İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

THE EFFECT OF HLA DPB1 ALLELES ON THE IMMUNE RESPONSE IN CHRONIC HEPATITIS B

M.Sc. THESIS

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Molecular Biology & Genetics and Biotechnology Programme

Thesis Advisor: Assoc. Prof. Dr. Gizem DINLER DOGANAY

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KRONİK HEPATİT B HASTALIĞINDA HLA DPB1 ALLELERİN HASTALIĞIN İMMUN YANITA ETKİSİ

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To My Dear Grandmothers and Grandfathers and Loved Ones,

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ABBREVIATIONS

Adv : Adefovir
ALT : Alanineaminotransferase
APC : AntigenPresentingCells
AST : Aspartateaminotransferase
cccDNA : Circularcovalentlyclosed DNA
DNA : Deoxyribonucleicacid
Etv : Entecavir
HBcAg : Hepatitiscoreantigen
HBV : Hepatitis B virus
HBsAg : Hepatitis B surfaceantigen
HCC : Hepatocellularcarcinoma
HLA : Human LeukocyteAntigen
IL : Interleukin
IFN : Interferon
LAM : Lamivudin
LdT : Telbivudine
MHC : Major Histocompatibility Complex
NA : Nucleotide Analogue
NK : Naturel Killer
NKT : Naturel Killer T cell
PCR : Polymerase Chain Reaction
PD : Programmed Death
RE : Restriction Endonucleases
RFLP : Restriction Fragment Length Polimorphism
RNA : Ribonucleic acid
SNP : Single Nucleotide Polymorphism
TcR : T cell receptor recognation
Tdf : Tenofovir
TLR : Toll Like Receptor
TNF : Tumor Necrosis Factor
GWAs : Genome Wide Association Study

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THE EFFECT OF HLA DPB1 ALLELES ON THE IMMUNE RESPONSE IN CHRONIC HEPATITIS B

SUMMARY

Chronic Hepatitis B Infection is a global health problem that affects more than 2 billion people all around the world. Furthermore, more than 400 million people are chronic carriers of this virus that can lead to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Annualy 2 million people die owing to the this virus throughout world. In Turkey, it is known that 3 million people suffer from Chronic Hepatitis B disease.

Hepatitis B virus (HBV) is a small DNA virus and is a member of hepadnaviruses that includes a partially double-stranded circular DNA containing approximately 3200 base pairs.

Once the Hepatitis B virus invades the body, it binds to the cell surface and penetrates to the host cells with the help of its envelope proteins. The virus is transported in to the nucleus from the plasma membrane of the cell. After that, the partially circular DNA is made into covalently closed circular DNA (cccDNA) to be used as a template for pregenomic RNA and mRNA. Even though, the negative strand of virus is almost a complete circle, the second strand, pozitive strand, is shorter in length. The negative strand encodes the structural proteins of the virus whereas the pozitive strand encodes some of the structural proteins and overlaps with most parts of the negative strand. Hepadnaviruses depends on reverse transcription of negative-strand DNA which is transcribed by the DNA polymerase from a “pregenomic” RNA intermediate. Viral proteins are translated by the mRNA, then the proteins and genome are packaged into virions and transported out of the cell to infect new hepatocytes.

This potentially life threatining virus is spread by contact with the infected blood, unprotected sexual activity, organ transplantation, horizontal, nosocomial injury and intravenous drug use. It can also transmitted from mother to infant at birth. Furthermore, host genetic factors play a significant role in the pathogenesis.

Mainly, the aim of the treatment for chronic hepatitis B is to supress viral replication and to induce remission before the significant irreversible liver damage. On the other hand, the long-term goal of treatment is to eliminate the virus, prevent disease

progression and to improve patient survival. Interferon and lamivudine are the most widely used agents for the treatment of chronic hepatitis B diseases. Other used agents are adefovir dipivoxil (ADV), tenofovir disoproxil fumarate (TDF), entecavir (ETV) and telbivudine (LdT).

The human leukocyte antigen (HLA) encodes the major histocompatibility complex (MHC) in humans and this region of the genome is critical for inflammation, infection and autoimmunity. Studies have shown that HLA class II molecules, which show high polymorphism, and cytokines are the susceptibility factors of chronic HBV infection.

