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Rapid and Accurate Genotyping of YMDD Motif Variants in the Hepatitis B Virus Genome by an Improved Reverse Dot Blot Method

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By using the "flow-through hybridization" principle, we developed a new, rapid and accurate reverse dot blot (RDB) method to detect lamivudine resistance-associated YMDD motif variants in hepatitis B virus (HBV) genome. The improved RDB method was very fast at simultaneously detecting HBV YMDD wild-type and mutant motifs. In a blind analysis, 100 samples previously genotyped by DNA clonal sequencing analysis were used to evaluate the sensitivity and specificity of this assay. Conventional restriction fragment length polymorphism (RFLP) data were also used to test our method. In blind experiments, our improved RDB method had an accuracy and specificity of 100%, which was much higher than RFLP, which had an accuracy and specificity of only 83.0%. In clinical detection practice, 49 patients highly suspected of lamivudine resistance were successfully diagnosed by this method. Our improved RDB assay is a simple, rapid, cheap, semiautomatic, accurate, sensitive, and contamination-proof method of detecting lamivudine resistance-associated mutants in the human hepatitis B virus genome.

Chronic hepatitis B is caused by hepatitis B virus (HBV) infection, which remains a major public health problem worldwide. Chronic, persistent HBV infection develops to end-stage liver disease, such as liver cirrhosis and hepatocellular carcinoma (21). More than 380 million people have been infected by chronic HBV in the world, and 1 to 2 million people have died from it (7, 15). Antiviral therapy to treat chronic HBV infection aims at interrupting the progress and clinical outcome of the disease. The nucleoside analogue lamivudine, which has antiviral activity by inhibiting viral DNA synthesis (13), is extensively used in patients with chronic hepatitis B. Despite a good safety profile and initial efficacy, lamivudine-induced decreases in viral load are difficult to sustain for a long time due to the occurrence of viral drug resistance. The antiviral effects of the drug are gradually reversed in most cases, and rebound occurs. Many patients became drug resistant after 6 months of lamivudine therapy. In lamivudine-resistant patients, serum HBV DNA and alanine and aspartate aminotransferases increased rapidly (4, 22). Some sequence analysis revealed the emergence of a specific mutation in the YMDD (tyrosine, methionine, aspartate, aspartate) motif of the HBV polymerase gene, where the methionine was replaced with either an isoleucine or a valine (8, 14). It is important to detect the YMDD motif mutation during lamivudine treatment. At present, many methods have been developed to monitor HBV drug resistance, such as mass spectrometric analysis (11), fluorescence polarization (3), peptide nucleic acid clamping (17), restriction fragment length polymorphism (RFLP) (2), LiPA (18), fluorescence quantitative PCR (19), microarray (10), and sequencing. Most of these techniques are accurate but time-consuming, labor intensive, and hard to adapt to high-throughput screening. They are only amenable to the analysis by those who are well trained and well equipped, which is not suitable for small hospitals. RFLP, simple and available in most hospitals, is mainly used for detecting mono-infection but cannot detect samples with infection by mixed virus populations, especially those in which 5% to 10% are mutant virus (2, 16). The procedure is long and fussy, with multiple PCRs and multiple enzyme digestions. A skillful technician is required because a specific endonuclease reaction mixture must be developed for each mutant analyzed. Such mixtures only tend to be "home brews" (6, 12).

The reverse dot blot (RDB) method can be used to detect many mutants simultaneously. Samples differing only in one nucleotide are easily distinguished (5, 19). We combined "flow-through hybridization" technology with RDB assay to develop a rapid RDB method that can simultaneously detect the YMDD wild type and its mutants. Compared with the conventional passive hybridization process that required hours or even overnight hybridization, the "flow-through hybridization" takes only several minutes to complete by directing the flow of the target molecules toward the immobilized probes.

MATERIALS AND METHODS

Equipment and reagent. The "flow-through hybridization" was performed on KaiPu DNA hybriMax Rapid Hybridization Machine (Hong Kong DNA Ltd., Hong Kong, China). *Taq* DNA polymerase was from Fermentas MBI (Jingmei Biotech Co., Ltd.). The membrane strip was a Bigdye C membrane (Pall Co.).

