully detected by COLD-PCR, including low-prevalence mutations (<10%), as confirmed by ultradeep pyrosequencing. In conclusion, COLD-PCR provides a highly sensitive, simple, inexpensive, and practical tool for significantly improving amplification efficacy and detecting low-level mutations in clinical CHB cases.

The hepatitis B virus (HBV) gene is prone to undergoing mutations due to the lack of stringent correction mechanisms in the transcription process. High rates of nucleotide (nt) mismatches occur during treatment with antiviral drug nucleoside (or nucleotide) analogues (NAs), especially in the reverse transcriptase (rt) region of the HBV polymerase gene (1). These mismatches are genotypic resistance mutations that lead to antiviral drug resistance (2). In order to improve HBV treatment by monitoring and reducing the occurrence of drug resistance, it is essential to detect rt mutations in chronic hepatitis B (CHB) patients over the course of treatment (3).

Current laboratory methods for the detection of HBV genetic mutations are nucleotide sequencing, reverse hybridization, PCR-restriction fragment length polymorphism (RFLP), allele-specific real-time PCR, restriction fragment mass polymorphism (RFMP), ultradeep pyrosequencing (UDPS), and gene chips (DNA microarrays). Direct PCR sequencing (Sanger sequencing) is considered to be the gold standard for detecting genotypic resistance mutations, because it can either detect multiple mutation sites during an assay or detect both known and unknown mutations simultaneously, with low false-positive rates (4). However, the sensitivity of population-based sequencing often decreases when the method is used to detect mutants present at <20% prevalence in a given sample (1, 4). The reverse hybridization assay has higher sensitivity, to about 5% prevalence, but the high cost and complex procedure make widespread implementation difficult in developing countries (5). PCR-RFLP and real-time PCR detect only known single mutations. With the application of multiple NAs and the occurrence of new HBV genotypic resistance mutations, these methods have been unable to meet requirements (6, 7). Some new technologies, such as RFMP, UDPS, and gene chips, have gradually come into use, but they bear the disadvantages of being expensive and cumbersome to perform, with heavy data analysis workloads and the possibility of

nucleotide mismatches, making them more suitable for research purposes than for clinical application (8–10). Therefore, it is necessary to establish a more practical method with high sensitivity to detect HBV genotypic resistance mutations.

Coamplification at lower denaturation temperature (COLD)-PCR was first reported by Li et al. in 2008 (11), as a PCR method to improve the sensitivity of Sanger sequencing. The method is based on changes in the melting temperature (T_m) of double-stranded DNA containing mismatched base pairs. It can be divided into full COLD-PCR and fast COLD-PCR, depending on whether the mutated base would reduce T_m (Fig. 1). Because of the low equipment cost of COLD-PCR and the increased detection sensitivity for mutants through optimization of the system, it has been widely used for detecting cancer-related genetic mutations, such as those in *p53*, *EGFR*, *KRAS*, and *MPL* genes (12, 13). However, its application to HBV genetic mutations has not been reported to date. In order to overcome limitations associated with conventional methods for detecting HBV genotypic resistance mutations, we have developed a COLD-PCR amplification method coupled with Sanger sequencing for the detection of known and unknown HBV genotypic resistance mutations.

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FIG 1 Schematic workflow for COLD-PCR. (A) Full COLD-PCR can potentially amplify all possible mutations. Several rounds of conventional PCR cycles produce the initial material for the target amplicons. After denaturation at approximately 95.0°C, the PCR amplicons are incubated (e.g., at 70.0°C for 2 to 8 min) for cross-hybridization. Hybridization of the mutant and wild-type alleles forms heteroduplexed molecules (containing mismatches) that require lower T_m values than the homoduplexed molecules. The PCR temperature is raised to the T_c (e.g., $T_c = 86.5^\circ\text{C}$) to preferentially denature the heteroduplexed amplicons, followed by the annealing temperature (e.g., 55.0°C), and then increased to 72.0°C for primer extension, thus preferentially amplifying the mutation-containing alleles. (B) Fast COLD-PCR can be performed to amplify mutations with T_m values lower than that of the wild-type amplicon (such as G-C→A-T or G-C→T-A mutations). Denaturation at the T_c (rather than the standard 95.0°C) preferentially denatures strands containing the lower- T_m allele, which generates single-stranded DNA for primer annealing and extension.

