Direct detection of lamivudine-resistant hepatitis B virus mutants by a multiplex PCR using dual-priming oligonucleotide primers

Jong-Kee Kim, Hyeon-Ji Lee, Young-Jo Lee, Jong-Yoon Chun, In-Kyoung Lee, Young-Suk Lim, Dong Jin Suh, Sun-Young Ko, Myeong Hee Kim, Heung-Bum Oh

Abstract

Mutations in the YMDD motif of the hepatitis B virus (HBV) polymerase gene increase lamivudine resistance of HBV, highlighting the clinical importance of accurate and sensitive detection of HBV mutants. Using dual-priming oligonucleotide primer technology, an assay that can detect mutations at codons 180 (L528M) and 204 (YVDD, YIDD, and YSDD) by a single-step multiplex PCR was developed. This Seeplex Lami-DR assay was sufficiently sensitive to detect 10^3 HBV/ml and was able to detect minor mutants comprising as little as 2% of the viral population. Mutants were detected in 57 of 65 serum samples (88%) from patients with chronic hepatitis B who had been treated with lamivudine (median, 32 months; range, 1–83 months). The agreement with direct sequencing was only 38.5% (25/65). Discrepancies between these methods resulted from detection of additional mutants by the Seeplex Lami-DR assay, as confirmed by a novel verification analysis. This assay is not only highly accurate and sensitive, but is also simple and cost-effective, requiring no expensive probes, laborious sequencing procedures, or digestion with restriction enzymes. Accordingly, the Seeplex HBV Lami-DR assay should be considered as a first-line, cost-effective tool for detecting viral mutations in patients with chronic hepatitis B receiving lamivudine therapy.

Keywords: HBV; Chronic hepatitis B; Lamivudine resistance; Seeplex Lami-DR assay

1. Introduction

Hepatitis B virus (HBV) infection is one of the main causes of liver disease, and chronically infects an estimated 350 million people worldwide (Mahoney, 1999; Mast and Alter, 1993). Although the progression of chronic HBV infection is variable, 15–40% of HBV carriers will develop serious sequelae, including cirrhosis, hepatic decompensation, and hepatocellular carcinoma (Lim and Suh, 2004). For patients suffering from chronic hepatitis B, only antiviral treatments are beneficial. Lamivudine is the first oral nucleoside analogue approved for the treatment of chronic hepatitis B. Owing to its relative affordability, one-pill-per-day regimen, and low incidence of side effects, lamivudine has been used extensively in patients with chronic hepatitis B (Merle and Trepo, 2001). Long-term lamivudine therapy, however, is associated with a high rate of emergence of lamivudine resistance. This resistance increases at a rate of 14–32% annually and exceeds 70% after 48 months of treatment (Lai et al., 2003; Papatheodoridis et al., 2002; Rapti et al., 2007). Selection for drug-resistant HBV is associated with disease progression and leads to a rapid loss of initial therapeutic benefits (Lai et al., 1997). In some cases, lamivudine-resistant HBV can progress to fulminant hepatic failure and death (Liaw et al., 1999; Wang et al., 2002).

It is well known that resistance to lamivudine is correlated with mutations in the highly conserved YMDD motif of the HBV polymerase (Allen et al., 1998; Bowden et al., 2003; Hussain and Lok, 1999; Niesters et al., 2002; Pillay et al., 1998).
The most commonly described mutations are substitutions of valine, isoleucine, or serine for methionine at position 204 in the C domain of the polymerase (Stuyver et al., 2001). Such lamivudine-resistant HBV mutants are known to replicate less efficiently than the wild type \textit{in vitro}. However, an additional substitution of methionine for leucine at position 180 in the B domain of the polymerase is often co-selected and compensates for the loss of \textit{in vitro} replication efficiency (Allen et al., 1998; Delaney et al., 2003; Fu and Cheng, 1998; Seigneres et al., 2002).