Primer and probe design. We aligned the HBV genome DNA sequences extracted from the international DNA data banks (GenBank/EMBL/DDBJ) and selected the conserved region to design primers. Primers and probes (Table 1) were designed with the aid of bio-software Primer Express 2.0 (Applied Biosystem, Inc., Co.), DNAMAN 4.0 (Lynnon BioSoft), and Oligo 6.31 (Molecular Biology Insights, Inc., Co.). The reverse primer was labeled with biotin at the 5′ end, and the hybridization probes were labeled with amino group at the 5′ end. In order to judge the validity of hybridization process, we designed a color

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TABLE 1. Preferentially selected PCR primers for amplification of YMDD motif and probes for RDB analysis

Primer or probe	Strand	Sequence (5′→3′)	Nucleotide position	GenBank accession no.
Primers				
Forward	+	GTATTCCCATCCCATCATC	600–618	AB106885
Reverse	−	AATGTATACCCAAAGACAAAAGA	832–810	AB106885
Probes				
YMDD	+	GGCTTTCAGTTATATGTAT	726–744	AB106885
YIDD	+	GGCTTTCAGTTATATTGAT	726–744	AY800389
YVDD	+	GGCTTTCAGTTATGTGGAT	726–744	AY596107
Color control	−	TCTTCCAATATCCGGTC	3391–3375	AP003378

control probe (Table 1) for the hybridization control, which was labeled with a biotin group at the 5′ end and an amino group at the 3′ end. The color control probe can bind only with the chromogen but not with the targeting molecule.

Sample preparation. Six DNA samples with the YMDD wild type and two mutant types from the Affiliated Hospital of AnHui Medical University (HeFei, China) were used to develop the method. Each of these known serum HBV DNA sequences was previously characterized by DNA sequence analysis. One hundred archived serum samples from Guangzhou Liver Hospital (Guangzhou, China) were used to test this method; all of the sera were HBV DNA positive, with viral loads from 1×10^3 to 1×10^8 copies/ml. Eighteen samples had a viral load of 1×10^3 copies/ml. Forty-nine blood samples for clinical detection came from patients with clinically suspected lamivudine resistance in Guangzhou Liver Hospital based on the following criteria. (i) The patients had a virological (serum viral load of at least 1×10^3 copies/ml detected with an HBV fluorescence quantitative PCR detection kit from DaAn Gene Corporation, Guangzhou, China) and biochemical (alanine and aspartate aminotransferases) evidence of treatment failure. (ii) All patients were treated with lamivudine for at least 8 months, with an average of 16.9 months. Of the 49 clinic serum samples, 5 had a viral load of 1×10^3 copies/ml. All clinically suspected resistant samples were further tested by DNA clonal sequencing analysis.

Serum HBV DNA extraction and PCR conditions. From each serum sample, 50 μl was incubated at 100°C for 10 min in the same volume of DNA extraction buffer containing 50 mM NaOH, 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 1% NP-40, and 0.5 mM EDTA (pH 8.0), which was then centrifuged at 10,000 rpm for 5 min. Five microliters of the supernatant was amplified by PCR in 50 μl of 1× PCR buffer containing 200 μM of each deoxynucleoside triphosphate (dNTP), 2 U of *Taq* DNA polymerase, and 0.2 μM of each primer. In order to prevent contamination, we replaced dTTP with dUTP and added 0.5 U of uracil-DNA glycosylase (UDG) to the PCR system. The amplification was performed by using an Applied Biosystems 9700 thermal cycler (Perkin-Elmer) under the following conditions: incubation at 50°C for 3 min before an initial denaturation step at 93°C for 3 min, followed by 40 cycles of 93°C for 30 s, 55°C for 30 s, and 72°C for 35 s. A final extension was performed at 72°C for 5 min.

