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Application of PCR-LDR-Nucleic Acid Detection Strip in Detection of YMDD Mutation in Hepatitis B Patients Treated With Lamivudine

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Chronic hepatitis B virus (CHBV) infection causes cirrhosis and hepatocellular carcinoma. Lamivudine (LAM) has been successfully used to treat CHBV infections but prolonged use leads to the emergence of drug-resistant variants. This is primarily linked to a mutation in the tyrosine–methionine–aspartate–aspartate (YMDD) motif of the HBV polymerase gene at position 204. Rapid diagnosis of drug-resistant HBV is necessary for a prompt treatment response. Common diagnostic methods such as sequencing and restriction fragment length polymorphism (RFLP) analysis lack sensitivity and require significant processing. The aim of this study was to demonstrate the usefulness of a novel diagnostic method that combines polymerase chain reaction (PCR), ligase detection reaction (LDR) and a nucleic acid detection strip (NADS) in detecting site-specific mutations related to HBV LAM resistance. We compared this method (PLNA) to direct sequencing and RFLP analysis in 50 clinical samples from HBV infected patients. There was 90% concordance between all three results. PLNA detected more samples containing mutant variants than both sequencing and RFLP analysis and was more sensitive in detecting mixed variant populations. Plasmid standards indicated that the sensitivity of PLNA is at or below 3,000 copies per ml and that it can detect a minor variant at 5% of the total viral population. This warrants its further development and suggests that the PLNA method could be a useful tool in detecting LAM resistance. ***J. Med. Virol. 82:1143–1149, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: HBV; ligase detection reaction; nucleic acid detection strip

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INTRODUCTION

The Hepatitis B virus (HBV) inflicts nearly 2 billion people and accounts for 600,000 deaths annually. Three hundred fifty million people suffer from chronic HBV (CHBV) which causes cirrhosis and hepatocellular carcinoma [WHO, 2009]. A majority of CHBV patients reside in developing world countries, particularly in Southeast Asia and the Western Pacific [Gust, 1996; Custer et al., 2004]. Although a highly effective vaccine exists, treatment options remain limited for chronically infected patients. Nucleotide-analogue inhibitors such as lamivudine (LAM) effectively reduce HBV DNA levels in serum and prevent liver damage through HBV RT inhibition [Dienstag et al., 1995]. However, prolonged LAM therapy is associated with the emergence of LAM-resistant mutant strains and viral breakthrough has been observed in 16–55% of patients after 1 year of treatment [Lai et al., 1998; Dienstag et al., 1999; Jardi et al., 1999; Lau et al., 2000; Leung et al., 2001; Zoulim, 2001; Bozdayi et al., 2003].

LAM resistance is primarily linked to a mutation at amino-acid position 204 in the tyrosine–methionine–aspartate–aspartate (YMDD) motif of the HBV polymerase gene [Ling et al., 1996; Tipples et al., 1996; Allen et al., 1998, 1999]. The most common mutations result in an amino acid change from methionine to valine

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(rtM204V) or isoleucine (rtM204I) and less commonly to serine (rtM204S) [Niesters et al., 1998].

LAM-resistant HBV mutants have a decreased replication capacity compared to wild-type (WT) HBV. Therefore breakthrough viral infections are precluded by the presence of small amounts of mutant virus in a larger population of WT HBV [Chayama et al., 1998]. Low levels of mutant DNA have also been found in treatment-naive patients [Kobayashi et al., 2001; Zoulim, 2001; Kirishima et al., 2002; Heo et al., 2004]. Therefore, a highly sensitive molecular diagnostic for early diagnosis and treatment monitoring is required for the success of long-term treatment regimens.

The two most widely used methods for drug-resistance detection are direct sequencing and restriction fragment length polymorphism (RFLP) analysis. Direct sequencing allows detection of all drug-resistance conferring mutations, but requires the minor species to be present at 20% of the total HBV population. RFLP is more sensitive but requires a unique endonuclease for each mutation. Both methods are not amenable to routine diagnosis in low-resource settings. A number of new methods such as the line probe reverse hybridization, oligonucleotide microarray, MALDI-TOF mass spectroscopy, oligonucleotide chip, pyrosequencing, real-time PCR, multiplex PCR, and invasive nuclease cleavage all allow increased sensitivity compared to sequencing and RFLP [Stuyver et al., 2000; Aberle et al., 2001; Lok et al., 2002; Zhang et al., 2002; Hong et al., 2004; Jang et al., 2004; Lindstrom et al., 2004; Chen et al., 2005; Xiao et al., 2006; Woo et al., 2008; Degertekin et al., 2009; Ntziora et al., 2009]. The commercially available reverse hybridization assay LiPA DR can detect a minor virus population that is prevalent at 5% of the total HBV population using membrane-bound oligonucleotide probes. However, total processing time of this method is over 5 hr and is primarily being marketed to high-income markets.

