

# GENOTYPE ANALYSIS OF HEPATITIS B VIRUS IN THE TURKISH POPULATION USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM PATTERNS

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## ABSTRACT

**Objective:** Six different HBV genotypes have been defined comparing the complete HBV genome. The distribution of genotypes among infected populations varies geographically. A relationship between HBV genotypes and the development of chronic liver disease has been proposed. Therefore, this study was designed to classify HBV genotypes in the Turkish population using restriction fragment length polymorphism (RFLP) analysis of the S gene region.

**Methods:** The HBV DNA extracted from forty-eight serum samples were subjected to polymerase chain reaction (PCR) using primers from the S region of HBV genome. Restriction enzymes, Hph I (Genotype F), Nci I (Genotype E), Alw I (Genotype C), Ear I (Genotype B), and Nla IV (Genotype A and D), were used for RFLP analysis of amplified product of HBV DNA.

**Results:** In the 48 serum samples investigated, 3 different genotypes were detected. These were as follows; Genotype D: 41 (85.41%), Genotype A: 5(10.41%), and Genotype F: 2 (4.16%).

**Conclusion:** This study shows a molecular heterogeneity of HBV in the Turkish population

with at least 3 different genotypes observed in the 48 samples studied. The genotype D was by far the most common genotype in the Turkish population as is the case in all other Mediterranean countries.

**Key Words:** Hepatitis B virus, Genotypes, S gene, Restriction fragment length polymorphism.

## INTRODUCTION

Hepatitis B virus (HBV) can cause acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. HBV is a member of the hepadnavirus family, characterized by a small, circular, partially double-stranded DNA. The genome contains four major open reading frames (ORFs); preS/S, which encodes the surface antigen proteins of virion envelope; preC/C, which encodes the core antigen of the nucleocapsid; X, which encodes the X protein; and P, which encodes the DNA polymerase activities of the virus (1).

The serological heterogeneity of hepatitis B surface antigen (HBsAg) has long been established, and the HBV isolates have been

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classified into nine different subtypes, ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq<sup>-</sup>, and adrq, according to the antigenic determinants of their HBsAg (2,3).

The entire nucleotide sequences of HBV genomes of various subtypes have been classified into six genetic groups, named A to F, based on an intergroup divergence of 8% or greater of the complete nucleotide sequence. The relationship of the nine subtypes to genomic groups A to F has been established (4-6).

The distribution of genotypes among infected populations varies geographically and there has been speculation about the relationship between HBV genotypes and development of chronic liver disease.

The aim of the present study, is to classify the HBV genotypes in Turkish population using restriction fragment length polymorphism (RFLP) analysis of the S gene region.

## **MATERIALS AND METHODS**

Serum samples were provided by Microbiology and Clinical Microbiology Laboratories of Haydarpasa Numune Hospital, Istanbul, and selected from chronically infected adults. All were HBsAg and anti HBc IgG positive.

### **Extraction of viral DNA from serum.**

Forty-eight serum samples were collected from Turkish patients with chronic HBV infection. A 100 µl portion of serum was incubated at 45°C for 2 h in a mixture of proteinase K (100 µg/ml), 0.5% sodium dodecyl sulfate, 5mM EDTA, and 10 mM Tris HCl, pH 8.0. The solution was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol, followed by an ethanol precipitation. The precipitate was dissolved in 50 µl of distilled water.

### **PCR amplification of HBV DNA.**

A 10 µl aliquot of serum DNA was subjected to polymerase chain reaction (PCR) with the following amplification mixture: 2.5 µl of 10xTaq polymerase buffer (MBI, Fermentas), 2.5 µl of 2.5 mM deoxyribonucleotide triphosphate, 0.1 µl of

Taq polymerase (5 units, MBI, Fermentas), 10 pmol of sense HBVF1 (CCCTGCTGGTGG CTCCAAGTTC) and antisense primers HBVR2 (AAGCCAAACAGTGGGGGAAAGC) in a 25 µl reaction volume (7). The amplification profile was 2 min 96°C, followed by 25 cycles at 94°C for 15 sec (denaturation), 45 sec at 60°C (annealing) and 45 sec at 72°C (extension) and was performed in a thermal cycler (Biometra-Thermoblock).

