A Nested PCR Method for the Identification of Hepatitis B Virus Genotype in Paraffin Blocks of Formalin-Fixed Liver Biopsies

Bita Geramizadeh MD*, Roghieh Kaboli MD*, Abbas Behzad-Behbahani PhD**, Marjan Rahsaz MSc***, Negar Azarpira MD*, Mahdokht Aghdai MSc***, Maryam Aytollahi MSc***, Ramin Yaghoobi PhD***, Mehrzad Baneehashemee BSc**

Hepatitis B virus is a hepatotropic virus that causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma; it is responsible for more than one million deaths annually worldwide despite hepatitis B vaccination. Until now, there are eight known genotypes (A-H). Clinical course of chronic hepatitis B varies according to the genotype of Hepatitis B virus. Liver biopsy becomes necessary to judge the degree of liver lesions and to make the final diagnosis, especially to make the diagnosis for latent liver damage and early-compensated cirrhosis.

Genotype is very important for prognostication, but it has not yet been reported on liver tissues. Sometimes, it can be helpful to do genotyping of Hepatitis B virus of the liver tissue; such conditions include research programs, when serum is not available or when serum is negative for Hepatitis B virus DNA. In this study, we wanted to evaluate the feasibility of a simple method for genotyping of liver tissue samples. We performed genotyping of the liver biopsies and intended to find out a simple and reliable method for genotyping in the paraffin-embedded formalin-fixed liver tissue.

Genotype D was the only isolated genotype in all the liver biopsies. The tissue genotype was just the same as that found in serum. The procedure was easy and good for large scale studies.

Genotyping in the paraffin-embedded formalin-fixed stored liver tissue can be done with the same accuracy of the serum.

Keywords: Genotype • HBV • liver tissue • nested PCR

Introduction

Hepatitis B virus (HBV) is a hepatotropic virus that causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. It is one of the most significant global health problems and is responsible for more than one million deaths annually worldwide despite hepatitis B vaccination. Until now, there are eight known genotypes (A-H). Clinical course of chronic hepatitis B varies depending on genotype of the infecting HBV. Current approaches to genotyping include sequencing and phylogenetic analysis, differential hybridization, hemi-nested and multiplex PCR, and restriction fragment length polymorphism (RFLP). However, to the best of our knowledge, all of the current methods in use are only applicable to serum samples. Nonetheless, sometimes, especially for research purposes, serum may not be available for genotyping. Furthermore, some studies showed that some potential pathogenic strains, although replicate actively in hepatocytes, are not released into the circulation. Liver biopsy becomes necessary to assess the
extent of liver damage and to make the final diagnosis, especially to diagnose the early-compensated cirrhosis.\textsuperscript{6}

We performed this study to assess the feasibility of a new method for genotyping of the paraffin-embedded formalin-fixed liver tissues.

**Patients and Methods**

**Patients**

All patients from whom a liver biopsy was taken in the affiliated hospitals of Shiraz University of Medical Sciences, between 2000 and 2005, were included in this study. Those biopsies which had enough tissue for DNA extraction and had available serum were selected.

None of these cases have received antiviral treatment before liver biopsy. All of them were tested for HBs and HBe antigens in the serum as well as genotyping. Patients with other histories like alcohol consumption, diabetes, or drug abuse were excluded from the study.

Patients with clinical evidence of liver cirrhosis and any other positive serology (hepatitis A, C) were also excluded.