Genome-wide association study (GWAS) can methodically investigate hundreds of thousands of single nucleotide polymorphisms (SNPs) that are located near *HLA* loci in chromosome 6p21.According to previous studies, GWAS determined candidate SNPs of the HLA loci that are related with chronic HBV or HCV infection, hepatic fibrosis and hepatocellular carcinoma. Moreover, recent GWAS done on chronic hepatitis B infected patients revealed SNPs in HLA class II genes, especially HLA-DPA1 and HLA-DPB1 region. In this line, our work aims to determine alterations in the HLA-DPB1 site of the genome of HBV infected Turkish patients and link these possible polymorphisms to disease associations in Turkish population.

In this study, a total of 94 patients (Group A) were included, from Department of Gastroenterology, Goztepe Teaching and Research Hospital. These hepatitis B patients who were followed up at hepatology clinic and, age and gender matched 85 HBsAg negative, anti-HBs positive and anti –Hbc IgG positive spontaneous seroconverted healthy subjects (Group B) were enrolled. We also used historical controls as Group C by taking disease related information directly from the published literature, which includes Turkish and German populations. Group C is used to compare HLA-DPB1 frequencies from general population with the disease population of this study. Local ethics committee approval was taken. Genomic DNA was extracted from peripheral blood samples. HLA DPB1 alleles were determined by restriction fragment length polymorphisms–polymerase chain reaction at medium resolution. 9 different restriction endonucleases were selected for digestion to detect allele-specific cleavage after PCR-amplification of the DPBl alleles.The distribution of the alleles among patients and control subjects were analyzed.

Among the 19 analyzed DPB1 alleles in this study, DPB1*02:01 was the most prevalent (genotyped in 30.2% of patient and control groups). DPB1*15:01 allele was more frequent in the spontaneous seroconverted control group compared to chronic hepatitis B patients (15.3% vs. 1.1%, χ^2=12.5, OR=0.06, 95% CI=0.08-0.046 p<0.001,pc<0.001). DPB1*02:01 and DPB1*10:01 were the other alleles genotyped more frequently found in the control group (38.8% vs. 22.3% p=0.02 and 16.5% vs. 5.3% p=0.02, respectively). However these two alleles' associations lost their significance after Bonferoni's correction (pc=0.4 for all).HBsAg seroconversion is the ultimate target in chronic hepatitis B. This study revealed an association of HLA DPB1*15:01 allele with spontaneous HBsAg seroconversion. However, after Bonferroni correction, only DPB1*1501 maintained its significance. It can be said that, presence of DPB1*1501 was the protective allele at Turkish population just looking at the above data without Hardy Weinberg Analysis.

KRONİK HEPATİT B HASTALIĞINDA HLA DPB1 ALLELERİN HASTALIĞIN İMMUN YANITA ETKİSİ

ÖZET

Kronik Hepatit B hastalığı evrensel bir sağlık problemidir. Hepatit B hastalığına sebep olan Hepatit B virüsünün, tüm dünyada 2 milyardan fazla kişiyi enfekte ettiği bilinmektedir. 400 milyondan fazla kişi ise kronik olarak hepatit B taşıyıcısıdır ve bu taşıyıcılık, kronik hepatit başta olmak üzere, karaciğer kanseri ve karaciğer sirozunun oluşmasına sebep olabilmektedir. Yıllık olarak bakıldığında tüm dünyada, 2 milyon kişinin bu virüsten dolayı hayatlarını kaybettiği kayıtlara geçmektedir. Türkiye'de ise yaklaşık 3 milyon kişinin bu hastalıkla mücadele ettiği biliniyor.

Hepatit B virüsü küçük bir DNA virüsüdür ve yaklaşık olarak 3200 baz çiftine sahiptirler. Hepatit B virüsü Hepadonovirus ailesinin bir üyesidir ve kısmi olarak çift sarmallı, dairesel DNA içerir.

Genom üzerinde 4 tane protein kodlayacak nükleik asit dizisi vardır. Bunlar S, C, X ve P bölgeleri olarak adlandırılan yüzey, kor, X ve polimeraz proteinleridir. C geni, HBcAg proteinlerini kodlar ve bu kor nükleokapsid oluşumuna sebep olan yapısal proteinleri içerir. Bu proteinler HBeAg antijenine translasyon sonrası dönüşür. S genini içeren Pre S1 ve Pre S2 yüzey proteinleri HBsAg anijenlerini kodlar. P geni DNA polimerazı kodlayan protein, ters transkripsiyon aktivitesine sahiptir ve son olarak X geni hücrenin potansiyel transaktivatörüdür. X geninin görevi ise tamamen bilinmemektedir. Genom üzerinde, 6 başlangıç kodonu, 4 promotör ve 2 tane hızlandırıcı element bulunmaktadır.

