Evaluation of antiviral resistant hepatitis B virus subpopulations in patients with chronic hepatitis B by using terminal restriction fragment length polymorphism

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Abstract Antiviral therapies with nucleotide analogues (NA) is crucial in the treatment of chronic hepatitis B as it substantially protects patients from the complications of the disease. However in most of the available NA therapies, resistance emerges in the patients' HBV populations. Therefore, detection of antiviral resistance as early as possible by means of genotypically monitoring the patients' HBV pool during NA therapy is critical to manage treatment regime. In this research study we have investigated the sensitivity and specificity of the terminal restriction fragment length polymorphism (T-RFLP) method in detecting HBV subpopulations carrying antiviral resistance mutations. For this aim, differentiation of mutant strains from wild type strains was demonstrated by PCR-RFLP method. With using recombinant plasmids containing mutant and wild type HBV genomes, we constructed artificial HBV genome populations in order to determine the sensitivity of PCR-T-RFLP method in detecting antiviral resistant minor HBV populations. Finally by comparing with the DNA sequencing method, we demonstrated the specificity of T-RFLP method in genotyping HBV populations. As a result we showed that T-RFLP is able to detect HBV subpopulations representing as low as 1 % of the whole viral population. Additionally T-RFLP showed 100 % concordance with the DNA sequencing

method in genotyping HBV populations. As a conclusion, considering the other genotyping methods used in evaluating HBV populations, T-RFLP showed high sensitivity and specificity profiles in detecting antiviral resistant HBV subpopulations. Therefore T-RFLP method can be easily employed in genotypic evaluation of patients' HBV populations during the course of antiviral treatment.

Keywords Chronic hepatitis B · nucleotide analogue · Antiviral resistance · HBV quasispecies · T-RFLP

Introduction

Hepatitis B virus (HBV) is one of the major global pathogens that causes acute and chronic infections in human. With an estimation, about 2 billion individuals have encountered with HBV infection worldwide. According to the World Health Organization more than 240 million individuals are chronically infected with HBV and every year approximately 600,000 individuals die due to the acute and chronic complications of HBV infection (<http://www.who.int/mediacentre/factsheets/fs204/en/>).

In regards to the viral diagnosis of the hepatitis B, several serologic assays were developed for hepatitis B surface antigen (HBsAg) and other HBV antigens and antibodies during the last two decades. Additionally with the more recent advances in molecular biology techniques, nucleic acid hybridization and polymerase chain reaction (PCR) based assays have been established for the direct detection of HBV DNA. Furthermore the HBV infections can also be diagnosed by the detection of HBsAg or hepatitis B core antigen (HBcAg) in liver tissues by using immunohistochemical assays and of HBV DNA by in situ hybridization, PCR or southern blot assays.

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To treat chronic HBV infections, currently available therapies are immunomodulatory agents including interferon α and pegylated interferon α , and antiviral agents that suppress viral replication which consist of nucleoside and nucleotide analogues (NA) [16]. Antiviral therapy of chronic hepatitis B is applied with the goal of suppressing viral genome replication and so the virus production in the infected liver, which subsequently prevents chronic complications of the HBV infection such as fibrosis, cirrhosis and hepatocellular carcinoma. Although antiviral therapies significantly reduce the risk of developing cirrhosis and hepatocellular carcinoma in patients with chronic hepatitis B, viral clearance usually is not achieved after a year of intensive antiviral therapies. The major difficulties of long term therapies with most of the available antiviral agents is the development of resistance in the patients' HBV pool which leads to virological breakthrough, progressive liver injury and increased disease complications. The antiviral resistance of HBV is associated with the resistance mutations that arise in the HBV polymerase gene. According to this phenomenon, the antiviral resistance mutations in the polymerase gene cause amino acid substitutions in the nucleotide binding regions of the polymerase enzyme, which are the sole targets of NAs. As a result of these amino acid substitutions, NAs are no longer able to bind to these regions and function as replication inhibitors. Some well documented primary antiviral resistance mutations of HBV include L180M and M204V/I for lamivudine and telbivudine, A181V and N236T for adefovir dipivoxil and I169T, T184, S202 and M250V for entecavir [16].

To manage the optimal NA treatment type and regime for the antiviral therapy of patients with chronic hepatitis B, it is critical to know when the antiviral resistance arises in the patients' HBV pool. To determine the antiviral resistance mutations as early as possible after their occurrence in the HBV genome, a genotyping method with relatively high sensitivity and specificity is required.

