

## Detection of YMDD mutation using mutant-specific primers in chronic hepatitis B patients before and after lamivudine treatment

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### Abstract

**AIM:** To develop a PCR assay using mutant-specific primers to detect mutation of tyrosine-methionine-aspartate-aspartate (YMDD) motif of HBV to tyrosine-valine-aspartate-aspartate (YVDD) or tyrosine-isoleucine-aspartate-aspartate (YIDD).

**METHODS:** Cloned wild-type and mutant HBV sequences were used as templates to test the sensitivity and specificity of the assay. A variety of primer construction, primer concentration, dNTP concentration, and annealing temperature of primers were systematically examined. Pair primers specific to rtL180M and rtM204V were selected for YVDD detection. Primer specific to rtM204I with an additional 3'-penultimate base mismatched to both the mutant and wild-type sequence was selected for YIDD detection. We applied this assay to study YMDD mutants in 28 chronic hepatitis B patients before and after lamivudine treatment.

**RESULTS:** We could detect as little as 0.001%-0.00001% of mutant viruses coexisting in  $10^8$ - $10^9$  copies of wild-type HBV using this assay. YMDD mutants were detected in 8 of 12 HBeAg-positive patients and 8 of 16 HBeAg-negative patients before lamivudine treatment. After treatment, two more patients in HBeAg-positive patients and seven more patients in HBeAg-negative patients developed YMDD mutations.

**CONCLUSION:** We developed a highly sensitive and specific assay for detecting YMDD mutants. This assay can be applied to monitor chronic hepatitis B patients before and during lamivudine treatment.

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**Key words:** Hepatitis B virus; Lamivudine; Tyrosine-

### INTRODUCTION

Hepatitis B virus (HBV) is one of the most common infectious diseases in the world. More than 300 million people worldwide are estimated to have chronic HBV infection. Ten percent of these patients will die as a direct consequence of persistent viral infection<sup>[1]</sup>. Nucleoside analogue therapy allows safe, long-term suppression of HBV and is a major milestone in the treatment of chronic hepatitis B. Lamivudine, the first of these agents approved worldwide, effectively suppresses viral replication, reduces disease activity, improves liver histology, and delays clinical progression<sup>[2-4]</sup>. However, the development of lamivudine resistant mutations occurs in 14%-32% of patients after 1 year of therapy<sup>[5,6]</sup>. The longer the treatment is continued, the more frequently resistance is seen (65% at 5 years)<sup>[7]</sup>. Lamivudine-resistant HBV is associated with mutations of the YMDD motif in the polymerase gene. The key mutations are the substitutions of methionine at the rtM204 (domain C) to either isoleucine (rtM204I, YIDD variant) or valine (rtM204V, YVDD variant)<sup>[8,9]</sup>. The rtM204V variant is almost always accompanied by an additional rtL180M mutation in the domain B<sup>[10,11]</sup>.

Several assays, including DNA sequencing<sup>[12]</sup>, restriction fragment length polymorphism (RFLP)<sup>[13,14]</sup>, peptide nucleic acid (PNA) mediated PCR clamping<sup>[15]</sup>, line probe assay<sup>[16,17]</sup>, and oligonucleotide microarray<sup>[18,19]</sup>, have been developed to detect YMDD mutations. The best sensitivity achieved so far was  $10^4$  copies of variant in the mixture of  $10^9$  copies of the wild-type virus and  $10^1$  in  $10^5$  of wild-type<sup>[15]</sup>.

In this study, we developed a simple but highly sensitive and specific assay using mutant-specific primers to detect lamivudine-resistant mutations. We applied this method to detect YMDD mutants in chronic hepatitis B patients before and after lamivudine treatment.

## MATERIALS AND METHODS

### Clinical samples and method for extraction of HBV DNA

Serum samples were collected from 28 chronic hepatitis B patients who had histologically confirmed cirrhosis and had received lamivudine treatment for 12 to 48 mo. Informed consent was obtained from all patients according to the ethical guidelines of the 1975 Declaration of Helsinki. HBV DNA was extracted from serum using a DNA extraction kit (Qiagen, Hilden, Germany).

