

Evaluation of dual priming oligonucleotide (DPO)-based multiplex PCR for detection of HBV YMDD mutants

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Abstract We evaluated the usefulness of dual priming oligonucleotide (DPO)-based multiplex PCR, Seeplex HBV Lami-DR assay (Seegene Institute of Life Sciences, Seoul, Korea), to detect lamivudine-resistant HBV mutants in a comparison with the use of TRUGENETM HBV genotyping and restriction fragment mass polymorphism (RFMP). Sera from 44 chronic hepatitis B patients were analyzed for the presence of mutations at codons 180 and 204 by performing DPO-based multiplex PCR, RFMP, and TRUGENE. The overall concordance rate among the three assays was 40.9% (18/44). Concordance rates between multiplex PCR and RFMP or multiplex PCR and TRUGENE were 61.4% (27/44) and 50.0% (22/44), respectively. In ten patients, multiplex PCR identified additional mutants not found using the other two methods. DPO-based multiplex PCR is a highly sensitive method to identify minor mutant populations and could be a practical tool in the monitoring of lamivudine resistance.

Introduction

Lamivudine is a nucleoside analogue that suppresses the replication of hepatitis B virus (HBV) by inhibiting viral RNA-dependent DNA polymerase, and it has been used as a primary antiviral agent for treating HBV infection. However, long-term lamivudine therapy induces emergence of viral mutants that exhibit changes in a highly conserved tyrosine, methionine, aspartate and aspartate (YMDD) motif of HBV DNA polymerase; these mutants are denoted HBV YMDD mutants. The most commonly described mutations are substitutions of valine or isoleucine for methionine at codon 204 (rtM204I/V) with or without the rtL180M mutation [1–3]. As there is an increasing need for early detection of emerging YMDD mutants, several molecular methods have been introduced. Direct sequencing of HBV DNA and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analyses have been commonly used for detecting YMDD mutants. However, these methods are time-consuming and labor intensive [4, 5]. There are also two widely-used, standardized assays for the identification of YMDD mutants, a direct sequencing-based assay, TRUGENETM HBV genotyping (Visible Genetics/Bayer, Tarrytown, NY, USA), and a reverse hybridization-based assay, the INNO-LiPA HBV DR (Innogenetics, Ghent, Belgium). A matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) assay that is termed restriction fragment mass polymorphism (RFMP) has also been developed, and we have previously evaluated its usefulness in the monitoring of lamivudine resistance in chronic HBV hepatitis patients in comparison with the use of TRUGENE [6]. Recently, a novel dual priming oligonucleotide (DPO) primer was developed to detect single nucleotide polymorphisms (SNP) in the

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CYP2C19 gene with a one-step PCR assay [7], and the Seplex HBV Lami-DR assay (Seegene Institute of Life Sciences, Seoul, Korea) using the DPO primer system was also developed to detect HBV YMDD mutants [8]. The DPO primer system differs from a conventional system by including a poly(I) linker between two unequal segments of primer sequences. The poly(I) linker forms a bubble-like structure and separates a single primer into two functional regions, which increases the specificity sufficiently to discriminate single base changes by the use of one-step PCR. Furthermore, DPO allows accurate multiplex PCR to be undertaken as the bubble-like structure of the poly(I) linker in DPO efficiently prevents primer competition.

There has been only one study evaluating the performance of the DPO-based PCR method to detect HBV YMDD mutants in comparison with direct DNA sequencing analysis [8]. In this study, we have evaluated the usefulness of DPO-based multiplex PCR to detect lamivudine resistant HBV mutants in a comparison with TRUGENE and RFMP.

Patients and methods

Patients

Sera were collected from 44 chronic hepatitis B patients that had received lamivudine (Glaxo-Wellcome, Greenford, UK) therapy for more than three months at the Department of Internal Medicine, Kangbuk Samsung Hospital in Korea, from January through April 2005, and from March through May 2007. Among the 44 patients, 36 were the subjects of a previous study [6]. None of the patients were positive for either anti-hepatitis C virus antibody or anti-human immunodeficiency virus antibody. The 44 patients included 33 males and 11 females, with a median age of 44 years (range, 29–72 years). The median duration of lamivudine therapy was 32 months (range, 1–81 months); it was less than one year in eight patients, 1–2 years in seven patients, 2–3 years in ten patients, and over 3 years in 19 patients. Written informed consent to participate in the study was obtained from all of the patients, and the Institutional Ethics Committee of Kangbuk Samsung Hospital approved this

study. The number of copies of HBV DNA was measured using the COBAS AmpliCor HBV Monitor Test (Roche Diagnostics, Branchburg, NJ, USA) and COBAS Taqman 48 Analyzer (Roche Diagnostics) with a lower detection limit of 300 copies/ml and 70 copies/ml, respectively.