The apparent incidence of genotypic resistance may vary according to the sensitivity of methods used to detect resistant mutations. Thus, methodological options for detecting HBV mutations are an increasingly important issue in clinical management. Sequencing remains the best approach for identifying new mutation patterns. However, because of its low sensitivity, this method can rarely detect mixed populations of two or more HBV genotypes. Generally, sequencing cannot detect a minor subpopulation comprising less than 25% of a total viral population and is not practical for use in large cohort studies or clinical laboratories because it is labor-intensive and time-consuming (Allen et al., 1998; Sablon and Shapiro, 2005). Several other methods have been used to detect lamivudine-resistant HBV, including PCR restriction fragment length polymorphism (PCR-RFLP) (Allen et al., 1999; Jardi et al., 1999; Sablon and Shapiro, 2005), PCR restriction fragment length polymorphism (PCR-RFMP) (Hong et al., 2004), real-time PCR (Chieochansin et al., 2006; Wightman et al., 2004), a line probe assay (Aberle et al., 2001; Lok et al., 2002), an oligonucleotide chip assay (Jang et al., 2004), and a 5' -nuclease assay (Allen et al., 1999).

Recently, a novel dual-priming oligonucleotide (DPO) primer method was developed that can detect single base mutations with greater specificity (Chun et al., 2007). The DPO primer system differs from conventional primer systems in that it includes a poly(I) linker between two unequal segments of primer sequences. The poly(I) linker forms a bubble-like structure that separates a single primer into two functional regions, thereby increasing specificity. Furthermore, because the bubble-like structure of its poly(I) linker efficiently prevents primer competition, the DPO system is more accurate for multiplex PCR applications.

In this study, the Seeplex HBV Lami-DR assay (Seegene Inc., Seoul, Korea) was evaluated using two DPO-based multiplex PCR sets to detect four lamivudine-resistant HBV mutants. Assay results were compared with direct sequencing. To demonstrate that the Seeplex HBV Lami-DR assay is more accurate than direct sequencing, a new verification method was developed and used to confirm that the Seeplex HBV Lami-DR assay could detect the presence of minor amounts of mutant virus in patient samples that were not detectable by direct sequencing.

2. Materials and methods

2.1. Patient samples and extraction of HBV DNA

A total of 65 serum samples collected from patients receiving lamivudine therapy to treat chronic HBV infection were used in this study. The median duration of lamivudine therapy was 32 months (range 1–83 months). Patients included in this study had been hepatitis B surface antigen-positive for 6 months or longer and had elevated ALT values, with HBV DNA levels greater than 20,000 copies/ml before commencement of lamivudine therapy. All patients underwent medical examinations at the Department of Gastroenterology, Asan Medical Center (AMC), Seoul, Korea. The patients received regular follow-up examinations in the outpatient clinic at 2- to 3-month intervals. Viral loads were measured during each clinic visit. Serum HBV DNA levels were determined by quantitative PCR using a commercially available, TaqMan probe-based assay (Real-Q HBV Quantitation Kit, BioSerum Inc., Seoul, Korea). Genotyping for YMDD mutants was performed on patients exhibiting virologic breakthrough, defined as a 10-fold (>1 log 10) increase in serum HBV DNA from nadir after an initial virologic response to treatment.

Serum samples for mutant genotyping were divided into 200-μl aliquots and stored at −80°C until used. HBV DNA was extracted from each aliquot using Viral Gene-spin™ Viral DNA/RNA Extraction Kit (iNtRON, Seoul, Korea), according to the manufacturer’s instructions. The study was approved by the Asan Medical Center Institutional Review Board (AMC-IRB), and written informed consent was obtained from all patients.

2.2. DPO primer design

To detect lamivudine-resistant HBV mutants, three forward and three reverse DPO primers were designed against the HBV polymerase gene (GenBank accession no. AY247031) (Fig. 1). A single forward and reverse primer was designed against the \textit{rbcL} gene of rice (GenBank accession no. X15901) to detect internal control plasmids. Multiplex PCR set A contains a six-primer combination mixture (HBVF, HBVR, YIDDF, YVDDR, rbcLF, and rbcLR) that amplifies four fragments: HBV common, YVDD mutant, YIDD mutant, and the internal control. A second six-primer combination mixture (HBVF, HBVR, YSDDF, L528MR, rbcLF, and rbcLR) was used for multiplex PCR set B, which amplifies four fragments: HBV common, L528M mutant, YSDD mutant, and the internal control.