PCR products, clones, and sequencing. The PCR products were purified by phenol-chloroform (1:1) extraction, precipitated, and dissolved in water; fragments 233 bp in length were cloned into pMD 18-T simple vector (TaKaRa, Japan) according to the manufacturer's instructions. Cloned plasmids were sequenced at BioAsia Corporation (Shanghai, China).

RDB and RFLP analysis. RFLP was used according to Allen et al. (2). We used NdeI and NlaIII to discriminate the YMDD motif and YVDD motif, respectively. Based on the method of Zhang et al. (20), the improved RDB was used according to the principle of "flow-through hybridization," which was performed on the KaiPu DNA hybriMax Rapid Hybridization Machine. Its detailed steps are as follows. (i) Make the hybrid membrane strip by activating the membrane with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich, commercial grade) and then dotting the YMDD, YIDD, and YVDD motif probe and the color control probe at the given positions on the membrane; the amino group of the probe may bind with the carboxyl group of the membrane. (ii) Denature the PCR products. (iii) Prehybridize the membrane. (iv) Hybridize the target PCR products with the specific probes. (v) Wash the unhybridized PCR products. (vi) Combine peroxidase (POD) with the biotin group on the PCR products or on the color control probe. (vii) Wash the membrane to eliminate the uncombined POD. (viii) Color with 3,3′,5,5′-tetramethylbenzidine (TMB) chromogen. We set positive and negative controls for all detection. The machine works on the basis of the particular principle of "flowthrough hybridization"; there is a negative pressure under the airproof hybridization membrane, which was produced by pumping. All of the hybridization solution, washing solution, POD solution, and coloring solution flows through the membrane automatically. The improved RDB method actively directs the flow of the targeting molecules toward the immobilized probes within

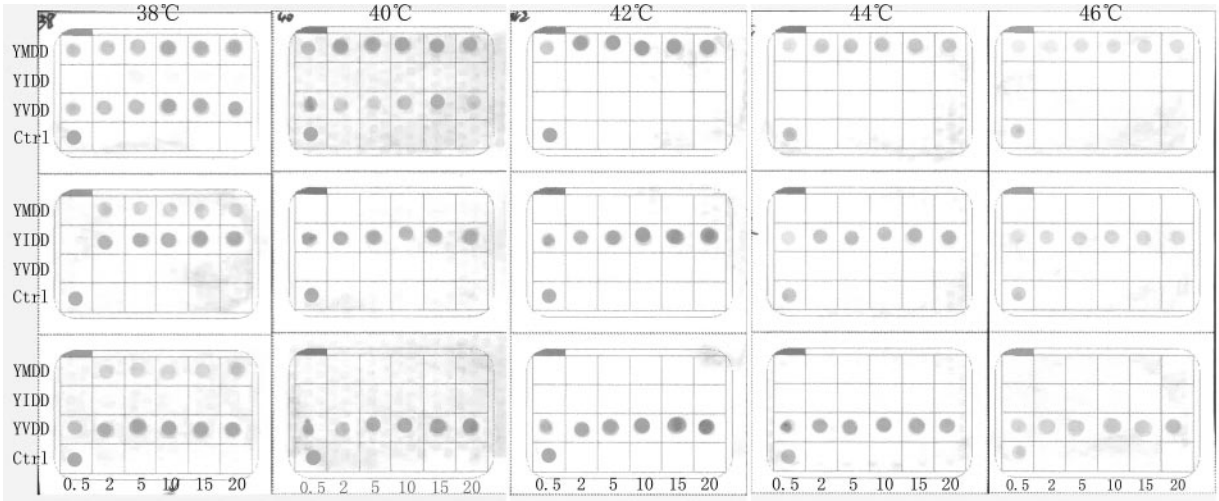

FIG. 1. Dynamic RDB assay profiles of the YMDD wild type and mutants at different probe concentrations and hybridization temperatures. The numbers (0.5, 2, 5, 10, 15, and 20) represent the probe concentrations (μmol/liter) of the HBV YMDD, YIDD, and YVDD motifs; the left dot in the last row in each hybrid membrane is the color control probe (Ctrl); the concentration is 2 μmol/liter.