DNA extraction

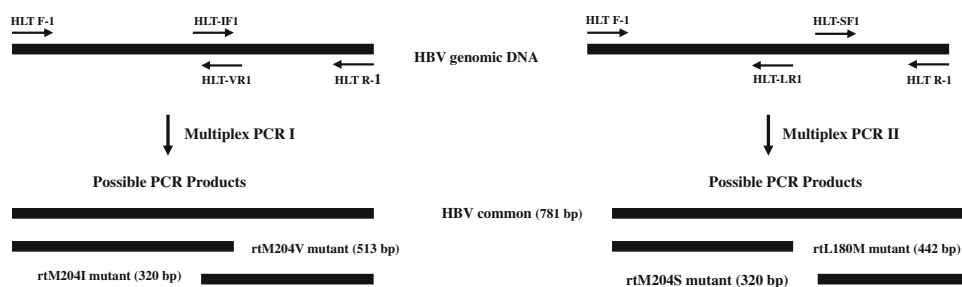
The HBV DNA was extracted from serum using the GenespinTM viral DNA/RNA Extraction Kit (iNtRON, Seoul, Korea) for the DPO-based multiplex PCR and by use of the QIAmp Blood Mini Kit (QIAGEN Inc., Valencia, CA, USA) for the RFMP and TRUGENE assays according to the manufacturer's instructions.

Design of DPO primers and construction of positive and internal control plasmids

Three forward and three reverse DPO primers were designed for the HBV polymerase gene (GenBank accession number AY247031) to detect lamivudine-resistant HBV mutants (Fig. 1). One forward and one reverse primer were designed for the *rbcL* gene of rice (Genbank accession number X15901) to detect the internal control plasmid. A six-primer-combination mixture (HBVF, HBVR, YIDDF, YVDDR, *rbcLF*, and *rbcLR*) was used for multiplex PCR set A, which amplifies four fragments: HBV common, rtM204V mutant, rtM204I mutant, and the internal control. Another six-primer-combination mixture (HBVF, HBVR, YSDDF, L528MR, *rbcLF*, and *rbcLR*) was used for multiplex PCR set B, which amplifies four fragments: HBV common, rtL180M mutant, rtM204S mutant, and the internal control. The mutant-specific DPO primers (YIDDF, YVDDR, YSDDF, and L528MR) were designed to have a single or two-base variations in the middle of the 3'-segment as such a position has been shown to maximize the ability of DPO to discriminate single base changes by disrupting 3'-segment annealing [7].

Standard positive control sequences for rtM204I, rtM204V, and rtL180M mutations were obtained by PCR with the HBVF-HBVR primer pair from patient samples containing each type of HBV DNA. We could not identify YSDD mutants from patient samples and performed site-

Fig. 1 Schematic representation of DPO primer design to detect HBV YMDD mutants at codons 180 and 204. Two forward and two reverse DPO primers were designed for each codon



directed mutagenesis to generate an rtM204S mutant as a positive control sequence. The internal control sequence was obtained by PCR with the rbcLF-rbcLR primer pair from rice DNA. The PCR products were subsequently cloned into the pCR2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications to generate the control plasmids. DNA sequencing analysis confirmed each standard positive control and the internal control plasmid.

DPO-based multiplex PCR

The Seeplex HBV Lami-DR assay, consisting of two multiplex PCR sets, was conducted with 3- μ l HBV DNA samples using a 2 \times Master Mix kit (Solgent, Seoul, Korea) according to the manufacturer's instructions. After an initial incubation at 94°C for 5 min, 40 amplification cycles were carried out in an Applied Biosystems 9700 thermal cycler (Perkin-Elmer, Boston, MA, USA) using the following amplification parameters: 94°C for 30 s, 60°C for 30 s, 72°C for 1 min. A final extension was performed at 72°C for 5 min. PCR amplicons of the expected sizes were visualized using 2% agarose gel electrophoresis. The same PCR parameters and gel electrophoresis conditions were used throughout in this study. An image of an agarose gel showing PCR amplified products is seen in Fig. 2.

RFMP genotyping

PCR followed by restriction enzyme digestion and analysis using a linear MALDI-TOF MS (Bruker Daltonics Biflex IV, Billerica, MA, USA) was performed as previously described [6]. Investigations were performed for the rtM204I/V and rtL180M mutations.

TRUGENE HBV genotyping

The assay was performed by using the TRUGENE HBV genotyping kit v1.0 (Visible Genetics/Bayer, Tarrytown,

NY, USA) as previously described [6]. Investigations were performed for the rtM204I/V/S and rtL180M mutations.