MATERIALS AND METHODS

Patients. A total of 30 CHB patients were enrolled in the First Affiliated Hospital of Fujian Medical University between September 2012 and February 2013, including 9 outpatients and 21 inpatient (19 male and 11

female patients; age range, 20 to 76 years [average, 38.2 ± 14.9 years]). Patients 1 to 5 were treated with adefovir (ADV), patients 6 to 15 were treated with lamivudine (LMV), patients 15 to 22 were treated first with LMV and then with entecavir (ETV), and patients 22 to 30 were treated

with ETV. All patients had virological breakthrough, i.e., $\geq 1 \log_{10}$ IU/ml increases in serum HBV DNA levels from the nadir in two consecutive samples 1 month apart for patients who had responded and had been compliant with antiviral treatment (1), and biochemical breakthrough, i.e., serum alanine aminotransferase levels exceeded the upper limit during treatment for patients who had achieved initial normalization (1). No HBV drug-resistant mutations were revealed by conventional PCR-Sanger sequencing. Serum samples from the patients were separated and stored at -80°C for future experiments.

Source of genomic DNA. For wild-type DNA, pUC-HBV14Kc was used as the template (gift from Lin Xu [Fujian Medical University], containing the full-length HBV DNA genome [3,215 bp]; GenBank accession no. AF411408.1; genotype C, serotype adr). For mutant DNA, HBV DNA with rt mutations rtM204I, rtA181T, rtM204V, rtL180M+rtM204V, and rtL180M+rtM204I, discovered by Sanger sequencing were used as templates (genotype C, serotype adr). An amplicon of 141 bp was amplified using the forward primer 5'-GCCTCAGTCCGTTTCTC-3' (nucleotides [nt] 650 to 666) and the reverse primer 5'-AAAGGGACTCAAGATGT T-3' (nt 773 to 790). The polymorphism analysis of the most common genotypes (genotypes B and C) in China obtained from GenBank was performed with DNAMAN software (Lynnon BioSoft, Canada). The target amplification fragment covered common mutations at rt positions 180 to 215. The PCR conditions were consistent with the conventional PCR system, as shown below. The PCR products were subjected to a serial procedure of recovery, purification, vector cloning, transformation, screening, and identification. The concentration of each recombinant plasmid DNA was measured with a BioPhotometer UV spectrophotometer (Eppendorf, Germany).

Conventional PCR coupled with Sanger sequencing. The PCR procedure was performed in a 25- μl reaction volume containing 50 ng DNA, $1\times$ SYBR Premix *Ex Taq* (TaKaRa Corp., Japan), and 0.2 $\mu\text{mol/liter}$ of each primer, in a StepOne Plus real-time fluorescence quantitative PCR instrument (Applied Biosystems, USA). Conventional PCR cycling conditions were as follows: 95°C for 30 s and 40 cycles of 95°C for 10 s and 56°C for 30 s. PCR products were processed for Sanger dideoxy sequencing with an ABI 3130 genetic analyzer (Applied Biosystems) with a general PCR purification kit (Tiangen Ltd., China). To achieve accurate full coverage of each amplicon, PCR products were sequenced using forward and reverse primers.

Ultradeep pyrosequencing. UDPS was performed using a Roche 454 GS FLX+ system (Roche), following the manufacturer's protocols. This instrument offers quantitative single-nucleotide polymorphism (SNP) and mutation analyses by rapidly sequencing short stretches of DNA directly from PCR templates. PCR amplification and pyrosequencing primers were designed using PyroMark Assay Design 2.0 software. The following primers were designed to amplify a 442-bp fragment of the HBV rt polymerase domain containing positions 180 to 215: forward primer, 5'-CTCATGTTGCTGTACAAAACC-3' (nt 559 to 579); reverse primer, 5'-CAATTCKTTGACATACTTTCCA-3' (nt 979 to 1,000). Thirty-three samples, including 30 clinical samples and 3 plasmid control samples, were sequenced using the Roche 454 GS FLX platform. The median sequence read length was 405 bp, and the median coverage was $\sim 1,500$ sequence reads per nucleotide. Data analysis was performed using Bowtie2 and SAMtools software (14).