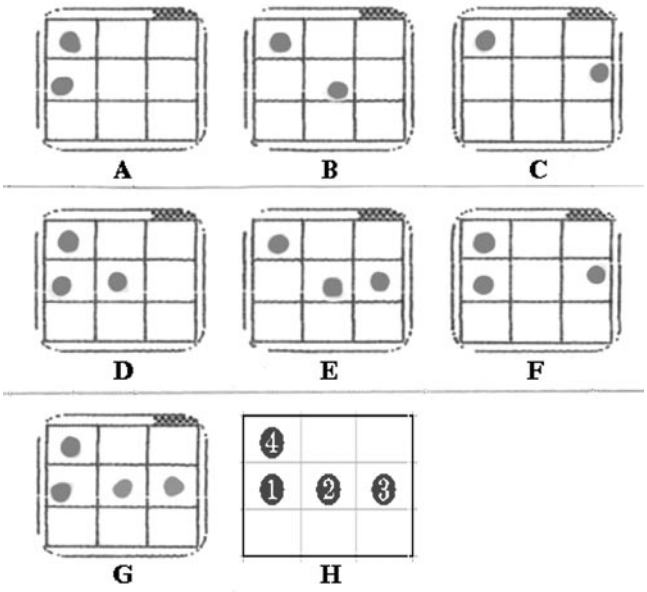

FIG. 2. Representative RDB profiles of infection with different YMDD motifs. (A) HBV YMDD. (B) HBV YIDD. (C) HBV YVDD. (D) Mixed HBV YMDD plus YIDD. (E) Mixed HBV YIDD plus YVDD. (F) Mixed HBV YMDD plus YVDD. (G) Mixed HBV YMDD plus YIDD plus YVDD. (H) Probes in the aligned mode in the hybridization membrane strip. Circled numbers: 1, HBV YMDD probe; 2, HBV YIDD probe; 3, HBV YVDD probe; and 4, color control probe.

the membrane fibers. The complementary molecules will be hybridized and form duplexed DNA; at the same time, any unbound molecules will be removed by passing through the membrane. This speeds up the interaction between the complementary molecules, reduces the hybridization time from hours down to minutes, and provides results hundreds of times faster than traditional passive hybridization methods.

Determination of the optimum probe concentration and hybridization temperature for the RDB assay. We used different concentrations of YMDD, YIDD, and YVDD motif-specific probes (0.5, 2, 5, 10, 15, and 20 μmol/liter, respectively) on the same membrane strip to decide the optimal probe concentration. The RDB experiments were carried out at different hybridization temperatures (38, 40, 42, 44, and 46°C) to determine the optimal hybridization temperature.

RESULTS

Validation of RDB and sequence analysis. To enable the unequivocal interpretation of data according to RDB analysis, probes used in the RDB assay were selected and optimized to produce a clear and specific dot pattern. The probe concentration and hybrid temperature were optimized for the RDB analysis (Fig. 1). The results indicated that all of them had nonspecific hybridization blue dots that appeared at 38°C and were reduced with the increase of temperature. A clear hybridization blue dot and clean background appeared at 42°C; YMDD and YIDD had a weak hybridization dot at 44°C and a weaker dot at 46°C. This indicated that 42°C was the optimal temperature for hybridization. The hybridization dots became bright and clear with the increase in probe concentration. At a concentration of 5 μmol/liter, all of them had a clear blue signal that was similar to the results at 10, 15, and 20 μmol/liter. So we concluded that 5 μmol/liter was an economically optimal concentration for hybridization.

TABLE 2. Concordant results obtained by blind analysis by three methods of 100 archival serum samples

Infection type	Genotype[a]	No. of results by:		
		RDB	RFLP	Sequence analysis
Mono	M	12	12	12
	I	4	4	4
	V	20	20	20
Mixed	M+I	3	3	3
	M+V	40	38	40
	I+V	6	4	6
	M+I+V	15	2	15
Total		100	83	100

[a] M, HBV YMDD motif; I, HBV YIDD motif; V, HBV YVDD motif.