The mutant-specific DPO primers, YIDDF, YVDDR, YSDDF, and L528MR, were designed to contain either a single or two-base variation in the middle of the 3'-segment so as to maximize the ability of DPO to discriminate single base changes resulting from disruptions in 3'-segment annealing (Chun et al., 2007).

2.3. Construction of positive- and internal-control plasmids

Standard positive-control sequences for YMDD, YIDD, YVDD, and L528M mutations were amplified from patient samples containing the corresponding types of HBV DNA by PCR using the HBVF-HBVR primer pair. The YSDD mutant was not identified in patient samples; thus, a YSDD mutant positive-control sequence was generated from wild-type sequence by site-directed mutagenesis. The internal control sequence was generated by PCR from rice DNA using the rbcLF–rbcLR primer pair. PCR products were subsequently cloned into the pCR2.1-TOPO plasmid vector (Invitrogen, California, USA).
Fig. 1. Schematic representation of DPO primer positions and predicted PCR products for Seeplex HBV Lami-DR assay multiplex PCR sets A and B. The relative positions of the DPO primers are represented by arrows. The asterisk indicates the position of the mutation in the HBV DNA polymerase gene. The relative sizes of the predicted PCR products for each multiplex PCR set are shown by three thick horizontal bars. Internal control primers and plasmids included in the Seeplex HBV Lami-DR assay are not shown in this figure.

according to the manufacturer’s instructions, to generate the control plasmids. All standard positive-control and the internal-control plasmids were confirmed by sequencing analysis.

2.4. Seeplex HBV Lami-DR assay

Seeplex HBV Lami-DR assays employing two multiplex PCR sets were performed using 3 μl of HBV DNA samples and a 2X Master Mix kit (Solgent, Seoul, Korea). After initial incubation at 94 °C for 5 min, 40 amplification cycles of 94 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 1 min were carried out using an Applied Biosystems 9700 thermal cycler (Perkin-Elmer, Boston, MA, USA). A final extension was performed at 72 °C for 5 min. PCR amplicons of the expected sizes were visualized by ethidium bromide staining following electrophoresis on 2% agarose gels.

Fig. 2. Generation of wild-type and mutant HBV PCR products of different sizes. Step 1: The HBV polymerase region containing codons 204 and 180 was amplified using primer sets MF1/HBVR (codon 204) and MF2/HBVR (codon 180). Step 2: PCR products were analyzed by restriction digest. Upon digestion, the PCR products from wild-type HBV containing ATG at codon 204 and CTG at codon 180 were cleaved by NdeI and ScrFI, respectively; the PCR products from mutant HBV (YIDD, YVDD, and L528M) containing different sequences at those codons were not digested by the enzymes.
2.5. Specificity and sensitivity of Seeplex HBV Lami-DR assay

The specificity of the Seeplex HBV Lami-DR assay was determined using six samples of known mutant types. Of the six samples, three were patient samples in which the mutant types had been characterized by direct sequencing and three were serum samples spiked with one or more positive control plasmids (2 x 10^13 copies/ml for each). The sensitivity of the Seeplex HBV Lami-DR assay was determined by 10-fold serial dilutions of the positive control plasmid (10^1 to 10^9 copies of plasmid/ml). Assay sensitivity was defined as the lowest template copy number at which an amplified product was detected. In addition, the assay was evaluated against mixtures containing various ratios of the wild-type positive-control plasmid and the mutant positive-control plasmid to determine the ability of the assay to detect minor subpopulations.

2.6. Direct sequencing of HBV DNA

PCR fragments containing the HBV mutant sites were amplified and sequenced using the primer pair, HBVF1–HBVR1. Sequencing was performed using an Applied Biosystems 9700 thermal cycler (Perkin-Elmer) and an ABI PRISM 3110 Avant Genetic Analyzer (Perkin-Elmer). Sequence chromatograms were analyzed with Chromas Lite equipment (Technelysium Pty. Ltd., Australia).