**Methods**

**DNA extraction procedure**

- Deparaffinizing in one milliliter of xylol, incubate for 30 min at room temperature;
- Centrifuge at 12,000 rpm for five min, collect supernatant;
- Repeat step two again;
- Add one milliliter 100% ethanol and incubate for 30 min;
- Centrifuge at 12,000 rpm for 10 min, discard the supernatant;
- Add 200 µL of digestion buffer (50 mmol/L Tris-HCl, pH: 8, one milimole per litter EDTA, and 0.5% Tween 20) and one milliliter proteinase K 10 mg/mL;
- Incubate at 48°C overnight;
- Centrifuge at 7000 rpm for five min;
- Boiling of supernatant for 10 min;
- Add 10 µL sodium acetate;
- Add 200 µL 100% ethanol and incubate for one hour at -80°C;
- Centrifuge at 14,000 rpm at 4°C for 10 min;
- Discard the supernatant and wash with ethanol; and
- Centrifuge at 12,000 rpm for five min, collect DNA. Dry completely and dissolve in 20 µL distilled water.

**Amplification**

The first-round PCR primers (outer primer pairs) and the second-round PCR primers (inner primer pairs) were designed on the basis of the conserved nature of the nucleotide sequences in regions of the pre-S1 through S genes, irrespective of the six HBV genotypes.\textsuperscript{7}

P1 (sense) and S1-2 (antisense) were universal outer primers (1,063 bases). B2 was used as the inner primer (sense) with a combination called mix A for genotypes A, B, and C. Mix A consists of antisense primers BA1R (type A specific), BB1R (type B specific), and BC1R (type C specific). B2R was used as the inner primer (antisense) with a combination called mix B for genotypes D, E, and F. Mix B consists of sense primers BD1 (type D specific), BE1 (type E specific), and BF1 (type F specific). After optimizing the process, the first PCR was carried out in a tube containing 40 µL of the reaction mixture made up of 0.5 µM of each of the outer primers, 200 µM of each of the four deoxynucleotides, 2.5 units of Taq DNA polymerase, five µL of reaction buffer and 1.5 mM MgCl\textsubscript{2}. Then, the sample was added. The samples were denatured for two min at 94°C followed by 40 cycles each consisted of 40 s at 94°C, one min at 57°C, and one min at 72°C in an Eppendorf thermal cycler (master cycler 5330). One-microliter aliquot of the first-round PCR product was added to two tubes containing the second sets of each of the inner primer pairs, each of the deoxynucleotides, Ampli Taq Gold DNA polymerase, and PCR buffer as in the first reaction. The samples were amplified for 35 cycles consisting of preheating at 95°C for two min, 20 cycles of amplification at 94°C for 20 s, and 68°C for 50 s (Table 1).

**Detection of PCR product**

Ten microliter of the reaction product was electrophoresed on a 3% agarose gel, stained by ethidium bromide, and evaluated under UV light. Negative and positive controls were also treated as samples. We used DNA samples of HBV, with different genotypes which were confirmed by genotype sequencing, as controls.

Beta actin was used as an internal control (house-keeping gene).
In this study, we used some genotype D sera for control, all of which had been sequenced.

Results

In our study, nested PCR was performed on freshly-cut sections of the liver biopsy paraffin blocks.

These were 80 cases all with chronic hepatitis B.

Genotype D was the only detected genotype in different clinical forms of chronic HBV infection, regardless of seropositivity for HBe Ag (Figure 1). No case with genotype other than D was found which was completely compatible with serum results of patients and also previous reports from Iran.8

The method of the isolation was simple. The PCR method was easy to use, especially in our country where all of the reported genotypes until now have been genotype D.

