The detection of YMDD mutation of hepatitis B virus by allele-specific PCR and a cross-contamination proofed device

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Abstract: Lamivudine (LAM) is commonly used to treat Hepatitis B virus (HBV) infection, but its use frequently induces drug resistance. Therefore, rapid and correct detection of drug-resistant HBV is important for effective treatment of HBV infection. In this study, we aimed to develop a novel, simple, and user-friendly method for the detection of LAM resistant HBV. Samples were collected from 60 HBV infected patients for the analysis by allele-specific polymerase chain reaction (AS-PCR), nucleic acid detection strip (NADS) and a cross-contamination proofed device. HBV YMDD mutations were detected by AS-PCR, restriction fragment length polymorphism (RFLP) and DNA sequencing. A 91.7% concordance between all three methods was obtained. Compared to sequencing and RFLP, AS-PCR detected more samples with mutant variants and was more sensitive. This novel method had a detection limit of approximately 10^3copies/ml and detected a variant of only 5% of total HBV population. In conclusion, we develop a new assay which could be useful for the detection of HBV LAM resistance, especially in resource-poor settings.

Keywords: Hepatitis B virus, clinical assay, allele-specific PCR, molecular assay, antiviral resistance

INTRODUCTION

Lamivudine (LAM) is a nucleotide-analogue inhibitor that prevents genomic replication of Hepatitis B virus (HBV) and has been widely used for the treatment of HBV (Dienstag *et al*., 1995). Nevertheless, LAM frequently promotes the appearance of drug resistant HBV strains, mainly due to the mutations in the Tyrosine-Methionine-Aspartate-Aspartate (YMDD) motif located at the C-terminal domain of the polymerase. The most common mutation is the change of methionine 204 to Valine (YVDD mutant) or Isoleucine (YIDD mutant), and less commonly to Serine (Zoulim 2001).

Various methods have been developed for the detection of HBV LAM resistance mutations, including restriction fragment length polymorphism (RFLP), DNA sequencing, gene chip, allele-specific polymerase chain reaction (AS-PCR), PCR-ligase detection reaction (LDR) and mass spectroscopy (Lok *et al.,* 2002; Hong *et al*., 2004; Jang *et al.,* 2004; Woo *et al.,* 2008; Xu, *et al.,* 2010; Tauseef *et al*., 2012). However, few simple and low-cost methods have been designed for the application in developing countries where the majority of HBV patients occur. Therefore, a highly sensitive and user-friendly diagnostic method of HBV LAM resistance is required.

In this study, we described the development and application of an allele-specific PCR (AS-PCR), nucleic acid detection strip (NADS) and cross-contamination-proofed device based method to detect YMDD mutations in HBV. AS-PCR is used to discriminate a single-base mismatch using a 5'-end biotin labeled primer, and this extended product of the biotin labeled primer is then able to hybridize with a 3'-end fluorescein isothiocyanate (FITC) labeled probe in the reaction tube. The hybridized complex can be directly detected in the cross-contamination proofed and disposable device. Finally, the device allows for an easy and quick read-out, usually within 15 min.

MATERIALS AND METHODS

Patients and samples

Sixty patients with HBV infection were included in this study. These patients received LAM treatment at Huai'an First Hospital affiliated to Nanjing Medical University (Huai'an, China) from September 2008 to October 2011. The duration of LAM therapy was 20 months (range of 12 to 40 months). All samples were collected after obtaining informed consent from each patient, and the study protocol was approved by Ethics Committee of Huai'an First Hospital. 5ml blood samples were collected from each patient and the serum was separated and distributed as 0.5ml aliquots.

Oligonucleotides and DNA extraction

Oligonucleotides were synthesized by Shanghai Sangon Tech (Shanghai, China), with the sequences shown in table 1. HBV DNA was extracted from 0.5ml serum by

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phenol-chloroform method and used for AS-PCR, PCR-RFLP and DNA sequencing. OptiQuant HBV Viral DNA Panel was purchased (Acrometrix, Canada) as the wild-type plasmid for AS-PCR.

AS-PCR

AS-PCR was performed with 10μl *Premix* Ex Taq™ (Takara, Japan) and 4μl of extracted DNA, 200nM biotin labeled primer (HBVPF), 50nM unlabeled primer, 100 nM FITC-labeled probe (HBVDR3F). The mixture was held at 95°C for 5 min, followed by 45 cycles of 94°C for 10 sec, 62°C for 15 sec and 72°C for 15 sec and extended at 72°C for 5min, then kept at 95°C for 5min before cooling to room temperature.

The cross-contamination proofed device

During the AS-PCR, the biotin labeled primer extended, and the amplicon of the biotin labeled primer was then able to hybridize with a 3'-end FITC labeled probe in the reaction tube after the reaction. The FITC/biotin labeled amplicons were detected in the cross-contamination proofed device (shown in fig. 1). After 10 min, the results were read, and the control line on the NADS was used to monitor the availability of the strip in the cross-contamination proofed device. If both of T-line and C-line were visible, the result was positive. A negative read was scored when only C line was visible. If no T-line nor C-line was visible, the assay was regarded as invalid.