2.7. Verification analysis

To confirm the presence of minor fractions of mutant virus uniquely detected by the Seeplex HBV Lami-DR assay, a verification analysis was performed as follows: for codon 204, PCR was conducted with the DPO primer pair MF1-HBVR (Fig. 2). A long forward MF1 primer (52-mer) was designed against the region immediately upstream of the methionine codon of the YMDD motif such that a single T-to-C substitution in the 3′-end of the primer generated an NdeI site (CA↓TATG) in the wild-type virus. The NdeI site is absent in the DNA amplified from the YMDD mutants, owing to the presence of ATG to GTG or ATT mutations at codon 204. The length of the MF1 primer is such that its 5′-end point is located far enough from the NdeI site to generate a size difference between the amplified wild-type and mutant virus fragments. For codon 180, PCR was performed using the DPO primer pair, MF2-HBVR (Fig. 2). The MF2 primer generates a ScrFI restriction site (CC↓TGG) only in the fragment amplified from the wild-type virus. The L528M mutant has a CTG to ATG mutation at codon 180 and therefore lacks the ScrFI site. A 5-μl aliquot of each PCR product was digested with NdeI or ScrFI for 4 h at 37 °C, and the resulting DNA fragments were visualized on 2.5% agarose gels. Since no other NdeI or ScrFI sites are present in the amplified regions, a unique 51-base-pair product corresponding to the difference between the undigested PCR product and digested PCR product is generated. The larger bands, representing HBV mutants, were eluted from the gel, re-amplified using the same PCR primer pairs, and sequenced with the HBVR primer to confirm the sequence of the minor mutant population.

3. Results

3.1. Specificity of the Seeplex HBV Lami-DR assay

To test the specificity of the Seeplex HBV Lami-DR assay, multiplex PCR reactions employing two primer sets (set A and B) were performed on six samples of known mutant types (Fig. 3A and B). An internal control band was detected in all lanes, confirming that PCR reagents and reaction conditions were satisfactory. The HBV common band was detected in lanes 1–6, indicating the presence of HBV in all samples. Two mutants were correctly identified in two patient samples (YIDD and L528M in lane 1, Fig. 3A and B, respectively; YIDD and YVDD in lane 3, Fig. 3A), and three mutants were correctly identified in another (YIDD, YVDD, and L528M in lane 2 of Fig. 3A and B). In the plasmid samples, wild-type HBV was correctly and uniquely identified in a sample containing only wild-type HBV (note absence of mutant in lane 4 of Fig. 3A and B); in two samples containing two mutants each, both mutants were correctly identified (YVDD and YSDD in lane 5 and YIDD and L528M in lane 6 of Fig. 3A and B). Nonspecific products were not generated, despite the addition of excess HBV plasmid templates (2 x 10^13 copies/ml). Finally, no amplification was observed when the Seeplex HBV Lami-DR assay was performed on the HBV-negative sample (lane N). These results are in agreement with the known components of the test samples and indicate that the Seeplex HBV Lami-DR assay can accurately detect individual mutants in a mixed population of mutants.
3.2. Sensitivity of the Seeplex HBV Lami-DR assay

The sensitivities of the two multiplex PCR sets were determined using a 10-fold dilution series (10<sup>1</sup> to 10<sup>8</sup> copies of plasmid/ml) of the positive-control plasmids. In both multiplex PCR assays, PCR products were detected readily on agarose gels from samples containing 10<sup>3</sup> copies/ml (Fig. 4). In addition, the ability to detect minor populations in mixtures of wild-type and mutant viruses was evaluated. As shown in Fig. 5, the YIDD, YVDD, and L528M mutants were detected
Table 1
Comparison of the results obtained by direct sequencing and Seeplex HBV Lami-DR analysis of 65 patient samples

<table>
<thead>
<tr>
<th>Agreement</th>
<th>Direct sequencing assay</th>
<th>Seeplex HBV Lami-DR assay</th>
<th>Patients (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Codon 180</td>
<td>Codon 204</td>
<td>Codon 180</td>
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<tr>
<td>Concordant</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>I</td>
<td>L</td>
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<tr>
<td></td>
<td>L</td>
<td>V</td>
<td>L</td>
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<tr>
<td></td>
<td>L</td>
<td>I, V</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>I</td>
<td>M</td>
</tr>
<tr>
<td>Sub-total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discordant</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>M</td>
<td>M</td>
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<td>V</td>
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<tr>
<td>Sub-total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M: YMDD; I: YIDD; V: YVDD; L: L528M.