Hepatit B virüsünün tüm genomik sekansının üzerinde 8 genotipi tanımlanmıştır ve bunlar A'dan H'ye kadar düzenlenmiştir. Bağlantılı olarak, HBV'nin alt tipleri (adw, adr, ayw ve ayr) de mevcuttur. A'dan H'ye düzenlenen bu 8 genotip farklı coğrafik bölgelerde yayılım göstermiştir.

Hepatit B virüsü konak hücreye invaze olduğu zaman, hücre yüzeyine bağlanır ve bu hücreye kendi zarf proteinleri yardımıyla girer. Virüs, hücrenin plazma membranından nükleusa taşınır. Bu şekilde nükleusa giren virüs kısmen sirküler formdan sirküler DNA (cccDNA) formuna dönüşerek öncül RNA ve haberci RNA'ya kalıp oluşturur. Virüsün negatif zinciri neredeyse tamamen halkasal yapıda olmasına rağmen, pozitif zincirin uzunluğu daha kısadır. Negatif zincir virüsün yapısal proteinlerini kodlarken, pozitif zincir bazı yapısal proteinleri kodlar. Translasyon sonucu oluşan viral proteinler nükleokapsid içinde toplanır ve paketlenir. Daha sonra yeni hepatositleri işgal etmek üzere sitoplazmadan salınır.

Hayatı tehdit eden bu virüsün bulaşma yolları şu şekildedir: Enfekte olmuş kan temasları, korunmasız olarak yapılan cinsel aktivite, organ nakilleri, yatay olarak meydana gelen bulaşma, hastane yaralanmaları ve intravenöz ilaç kullanımı olarak özetlenebilir. Ayrıca bu hastalık anneden bebeğe doğum esnasında da geçebilir. Yapılan çalışmalar, hepatit B virüs enfeksiyonunun progresyonunda sadece viral genotip ve vireminin değil, aynı zamanda konak genetik faktorlerinin de oldukça önemli rol oynadığını göstermiştir. Aynı zamanda, hem hücresel hem de hümoral immün yanıtın virüsün eredike edilmesi için gerekli olduğu birçok deneysel çalışmada gösterilmektedir.

Temel olarak kronik hepatit B hastalığının tedavisinin amacı, viral replikasyonu baskılamak ve geri dönülemez karaciğer zararlarını en aza indirgemektir. Öte yandan, uzun süreli tedavinin amacı ise virüsü elimine ederek hastalığın progresyonunu önlemek. İnterferon ve lamuvidin kronik Hepatit B hastalığının tedavisinde kullanılan yaygın ajanlardır. Tedavide kullanılan diğer ajanlar ise adefovir dipivoksil (ADV), tenofovir disoproksil fumarat (TDF), entecavir (ETV) and telbivudin(LdT)dir.

Kromozomun 6p21.3 bölgesinde yer alan yüksek derecede polimorfik insan lökosit antijen (HLA) geni immün yanıtla çok yakından ilişkilidir.Ayrıca bu polimorfik bölge inflamasyondan, enfeksiyondan ve otoimmüniteden de sorumludur.

Genomun tarandığı (genome wide association-GWA) çalışmalar kromozomun 6p21 noktasının yakınında bulunan insan lökosit antijende ki, binlerce tek nükleotid polimorfizmini ortaya çıkartmıştır. Daha önceki çalışmalara göre HLA lokusunda bulunan ve kronik HBV, HCV, hepatik fibroz ve gelişen karaciğer kanser

hücreleriyle bağlantılı olabilen aday tek nükleotid polimorfizmleri belirlenmiştir. Genomun tarandığı çalışmalarda HLA-DPB1 gen bölgesinin Hepatit B hastalığı ile ilişkili olabileceği üzerine veriler elde edilmiştir. Bu bölgenin polimorfik bir bölge olmasından dolayı Hepatit B hastalarında hastalık direncine bakarak DPB1 allel tayini yapmak ve elde edilecek verilere göre allellerin hastalık için direnç ya da yatkınlıklarını incelemek bu çalışmanın temel amacıdır.Biz bu çalışma ile Türk populasyonunda, Hepatit B hastalığının immün yanıtta önemli bir rol oynayan HLA sınıf II bölgesi genlerinden HLA-DPB1 polimorfizmleri ile ilişkisini araştırarak, aday alellerin ve koruyucu alellerin belirlenmesini hedefledik.