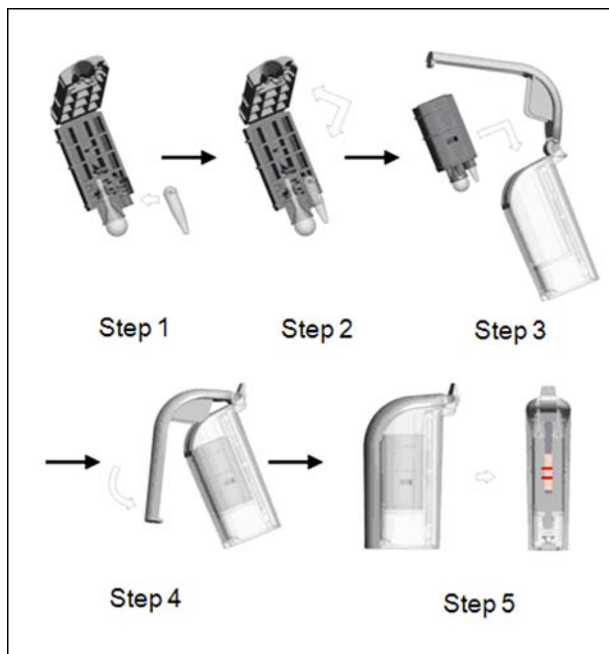

Fig. 1: The procedure of using cross-contamination proofed device. The reaction tube was placed in the cartridge (step 1); the cartridge was closed to immobilize the reaction tube (step 2); closed cartridge was inserted into the detection chamber (step 3); the handle of the detection chamber was closed to seal the vessel into the chamber and open the buffer in reservoir and amplicon in the reaction tube (step 4); the assay result was read and scored by naked eye after 10 to 15 min (step 5).

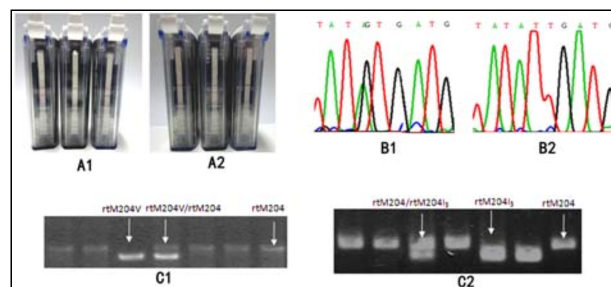

Fig. 2: Representative results of AS-PCR, sequencing, RFLP analysis. (A) AS-PCR: Three devices were used to detect each strain (rtM204, rtM204I and rtM204V), A1 indicated rtM204V/WT mixed strains; A2 indicated rtM204I. (B). Sequencing: B1 indicated rtM204V/WT mixed strains; B2 indicated rtM204I3. (C). PCR-RFLP: C1 indicated the presence of rtM204V and WT; C2 indicated the presence of rtM204I and WT strains.

PCR-RFLP and sequencing analysis

PCR products were isolated and used for RFLP analysis, as previously described (Chayama *et al.*, 1998). In addition, PCR products were subjected to sequencing analysis by ABI Prism 3700 genetic analyzer.

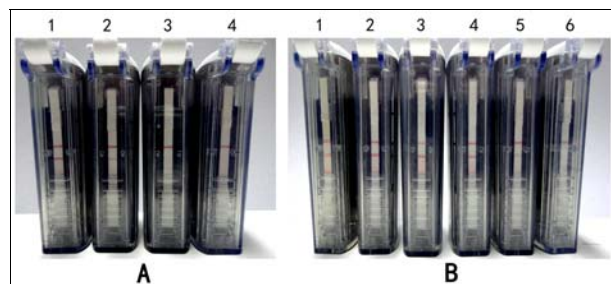

Fig. 3: Sensitivity and specificity of AS-PCR. (A) rtM204I3 plasmid was serially diluted from 10^4 to 10^2 copies /ml: 1, 10^4; 2, 10^3; 3, 10^2; 4, negative control. The detection limit was 10^3 copies/ml. (B) Plasmids were mixed with the mutant type (rtM204I) and wild type at various ratios: 1, 50:50; 2, 30:70; 3, 20:80; 4, 10:90; 5, 5:95; 6, 0:100. The mutant viral population present at 5% of the total viral load was detected.

STATISTICAL ANALYSIS

Statistical analysis were performed using McNemar`s Chi-squared test. Statistical significance was accepted at $P< 0.05$.

RESULTS

Sensitivity of AS-PCR

To evaluate the sensitivity of AS-PCR for the detection of mutant and wild type (WT) HBV strains, we made 10-fold serial dilutions of plasmid controls from 10^4 to 10^2

copies/ml. The detection limit of the AS-PCR was determined to be 10^3 copies/ml (fig. 2A). Mixtures of 10^5 copies/ml of resistant rtM204I or rtM204V and WT viruses were prepared at the following ratios: 50:50, 30:70, 20:80, 10:90, 5:95, 0:100. AS-PCR was able to detect the minority HBV population at only 5% of total HBV population (fig. 2B), which represented $5x10^3$ copies/ml of mutant DNA in $9.5x10^4$ copies/ml of WT DNA.