Table 2
Summary of results obtained by direct sequencing and Seeplex HBV Lami-DR analysis of 65 patient samples

<table>
<thead>
<tr>
<th>Detection type</th>
<th>Genotype</th>
<th>Number detected by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct sequencing assay</td>
</tr>
<tr>
<td>Wild</td>
<td>M</td>
<td>31</td>
</tr>
<tr>
<td>Mono mutant</td>
<td>I</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>1</td>
</tr>
<tr>
<td>Mixed mutants</td>
<td>I + V</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>V + L</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>I + L</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>V + I + L</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>65</td>
</tr>
</tbody>
</table>

M: YMDD; I: YIDD; V: YVDD; L: L528M.

when present at frequencies as low as 2% of the total viral load.

3.3. Comparison of Seeplex HBV Lami-DR assay with direct sequencing

To evaluate the accuracy of the Seeplex HBV Lami-DR assay in detecting the four lamivudine-resistant HBV mutants, assay results were compared with direct sequencing of all 65 patient samples (Table 1). Surprisingly, the overall agreement between direct sequencing and the Seeplex HBV-DR assay was only 38.5% (25/65), with discrepancies detected in 40 patient samples (61.5%). All mutants detected by direct sequencing were detected by the Seeplex Lami-DR assay, but additional mutants in the 40 patient samples were detected only by the Seeplex Lami-DR assay (Table 1). In 23 of 40 patient samples, direct sequencing detected only the wild type, whereas the Seeplex Lami-DR assay detected one or two mutants. In 17 of 40 patient samples, direct sequencing detected one mutant but the Seeplex Lami-DR assay detected an additional mutant. Altogether, direct sequencing detected only 34 mutants (12 mono and 22 mixed mutants), whereas the Seeplex Lami-DR assay detected 57 mutants (7 mono and 50 mixed mutants) (Table 2).

3.4. Verification of the presence of minority mutant populations

To confirm that mutants detected uniquely by the Seeplex HBV Lami-DR assay were indeed present in the samples, a new verification method was developed, as outlined in Section 2.
Fig. 6. Verification of YIDD (top panel), YVDD (middle panel), and L528M (bottom panel) mutants, present as a small percentage of the total HBV population, in the sera of patients 14, 34, and 23, respectively. Each mutant type was detected by the Seeplex Lami-DR assay (A) but not by direct sequencing (B), and was confirmed by the verification analysis (C). Arrows represent the point mutation sites, indicating the presence of mutant virus.

This method demonstrated that mutants detected by the Seeplex Lami-DR assay (Fig. 6A) but not by direct sequencing (Fig. 6B) were present definitively (Fig. 6C).

4. Discussion

It is current practice to perform mutant genotyping for Lamivudine and consider rescue therapy with Adefovir or Entecavir in cases of virologic breakthrough, defined as a 10-fold increase in serum HBV DNA from nadir during treatment in a patient who had an initial virologic response (Lok and McMahon, 2007). However, there is a time lag between genotypic and phenotypic resistance to lamivudine ranging from 3 to 24 months (Hadziyannis et al., 2000). A recent, well-designed study showed that HBV is more rapidly and consistently suppressed in patients who start Adefovir early during the genotypic resistance phase than in patients starting Adefovir later after virologic breakthrough is evident (Lampertico et al., 2005). These findings clearly indicate that rescue therapy should be instituted as soon as genotypic resistance is detected, when serum HBV DNA level has not usually increased to a level that is too high to be successfully suppressed. Therefore, careful regular monitoring for genotypic resistance throughout treatment is mandatory to ensure early detection and early rescue of drug resistance (Liaw, 2007).