Unfortunately most methods are either too complicated or costly to perform in low-resource settings where a majority of CHBV patients live. A simple method to rapidly diagnose drug resistance is needed.

To meet these needs, a method that utilizes polymerase chain reaction (PCR) and ligase detection reaction (LDR) in a single tube to amplify and discriminate single base mismatches associated with LAM resistance has been developed. The resulting ligated oligonucleotides are visually detected with a nucleic acid detection strip (NADS) [Chow et al., 2008; Goldmeyer et al., 2008]. Asymmetric PCR is used to amplify the region of the HBV genome that contains the YMDD motif. Single nucleotide variations are detected in the same tube through a thermal DNA–ligase mediated ligation of two probes that are labeled with biotin at the 5′ end and fluorescein isothiocyanate (FITC) at the 3′ end. The NADS allows easy visual read-out following probe conjugation with streptavidin-coated red nanoparticles and subsequent capture by an anti-FITC test line. This entire method requires just 3 hr to perform and involves no significant post-amplification processing of the sample.

The results comparing the PCR-LDR-NADS assay (PLNA) for LAM-mutant detection to RFLP and genetic sequencing show comparable sensitivity between all three methods.

MATERIALS AND METHODS

Synthesis of Oligonucleotides

Two PCR primers (HBVF1 and HBVB1) were designed to flank a 92 bp region of the HBV genome that includes rtM204. Two LDR detector probes and five LDR discrimination probes were designed to detect WT and LAM-resistant mutants (rtM204V, $rtM204I_1$, $rtM204I_2$, $rtM204I_3$). Each LDR reaction mixture contained one 5′ biotin-labeled discrimination probe specific for the mutated nucleotide and one 3′ FITC conjugated detector probe. The primers and probes were sequenced by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd, and the sequences are shown in Table I.

In order to allow single-tube PCR-LDR amplification and base-pair discrimination, primers and probes were designed with the following requirements: (1) to ensure the melting temperatures (Tm) of PCR and ligation oligonucleotides were significantly different. (2) To ensure that the mutation site was at the n − 1 site near the 3′ end of the detector probe used for ligation.

TABLE I. The Sequences of the Primer and Probes Used in the PLNA

Type	Primer name	Sequence
	HBVF1	5′-TCAGTGGTTCGTAGGGCTTTCCCCC-3′
	HBVB1	5′-GGACTCAAGATGTTGTACAGACTTGGC-3′
YMDD		
	$rtM204_1$	5′-ATGATGTGGTATTG(FITC)-3
	$rtM204_2$	5′-(Biotin) TTTCAGCTATACGG-3
YIDD		
	$rtM204_1$	5′-ATGATGTGGTATTG(FITC)-3
	$rtM204I_1$	5′-(Biotin) TTTCAGCTATATAG-3
	$rtM204I_2$	5′-(Biotin) TTTCAGCTATATCG-3
	$rtM204I_3$	5′-(Biotin) TTTCAGCTATATTG-3
YVDD		
	$rtM204V_1$	5′-GGATGATGTGGTATTG(FITC)-3
	$rtM204V_2$	5′-(Biotin) CTTTCAGCTATGT-3