We had good results in the biopsies which have

Table 1. Primer sequences used for HBV genotyping by nested PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence a (position, specificity, and polarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First PCR</strong></td>
<td></td>
</tr>
<tr>
<td>P1b</td>
<td>5’-TCA CCA TAT TCT TGG GAA CAA GA-3’ (nt 2823-2845, universal, sense)</td>
</tr>
<tr>
<td>S1-2</td>
<td>5’-CGA ACC ACT GAA CAA ATG GC-3’ (nt 685-704, universal, antisense)</td>
</tr>
<tr>
<td><strong>Second PCR</strong></td>
<td></td>
</tr>
<tr>
<td>Mix A</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>5’-GGC TCM AGT TCM GGA ACA GT-3’ (nt 67-86 types A to E specific, sense)</td>
</tr>
<tr>
<td>BA1R</td>
<td>5’-CTC GCG GAG ATT GAC GAG ATG T-3’ (nt 113-134, type A specific, antisense)</td>
</tr>
<tr>
<td>BC1R</td>
<td>5’-CAG CCT AGG AAT CCT GAT GTT G-3’ (nt 165-186, type C specific, antisense)</td>
</tr>
<tr>
<td>Mix B</td>
<td></td>
</tr>
<tr>
<td>BD1</td>
<td>5’-GCC AAG GTA GGA GCT-3’ (nt 2979-2996, type D specific, sense)</td>
</tr>
<tr>
<td>BE1</td>
<td>5’-CAC CAG AAA TCC AGA TTG GGA CCA –3’ (nt 2955-2978, type E specific, sense)</td>
</tr>
<tr>
<td>BF1</td>
<td>5’-GYT ACG GTC CAG GGT TAC CA-3’ (nt 3032-3051, type F specific, sense)</td>
</tr>
<tr>
<td>B2R</td>
<td>5’-GGG GGC GGA TYT GCT GGC AA-3’ (nt 3078-3097, types D to F specific, antisense)</td>
</tr>
</tbody>
</table>

An “M” represents a nucleotide that could be either an A or a C; a “Y” represents a nucleotide that could be either a C or a T. “nt” stands for nucleotide.

Figure 1. PCR of the patients. Well 1: patient No. 1 Mix A; Well 2: patient No. 2 Mix A; Well 3: patient No. 3 Mix A; Well 4: patient No. 4 Mix A; Well 5: patient No. 5 Mix A; Well 6: 50-bp DNA ladder; Well 7: genotype D control Mix A; Well 8: patient No. 1 Mix B; Well 9: patient No. 2 Mix B; Well 10: patient No. 3 Mix B; Well 11: patient No. 4 Mix B; Well 12: patient No. 5 Mix B; Well 13: genotype D control Mix B; Well 14: genotype D control Mix B.
been stored for more than five years. Therefore, neither formalin fixation nor paraffin embedding has been interfering with our method of DNA isolation and genotyping. It can be used on old samples from those patients who might not be available for doing serum genotyping; this is particularly good for research purposes and also for genotyping in the HBV-negative patients.

Discussion

The association among HBV genotype, severity of disease, cirrhosis, hepatocellular carcinoma, and response to treatment has recently been the focus of several investigations.1

In nested PCR there are two sequential PCR amplifications with the primary PCR product being used as the template for the second PCR using primers internal to the primers used for the first PCR. This method is used to increase the assay specificity and sensitivity.9,10

Although there are several methods for HBV genotyping (e.g., RFLP, multiplex PCR, and DNA sequencing), none of them are applicable for testing the liver tissue.

Recently, DNA detection has been done in the liver tissue in patients with chronic hepatitis. In this report, HBV DNA was found in the liver tissue of some patients with negative viremia and positive HBs antigen, a condition the so-called “hepatitis B in situ.”5,6 Although in our study all of the patients were both HBV DNA- and HBs antigen-positive, this method is also applicable for those with hepatitis B in situ.

The method of genotyping in the liver tissue is also reported for those with a mix up between the liver biopsies in two HCV-positive cases who have different genotypes of HCV. However, such condition has not already been reported in hepatitis B.11

The PCR method described in this study provides a rapid and sensitive way for distinguishing HBV genotypes. This method may be suitable for large-scale clinical and epidemiologic studies, especially in high prevalence areas like Mediterranean and Middle East regions, and Asia, as a whole. It is the first study of genotyping in the liver tissue of hepatitis B patients with very good results.

It was used successfully even for tissues stored in paraffin blocks for more than five years. This is comparable with other studies on genotyping of HCV-positive liver tissue samples which had been stored for more than 20 years.12

References