COLD-PCR conditions. To determine the critical denaturation temperature (T_c) for both full and fast COLD-PCR of a given amplicon, a wild-type sample was first amplified via conventional PCR and then subjected to melting-curve analysis (ramping at 0.2°C/s from 65°C to 98°C) to identify the T_m with the StepOne real-time fluorescence quantitative PCR instrument (Applied Biosystems). Determination of T_c was achieved by using strains with wild-type DNA as templates with the same PCR system as described above, at 95°C for 30 s, 5 cycles of 95°C for 10 s and 56°C for 30 s, and 35 cycles of T_x for 20 s and 56°C for 30 s, where T_x refers to the temperature for tests and adjustment (generally starting from high to low temperatures, T_c is determined by changing the T_x value and examining

the results). It is preferred to set the initial T_x value 1°C to 2°C higher than the T_m value. Once amplification is successful, the T_x value can be further reduced by 0.5°C to 1°C each time, until no more PCR products are obtained with the lowest T_x value. Therefore, the last temperature tested before the lowest T_x value would be chosen as the T_c value for this system. After the T_c value was determined, the same reaction system was used for COLD-PCR. The conditions for fast COLD-PCR were 95°C for 30 s, 5 cycles of 95°C for 10 s and 56°C for 30 s, and 35 cycles of T_c for 20 s and 56°C for 30 s. The conditions for full COLD-PCR were 95°C for 30 s, 5 cycles of 95°C for 10 s and 56°C for 30 s, and 35 cycles of 95°C for 3 s, 70°C for 120 s (hybridization), T_c for 20 s, and 56°C for 30 s. The same purification and sequencing methods as used for conventional PCR products were used for COLD-PCR products, as described above.

Evaluation and comparison of conventional PCR and COLD-PCR assays. Plasmids containing wild-type DNA and mutant DNA were diluted to the same concentrations after being measured with the UV spectrophotometer. The mutant DNA was mixed with the wild-type strains of DNA using serial dilutions (e.g., 1:5, 1:10, 1:20, 1:50, 1:100, and 1:200). The mixed DNA was then subjected to conventional PCR-Sanger sequencing and COLD-PCR-Sanger sequencing, to compare the sensitivities of the two methods. At the same time, the same DNA mixture was diluted with double-distilled water (e.g., 1:100 and 1:10) to test the performance of COLD-PCR-Sanger sequencing in detecting mutant DNA in samples with different HBV DNA concentrations. To determine the analytical specificity of the COLD-PCR assay, we used all wild-type and mutant plasmids as templates, and the PCR products were subjected to Sanger sequencing.

DNA samples extracted from 30 patients with chronic hepatitis B were compared using conventional PCR-Sanger sequencing, COLD-PCR-Sanger sequencing, and UDPS methods. The results obtained with the UDPS method were used as the gold standard.

Statistical analysis. To evaluate and to compare the results obtained with conventional PCR and fast and full COLD-PCR in conjunction with Sanger sequencing, the sensitivity, the limit of detection for HBV genotypic resistance mutations, and the proportions of mutations in CHB cases were calculated. Comparisons between groups were performed using the χ^2 test. P values of <0.05 were considered significant. The statistical analysis was performed using SPSS version 12.0.

RESULTS

Confirmation of critical denaturation temperatures. First, the T_m value for the product of interest in the reaction system, as determined from the melting curve, was 82.5°C . The calculation of T_x for this experiment started at 84.0°C , which resulted in PCR product. Reductions to 83.5°C and 83.0°C still produced PCR products. The melting curve showed no PCR product when the temperature was reduced to 82.5°C . Therefore, 83.0°C was picked as the T_c for both full and fast COLD-PCR systems (wild-type double-stranded DNA merely unwinds; mutant double-stranded DNA chains or heteroduplex chains with wild-type and mutant DNA can successfully unwind).