Bu çalışma 94 hasta içermektedir. Bu hastalar Göztepe Eğitim ve Araştırma Hastanesi'nde hepatoloji kliniğinden takipli hastalardır. Yaş ve cinsiyetle eşleşmiş 85 HBsAg negatif, anti- HBs pozitif ve anti-Hbc IgG spontan serokonverted pozitif hastalar katılmıştır. Yerel etik kurulu onayı alınmştır. Kan örneklerinden genomik DNA izolasyonu sonucu HLA-DPB1 bölgesine spesifik primerler ile istenilen bölge polimeraz zincir reaksiyonu (PZR) ile çoğaltılmıştır. Bu çalışmanın ardından PZR-RFLP (Restriction Fragmen Length Polimorpism) metodu uygulanarak restriksiyon enzimi ile kesim gerçekleştirilir. Bu çalışmada 9 farklı restriksiyon endonukleaz kullanılmıştır: Bunlar, Bsp12861, FokI, DdeI, BsaJI, BssHII, Cfrl31, RsaI, EcoNI ve AvaII'dir. Restriksiyon enzim kesiminden sonra kesim bölgeleri incelenerek, allel tayini yapılmıştır.

Çalışmaya 94 kronik hepatit B hastası (Grup A) alınmıtır. Bu hastalar Göztepe EAH karaciğer hastalıkları polikliniğinde kayıtlı 6 aydan uzun süredir serum HBsAg (hepatit B yüzey antijeni) pozitif olan hastalardır. Hastaların ortalama yaşı 47,9±14,7 dir. Hastaların %61.7'si (n=58) erkek, %75,5 (n=71) HBeAg (hepatit B early antigen) negatiftir. Hastaların %57,4(n=54) 'e karaciğer biopsisi yapılmıştır, Biyopsi yapılan hastalarda HAI (hepatik aktivite skoru) 6,5 ±3,3 Fibroz skoru 2±1,5 dir. Hastaların %21,3'ünde (n=20) karaciğer sirozu mevcuttur, %83'ü(n=78) hepatit B'ye yönelik tedavi almaktadırlar. Ortalama serum ALT düzeyleri 102,1±113, AST 70±96 dir. Hastaların ortalama izlem süreleri 76 ±52 aydır.

Kontrol grubu olarak (Grup B) hepatit B geçirmiş, spontan HBsAg serokonversiyonu olmuş sağlıklı kişiler alınmıştır (HBsAg negatif, Anti HBs pozitif, Anti HBcIgG pozitif). Kontrol grubunda erkek oranı %54 yaş ortalaması 54±13.

Hastalıkla ilişkili olduğu düşünülen daha önce yapılmış çalışmalardan, Türk ve Alman populasyonlarından oluşan tarihsel kontrol (Grup C) olarak adlandırılan çalışma grubunu inceledik. Bu çalışma grubu Hepatit B hastalığıyla ilintili olan populasyonlarda HLA-DPB1 frekansını karşılaştırmak için kullanıldı.

Analizi yapılan 19 DPB1 alelinde, DPB1*15:01 aleli, kontrol grubunda hepatit B'ye sahip olan hastalara göre daha fazla gözlemlenmiştir. DPB1*02:01 ve DPB1*10:01 ise kontrol grubunda fazla görülen diğer alellerdir. Bu iki alel Bonferoni düzeltmesinden sonra önemini kaybetmiştir. Sadece DPB1*15:01 aleli $p<0,05$ den küçük olduğu için önemini sürdürmeye devam ettirmiştir. Türk populasyonunda Hardy Weinberg analizi yapılmadan DPB1*15:01 alelinin hepatit B hastalığına karşı koruyucu özelliğe sahip olduğunu söyleyebiliriz.

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1. INTRODUCTION

1.1 Purpose of the Thesis

Human Leukocyte antigens are highly polimorphic region (Chien *et al.*, 2010).