### Subcloning of the HBV gene

Sera with wide-type HBV DNA, YVDD or YIDD mutants were amplified by PCR with a primer set (sense primer: 5'-GATGTGTCTGCGGCGTTTTA-3', antisense primer: 5'-CAGCAAAGCCCCAAAAGACCCAC-3'). The obtained PCR products (625bp, containing site codons rt180 and rt204) were then cloned into pOSI-T vector (GeneMark, Taichung, Taiwan) using the standard method.

### Detection of YMDD mutation using PCR with mutant-specific primers

We used cloned YVDD, YIDD and wild-type HBV genomes as templates to determine the optimal conditions of mutant-specific PCR. A variety of primer construction (Table 1), primer concentration, dNTP concentration and annealing temperature of primers were systematically examined (data not shown). The primers finally selected were listed with boldface in Table 1. The single-step PCR for YVDD detection was carried out with the primers YVDD669 and YVDD758R in a 25 L reaction volume containing 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl, 2.0 mmol/L MgCl<sub>2</sub>, 0.03 mmol/L of each dNTP, 2.5 pmol of each primer, 1.5 units of Taq polymerase (Viogene, Taipei, Taiwan). The PCR condition included initial denaturation for 3 min at 94°C, 48 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 57°C for 30 s, extension of primer at 72°C for 25 s, and final extension at 72°C for 1 min. The nest-PCR was performed with HBV376 and HBV1021R as outer primers. The first PCR was conducted in a 20 L reaction volume with 0.0375 mmol/L dNTP and 1 pmol of each outer primer. The PCR condition was initial denaturation for 3 min at 94°C, 30 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 53°C for 30 s, extension of primer at 72°C for 50 s, and final extension at 72°C for 2 min. One microliter of the first reaction was used for the second PCR. The second PCR condition was the same as the single-step PCR except the annealing temperature was 55°C. The single-step PCR for YIDD detection was carried out with primers HBV379 and YIDDR2T in the same reaction condition as for YVDD detection except the annealing temperature was 59°C. The first PCR of the nest-PCR for YIDD detection was performed in the same condition as for YVDD detection. One microliter of the first reaction was used for the second PCR. The second PCR was carried out in a 25 L reaction volume with 0.03 mmol/L of each dNTP, 2.5 pmol of each primer (HBV485 and YIDDR2T) and 0.1U of Perfect Match PCR enhancer (Stratagene, La Jolla, California, USA), 1.5 units of Taq polymerase. The reaction condition was initial one cycle of

Table 1 DNA sequence of the primers tested for detection of YMDD mutation

<sup>1</sup> Primers	<sup>2</sup> DNA sequence	Nuclidean
<b>YVDD758</b>	5'-CACIGTTTGGCTTTCAGTTATG (A)	737-758 Sense
<b>YVDD758R</b>	5'-CCCCAAAACCACATCATCCAC (T)	758-739 Antisense
<b>YVDD669</b>	5'-GGGCCTCAGICCGTTTCTCA (T)	650-669 Sense
<b>YIDD</b>	5'-CTGTTTGGCTTTCAGTTATATT (G)	739-760 Sense
<b>YIDD2G</b>	5'-GTTTGGCTTTCAGTTATAGT (TG)	741-760 Sense
<b>YIDD2A</b>	5'-GTTTGGCTTTCAGTTATAAT (TG)	741-760 Sense
<b>YIDD2C</b>	5'-GTTTGGCTTTCAGTTATACT (TG)	741-760 Sense
<b>YIDDR</b>	5'-CCCCAAAACCACATCATCA (C)	760-745 Antisense
<b>YIDDR2T</b>	5'-CCCCAAAACCACATCATTA (CC)	760-745 Antisense
<b>YIDDR2A</b>	5'-CCCCAAAACCACATCATAA (CC)	760-745 Antisense
<b>YIDDR2G</b>	5'-CCCCAAAACCACATCATGA (CC)	760-745 Antisense
<b>HBV376</b>	5'-GATGTGTCTGCGGCGTTTTA	376-395 Sense
<b>HBV485</b>	5'-CCAGGAACATCAACCACCAG	485-504 Sense
<b>HBV1021R</b>	5'-CAGCAAAGCCCCAAAAGACCCAC	1021-1000 Antisense

<sup>1</sup> The primers finally selected are indicated in boldface; <sup>2</sup> The engineered mismatched nucleotides are underlined; the wild-type nucleotides are shown in parenthesis.