2.5 µl of the first-round PCR products was then added to a second-round PCR mixture with the same condition but with a set of inner sense HBVF2 (GTCTAGACTCGTGGTGGACTTCT CTC) and antisense HBVR2 (AAGCCAAAC AGTGGGGGAAAGC) primers. The second-round PCR products which 485 bp long were analyzed by electrophoresis in 3 % agarose gels in (89mM Tris-HCl, 89mM boric acid, 2mM EDTA) stained with ethidium bromide, and then visualized under ultraviolet light. Standard precautions for avoiding contamination during PCR were observed. A negative control serum was also included in each run to ensure specificity.

### **The RFLP analysis for amplified S region of HBV DNA.**

The RFLP analysis for HBV genotyping was performed by restriction digestions of 20 µl second-round PCR products for 3 h after adjustment with 10X enzyme reaction buffer according to the manufacturer's recommendations. Reactions were carried out with 10 units of Alw I, Nci I, Ear I, Nla IV (New England BioLabs) or Hph I (MBI Fermentas) at 37°C. The digested PCR products were electrophoresed on 3.0 % Nusieve GTG (3:1) agarose gel in 1xTBE buffer containing ethidium bromide. The RFLP pattern was then evaluated under ultraviolet light.

## **RESULTS**

We used a genotyping method based on analysis of the restriction fragment length polymorphism (RFLP) of an S gene amplicon.

The HBV genotype specific regions were identified and digested by the restriction enzymes,

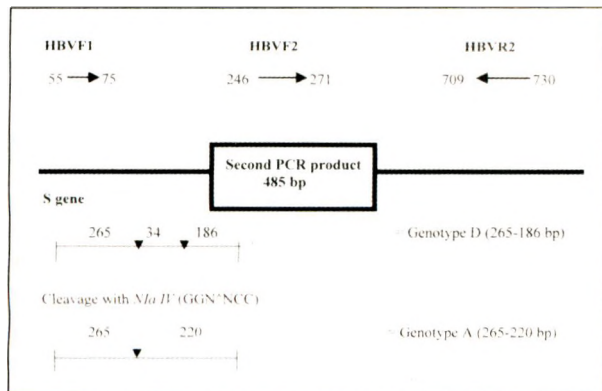
Hph I (Genotype F), Nci I (Genotype E), Alw I (Genotype C), Ear I (Genotype B), and Nla IV (Genotype A and D). Genotype D was identified by Nla IV digestion at position 265 and 299, therefore the expected bands for genotype D are 265, 186, and 34 bp. For genotype A, expected bands are 265 and 220 bp since the specific restriction enzyme site for Nla IV was not found at position 299. Genotype B and C were distinguished by Ear I and Alw I restriction

enzymes respectively, since they do not have restriction site in S gene. Nci I was used for identification of genotype E which contains a restriction site at position 461, and genotype F was digested at position 82 by Hph I restriction enzyme. The pattern observed with these restriction enzymes is shown in Fig. 1 and 2.