Results

Detection of the YMDD motif mutations

Mutations were detected in 42 (95.5%) out of 44 patients by at least one of the three assays. The frequencies of mutations were 88.1% for rtL180M, 78.6% for rtM204I, and 57.1% for rtM204V in 42 patients. Mixed virus populations were detected by any of the three methods in all 42 patients except for five patients that showed single rtM204I or rtM204V mutants without wild-type virus (marked as "a" in Table 1). Among the double mutations, rtM204I plus rtL180M with or without the wild-type was observed in 15 patients, and rtM204V plus rtL180M with or without the wild-type was observed in seven patients. A triple mutation, simultaneous mutations in three separate codons, i.e., rtM204I plus rtM204V plus rtL180M with or without the wild-type, was observed in 15 patients.

Concordance among DPO-based multiplex PCR, RFMP and TRUGENE

The results obtained for the analyzed codons (codons 180 and 204), as determined by three assays, are summarized in Table 1. The overall concordance rate among the three assays was 40.9% (18/44). Concordance rates between multiplex PCR and RFMP, multiplex PCR and TRUGENE, and RFMP and TRUGENE were 61.4% (27/44), 50.0% (22/44), and 68.2% (30/44), respectively (Table 2). As analyzed for the codons, the concordance rates between multiplex PCR and RFMP, multiplex PCR and TRUGENE, and RFMP and TRUGENE were 86.4% (38/44), 93.2% (41/44), and 88.6% (39/44), respectively, for codon 180. For codon 204, the concordance rates between multiplex

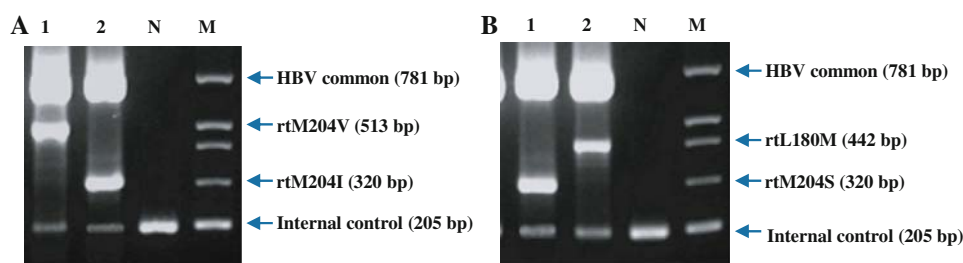


Fig. 2 Image of an agarose gel of the amplicons of DPO-based multiplex PCR for the detection of HBV YMDD mutants at codons 180 and 204. *N* negative control, *M* molecular marker. **a** PCR

designed to detect the presence of rtM204I and rtM204V. **b** PCR designed to detect the presence of rtL180M and rtM204S

Table 1 Comparison of the results obtained by DPO-based multiplex PCR, TRUGENE and RFMP from 44 patients

Results (number of patients)	Multiplex PCR		RFMP		TRUGENE		Patients	Viral loads (copies/mL)
	Codon 180	Codon 204	Codon 180	Codon 204	Codon 180	Codon 204		
All equal (18)	L	M	L	M	L	M	2	<300 in both patients
	L	I	L	I	L	I	2 ^a	>200,000 in both patients
	M	I	M	I	M	I	8	>200,000 in all patients
	M	V	M	V	M	V	6	>150,000 in all patients
Only multiplex PCR detected (10)	L	V	L	M	L	M	2 ^{a,b}	<300 and >200,000
	M	I	L	I	L	I	2	>200,000 in both patients
	M	I, V	M	I	M	I	2	>200,000 in both patients
	M	I, V	M	V	M	V	3	>200,000 in both patients
Multiplex PCR and RFMP detected (8)	M	I, V	L, M	I	M	I	1	>200,000
	L	I	L	I	L	M	1 ^a	>200,000
	M	I, V	M	I, V	M	I	3	>200,000 in all patients
	M	I, V	L, M	I, V	M	I	1	>200,000
	M	I, V	L, M	I, V	M	V	2	>200,000 in both patients
Only RFMP detected (5)	M	I, V	L, M	I, V	L	I	1	>200,000
	L	M	L, M	M, I	L	M	2 ^c	446 and <300
	L	M	L, M	M, I, V	L	M	1 ^c	<300
	M	I	M	M, I, V	M	I	1	38,300
RFMP and TRUGENE detected (2)	L	V	L, M	M, V	L	M	1	19,300
	M	M	M	I	M	I	2	5,104 and 86,700
Multiplex PCR and RFMP differ from TRUGENE (1)	M	I	M	I	M	V	1	>200,000
Total							44	

Amino acids are indicated by one-letter codes. In codon 180, the wild-type and the mutant show leucine (L) and methionine (M), respectively. In codon 204, the wild-type and the mutant show M and isoleucine (I) or valine (V), respectively

^{a, b, c} Patients are described in “[Results](#)”

PCR and RFMP, multiplex PCR and TRUGENE, and RFMP and TRUGENE were 68.2% (30/44), 54.5% (24/44), and 68.2% (30/44), respectively.