Sensitivity for detection of single mutations. When the mutation rtM204I (G $\rightarrow$ T at nt 741) or rtA181T (G $\rightarrow$ A at nt 670) occurred, the minimum ratio of mutant/wild-type DNA that was detected by conventional PCR-Sanger sequencing was 1:10 (10%). Fast COLD-PCR-Sanger sequencing had a detection limit of 1:100 (1%), which was 10 times more sensitive than conventional PCR-Sanger sequencing. The lowest ratio detected by full COLD-PCR-Sanger sequencing was 1:50 (2%), which was 5 times more sensitive than conventional PCR-Sanger sequencing. For detection of the mutation rtM204V (A $\rightarrow$ G at nt 739), the lowest mutant/wild-type DNA ratio detected by conventional PCR-Sanger sequencing was 1:10 (10%), which was sim-

FIG 2 Sensitivity of conventional PCR and COLD-PCR for detection of single mutations. Mutant and wild-type DNAs were diluted in 1:10, 1:50, 1:100, and 1:200 serial dilutions. The diluted HBV-DNA concentration was 1.0E+05 IU/ml. When the rtM204I or rtA181T mutations occurred, the sensitivities of conventional PCR and fast and full COLD-PCR for mutant detection were 10%, 1%, and 2%, respectively. When the rtM204V mutation occurred, the sensitivities of conventional PCR and fast and full COLD-PCR for mutant detection were 10%, 10%, and 2%, respectively. Red arrows, positions of mutated bases.

ilar to the performance of fast COLD-PCR-Sanger sequencing (10%). However, the lowest mutant/wild-type DNA ratio detected by full COLD-PCR-Sanger sequencing was 1:50 (2%), which was 5 times more sensitive than conventional PCR-Sanger sequencing (Fig. 2).

Sensitivity for detection of combination mutations. For detection of the rtL180M (C→A at nt 667) plus rtM204V (A→G at nt 739) combination mutations, the lowest mutant/wild-type DNA ratio detected by conventional PCR-Sanger sequencing was 1:10 (10%), which was similar to the performance of fast COLD-PCR-Sanger sequencing (10%). However, the lowest mutant/wild-type DNA ratio detected by full COLD-PCR-Sanger sequencing was 1:100 (2%), which was 10 times more sensitive than conventional PCR-Sanger sequencing (Fig. 3). For detection of the rtL180M (C→A at nt 667) plus rtM204I (G→T at nt 741) combination mutations, the lowest mutant/wild-type DNA ratio detected by conventional PCR-Sanger sequencing was 1:10 (10%). Fast COLD-PCR-Sanger sequencing had a detection limit of 1:200 (0.5%), which was 20 times more sensitive than conventional PCR. The lowest mutant/wild-type DNA ratio detected by

FIG 3 Sensitivity of conventional PCR and COLD-PCR for detection of the rtL180M (C→A at nt 667) plus rtM204V (A→G at nt 739) combination mutations. Mutant and wild-type DNAs were diluted in 1:10, 1:50, 1:100, and 1:200 serial dilutions. The diluted HBV-DNA concentration was 1.0E+05 IU/ml. The top line for each dilution represents the rtL180M mutation (green peaks identified by arrows). The bottom line represents the rtM204V mutation (black peaks identified by arrows).

full COLD-PCR-Sanger sequencing was 1:100 (1%), which was 10 times more sensitive than conventional PCR-Sanger sequencing (Fig. 4).