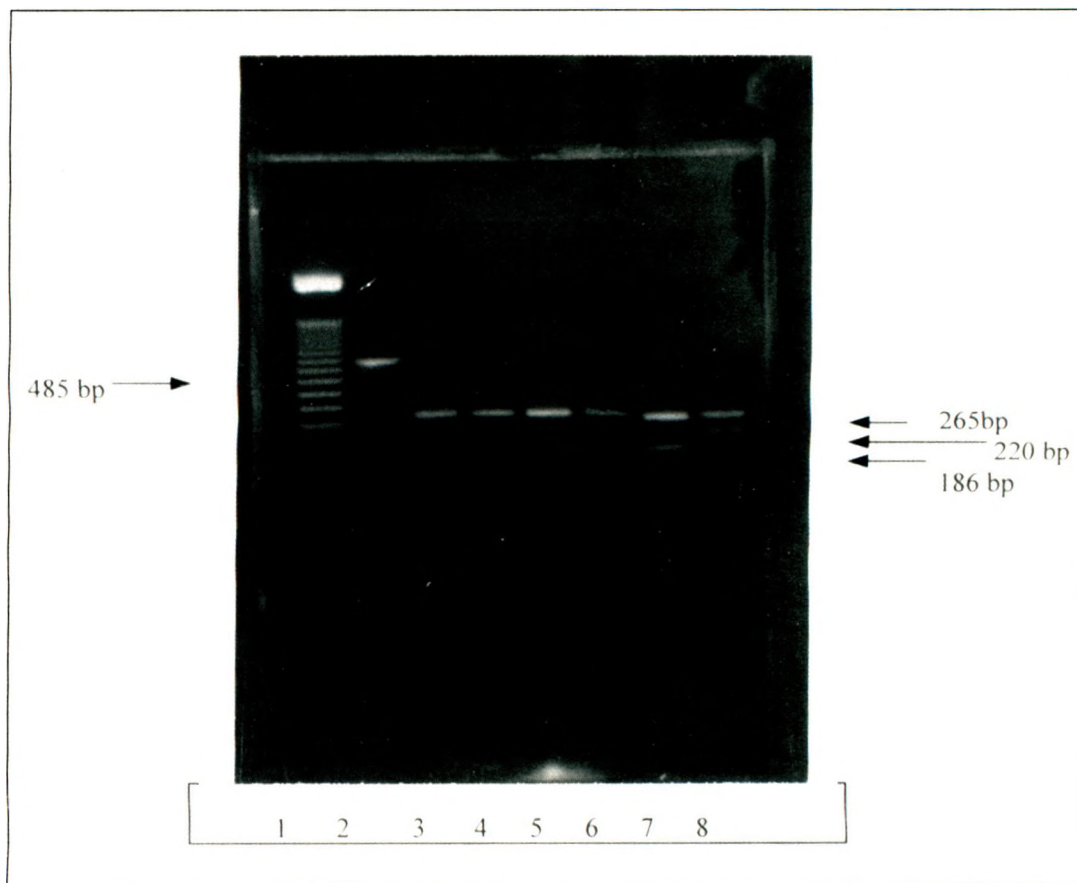
In the 48 serum samples investigated, 3 different genotypes D, A, and F were detected. Genotype D (85.41%) was found in 41, genotype A (10.41%) in 5 and genotype F (4.16%) in only 2 of 48 samples examined.

### DISCUSSION

Although a high prevalence HBV infection is present in Turkey, the genotype distribution is still



**Fig. 1 :** Restriction pattern of HBV S region with *Nla IV*.



**Fig.2 :** RFLP pattern of HBV S region with *Nla IV* restriction enzyme. Lane 1: Marker (*OX 174*), Lane 2: PCR product. Lane 3,7: digestion with *Nla IV* (genotype D), Lane 8: digestion with *Nla IV* (genotype A). Genotype A was digested into 220 and 265 bp fragments while genotype D was 186 bp.



unclear. The HBV is classified into six genotypes based on intergroup divergence, however, the genotype-related differences in the pathogenicity of HBV remains unknown. The aim of this study was to investigate the epidemiological distribution of HBV genotypes in Turkey and further to clarify the genotype-related differences in the pathogenicity of HBV.

The clinical value of HBV genotyping remains uncertain at present. But previous data suggest an association C-1858 strains with severe liver damage (8). The results of a study performed by a Japanese study group showed that liver disease was more common in genotype C carriers than those mainly genotype B in Japan (9,10). A recent report concluded that patients infected with genotype A (C-1858) responded better to interferon therapy than those with non-A genotypes (T-1858) (11) do, further, indicate that genotyping may be of clinical importance.

In this study, PCR product of the S gene is preferred for HBV genotyping using RFLP analysis. Although HBV genotyping using RFLP analysis of a PCR product of the S gene or pre-S sequence have been reported, it is also shown that the sequence of the S gene is more conserved than the pre-S region. Because the S gene overlaps the reverse transcriptase active site in the P gene which encoded in a different frame. Therefore, the S gene is more suitable for genotyping than the pre-S region.

Full-genomic sequences are more ideal for genotype assignment, on the other hand sequence determination is costly and time-consuming. Therefore, RFLP analysis has been developed to simplify classification of HBV genotypes (7). HBV genotyping by RFLP is less complicated compared to direct sequencing and sequence analysis.

This study showed a molecular heterogeneity of HBV in the Turkish population with at least 3 different genotypes observed in the 48 samples studied. The genotype D is by far the most common genotype in the Turkish population as is the case in all other Mediterranean countries. Although the frequency of the percentage of genotype A and F is rather low, the presence may be result of relation of the Turkish people with Northern European (A) and South American (F) countries.

Our further aim is to investigate the association between HBV genotype A, D and F and their clinical outcome.

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