Multiplex PCR and RFMP identified additional mutants not found by the other two methods for ten and five patients, respectively, while TRUGENE could not. Multiplex PCR and RFMP revealed the presence of mutant virus populations, while the other two methods detected only wild-type virus in two (marked as “b” in Table 1) and three patients (marked as “c” in Table 1), respectively. The mutants identified only by multiplex PCR in two patients were mutants with rM204V, and the viral loads were <300 copies/ml and >200,000 copies/ml. From direct DNA sequencing analysis, one of the two patients, who showed a viral load of <300 copies/ml, exhibited mutants

with rM204V, while only wild-type virus was identified in the other patient with a viral load of >200,000 copies/ml. We also confirmed the results of another eight patients who carried additional mutants that were identified only by multiplex PCR by performing direct DNA sequencing analysis or rechecking specimens sampled on the next follow-up. Three out of eight patients showed the same results as those of multiplex PCR, another three patients showed the same results as those of TRUGENE or RFMP, one patient was identified with only wild-type virus (disappearance of the mutants), and isolates from one patient could not be genotyped. The viral loads of the three patients with mutants found only by RFMP (marked as “c” in Table 1) were all very low (<500 copies/ml), and the proportions of the mutants were below half (10, 50, 50%).

Table 2 Concordance of DPO-based multiplex PCR, TRUGENE, and RFMP among 44 patients

Results		Number of patients with concordant results by			
Codon 180	Codon 204	All three methods	Multiplex PCR and RFMP	Multiplex PCR and TRUGENE	RFMP and TRUGENE
Wild-type	Wild-type	2	2	5	4
Wild-type	Mutant	2	3	2	4
Mutant	Wild-type	0	0	0	0
Mutant	Mutant	14	22	15	22
Total no. of patients (%)		18 (40.9)	27 (61.4)	22 (50.0)	30 (68.2)

On the following tests, the original mutant virus populations were detected only by RFMP in two patients, including one patient in which an additional mutant virus was identified, and the analysis failed for the other patient. A wild-type virus population for either codon was additionally identified only by RFMP in five patients.

TRUGENE detected one more additional mutant than multiplex PCR in two patients. Different mutant populations for codon 204 were found in one patient; multiplex PCR and RFMP detected rtM204I, while TRUGENE detected rtM204V. Direct DNA sequencing analysis confirmed the presence of rtM204I.

Discussion

This study is the first to compare DPO-based Multiplex PCR to RFMP and TRUGENE for detection of HBV YMDD mutations in chronic hepatitis B patients.

Among the three methods, TRUGENE showed the lowest sensitivity for detecting minor virus populations in a mixed infection. TRUGENE could not identify additional YMDD mutant populations, whereas the other two methods detected the same mutant populations in eight patients, including two patients with no mutant detected by TRUGENE. In addition, TRUGENE could not identify one of the two mutations detected on two nucleotides of codon 204 (rtM204I and rtM204V), a finding that was also observed in our previous study comparing RFMP to TRUGENE [6]. TRUGENE could not detect the simultaneous presence of rtM204I and rtM204V in samples with viral loads of 4.6–9.4-log copies/ml in another study [9]. Kim et al. [8] also reported that the agreement between DPO-based multiplex PCR and direct DNA sequencing was only 38.5% (25/65), and discrepancies resulted from the detection of additional mutants by the use of multiplex PCR.

Confirmation tests such as direct DNA sequencing analysis or a re-check revealed that multiplex PCR succeeded in earlier detection of additional mutants than the other two methods in four patients. Among these four patients, multiplex PCR identified a single rtM204V

mutation in one patient with fluctuation of viral loads at a low level, while other two methods detected only wild-type. Earlier detection of mutants would be helpful when deciding to change the drug used for therapy. Choosing the proper drug in a timely manner is very important, as HBV that develops lamivudine resistance can be rapidly suppressed in patients that start adefovir earlier than in patients that start adefovir later, after viral breakthrough [10]. Continuing very high viral loads were also observed in another patient with rtM204V mutation that was identified only by multiplex PCR although the presence of an rtM204V was not confirmed by a direct DNA sequencing analysis. We suppose that a mutant virus existed in this patient, and a follow-up check will be required. Ohishi et al. [11] reported that lamivudine therapy was effective in a patient who was found to have the rtM204V mutant before therapy, but lamivudine was ineffective in our two patients, and the viral loads were increased. We could not confirm the mutations initially detected exclusively by the multiplex PCR in four other patients although we performed direct DNA sequencing analysis. Direct DNA sequencing analysis of multiple plasmid clones after cloning of PCR products rather than direct sequencing of PCR products would be necessary to clarify the multiplex PCR results.