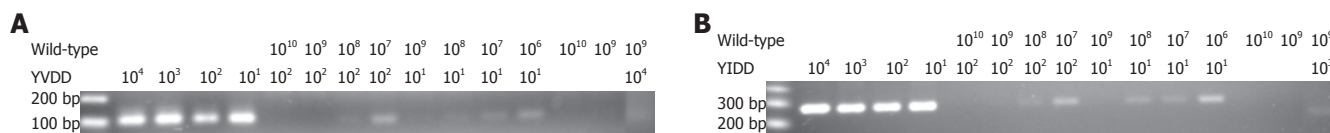
94°C for 5 min, 62°C for 5 min and 72°C for 30 s, 48 cycles of amplification with denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s, extension of primer at 72°C for 30 s, and final extension at 72°C for 1 min. The PCR products were subjected to electrophoresis in a 3% agarose gel.

### HBV DNA quantification and Sequencing

HBV DNA quantification was performed using the Lightcycler real-time PCR assay<sup>[20]</sup>. PCR amplification of the HBV genome was carried out using the same primers as subcloning of HBV DNA described above. PCR-amplified HBV DNA was purified and sequenced using specific sequencing primers and a commercial sequencing Kit (ABI Prism Dye Terminator Cycle Sequencing, Perkin Elmer, Warrington, UK). The sequencing reactions were analyzed on an automatic DNA sequencer.

## RESULTS

We assessed the sensitivity and specificity of this method using mutant type and wild-type HBV plasmid. For single-step PCR, the detection limit was 10<sup>4</sup> copies for YVDD variant or 8 × 10<sup>4</sup> copies for YIDD variant (data not shown). For nest-PCR, the detection limit was up to 10<sup>1</sup> copies for both variants (Figure 1A, B). Wild-type HBV, even up to 10<sup>10</sup> copies, was not detected using these mutant-specific primers for both single-step PCR (data not shown) and nest-PCR (Figure 1A, B). We then tested the mixture of wild-type and mutant HBV using nest-PCR. Various copies of wild-type plasmid (10<sup>7</sup>-10<sup>10</sup> copies) were mixed with YVDD variant at the indicated concentrations (10<sup>1</sup>-10<sup>4</sup> copies). In the presence of 10<sup>8</sup> wild-type sequences, a total of 10<sup>1</sup> copies of both variants could be detected (Figure 1A, B). A total of 10<sup>4</sup> copies of YVDD variants or 10<sup>3</sup> copies of YIDD variants were detected in the mixture of 10<sup>9</sup> copies of the wild-type sequences (Figure 1A, B). Thus, the detection limit was



**Figure 1** Sensitivity and specificity of the YMDD mutant detection by mutant-specific PCR. **A:** Various copies of wild-type sequence, YVDD mutant sequence, and mix of both sequences at the indicated concentrations. The left most lane contains molecular weight marker in base pair unit. The detection limit was 10<sup>1</sup> copies of YVDD mutant in 10<sup>8</sup> copies of wild-type HBV or 10<sup>4</sup> copies of YVDD mutant in 10<sup>9</sup> copies of wild-type HBV; **B:** Various copies of wild-type sequence, YIDD mutant sequence, and mix of both sequences at the indicated concentrations. The left most lane contains molecular weight marker in base pair unit. The detection limit was 10<sup>1</sup> copies of YIDD mutant in 10<sup>8</sup> copies of wild-type HBV or 10<sup>3</sup> copies of YIDD mutant in 10<sup>9</sup> copies of wild-type HBV.