Terminal restriction fragment length polymorphism (T-RFLP) was originally developed in the late 90s for genotypic analysis of complex bacterial communities, which are found in the environments such as soil, saliva and feces [2, 14]. Differently from the conventional polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, in the PCR-T-RFLP method, the oligonucleotide PCR primers are fluorescently labeled from their 5' terminals. With this aspect, the fluorescently labeled terminal restriction fragments can be analyzed in the automatized capillary gel electrophoresis systems with better sensitivity and specificity compared to the conventional PCR-RFLP method. Here in this study, we investigated the sensitivity and specificity

of PCR-T-RFLP method in detecting HBV subpopulations carrying antiviral resistance mutations. We first demonstrated the sensitivity of the PCR-T-RFLP method in detecting HBV subpopulations carrying antiviral resistance mutations. To this end, we artificially constructed HBV genome populations by using plasmids containing wild type and mutant HBV genomes. Next we determined the specificity of the PCR-T-RFLP method in terms of genotyping HBV populations simply by comparing the method with the direct DNA sequencing.

In this research study, we chose hepatitis B virus infection as a model. Since its treatment with the small molecule based antivirals gives rise to a development of resistance and that this phenomenon makes the HBV a relevant candidate for monitoring its antiviral resistance status with the T-RFLP method. On the other hand, different viruses that are treated with small molecule based antivirals and exhibit quasispecies such as hepatitis C virus (HCV) or human immunodeficiency virus (HIV) can also be studied with T-RFLP method for monitoring drug resistance as well as for genotypically evaluating their quasispecies viral pool.

Materials and methods

Patient sera

Sera used in this research study were collected from patients with chronic hepatitis B who enrolled to Institute of Hepatology, Ankara University for routine diagnostics. Patient sera were obtained from total of 18 patients comprised of ten male and eight female and whose ages vary from 25 to 67. Informed consent was obtained from all of the patients, and the study protocol was approved by the University's ethics committee.

Extraction of HBV DNA

HBV DNA was isolated from patients' sera using high pure viral nucleic acid kit (Roche, Basel, Switzerland). HBV DNA isolation was performed according to the protocol specified in the user manual of the kit.

Cloning of HBV genomes and construction of artificial HBV genome populations

HBV full genomes bearing wild type and mutant polymerase gene sequences were cloned into pCR-XL-TOPO plasmid using TOPO XL PCR Cloning Kit (Life Technologies, Carlsbad, CA, USA). For the colony selection procedure, we selected ten colonies following multiple colony purification steps and sequenced their plasmids in

order to ensure the uniformity of the insert sequence in both cloning of wild type HBV genomes and cloning of mutant type HBV genomes. Constructed plasmids, which were transformed into appropriate competent *E. coli* strain were isolated by using nucleospin plasmid isolation kit (Macherey–Nagel, Düren Germany). Isolated plasmids were then quantified with spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE, USA). To calculate the plasmid quantity in terms of plasmid copy number per ml, the following formula was used:

$$\text{copy/ml} = \frac{\text{g plasmid/ml} \times 6022 \times 10^{23}}{649 \times \text{base pair of plasmid}}$$

To construct artificial HBV genome populations, we mixed plasmids containing wild type HBV genomes with plasmids containing mutant HBV genomes with the appropriate ratios.

HBV polymerase gene specific PCR

For the genotypic analysis of codons 169, 180, 184, 202, 204 and 236, the specific region of the HBV polymerase gene that includes these codons were amplified using standard PCR. The oligonucleotide primers used in the standard PCR were designed with the ABI prism primer express 2.0 software (Life Technologies, Carlsbad, CA, USA). Regarding the analysis of codon 202 and codon 204, restriction recognition sites were introduced into the sequences containing these codons using site directed mutagenesis approach. For this purpose, the forward primers used to analyze codon 202 and codon 204 were modified in order to bear appropriate restriction recognition sequences. The forward primers and reverse primers used for the PCR step of T-RFLP were labeled with FAM and HEX fluorescent dyes, respectively.

For the analysis of codons 169, 180, 184 and 236, the forward primer PE440: GTACCAAACCTTCGGACGGA and the reverse primer PE891: AGCAAAACCCAAAA-GACCCA were used. Amplification with PE440 and PE891 primer couple was performed with the following thermal cycling conditions: denaturation for 3 min at 94 °C; 45 cycles of 15 s at 94 °C, 10 s at 56 °C, and 45 s at 72 °C; and an elongation step of 7 min at 72 °C.