Limits of detection and specificity. The concentrations required for fast COLD-PCR-Sanger sequencing varied for different types of mutations; the limits of detection for mutant DNA ranged from 5.0E+01 IU/ml to 1.0E+03 IU/ml. The concentrations required for full COLD-PCR-Sanger sequencing were approximately the same for single mutations, and products were detectable with mutant DNA at concentrations of ≥2.0E+02 IU/ml; the detection limit for combination mutations was lowered to 1.0E+02 IU/ml. The specificity evaluation showed that the specificity of COLD-PCR in the detection of wild-type and mutant plasmids was 100% (Table 1).

Analysis of CHB samples. Three methods were used for detection of mutations in 30 specimens collected from patients with chronic hepatitis B (Table 2). Conventional PCR-Sanger sequencing did not reveal any mutation. However, COLD-PCR-Sanger sequencing found mutations in 18 cases. Fast COLD-PCR-Sanger sequencing detected mutations in 15 cases (6 cases of rtA181T plus rtM204I, 3 cases of rtM204I, 2 cases of rtV191I, 1 case of rtA181T,

FIG 4 Sensitivity of conventional PCR and COLD-PCR for detection of the rtL180M (C→A at nt 667) plus rtM204I (G→T at nt 741) combination mutations. Mutant and wild-type DNAs were diluted in 1:10, 1:50, 1:100, 1:200, and 1:400 serial dilutions. The diluted HBV-DNA concentration was 1.0×10^5 IU/ml. The top line for each dilution represents the rtL180M mutation (green peaks identified by arrows). The bottom line represents the rtM204I mutation (red peaks identified by arrows).

1 case of rtH197H, 1 case of rtQ182Q plus rtV191I, and 1 case of rtM204I plus rtK212K mutations); full COLD-PCR-Sanger sequencing detected mutations in 13 cases (4 cases of rtM204I, 2 cases of rtV191I, 1 case of rtA181T, 1 case of rtH197H, 1 case of rtS189S plus rtM204I, 1 case of rtA181T plus rtM204I, 1 case of rtA180T plus rtM204V, 1 case of rtQ182Q plus rtV191I, and 1 case of rtS213S mutations). UDPS detected mutations in 21 cases, among which variants present at levels of $>1\%$ were detected by fast or full COLD-PCR-Sanger sequencing; however, variants present at levels of $<1\%$ were not found by COLD-PCR-Sanger sequencing (Table 2).

DISCUSSION

HBV drug resistance is the greatest challenge in treating hepatitis B; there are high rates of drug resistance, multidrug resistance, and cross-resistance (15). It is important to avoid developing resistance to the drug in use or for future use by detecting genotypic

resistance using effective methods at an early stage. Various methods are currently available for detection of HBV genotypic resistance mutations, but they usually have poor sensitivity, are very expensive and complicated to use, and detect only single mutations. COLD-PCR has recently found application in mutation analyses, especially in the area of detecting mutations in tumor samples (16). It is a promising method because of its outstanding advantages of being simple, economical, and practical. In this research, the COLD-PCR-Sanger sequencing platform has been used for the detection of common HBV genotypic resistance mutations (at rt positions 180 to 215) for the first time, overcoming the limitations of conventional methods. This method shows very good prospects in the field of HBV drug resistance detection.

This study compared the sensitivity of conventional PCR and fast and full COLD-PCR for the detection of mutants. The minimum detectable amount of mutants for conventional PCR was 10%, which was lower than that reported in the literature (20%). This discrepancy was probably due to differences in amplification systems and the shorter fragments used in our study, which detected the mutations in the region with ease. For single mutations, when a T_m -reducing mutation occurred, fast COLD-PCR showed the highest sensitivity (1%), followed by full COLD-PCR (2%); when a mutation caused no change or raised T_m , only full COLD-PCR improved the mutant-detecting capability (5 times greater than that of conventional PCR), which is consistent with findings from Milbury et al. (17). For combination mutations, detection sensitivity was raised 10- to 20-fold with fast COLD-PCR and full COLD-PCR methods versus that of conventional PCR. The limits of detection of fast and full COLD-PCR-Sanger sequencing for mutant DNA were 50 IU/ml and 100 IU/ml, respectively, thus greatly improving the mutant-detecting capabilities (compared with the detection limit of conventional PCR-Sanger sequencing of about 1.0×10^4 IU/ml for HBV DNA). Moreover, the analytical specificity of the COLD-PCR assay for detection of wild-type and mutant DNA was 100%. Therefore, results can be used to guide the rational use of drugs or changes in treatment in a timely manner for patients with CHB prior to the development of drug resistance.