Table 1: The sequences of the primer and probes used in this assay

Primer name	Sequence
HBVPF	5'-BIOTIN-GGCTTTCGCAAGATTCCTAT-3'
HBVDR3F	5'-GCACTAGTAAACTGAGCCA-FITC-3'
YMDD	
rtM204	5'-AATACCACATCATCCCT-3'
YIDD	
$rtM204I_1$	5'-CCAATACCACATCATGGA-3'
$rtM204I_2$	5'-CCAATACCACATCATGAA-3'
$rtM204I_3$	5'-CCAATACCACATCATGTA-3'
YVDD	
rtM204V	5'-AATACCACATCATCCTCA-3'

The detection of YMDD mutants in serum from patients

We detected YMDD mutations in serum from HBV infected patients using our method, PCR-RFLP and direct sequencing. The typical results were shown in fig. 3. As shown in table 2, 32 mutants were detected by RFLP including 11 rtM204V mutants, 10 rtM204I mutants, 5 rtM204I/WT mixture and 6 rtM204V/WT mixture; 31 mutants were detected by sequencing including 11 rtM204V mutants, 11 rtM204I mutants, 4 rtM204I/WT mixture and 5 rtM204V/WT mixture. AS-PCR detected 35 mutant strains including 11 rtM204V mutants, 10 rtM204I mutants, 7rtM204I/WT mixture and 7 rtM204 V/WT mixture. The consistence of AS-PCR results with those of RFLP and sequencing was 95% and 91.7%, respectively. AS-PCR detected 3 and 2 mixed samples that could not be detected by RFLP and sequencing, respectively. Moreover, we found that no samples have both types of mutations.

DISCUSSION

Here we described a rapid and sensitive assay for the determination of YMDD mutations in HBV polymerase. It consists of two steps: AS-PCR for the discrimination, followed by the detection of product with a cross-contamination proofed device. The sensitivity of AS-PCR was 10^3 copies/ml, and the minority HBV population at only 5% of total HBV population could be detected in a mixed population.

To investigate the application of AS-PCR assay for the diagnosis of clinical samples, the results of AS-PCR were compared with those of RFLP and sequencing, the overall concordance between all methods was 95% for AS-PCR and RFLP, and 91.7% for AS-PCR and sequencing, respectively. However, for the samples containing mixed mutant and WT HBV, the concordance was 64.3% (9/14). 4 samples containing rtM204I/wt and 5 samples containing rtM204V/WT were detected by sequencing, while 5 samples containing rtM204I/WT and 6 samples containing rtM204V/WT were detected by RFLP. In contrast, 7 samples containing each mix were detected by AS-PCR. The discordance of the results is perhaps due to the different sensitivity for mixed viral species, AS-PCR can detect a 5% minor population, while RFLP and sequencing can only detect 10% and 20%, respectively (Allen *et al*., 1999). AS-PCR detected both strains, which indicates the superiority of this method.

Two of the most attractive features of our method are that: first, AS-PCR can be performed in less than 3 hours; second, the products can be detected in a contamination proofed device which contains NADS. The detection procedure reduces potential cross-contamination of the amplicon by inserting the reaction tube in a closed device directly. However, more studies are needed to further optimize the assay, for instance, it only provides qualitative results. Furthermore, to discriminate whether YMDD region has mutations, the current assay requires three devices for YMDD, YIDD mutation and YVDD mutation, respectively, since it cannot simultaneously discriminate YIDD and YVDD mutations.

Both INNO-LiPA HBV DR line probe and PLNA could detect different types of mutation and have been used to

Table 2: Comparison of the results of AS-PCR, PCR-RFLP, and sequencing

Genotype	Mutation Detected by (No. of Samples)		
	PCR-RFLP	AS-PCR	Sequencing
YMDD	28	25	29
YVDD	11	11	11
YIDD	10	10	11
YMDD+YIDD	5	7	4
YMDD+ YVDD	6	7	5

Note: A total of 60 samples were tested.

detect HBV YMDD mutations on the strip (Lok *et al.,* 2002; Xu *et al.,* 2010). INNO-LiPA takes over 5 hours and requires strict hybridization and washing conditions (Alagozlu *et al*., 2013; Mese *et al*., 2013). In addition, the contamination of amplicon makes it difficult to use in normal laboratory (Ozekinci *et al*., 2014). Although PLNA is finished within 3 hours without significant post-amplification processing, it has the problem of amplicon contamination. In contrast, our assay can be performed in less than 3 hours with no use of expensive instrument and solve the problem of amplicon contamination due to the use of the disposal device.

In summary, a rapid and effective and cross contamination proofed method for the detection of YMDD mutants in HBV was developed. The assay has high sensitivity and good performance with clinical sample. Therefore, it has the potential as a routine assay for early detection of HBV YMDD mutation, especially in developing countries where there are a high HBV burden and limited resources.

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