**Table 2** Clinical Characteristics of 28 patients with chronic hepatitis B before and after long-term treatment of lamivudine

No.	Before treatment				After treatment				
	AST/ALT (IU/L)	HBeAg/anti-HBe	<sup>1</sup> HBV-DNA	<sup>2</sup> Nest-PCR	AST/ALT (IU/L)	HBeAg/anti-HBe	<sup>1</sup> HBV-DNA	Nest-PCR	<sup>3</sup> Sequencing
1	61/62	-/+	39.93	-	29/16	-/+	0.25	V	V
2	31/43	+/-	3574.29	V	29/54	+/-	4400.65	M	V
3	85/86	-/+	348.66	-	20/11	-/+	0.20	-	W
4	45/90	-/+	8.50	-	23/22	-/+	0.03	-	W
5	74/75	-/+	256.99	I	63/38	-/+	0.01	I	I
6	82/163	-/+	1596.12	-	38/64	-/+	3356.38	V	V
7	37/43	+/-	13.07	-	25/39	+/-	0.26	-	W
8	26/62	-/+	49.16	-	24/40	-/+	0.03	-	W
9	82/102	-/+	3313.93	-	29/17	-/+	0.60	V	W
10	40/59	+/-	546.76	I	24/28	-/+	0.11	M	I
11	117/232	+/-	49.50	I	26/27	+/-	346.96	M	I
12	46/61	-/+	47.40	-	24/32	-/+	0.06	V	V
13	72/107	-/+	5.37	M	66/92	-/+	2389.09	M	V
14	155/159	+/-	2325.13	I	22/14	+/-	0.10	M	W
15	65/54	-/+	1461.13	-	42/29	-/+	0.12	V	W
16	133/157	+/-	7943.81	I	73/64	+/+	2.05	I	I
17	107/146	-/+	23.13	M	151/232	-/+	59.94	M	V
18	35/48	+/-	1666.59	-	22/21	+/-	0.15	-	W
19	247/183	+/-	9336.17	I	27/14	+/-	0.01	I	W
20	45/56	-/+	219.58	-	28/38	-/+	0.03	-	W
21	109/186	-/+	61.27	-	30/48	-/+	2.91	M	I
22	173/335	+/-	11.98	-	29/31	+/-	0.03	M	V
23	51/81	-/+	1403.11	I	28/42	-/+	303.09	I	I
24	43/68	+/-	365.07	-	22/27	+/-	1.28	V	V
25	45/57	+/-	7530.63	I	30/25	+/-	6228.83	I	I
26	130/286	-/+	126.67	-	21/32	-/+	1.46	I	I
27	64/91	+/-	4700.63	I	52/73	+/-	6.12	I	I
28	88/164	-/+	0.38	-	30/38	-/+	0.07	-	W

<sup>1</sup> Unit (10<sup>5</sup>copies/mL); <sup>2</sup> M: YVDD + YIDD, V: YVDD, I: YIDD, -: YVDD and YIDD not detectable; <sup>3</sup> W: wild-type, V: YVDD, I: YIDD.

0.0001%-0.00001%.

We then applied this method to 28 chronic hepatitis B patients who had been treated with lamivudine for 12 to 48 mo (Table 2). Before treatment, there were 12 patients with positive HBeAg and 16 patients with negative HBeAg and positive anti-HBe. Among 12 HBeAg-positive patients, four were free of YMDD mutant, seven with YIDD mutant, and one with YVDD mutant. One developed HBeAg sero-conversion after treatment. Among 4 patients with initial negative YMDD mutant, two developed mixed mutation, and two remained free from YMDD mutation. Among 7 patients with initial YIDD mutant, three developed mixed mutations, and four remained with

YIDD mutation. The patient with initial YVDD mutant developed mixed mutation. Among 16 HBeAg-negative patients, before treatment, twelve were free of YMDD mutation, two with YIDD mutation, and two with mixed mutation. Among 12 patients with initial negative YMDD mutant, five developed YVDD mutation, one developed YIDD mutation, one developed mixed mutation, and five remained free of YMDD mutation after treatment. The other four patients, two with YIDD mutant, two with mixed mutation, remained with the same mutation. Conventional sequencing of post-treatment sera showed 11 cases of wild-type HBV, 8 cases of YVDD mutation, and 9 cases of YIDD mutation. Among 11 cases of wild-

type HBV, eight were wild-type, two were YVDD, one was YIDD, and one was mixed mutation by nest-PCR. Among 8 cases of YVDD mutation, four were YVDD and four were mixed mutation by nest-PCR. Among 9 cases of YIDD mutation, six were YIDD and three were mixed mutation by nest-PCR (Table 2). Overall, mutant-specific PCR agreed with sequencing on YMDD mutation detection, but was more sensitive in detecting additional mutations.