Our study, in this line, is concentrated on host immune response of chronic hepatitis B virus infection to analyze the association of human leukocyte antigen class II molecules (HLADPB1 Alleles) with the clinical outcome of chronic HBV infection in Turkish population**.**

In this line, our work aims to determine alterations in the HLA-DPB1 site of the genome of HBV infected Turkish patients and link these possible polymorphisms to disease associations in Turkish population.

Findings from this thesis, in general, aim to understand the relationship between immunopathogenesis of chronic hepatitis B infection and HLADPB1 Alleles.

1.2 Chronic Hepatitis B Disease

Hepatitis B virus (HBV) is a non-cytopathic hepatotropic viral pathogen (Chang *et al*.,2007). Also, Chronic Hepatitis B virus (HBV) infection is apotentially life-threatening liver infection worldwide, and approximately two billion people get infected with HBV (Huang *et al*., 2013). Additionally, more than 370 million people are chronic carriers of this virus that can lead to chronic hepatitis, hepatocellular carcinoma and liver cirrhosis (Ozaslan *et al.,* 2007; Chen*et al.,* 2013; Ciftci S *et al.,* 2012). Many epidemiological and molecular studies have shown thatHBV infection is one of the most significant infectious diseases throughout the world (Mauss *et al.,* 2013). Annualy, almost 1.2 million people die owing to the acute or chronic consequences of hepatitis B and HBV-related chronic liver diseases (Huang *et al*., 2013,Ishikawat *et al.,* 2012).

According to the World Health Organization (WHO), approximately 54 billion people are infected with the hepatitis B virus (HBV) and more than 240 million are

chronic hepatitis patients (Xu *et al.*, 2013). Recent studies have shown that nearly 3 million people have been infected with HBV in Turkey (Tozun *et al.*, 2012).

The progression rate from acute to chronic HBV infection reduces with age. For perinatally, the risk of Hepatitis B virus infection rate is nearly 90%, whereas for adults the risk of HBV infection rate is about 5% or even lower (Mauss *et al.*, 2013)

Viral recovery or persistence factors depend on age at infection, immunodeficiency states, gender and host genetic variation (Thomas *et al*., 2012).The natural history and clinical outcomes of chronic HBVinfection are determined by the viral replication cycle and the host immune response (Pramoolsinsup., 2002). Both viral factors and host immune response have been involved in the pathogenesis and clinical outcome of HBV infection (Baumert *et al*., 2007).

1.3 Genome Organization of Hepatitis B Virus

Hepatitis B virus (HBV), a member of hepadnaviruses is the smallest DNA virus whole the other animal viruses. The hepatitis B virus is classified as the type species of the *Orthohepadnavirus*. There are three different particule types of Hepatitis B virus; the infectious virions and the subviral particles (Tamori *et al.*, 2013) (Figure 1.1).

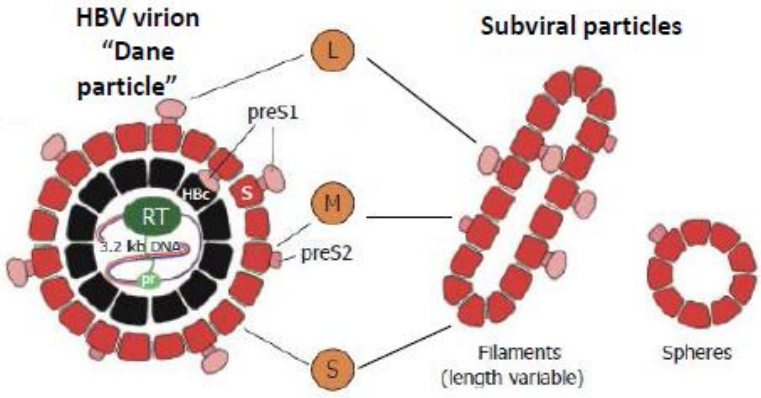


Figure 1.1 : Particules of Hepatitis B virus showing three different shapes (HBV virion and non-infectious empty subviral particles (filaments and spheres) (Mauss *et al.*, 2013)

The infectious virus particles are the so-called Dane particles, have a spherical, double shelled structure of 42-44 nm containing a single copy of the viral DNA genome. Also there are two additional types of particles, the spheres and the filaments,which are exclusively composed of hepatitis B surface proteins and host-

derived lipids (Tamori *et al.,* 2013). The subviral particles are non-infectious because they do not contain viral nucleic acids.