Asymmetric PCR and LDR

Concentrations of both excess and limiting primers were designed for asymmetric PCR. This ensures a higher concentration of available single stranded target for ligation. LDR probe concentrations were optimized by diluting the probe and observing the resulting reaction. Multiple reactions with different concentrations of primer, probe, Taq DNA polymerase and Taq DNA ligase were performed to define the optimal reaction mixture for efficient and accurate amplification and detection. The resulting 20 µl reaction mixture was as follows: 0.2 µM HBVF1 and 0.05 µM HBVB1, 0.1 µM discrimination probe and 0.05 µM detector probe (for YIDD, 0.015 µM detector probe was used), 1× Taq DNA ligase buffer (20 mM Tris–HCl (pH 7.6), 25 mM KAc, 5 mM $Mg(Ac)_2$, 10 mM DTT, 1 mM NAD (0.1% Triton X-100)) and 0.75× PCR buffer (100 mM Tris–HCl (pH 8.8), 500 mM KCl), 50 ng genomic DNA, 0.2 mM dNTP, 5 U Taq DNA polymerase (Sangon, Shanghai, P.R. China) and 10 U Taq DNA ligase (NEB). LDR was performed three times for the detection of three strains: mutant type (rtM204V, $rtM204I_3$) and WT (rtM204). Two PCR primers and seven ligation probes were used to amplify and discriminate between all variants (Table I).

The PCR conditions were as follows: 40 cycles of denaturation at 95°C for 10 sec and annealing and extension at 72°C for 15 sec. This was followed by LDR: two cycles of denaturation 95°C for 20 sec, and annealing and ligation at 45°C for 4 min. Temperatures were based off of the melting points for PCR primers and LDR probes.

NADS Preparation

NADS consisted of a sample pad overlaid onto a conjugate pad containing red nanoparticles, and a nitrocellulose membrane containing both a test and control line. An absorbent pad overlaid onto the nitrocellulose membrane provides sufficient capillary pressure for sample flow (Fig. 1). NADS were prepared as follows: red uncoated nanoparticles were incubated in streptavidin (10 mg/ml) containing glutaraldehyde buffer for 2–3 hr at 20°C and centrifuged at 8,000 rpm for 10 min. The resulting streptavidin-coated nanoparticles were briefly incubated in hydroxylamine and immobilized on a conjugate pad. For the test line anti-FITC was diluted in 1× PBS and dispensed on the nitrocellulose membrane. Biotin was dispensed on the nitrocellulose membrane for the control line.

Preparation of DNA Standards

Plasmids containing one copy each of WT, rtM204V, or rtM204I HBV polymerase gene were constructed for PLNA. Tenfold serial dilutions from 10^8 to 10^3 copies/ml were used for direct detection and to create mixed controls containing both mutant and WT HBV DNA. Mixed variant samples were prepared by mixing 10^5 copies/ml of resistant (rtM204I or rtM204V) and WT viral DNA in the following ratios: 70:30, 50:50, 30:70, 20:80, 10:90, 5:95, 0:100. Triplicate samples of serial dilutions and mixed controls were used to test the sensitivity of PLNA.

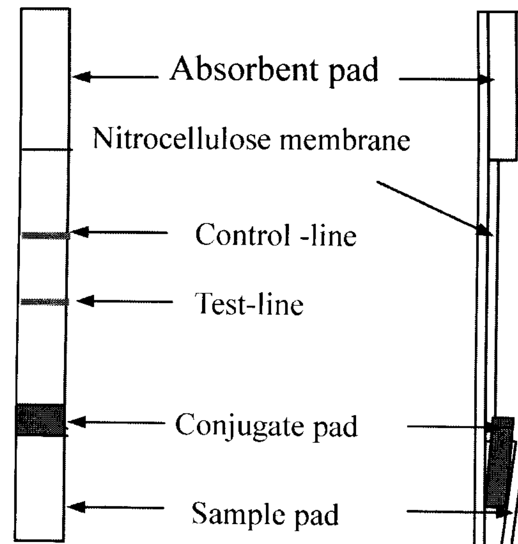

Fig. 1. The structure of the nucleic acid detection strip (NADS) is made up of five main parts: a plastic backing, nitrocellulose membrane, sample pad, conjugate pad, and absorbent pad. The conjugate pad contains streptavidin coated red nanoparticles (20–300 nm). The test line was labeled with anti-fluorescein isothiocyanate (FITC), and control line was labeled with biotin.

Detection of Mutants in DNA Extracted From Clinical Samples

Sera from 50 patients infected with HBV confirmed by direct DNA sequencing, who received long-term LAM therapy were used for analysis. The duration of LAM therapy for the patients infected with HBV was 20 months (range of 12–40 months). HBV DNA was extracted from 200 µl of each serum sample using the phenol–chloroform method and eluted in 50 µl (final volume) of 10 mM Tris–HCl. These samples were then directly used for PLNA, PCR-RFLP and sequencing.