For the analysis of codon 202, the forward primer PE202: CCCCCACTGTTTGGCTCTC and the reverse primer PE675: GGTAACAGCGGTAAAAAGGGACT were used. The underlined base in the forward primer PE202 was intentionally modified in order to generate DdeI restriction recognition site that enabled the differentiation of S202 wild type codon from S202C, S202I and S202G mutant forms. Amplification with PE202 and PE675 primer

couple was performed with the following thermal cycling conditions: denaturation for 3 min at 94 °C; 15 cycles of 15 s at 94 °C, 15 s at 52 °C, and 30 s at 72 °C; followed by 30 cycles of 15 s at 94 °C, 10 s at 56 °C, and 30 s at 72 °C; and an elongation step of 7 min at 72 °C.

For the analysis of codon 204, the forward primer PE204: CCCACTGTTTGGCTTTCAGTCAT and the reverse primer PE637: ACTTGGCCCCCAATACCAC were used. The underlined base in the forward primer PE204 was intentionally modified in order to generate *NdeI* and *Hin1II* restriction recognition sites that enabled the differentiation of M204 wild type codon from the M204I and M204V mutant forms. Amplification with PE204 and PE637 primer couple was performed with the following thermal cycling conditions: denaturation for 3 min at 94 °C; 15 cycles of 15 s at 94 °C, 15 s at 55 °C, and 30 s at 72 °C; followed by 30 cycles of 15 s at 94 °C, 10 s at 58 °C, and 30 s at 72 °C; and an elongation step of 7 min at 72 °C.

PCR was carried out in 0.2 ml tubes with the reaction mixture volume of 50 µl. The final concentrations of the PCR reagents for each type of PCR were: 1× PCR buffer (Fermentas, Waltham, MA, USA; 10 mM Tris HCl, pH 8.8, 50 mM KCl, and 0.08 % Nonidet P-40), 1.5 mM MgCl₂, 0.2 mM dNTPs, 15 pM forward and reverse primers and 2.5 U of hot start Taq DNA polymerase (Fermentas, Waltham, MA, USA) per reaction.

Restriction enzyme digestion

For the RFLP and T-RFLP analysis of polymerase gene codons that are associated with antiviral resistance, the restriction enzymes were determined as XapI (Fermentas, Waltham, MA, USA) for the analysis of codon 169, MvaI (Fermentas, Waltham, MA, USA) for the analysis of codon 180, BcuI (Fermentas, Waltham, MA, USA) for the analysis of codon 184, DdeI (Fermentas, Waltham, MA, USA) for the analysis of codon 202 and *DraI* (Fermentas, Waltham, MA, USA) for the analysis of codon 236 according to the restriction mapping of the HBV polymerase gene.

For the genotypic evaluation of codon 204, two restriction enzymes, *NdeI* (Fermentas, Waltham, MA, USA) and *Hin1II* (Fermentas, Waltham, MA, USA), were chosen. While *NdeI* recognizes wild type codon 204, which encodes for methionine amino acid present in the conserved YMDD motif of the HBV polymerase enzyme, *Hin1II* recognizes mutant type codon 204, which encodes for valine amino acid. Neither of the enzymes recognizes the other mutant type codon 204, which encodes for isoleucine amino acid.

For the restriction enzyme digestion, 40 units of restriction enzyme were used to digest 50 µl PCR

product in the presence of appropriate enzyme buffer. To ensure the complete digestion, the reaction mix was incubated overnight at 37 °C.

Agarose gel electrophoresis for RFLP

For the RFLP analysis of HBV polymerase gene codons, products of restriction enzyme digestion were separated electrophoretically using 2 % agarose gel containing 0.5 µg/ml ethidium bromide. For the agarose gel electrophoresis, TAE (Tris, acetic acid, EDTA) buffer consist of 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (pH 8) was used as the gel running buffer. For the sizing of the restriction fragments, ΦX 174 Hinf I DNA (Promega, Madison, WI, USA) was used as the size standard. Restriction fragments were visualized using UV trans illuminator.