In addition, we used two methods to detect the HBV genotypes of 30 patients with CHB. Among these 30 patients, 21 cases with variants present at prevalence levels of $<10\%$ were found by UDPS. Conventional PCR-Sanger sequencing suggested that all 30 cases had wild-type DNA and COLD-PCR-Sanger sequencing found mutations in 18 cases, as confirmed by UDPS. Among these cases, fast COLD-PCR-Sanger sequencing revealed 15 mutations, and full COLD-PCR-Sanger sequencing found 13 cases. As shown

TABLE 1 Limits of detection and specificities of COLD-PCR for mutant DNA

Mutation(s)	Fast COLD-PCR		Full COLD-PCR	
	Limit of detection (IU/ml) ^a	Specificity (%)	Limit of detection (IU/ml) ^a	Specificity (%)
rtM204I	1.0×10^2	100	2.0×10^2	100
rtM204V	1.0×10^3	100	2.0×10^2	100
rtA181T	1.0×10^2	100	2.0×10^2	100
rtL180M+rtM204V	1.0×10^3	100	1.0×10^2	100
rtL180M+rtM204I	5.0×10^1	100	1.0×10^2	100

^a The minimum mutant DNA concentration in an HBV wild-type background (1.0×10^4 IU/ml) detected by COLD-PCR.

TABLE 2 Comparison of conventional PCR, COLD-PCR, and UDPS for detection of HBV gene mutations

Case no.	HBV DNA level (IU/ml)	Mutation(s) detected with ^a :			Nucleotides detected by UDPS (%) ^b
		Conventional PCR	Fast COLD-PCR ^b	Full COLD-PCR ^b	
1	5.32E+04	—	rtA181T (G→A)	rtA181T (G→A)	181G (97.33), 181A (2.67)
2	5.90E+04	—	—	—	—
3	3.16E+05	—	rtA181T (G→A) + rtM204I (G→T)	rtA181T (G→A)	181G (98.67), 181A (1.33)
4	3.69E+04	—	—	—	—
5	2.53E+04	—	rtQ182Q (G→A) + rtV191I (G→A)	rtQ182Q (G→A) + rtV191I (G→A)	182G (97.97), 182A (2.03)
6	2.89E+07	—	rtM204I (G→T)	rtM204I (G→T)	204G (97.36), 204T (2.64)
7	1.68E+06	—	—	rtS213S (T→A)	213T (94.22), 213A (5.78)
8	1.28E+04	—	—	rtL180M (C→A) + rtM204V (A→G)	180C (94.58), 180A (5.42)
9	2.90E+07	—	—	—	—
10	1.12E+07	—	rtA181T (G→A) + rtM204I (G→T)	—	181G (99.01), 181A (0.99)
11	5.99E+07	—	rtA181T (G→A) + rtM204I (G→T)	rtM204I (G→T)	180C (98.95), 180A (1.05)
12	6.69E+05	—	—	—	—
13	9.92E+06	—	rtA181T (G→A) + rtM204I (G→T)	rtM204I (G→T)	181G (99.34), 181A (0.66)
14	1.08E+08	—	—	—	181G (99.28), 181A (0.72)
15	8.42E+06	—	—	rtS189S (A→T) + rtM204I (G→T)	189A (97.00), 189T (3.00)
16	1.42E+07	—	—	—	—
17	2.62E+07	—	—	—	—
18	8.12E+06	—	rtA181T (G→A) + rtM204I (G→T)	—	181G (99.45), 181A (0.55)
19	1.78E+05	—	rtH197H (C→T)	rtH197H (C→T)	197C (91.44), 197T (8.56)
20	5.18E+05	—	rtM204I (G→T) + rtK212K (G→A)	—	204G (99.28), 204T (0.72)
21	1.26E+06	—	—	—	208A (99.79), 208T (0.21)
22	5.46E+05	—	—	—	—
23	1.28E+08	—	rtV191I (G→A)	rtV191I (G→A)	191G (94.18), 191A (5.82)
24	6.86E+05	—	—	—	—
25	3.53E+06	—	rtM204I (G→T)	—	204G (98.27), 204T (1.73)
26	1.52E+06	—	—	—	—
27	2.17E+05	—	rtM204I (G→T)	rtM204I (G→T)	204G (95.88), 204A (4.12)
28	1.42E+05	—	—	—	208A (99.66), 208T (0.34)
29	7.38E+05	—	rtV191I (G→A)	rtV191I (G→A)	191G (96.53), 191A (3.47)
30	1.01E+06	—	rtA181T (G→A) + rtM204I (G→T)	—	181G (99.13), 181A (0.87)