The Detection of Mutants Using NADS

For detection, 10 µl of PCR-LDR product was diluted and denatured in 90 µl of 0.02 M NaOH, heated to 95°C for 5 min and allowed to cool to room temperature. The resulting 100 µl solution was applied directly to the sample pad of the NADS. After 15 min NADS were examined for a red control and test line. The control line indicated that the test was successful and the test line indicated the presence of a specific strain. To detect all three strains, three LDR and NADS assays were performed.

PCR-RFLP and Sequencing

PCR-RFLP and direct DNA sequencing were used to confirm PLNA results from clinical samples. Patient

DNA was first amplified with PCR [Jang et al., 2004] followed by RFLP analysis [Chayama et al., 1998]. PCR products were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 3700 genetic analyzer. Results were compared to PLNA.

RESULTS

Verification of LDR Product and NADS Detection

To verify the specificity of LDR and NADS for detecting single-base pair mismatches, WT DNA was amplified and LDR was performed in the presence of three different probes: (1) WT probes (2) rtM204V probes (3) rtM204I probes. Each of these three reactions was performed with and without Taq DNA Ligase. The resulting six LDR products were diluted with either 2× SSC or 0.02 M NaOH to encourage either hybridization or denaturation, heated to 95°C for 5 min, allowed to cool and applied to the NADS. When diluted with 2× SSC a positive result was found in both Taq DNA ligase positive and negative samples and did not depend on the type of probes present. When diluted in NaOH only the reactions containing Taq DNA ligase and the WT probes (perfectly base-paired target-probe complex) were positive. Similar results were found when these reactions were repeated with amplified mutant DNA (rtM204V and rtM204I): following NaOH dilution, only the reactions with Taq DNA ligase and the matching probe were positive.

Sensitivity of PLNA

The sensitivity of PLNA for both mutant and WT strains was evaluated using 10-fold serial dilutions of plasmid controls from 10^8 to 10^3 copies/ml. PLNA was 100% sensitive for all genomic types at each concentration level (Fig. 2).

In mixed samples PLNA was 100% sensitive for the mutant type and WT at all ratios (70:30–0:100). PLNA could detect the minority viral population at 5% of the total population. This represents 5×10^3 copies/ml of mutant type DNA in 9.5×10^4 copies/ml of WT DNA (Fig. 2).

Performance in Clinical Samples

PLNA was compared to results obtained from PCR-RFLP and sequencing (Fig. 3). Samples were positive for HBV using all methods (Table II). The RFLP method detected a mutant strain in 27 of the 50 samples: 11 rtM204V mutants, 8 rtM204I mutants, and 4 of both rtM204V/WT mix and rtM204I/WT mix. Sequencing detected 26 mutant strains: 11 rtM204V mutants, 9 rtM204I mutants, and 3 of both rtM204V/WT mix and rtM204I/WT mix. PLNA detected 30 mutants: 11 rtM204V mutants, 8 rtM204I mutants, 6 rtM204V/WT mix, and 5 rtM204I/WT mix. When PLNA was compared to RFLP and sequencing, concordant results were found in 94% and 90% of samples. PLNA reported three mixed-variant samples that were not detected by RFLP, and four mixed-variant samples that were not detected by sequencing. One sample was rtM204I/WT-positive by PLNA but was reported as containing only the rtM204I mutant strain by sequencing and only the WT by RFLP.

No samples containing both mutation types were found.

DISCUSSION

The utility of a new rapid and sensitive method for the detection of mutations in the YMDD motif of the HBV polymerase gene has been demonstrated in this study. PLNA consisted of two steps: asymmetric PCR and LDR in one tube, followed by NADS detection. PLNA was compared to two standard methods of diagnosis, RFLP and direct sequencing, and a high concordance between all three methods (90%) was observed. Individual concordance was 94% for PLNA and RFLP and 90% for PLNA and sequencing. Discordant results were due to the observed higher sensitivity of PLNA for mixed viral species. This is supported by sensitivity results using DNA plasmid standards. It was found that the overall sensitivity of PLNA was 10^3 copies/ml. In mixed standards the minor variant could be detected at 5% (5×10^3 copies/ml) of the total population (10^5 copies/ml). RFLP and direct sequencing can only detect the minor variant at 10% and 20% of the total viral population [Allen et al., 1999].