Four samples with YMDD mutations and two samples with the YMDD wild type previously characterized by DNA sequence analysis were used to develop the assay. Each of those two mutant types was represented in this panel of samples. Figure 2 showed the representative RDB assay profiles for the different motifs of HBV YMDD infection in serum. The results indicated that the assay could clearly distinguish each YMDD wild type and mutant in one RDB profile. For all detections, they were included in the following possible RDB profiles: (i) YMDD, YIDD, or YVDD mono-infection (Fig. 2A, B, and C); (ii) mixed infection with two of the three YMDD motifs, such as YMDD plus YIDD (Fig. 2D), YIDD plus YVDD (Fig. 2E), or YMDD plus YVDD (Fig. 2F); or (iii) mixed infection with YMDD, YIDD, and YVDD (Fig. 2G).

Blind analysis. To evaluate the accuracy of RDB for detecting YMDD motif mutations, we performed a comparison of this assay with sequencing and RFLP in a blind analysis. A total of 100 serum HBV DNA archival samples that had been previously characterized by RFLP or DNA clonal sequencing were detected. The RFLP and sequencing results were unknown until the genotypes obtained by RDB were scored. All of the samples tested by RDB were found to be in 100% (100/100) concordance with the sequencing data (Table 2) but only 83.0% (83/100) concordance with RFLP.

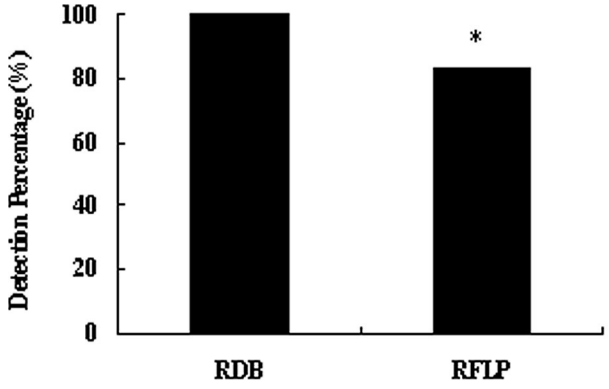

FIG. 3. Comparison of the accuracy and specificity of RDB and RFLP assays ($n = 100$; $P < 0.01$).

TABLE 3. Cases used in clinical detection

Infection type	Genotype	No. of cases
Mono	YMDD	4
	YIDD	2
	YVDD	10
Mixed	YMDD+YIDD	1
	YMDD+YVDD	26
	YVDD+YIDD	2
	YMDD+YIDD+YVDD	4
Total		49

Specificity and sensitivity. Compared to DNA sequencing and RFLP, the specificities of RDB were 100% and 83.0%, respectively (Fig. 3). As to the sensitivity, the improved RDB method can successfully detect all of the serum HBV with a virus load from 1×10^3 to 1×10^8 copies/ml; i.e., all of the positive HBV serum can be successfully tested.

Clinical detection. To evaluate the application of this assay, 49 patients highly suspected of clinical lamivudine resistance, including 5 subjects with a serum HBV viral load of 1×10^3 copies/ml, were detected. All of the samples were successfully detected by the methods; they were all included in the RDB assay profiles described above. There were 4 YMDD infections, 2 YIDD infections, 10 YVDD infections, 1 mixed YMDD and YIDD infection, 26 mixed YMDD and YVDD infections, 2 mixed YIDD and YVDD infections, and 4 mixed YMDD, YIDD, and YVDD infections (Table 3). The results were all confirmed by the DNA clonal sequencing analysis.

DISCUSSION

After long periods of antiviral treatment with lamivudine in HBV carriers, there develops a high risk of HBV drug resistance (8, 14). The sensitivity of lamivudine decreased after the emergence of resistance mutants leading to treatment failure (1). With the development of modern medicine, detailed virus serological and molecular biology information is required to know the status of virus variation, which is important to guide clinical practice. The method developed in this study can be used in detection of HBV YMDD, YIDD, or YVDD monoinfection and mixed infections. It can provide individual pieces of key molecular virology information for lamivudine therapy.