## DISCUSSION

Taq DNA polymerases extend mismatches much less efficiently than correct matches. Depending on the nature of the mismatch composition, the elongation efficiency of the mismatched duplex can vary from 0.0001% to 0.1% of the elongation efficiency of the perfectly matched duplex<sup>[21]</sup>. Reduced dNTP and primer concentration can increase the specificity of mutant-specific PCR<sup>[22]</sup>. We have lowered 2-4 fold the dNTP and primer concentration of conventional PCR and compensated for the reduced efficiency by increasing amplification cycles. With this modified PCR condition, we found that in the presence of  $10^6$  to  $10^7$  copies of wild-type HBV DNA, a single 3'-end mismatch primer still showed a false positive result (data not shown). For the YVDD mutation, rtL180M mutation is almost always accompanied by an additional the rtM204V mutation<sup>[10,11]</sup>. We therefore selected pair primers with 3'-end specific to rtL180M and rtM204V to detect YVDD mutation. For YIDD mutation detection, we designed primers containing a mutant-specific 3'-terminal base and a 3'-penultimate base mismatched to both the mutant and wild-type sequence. We checked 3 forward (YIDD2G, YIDD2A, and YIDD2C) and 3 reverse (YIDDR2T, YIDDR2A, and YIDDR2C) primer constructions. The YIDDR2T primer had the greatest specificity and sensitivity in detecting YIDD mutation. Besides, we add Perfect Match PCR enhancer to increase the specificity of the assay<sup>[23]</sup>. The present assay had a detection limit of 0.0001%-0.00001%, which was better than that of PNA mediated PCR clamping (0.001%-0.0001%)<sup>[15]</sup> and that of RFLP assay (1%-10%)<sup>[13]</sup>. However, the latter two methods have the advantage of detecting both wild-type and mutant HBV simultaneously in one reaction. The choice of assay should depend on the context of the application.

Hepatitis flare can occur during lamivudine treatment because of the emergence of YMDD mutants. The serum titer of the mutant viruses during hepatitis flare is well above  $10^5$  copies/mL<sup>[24]</sup>. The single-step PCR in this study only detected YMDD mutation at copy number greater than  $10^4$ , and can be applied in this clinical context. In fact, we have applied single-step PCR to check two cases of hepatitis flare during lamivudine treatment. It correlated well with HBV DNA quantification and sequencing (data not shown).

Because HBV employs reverse transcription to copy its genome, mutant viral genomes are found frequently. Under conditions of high levels of viral replication, it is likely that HBV mutations conferring resistance to lamivudine may pre-exist. Indeed, pre-existing YMDD mutants have been demonstrated in some lamivudine-naive asymptomatic

HBV carriers<sup>[25]</sup> or chronic hepatitis B patients<sup>[15]</sup>. The patients with pre-existing YMDD mutants were all positive for anti-HBe antibody<sup>[15,25]</sup>. In this study, twelve patients were shown to have pre-existing YMDD mutants, and eight of them were HBeAg-positive. Seven cases with pre-existing mutation had a serum HBV DNA level greater than  $10^8$  copies/mL. However there were also cases with a high level of HBV DNA but without a pre-existing mutation. We suspect that with more sensitive detecting methods, more cases of pre-existing mutation will be demonstrated. The patients with a pre-existing mutation had persistent mutation or developed mixed mutation during treatment, but only five of them had a poor virological response. Whether patients with a pre-existing lamivudine-resistant mutation should be treated with other nucleoside analogues requires further prospective studies.

In conclusion, we have developed a sensitive and specific method using mutant-specific primers to detect the YMDD mutation. This PCR assay might be an effective procedure for monitoring chronic hepatitis B patients treated with lamivudine or screening them before starting this anti-viral therapy.

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