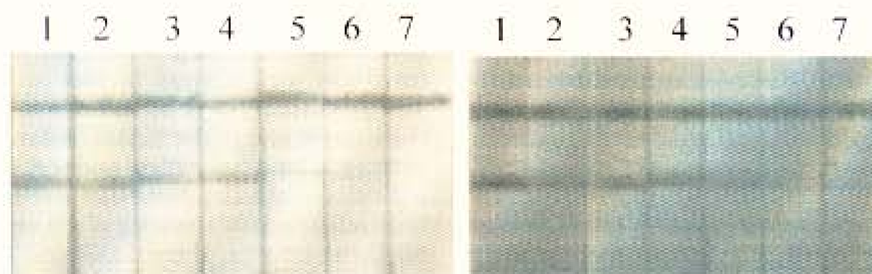

Fig. 2. Sensitivity and specificity of tyrosine–methionine–aspartate–aspartate (YMDD) mutant detection by PLNA. **A**: The detection limit of rtM204I$_3$ mutants by PCR-ligase detection reaction and nucleic acid detection assay strips (PLNA). **Lanes 1–6**: 10-fold serial dilutions of rtM204I$_3$ plasmid control from 10^8 to 10^3 copies/ml. **Lane 7**: No template control. PLNA detected the lowest concentration, 10^3 copies/ml (lane 6). **B**: The sensitivity and specificity of PLNA for a mutant strain in a mixed sample. Mixed viral populations were prepared by mixing 10^5 copies/ml of the mutant type (rtM204I) and wild-type (rtM204) at various ratios. Lanes 1–7: mixed mutant type and wild type plasmid controls in ratios of 70:30, 50:50, 30:70, 20:80, 10:90, 5:95, 0:100. PLNA could detect the mutant viral population present at 5% of the total viral load (lane 6) and did not report a positive result when no rtM204I was present (lane 7).

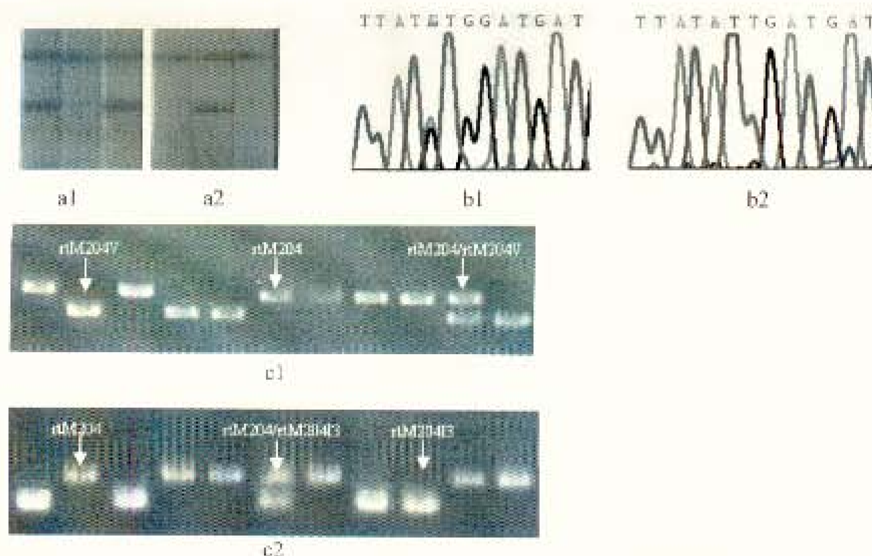

Fig. 3. Representative results of sequencing, PCR-restriction fragment length polymorphism (RFLP) analysis and PCR-ligase detection reaction and nucleic acid detection assay strips (PLNA) from 11 different samples. **A**: PLNA results from two samples: Three strips were used to detect the each strain (rtM204, rtM204I, and rtM204V), a1 indicates the presence of rtM204V and WT strains; a2 indicates the presence of rtM204I. **B**: Sequencing images from two samples: b1 indicates the presence of rtM204V and WT strains; b2 indicates the presence of rtM204I. **C**: PCR-RFLP analysis from the two samples: c1 indicates the presence of rtM204V and WT; c2 indicates the presence of rtM204I and WT strains.