In this study, we have described a simple, rapid, semiautomatic, reliable, and contamination-proof approach for genotyping the YMDD motif variants in the HBV genome. We developed a commercially prepared HBV YMDD motif variant detection kit equipped with the KaiPu DNA HybriMax Rapid Hybridization Machine. The machine was designed based on the particular principle of "flow-through hybridization." There is a negative pressure under the airproof hybridization membrane that was produced by pumping, so the improved method can actively direct the flow of the target molecules toward the immobilized probes within the membrane fibers, which enables rapid hybridization to occur. The dominant characteristic of the improved RDB method is that all of the PCR products, washing buffer, binding solution, and coloring solution flow through the hybrid membrane quickly and directly with the help of negative pressure, which is semiautomated and is essentially different from the traditional method. The complementary molecules will be hybridized and form duplexed DNA; at the same time, any unbound molecules will be removed through the membrane. This speeds up the interaction between the complementary molecules, reduces the hybridization time from hours down to minutes, and provides results hundreds of times faster than the traditional passive hybridization methods. For the study, we designed and optimized not only the specific probes for the specific virus types but also the color control probe for the hybridization operation to reach 100% specificity. The color control probe can bind only with the chromogen, but it can't bind with the target molecule, which helps to judge the validity of hybridization. Instead of using dTTP, we used dUTP and UDG in the PCR system to prevent PCR products from contaminating. As to the sensitivity, the improved method can detect a positive HBV serum DNA level as low as the viral load of 1 copy per microliter (i.e., 10^3 copies/ml), which is the critical value to judge if it is a positive HBV serum in HBV fluorescence quantitative PCR detection. All of these findings indicate the method is sensitive, specific, and ensures quality in clinical tests.

In addition, the improved RDB method is clean, versatile, and less expensive than traditional hybridization. PCR-based direct nucleotide sequence analysis (9) is considered as the gold standard for mutation detection. DNA sequencing is highly sensitive and specific for mutational scanning but is time-consuming and costly. The new RDB assay directs all of the PCR products and solution to directly flow through the hybrid membrane, which increases the diffusivity and local reaction concentration of the nucleic acid molecule and occurs in three-dimensional volumes rather than the conventional two-dimensional surface. Compared with the traditional RDB method and the commercially available Inno-LiPA assay, which require several hours to complete the molecular hybridization process and large volumes of sample and reagent, hybridization by the new technique can be done within 7 min with very small samples and reagent volumes, which increases the hybridization efficiency markedly and reduces the running cost. The cost of this assay is low: the KaiPu DNA hybriMax Rapid Hybridization Machine costs only $2,000, and the total cost is about $1.40 for a sample. It's available for most small hospitals. With the new method, the background of the hybridization membrane strip is very clean and nonspecific hybridization is reduced. The method can be used to rapidly detect not only a large number of clinical samples and genotypes simultaneously, but also single-base mutations. (Fifteen or more samples can be detected simultaneously; more than 24 probes can be used for a given sample.) All of the probes are integrated in a small hybridization membrane just like a low-density microarray, which makes high-throughput detection possible. The improved method can also be extensively used to rapidly detect other clinical microbes or genetic disorders if we change the probes in the membrane strip.

As for RFLP, we used two kinds of restriction enzymes to discriminate the YMDD, YIDD, and YVDD motifs. RFLP is a fussy and tedious technique, in which it is easy to make mistakes and hard to discriminate the small DNA fragments digested by the endonuclease. Skilled personnel are required because a specific endonuclease reaction mixture has to be

developed for each mutant. In addition, gel electrophoresis can discriminate limited concentrations of PCR products with much lower sensitivity than the RDB, which includes a cascade amplification reaction. The RFLP often leaves out the samples with mixed virus populations and the low-component virus population (Table 2), which is suited only for mono-infection; this may explain the lower specificity and sensitivity of the RFLP analysis.

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