Evaluation of fluorescently labeled terminal restriction fragments for T-RFLP

T-RFLP studies were carried out with ABI Prism 310 genetic analyzer. Before running the capillary gel for the analysis of terminal restriction fragments, the products of restriction enzyme digestion were desalted by using the high pure PCR product purification kit (Roche, Basel, Switzerland). The desalted digestion products were separated in the pop-4 polymer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) with denaturing conditions. For denaturation of the desalted digestion products, 1 µl of desalted digestion product was treated with 9 µl of HI DI formamide (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) at 94 °C for 2 min. For the evaluation of the sizes of the terminal restriction fragments, Genescan-500 ROX size standard (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used. For the calculation of the sizes of the terminal restriction fragments, the local southern method was preferred. The capillary electrophoresis conditions were determined as follows: the module was selected as GS STR POP4 (1M) F; the injection time was adjusted to 5 s; the injection voltage and the running voltage were adjusted to 15 kV; the temperature of the gel was adjusted to 60 °C; the gel running duration was adjusted to 24 min and the analysis parameter was selected as microsatellite analysis.

Quantification of serum HBV DNA

The quantification of the serum HBV DNA was carried out by performing COBAS Ampliprep-COBAS TaqMan Hepatitis B Virus (HBV) Test (Roche, Basel, Switzerland).

DNA sequencing of HBV polymerase gene

The direct DNA sequencing of HBV polymerase gene was performed according to the standard dideoxyribonucleotide chain-termination method by using ABI Prism 310 genetic analyzer. For the PCR step of the DNA sequencing of HBV polymerase gene, PE440 and PE891 primer couple was used.

Statistical analysis

Fisher's exact test was performed for the evaluation of mutant frequency distributions in T-RFLP and direct DNA sequencing methods. All statistical analysis was performed by SPSS software, version 22.0 (IBM, Armonk, NY, USA).

Results

PCR-RFLP analysis for codons associated with HBV antiviral resistance

For the PCR-RFLP studies of HBV polymerase gene codons, firstly the sequences of HBV polymerase genes obtained from The HBV Genome Data Base of Institute of Hepatology, Ankara University were aligned using MEGA4 software. Subsequently, by considering the common sequence data, the restriction map of the codons associated with antiviral resistance were determined. As a result of this study, antiviral resistance-related six codons, which are 169, 180, 184, 202, 204 and 236, were found to be suitable for being analyzed by RFLP or T-RFLP methods. For the PCR-RFLP analysis of codons 169, 180, 184, 202, 204 and 236, total of six sera collected from six patients with chronic hepatitis B whose HBV populations had developed antiviral resistance due to the long term antiviral therapy was used. The antiviral resistance mutations of the HBV populations of these six patients were predetermined with direct DNA sequencing of the HBV polymerase gene. Within the HBV populations of the six patients, three had single point mutation in the codons 169, 184 and 202, respectively, which were arisen due to the long term entecavir therapy. Two had single point mutation in the codons 180 and 204, respectively, which were arisen due to the long term lamivudine therapy and one had single point mutation in the codon 236, which was arisen due to the long term adefovir therapy. PCR-RFLP results of wild type and mutant HBV polymerase gene codons 169, 180, 184, 202, 204 and 236 are shown in Fig. 1a–f.

Fig. 1 PCR-RFLP analysis for codons associated with HBV antiviral resistance. **a** PCR-RFLP at codon 169. The *XapI* restriction enzyme recognizes the wild type codon 169 and forms 388 bp long restriction fragment upon digestion of the PCR product. **b** PCR-RFLP at codon 180. The *MvaI* restriction enzyme recognizes the wild type codon 180 and forms 353 bp long restriction fragment upon digestion of the PCR product. **c** PCR-RFLP at codon 184. The *BcuI* restriction enzyme recognizes the wild type codon 184 and forms 341 bp long restriction fragment upon digestion of the PCR product. **d** PCR-RFLP at codon 202. The *DdeI* restriction enzyme recognizes the wild type codon 202 and forms 74 bp long restriction fragment upon digestion of the PCR

product. **e** PCR-RFLP at codon 204. While the *HinIII* restriction enzyme recognizes the mutant type codon 204 which encodes for valine amino acid, the *NdeI* restriction enzyme recognizes the wild type codon 204 which encodes for methionine. Neither of the enzymes recognizes the other mutant type codon 204 which encodes for isoleucine amino acid. The *HinIII* and *NdeI* restriction enzymes form 57 and 59 bp long restriction fragments upon digestion of the PCR product. **f** PCR-RFLP at codon 236. The *DraI* restriction enzyme recognizes the wild type codon 236 and forms 264 and 187 bp long restriction fragments upon digestion of the PCR product. WT wild type