Of the 20 samples that were reported to have only the mutant strain (rtM204V-or-I) by the three methods 19 were in complete concordance. In samples with a mixed population of mutant and WT only 54.5% (6/11) were positive by all three methods. These discrepancies result in a difference of sensitivity between the three methods. PLNA detected six and five samples containing a mixture of either rtM204I or rtM204V and WT, while the RFLP detected four rtM204I/WT mix and four rtM204V/WT mix and sequencing detected three of each mix. This is similar to other results that showed RFLP was more sensitive than sequencing in mixed variant samples and suggests that PLNA may be more sensitive than both RFLP and sequencing for detecting minor viral populations [Allen et al., 1999]. For one sample RFLP indicated that only the WT was present while sequencing indicated the presence of only the rtM204I mutant and PLNA detected both WT and the rtM204I

TABLE II. Comparison of the Results Obtained by PCR-RFLP, PLNA and Sequencing

	Mutation detected by (no. of samples)		
Gene types	PCR-RFLP	PLNA	Sequencing
rtM204	23	20	24
rtM204V	11	11	11
rtM204I	8	8	9
rtM204 + rtM204I	4	6	3
rtM204 + rtM204V	4	5	3

A total of 50 samples were tested. The rate of concordance between the three assays was 90% (45 of 50 samples), 94% (47 of 50 samples) for PCR-ligase detection reaction and nucleic acid detection assay strips (PLNA) and PCR-restriction fragment length polymorphism (RFLP), and 90% for PLNA and sequencing.

mutant. RFLP did not detect the isoleucine mutant because the endonuclease used allows detection of only one of the three isoleucine codons. The fact that sequencing did not detect the WT strain suggests that the sample contained a minor WT population. PLNA detected both strains indicating the utility of this method. It is highly unlikely that the high sensitivity of PLNA is due to unspecific binding during LDR because it was shown that a positive result was only possible when a perfectly matching probe and target combination was co-present with DNA ligase and a denaturing agent (NaOH).

All three methods performed in this study utilize PCR to first amplify the target sequence. In PLNA, asymmetric PCR is used to amplify DNA and LDR is used to detect single-base pair mismatches. Asymmetric PCR utilizes a limiting and excess primer so that at higher cycle numbers one strand is preferentially amplified, creating ssDNA. Although this method typically results in efficiencies of 60–70% compared to 90% obtained with symmetric PCR, by optimizing probe design, efficiencies approaching that of symmetric PCR can be obtained [Sanchez et al., 2004]. In PLNA, asymmetric PCR is used to ensure that ssDNA is present even at the low temperatures (45°C) required for LDR probe binding. To increase the sensitivity of this method two to five cycles of LDR is performed. This could account for the increased sensitivity of PLNA compared to RFLP and sequencing. The higher efficiency of the PLNA due to the absence of significant post-PCR processing could also account for an increased sensitivity.

It should be noted that future studies on PLNA will need to be performed to further optimize and prove its utility and sensitivity. Particularly, PLNA will need to

be compared to a method of higher sensitivity so that minor virus populations not detected by sequencing or PCR-RFLP analysis can be confirmed. It is also not clear why the optimal concentration for the two probes used in LDR were different. Additionally, PLNA does not provide quantitative results, although the strength of the signal is theoretically related to the amount of original DNA present. In order to discriminate between all three viral types, PLNA was performed three times. This is because there is no way to discriminate between multiple mutations in the LDR reaction simultaneously. However, future methods could utilize three different colored particles for simultaneous detection. PLNA does not currently detect other mutations associated with LAM resistance or other drug resistance, although this capability could be developed in the future.

The INNO-LiPA HBV DR line probe assay is capable of detecting different mutation types on the same detection strip and has been recently used to detect mutations associated with resistance to several HBV drugs. While this method is powerful, it sometimes requires nested PCR as well as incubation with BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium chromogen and is mostly sold to higher income markets [Stuyver et al., 2000; Lok et al., 2002]. Total processing time of the line-probe assay is over 5 hr not including sample processing, and strict hybridization requirements and stringent washing conditions make this method difficult in areas with a low level of laboratory infrastructure. PLNA can be performed in less than 3 hr and requires no incubation or significant post-amplification processing. Therefore, this method could be useful for low-cost, rapid diagnosis and the large-scale screening of patient populations. Because of its high sensitivity and ease of use, PLNA could become a routine diagnostic for early diagnosis and treatment monitoring. Only then will countries with a high HBV burden be able to control and prevent the spread of this debilitating disease.

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