HBV includes a partially double stranded circular DNA of approximately 3200 base pairs for the genome organization. The small Hepatitis DNA virus contains a nucleocapsid that consists of HBsAg proteins. There are three various protein Hepatitis surface antigen; L, M and S. These are on the same sequencing. The surface proteins are named the preS1 (or large), the preS2 (or middle) and the S (or small), which correspond to the HBsAg. These surface antigens play a significant role in the identification of HBV infection (Baumert *et al.,* 2007).

The small genome encodes for four main genes by a series ofoverlapping reading frames (ORF) (Figure 1.2).

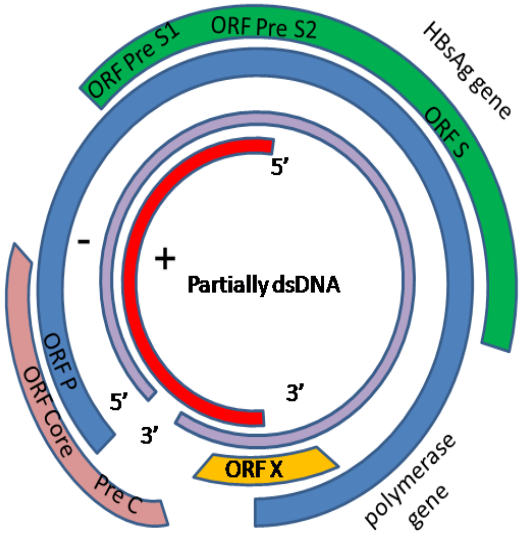


Figure 1.2: Genome Organization of Hepatitis B virus(adopted from, http://commons.wikimedia.org/wiki/File:HBV_genome.png)

The C gene is divided into a core and a precore region by 2 in-frame initiating AUG start codons. While the core region codes the structural protein of the nucleocapsid-HBcAg, the precore region codes the HBeAg. The function of nucleocapsid protein is to enclose the viral DNA and the DNA polymerase that is essential for HBV replication (Tan *et al.,* 2008; Shi *et al.,* 2009; Chang *et al*., 2007**)**

The S gene codes for the surface antigen (HBsAg) that encodes 3 polypeptides of the surface antigen (preS1, preS2 and S) produced from the alternative translation start sites (Figure 1.2). The most significant part of HBV genome among the four

ORFs (C, P, X and S genes), is the HBsAg gene, because the S protein product consists of an epitope that generates a protective immune response against the virus (Perillo *et al.*, 2009; Schaefer *et al.*, 2007; Chotiyaputta *et al.*, 2009)

The gene P also known as HBV polymerase is an enzyme that enables the synthesis of DNA using either RNA or DNA templates. Furthermore the gene P includes reverse transcriptase activityand its RNase H activity provides the degradation of the RNA strand of RNA-DNA hybrids, and the packaging of the RNA pregenome into nucleocapsids (Hepatitis B Fact sheet 2008; Perillo *et al.*, 2009).

The X gene is a transactivator of viral transcription and is thought to display a siginificant role in hepatocellular carcinoma (HCC) development. Also, its contribution to tumor invasion and metastasis in the course of HBV infection has not been established (Schaefer *et al.*, 2007; Coash *et al.*, 2009)