Sensitivity of the T-RFLP method in detecting HBV subpopulations carrying antiviral resistance mutations

In order to study the sensitivity of the PCR-T-RFLP method, we constructed artificial HBV genome populations. For this purpose, with varying ratios, we mixed pCR-XL-TOPO plasmid containing wild type HBV full genome with pCR-XL-TOPO plasmid containing mutant HBV full genome, which bear L180M mutation. To determine the sensitivity of the PCR-T-RFLP method in detecting HBV subpopulations, we performed PCR-T-RFLP assay with artificially constructed HBV genome populations of which

mutant (L180M)-to-wild type ratios were adjusted to 10, 5, 1, 0.1 and 0.01 %, respectively. The titer of the plasmids bearing HBV genome was adjusted to 10^6 copy/ml. As a result we showed that PCR-T-RFLP method is capable of detecting HBV subpopulations carrying antiviral resistance mutations that represent only 1 % of the total HBV population (Fig. 2). To validate the PCR-T-RFLP results, we performed the PCR-T-RFLP assay two more times with artificially constructed HBV genome populations of which mutant (L180M)-to-wild type ratios were adjusted to 1 %. As a result we were again able to detect the mutant (L180M) HBV subpopulations that represent 1 % of the total HBV population (data not shown). To further confirm

Fig. 2 PCR-T-RFLP assay with artificially constructed HBV genome populations that contain mutant (L180M) HBV subpopulations at various ratios. **a** Five electropherograms represent the T-RFLP assay results of artificially constructed HBV genome populations of which mutant (L180M) to wild type ratios were adjusted to 10, 5, 1, 0.1 and 0.01 % from top to the bottom respectively. Peaks (black color) that identify the mutant (L180M) HBV subpopulations representing the 10, 5 and 1 % of the total HBV population were indicated with thin arrows. The magnified view of the electropherogram belonging to the

mutant (L180M) HBV subpopulation representing 1 % of the total HBV population is shown within a black colored circle. The peaks that represent wild type (180L) HBV populations are indicated with thick arrows. **b** The negative and positive controls of the sensitivity experiments are shown in the upper and the lower electropherograms respectively. In the upper electropherogram, the black peak indicated with arrow represents the 100 % wild type (180L) HBV population. In the lower electropherogram, the black peak indicated with arrow represents the 100 % mutant type (L180M) HBV population

this 1 % sensitivity value, we performed the same PCR-T-RFLP experiments with the artificially constructed HBV genome populations bearing N236T mutation. We adjusted the mutant type/wild type ratios to 5, 1 and 0.66 %. As a result we were still able to detect the mutant (N236T) HBV subpopulations that represent 1 % of the total HBV population (Suppl. fig. 1).

Specificity of the T-RFLP method in genotyping HBV populations

To determine the specificity of the T-RFLP method, we randomly selected 12 patients with chronic hepatitis B who were on lamivudine therapy and registered to our clinics for routine diagnostics. We collected the patients' sera and isolated their serum HBV DNA. We then performed PCR-T-RFLP assay specific for the genotypic evaluation of the codon 204 and correlated the results with the direct DNA sequence analysis. As a result, the T-RFLP method exhibited 100 % concordance with the results of direct DNA sequencing in genotyping of the HBV populations (Table 1). Additionally we were able to detect antiviral resistant HBV minor populations from the patient sera that have a viral load of as low as 10^5 copy virion/ml (Table 1). Furthermore, due to the high sensitivity profile of PCR-T-RFLP method in detecting HBV minor populations, some HBV subpopulations with lamivudine resistance were identified in four patients (Table 1). In the three of them, HBV subpopulation bearing M204I mutation was detected within the wild type HBV major population. In the remaining one patient, HBV subpopulation bearing M204I mutation was detected within the HBV major population bearing M204V mutation. Once we compared the mutant

(M204I) detection capabilities of T-RFLP and direct DNA sequencing methods, T-RFLP method had significantly higher mutation detection frequencies compared to the direct DNA sequencing method (Fisher's exact test, $P < 0.1$, $P = 0.084$).