Multiplex PCR detected a single rtL180M without a mutation of codon 204 in two patients, while the other two assays identified rtM204I as well as rtL180M. We rechecked the viral loads of the stored sera from these two patients using COBAS Taqman, but direct DNA sequencing analysis failed due to the lack of a specimen for both patients. To our surprise, significant decreases in viral loads were observed (from 5,140 to 300 copies/ml and from 86,700 to 2,700 copies/ml). We assume that HBV DNA might be degraded during storage or delivery of the sera, because we performed multiplex PCR two years after the time we performed the other two assays in these two patients, and the sera were sent to another laboratory. In one study, the HBV DNA levels were decreased by 1.8, 3.4, and 20% per day at 23, 37, and 45°C, respectively [12]. Although Jerome et al. [13] reported that the levels of

purified viral DNA remain stable after storage for 16 months at 4°C, we did not store the purified viral DNA but stored the sera. Therefore, missed detection of rtM204I by multiplex PCR could be due to the lack or instability of HBV DNA, but the inherent inaccuracy of the multiplex PCR for specimens with low levels of virus also cannot be excluded. Gutfreund et al. [14] reported that rtL180M was not associated with viral breakthrough, and this finding was in accordance with an only an 18-fold increase in the inhibitory concentration 50 (IC₅₀) of lamivudine in cell culture systems as compared to a greater than 10,000-fold increase in the IC₅₀ as seen with rtM204I, rtM204I/rtL180M and rtM204V/rtL180M. Therefore, if a single rtL180M mutation is observed in patients with viral breakthrough, it is necessary to confirm the presence of other mutations using another sensitive method.

In six patients, RFMP identified wild-type virus populations in addition to the mutant virus populations that were also detected by DPO-based multiplex PCR. This is attributable to the characteristics of RFMP, which can determine the relative proportions of each mutant and the wild-type virus population. We could not predict viral breakthrough based only on the presence of the mutant virus, and the results of this highly sensitive molecular method should be interpreted carefully, as lamivudine could be effective despite the presence of mutants [6]. Lee et al. [15] reported that a 5-fold predominance of the YMDD mutant over wild-type virus was significantly associated with viral breakthrough. Therefore, the lack of ability to detect both the wild-type and mutant virus populations could be a disadvantage of employing DPO-based multiplex PCR. RFMP detected the mutant and wild-type virus populations, while multiplex PCR detected only the wild-type virus population in three patients with very low levels of viremia (446, <300 and <300 copies/ml). These findings may have resulted from the different analytical sensitivities of the two assays. TRUGENE, DPO-based multiplex PCR, and RFMP generally cannot detect a minor virus population that comprises less than 20% [9], 2% [8], and 1% [16] of the total population, respectively. In addition, the detection limits of TRUGENE, DPO-based multiplex PCR, and RFMP were 3,860 copies/ml [6], 1,000 copies/ml [8], and 122 copies/ml [6], respectively.

There have been several methods based on the PCR for detecting SNPs or mutations. However, all of the methods require additional verification steps such as DNA sequencing, enzyme digestion, or hybridization and are neither rapid nor easy to manipulate. Real-time PCR also can identify SNPs without additional steps, but it requires expensive equipment and showed inferior detection ability for detecting minor virus population (as little as 5% of the total population) in a mixed infection compared to DPO-based multiplex PCR [17]. The DPO-based multiplex PCR

method enabled SNP to be identified by a single multiplex PCR step with no need for performing additional steps or expensive equipment, or technicians with specialized skills [7, 8]. Furthermore, DPO-based multiplex PCR has a high throughput capacity. Therefore, DPO-based multiplex PCR can be easily established in average-sized clinical laboratories.

In summary, we have demonstrated the successful application of DPO-based multiplex PCR for detecting HBV YMDD mutants. While RFMP had an advantage of the ability to discriminate the relative proportions of each mutant and wild-type virus, multiplex PCR was more economical and easier to perform. Therefore, DPO-based multiplex PCR could be a practical tool for the monitoring of lamivudine resistance.

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