Point mutations at codons 204 and 180 in the HBV polymerase gene are known to be associated with reduced susceptibility to lamivudine. Many techniques have been previously developed for accurate detection of HBV mutants (Chieochansin et al., 2006; Jang et al., 2004; Kirishima et al., 2002; Ou et al., 2005; Sablon and Shapiro, 2005; Stuyver et al., 2001). Of these methods, sequencing, RFLP, DNA hybridization, and RFLP are used extensively at present. However, these methods require additional identification steps after PCR and have disadvantages in terms of specificity, sensitivity, operational complexity and/or cost.

As shown in Figs. 3–5, the results indicate that, in addition to its greater simplicity, the Seeplex Lami-DR assay has superior specificity and sensitivity. In terms of specificity, the Seeplex Lami-DR assay could accurately detect single or mixed populations in both patient and plasmid samples (Fig. 3A and B). Nonspecific amplification was not observed, even in the presence of excess plasmid template ($2 \times 10^{13}$ copies/ml; Fig. 3, lanes 4–6 of Fig. 3A and B). These results clearly indicate the high specificity of the Seeplex Lami-DR assay. The Seeplex Lami-DR assay could accurately detect as few as $10^3$ HBV copies/ml (Fig. 4A and B) and could discriminate minor mutant populations that comprised only 2% of the wild-type/mutant DNA mixture (Fig. 5A and B). This indicates that the Seeplex Lami-DR assay can detect resistant strains at a stage prior to viral breakthrough. In addition, the Seeplex Lami-DR assay did not give rise to nonspecific bands, even though only a single annealing temperature of $60^\circ\text{C}$ was used. This latter observation highlights the fact that the DPO primer technique is easy to optimize and is highly advantageous in various diagnostic applications (Chun et al., 2007).

When the performance of the Seeplex HBV Lami-DR assay was compared with direct sequencing results from samples of 65 chronically infected HBV patients who had received short- or long-term lamivudine therapy, the Seeplex HBV Lami-DR assay
detected additional mutations in 40 of 65 (61.5%) patients. This indicates that the Seeplex Lami-DR assay is more sensitive than direct sequencing (Tables 1 and 2). Indeed, previous reports have indicated that direct sequencing detects only the most prevalent viral type in mixed virus populations, thus limiting its usefulness in detecting resistant mutants at early stages (Aberle et al., 2001; Jang et al., 2004; Pas et al., 2002). In particular, Pas et al. have previously reported that a mutant virus must comprise approximately 50% of the viral population to be identified by direct sequencing (Aberle et al., 2001; Jang et al., 2004; Pas et al., 2002).

In this study, a novel method for verifying mutant viruses in patient samples has been described. To date, the labor-intensive clonal analysis technique has been the tool of choice to verify the presence and identity of viral quasi-species. Clonal analysis may not detect minor subpopulations unless a sufficient number of clones is analyzed (Stuyver et al., 2000; Zoulim, 2002). By contrast, the simple verification method described in this paper accurately detects the presence of minority mutant populations without the use of long and time-consuming cloning procedures.

In conclusion, the Seeplex HBV Lami-DR assay can accurately and sensitively detect lamivudine-resistant mutants. The assay is easy to perform and does not require any expensive probes or instruments, laborious sequencing procedures, or digestion with restriction enzymes. Accordingly, the Seeplex HBV Lami-DR assay should be considered as a first-line, cost-effective tool for detecting viral mutations in patients with chronic hepatitis B receiving lamivudine therapy.

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Liaw, Y.F., Chien, R.N., Yeh, C.T., Tsai, S.L., Chu, C.M., 2000. Accurate detection of additional mutations in 40 of 65 (61.5%) patients. This indicates that the Seeplex Lami-DR assay can accurately and sensitively detect lamivudine-resistant mutants. The assay is easy to perform and does not require any expensive probes or instruments, laborious sequencing procedures, or digestion with restriction enzymes. Accordingly, the Seeplex HBV Lami-DR assay should be considered as a first-line, cost-effective tool for detecting viral mutations in patients with chronic hepatitis B receiving lamivudine therapy.

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