Discussion

Antiviral resistance remains to be the major problem in the NA therapy of patients with chronic hepatitis B. In this respect, determining the antiviral resistance soon after its emergence in the patient's HBV pool is crucial to initiate the appropriate rescue therapies. Hence, there is a strong need for a monitoring method that enables the detection of resistant HBV strains as early as possible during the NA therapy period. Such a method provides great advantages in the NA therapy of patients with chronic hepatitis B. Primarily, by using such a monitoring method, the clinical resistance can be detected months before the emergence of a viral breakthrough and thus the treatment strategy can be manipulated appropriately. Additionally, with such a method, we are informed about the nature of the resistant HBV variant and thus we can determine the most relevant alternative NA therapy accordingly. Furthermore, this method enables us to verify the clinical resistance by demonstrating the antiviral resistance mutations in the patient's HBV pool and thus answer the question that whether it is still logical to proceed with the current NA therapy.

Due to the fact that an antiviral resistance can arise at any time during the course of an antiviral therapy and furthermore in some cases can be present even before the

Table 1 Comparison of the results of PCR-T-RFLP assay with the results of direct DNA sequencing

Patients	HBV viral load (copy/ml)	T-RFLP results	Direct DNA sequencing results
MC	6.2×10^7	204M	204M
SD	4.8×10^5	204M, <u>M204I</u>	204M
GY	7.3×10^5	204M, <u>M204I</u>	204M
HD	7.2×10^7	204M	204M
MU	1.2×10^8	204M	204M
NC	1.1×10^5	204M	204M
AA	7.4×10^5	M204I	M204I
SDA	7.5×10^4	204M, <u>M204I</u>	204M
NG	1.4×10^8	M204I	M204I
GG	1.2×10^8	M204I	M204I
Fi	1.9×10^6	M204V, <u>M204I</u>	M204V
NK	3.5×10^7	M204I	M204I

The underlined mutations were detected as HBV subpopulations according to the T-RFLP assay. T-RFLP method had significantly higher mutation detection frequencies compared to the direct DNA sequencing method (Fisher's exact test, $P < 0.1$, $P = 0.084$)

initiation of the antiviral therapy, it is crucial to monitor the resistance in infected patients from the very beginning of the antiviral therapy.

In this context, for the genotypic evaluation of antiviral resistant hepatitis B virus subpopulations, various monitoring methods have been developed as alternatives to commonly used direct DNA sequencing method. These include, PCR-RFLP method [1, 7], PCR-RFMP (restriction fragment mass polymorphism) method [4], methods using conventional PCR with allele specific primers [10, 19, 20], peptide nucleic acid (PNA) mediated PCR clamping method [8], methods based on real-time PCR [3, 5, 12, 13, 17, 18] and methods based on immobilized oligonucleotide probes [6, 9, 11, 15, 21, 22]. The sensitivity of these methods in detecting antiviral resistant HBV subpopulations has been reported to be 5 % in PCR-RFLP method [1], 1 % in PCR-RFMP method [4], 0.5 % in allele specific PCR method [10], 0.01 % in PNA mediated PCR clamping method [8], depending on the experimental settings 10–0.01 % in real-time PCR methods [3, 5, 12, 13, 17, 18] and 30–5 % in methods using immobilized oligonucleotide probes [6, 9, 11, 21, 22]. All these methods that are used to evaluate antiviral resistant HBV subpopulations involve PCR step, which is required for the amplification of the target HBV DNA sequence. Therefore, the sensitivities of these methods are mostly dependent on the sensitivity of the PCR step included in the methods.

To detect antiviral resistant HBV subpopulations in a large quasiespecies HBV pool, we employed PCR-T-RFLP method. With our PCR conditions set up for the T-RFLP assay, we were able to genotype HBV populations, which have a titer of 10^3 copy/ml (data not shown). By using PCR-T-RFLP method, we were able to detect antiviral resistant HBV subpopulations representing only 1 % of the total HBV population. Besides, PCR-T-RFLP method exhibited 100 % concordance with the commonly used direct DNA sequencing method in genotyping HBV populations. With the PCR-T-RFLP method we tested, lamivudine resistant (L180M, M204V/I) HBV subpopulations can be studied with high sensitivity and specificity profile. However, in addition to lamivudine resistant HBV subpopulations, with the same PCR protocols we used, entecavir resistant (I169T, T184A/G/I/L/S and S202G/I/C) and adefovir resistant (N236T) HBV subpopulations can be also easily studied with this method, with the same sensitivity and specificity profiles.

As a conclusion, the PCR-T-RFLP method we employed may be adopted in the follow-up of genotypic resistance in patients with chronic hepatitis B who are on lamivudine, entecavir or adefovir therapy.

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