^a The proportions of mutations detected were as follows: conventional PCR, 0%; fast COLD-PCR, 50.00% (15/30 cases); full COLD-PCR, 43.33% (13/30 cases); UDPS, 70.00% (21/30 cases). —, no mutation detected; +, combination mutations detected.

^b Statistically significant difference in comparison with conventional PCR ($P < 0.05$).

in Table 2, both fast and full COLD-PCR significantly improved the detection rate for HBV mutants, with consistency in detecting mutations in 10 cases (cases 1, 3, 5, 6, 11, 13, 19, 23, 27, and 29). The test results were statistically different from those for conventional PCR. We also found differences in the mutation types detected by the two COLD-PCR methods. For example, fast COLD-PCR detected mutations in samples 10, 18, 20, 25, and 30, whereas full COLD-PCR failed to. This is mainly due to the superior sensitivity of fast COLD-PCR versus full COLD-PCR for certain types of mutations (T_m -reducing mutations), with values as low as 0.5% for the former and 1 to 2% for the latter. The results were verified by UDPS. For samples 7, 8, and 15, full COLD-PCR but not fast COLD-PCR detected mutations. The reason for this finding involves the presence of combination mutations and T_m remaining unchanged or even being higher after mutation. Fast COLD-PCR has no or less ability to amplify this part of the mutant strain, in comparison with full COLD-PCR. For samples 14, 21, and 28, mutations were detected by UDPS but not COLD-PCR. This may be due to the low levels of mutations (<0.5%) in the samples, which were below the minimum detection limit of COLD-PCR. Because most cancer gene mutations are single mutations that

reduce T_m values, fast COLD-PCR is better than COLD-PCR in amplifying the mutants, which can be quantitatively measured with TaqMan probes (18). Therefore, fast COLD-PCR is used more frequently than full COLD-PCR. Due to the multiple sites and types of the HBV gene mutations, our results showed that, although the mutation detection rate for full COLD-PCR is not as good as that for fast COLD-PCR, more types of mutations can be detected by full COLD-PCR. Therefore, we recommend the combination of full COLD-PCR and fast COLD-PCR to increase the detection rate for HBV mutants. In a previous study, Li et al. reported that two-round COLD-PCR improved the detection limit for identification of cancer-related mutations in TP53 through the use of nested primers for the second round prior to sequencing (19). However, the region of HBV rt was not conserved, and the region for primer design was limited. Therefore, we used only one round of COLD-PCR. In this study, we found a set of new mutations such as rtV191I. These have not been reported to date, probably due to the low levels of these mutations, which were difficult to identify by conventional PCR-Sanger sequencing. We will increase the sample size in the follow-up study, to verify the clinical value of such mutations.

In general, COLD-PCR-Sanger sequencing shows higher sensitivity than conventional PCR-Sanger sequencing through optimization of conditions, without requirements for additional costs and equipment. The advantage of significantly reducing the proportion undetected by Sanger sequencing will be beneficial for the rational use of drugs or timely changes in treatment for CHB patients, prior to the appearance of clinical drug resistance.

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