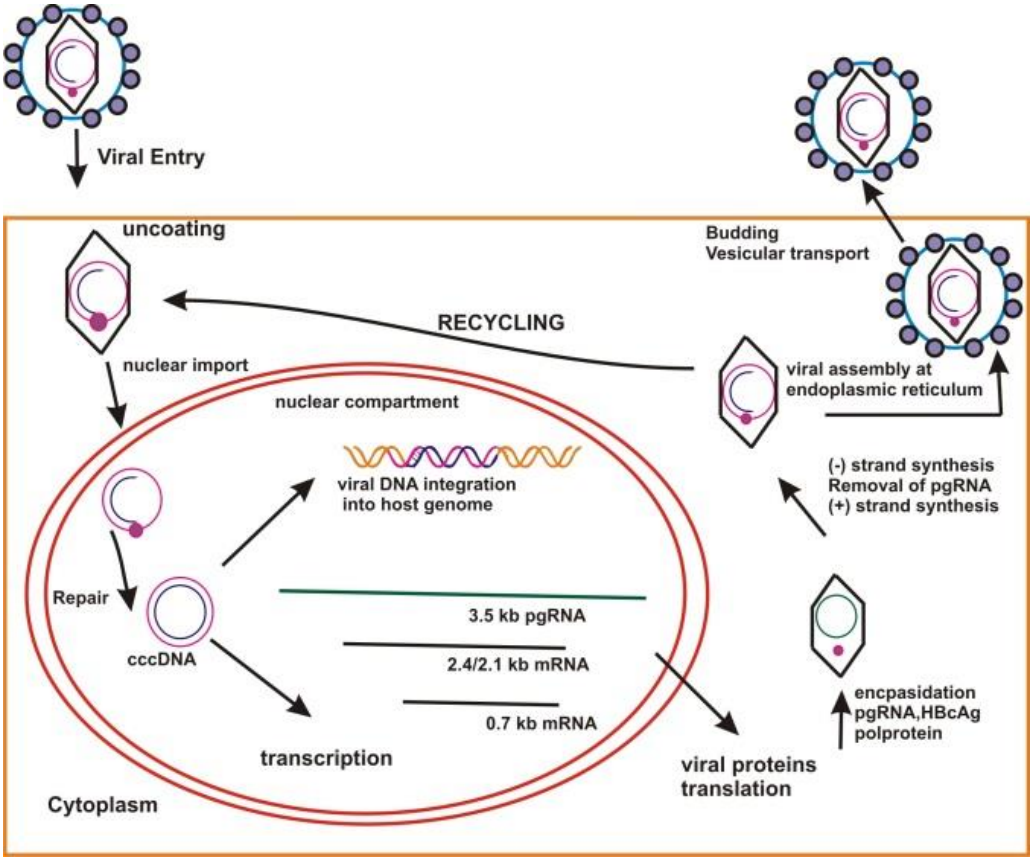


Figure 1.3 : Replication form of Hepatitis B virus (Jayalakshm *et al.*, n.d) Upon hepatocyte infection the nucleocapsid is released into nucleus where it is converted into the cccDNA. After transcription of the viral RNAs, the pgRNA is encapsidated. Through Golgi and ER, newly synthesized HBV DNA transported out of the cell.

Once the Hepatitis B virus invades the body, it binds to the cell surface and penetrates to the host cells with the help of its envelope proteins. The virus is transported to the nucleus fromthe plasma membrane of the cell. After that, the partially circular DNA is made into covalently closed circular DNA (cccDNA) to be used as a template for pregenomic RNA and mRNA. Even though, the minus strand of virus is almost a complete circle, the second strand, plus strand, is shorter in length. The minus strand encodes the structural proteins of the virus whereas the plus strand encodes some of the structural proteins and overlaps with most parts of the minus strand. Hepadnaviruses depends on reverse transcription of minus-strand DNA which is transcribed by the DNA polymerase from a "pregenomic" RNA intermediate. Viral proteins are translated by the mRNA, then the proteins and genome are packaged into virionsand transported out of the cell to infect new hepatocytes(Vierling *et al.*,2007; Bertoletti *et al.*, 2005; Das *et al.*, 2010;Özacar *et al*., 2008) (Figure 1.3).

1.3.1 Associations between HBV genotypes and mutations and their effect on clinical outcome

During viral replication due to the lack of proofreading activity of the viral polymerase, misincorporation of nucleotide mutations occur. This has led to the emergence of eight HBV genotypes and have been distinguished as A to H, according to overall nucleotide sequence variation. There are 9 HBsAg subtypes (4 major HBsAg subtypes, adw, adr, ayw and ayr) based on their antigenic determinants of the surface antigen and HBV genotypes.

Table 1.1 : Geographic distribution of HBV genotypes and the major serological subtypes (Tan *et al.*, 2008)

	HBsAg Subtype	Geographical Distribution
A	adw2, ayw1	USA, Northwestern Europe, Africa, India
B	adw2, ayw1	China, Indonesia, Taiwan, Japan
C	adw2,adrq+, adrq-, ayr, adr	East Asia, China, Korea, Japan, Vietnam, USA Australian,
D	ayw2, ayw3, ayw4	East Asia, China, Korea, Japan, Vietnam, USA Australian,
E	ayw4	West Africa
F	adw4q, adw2, ayw4	Central and South America, Polynesia,
G	adw2	Central America, France, USA, Germany
H	adw4	Central and South America

The HBV genotypes and serological subtypes have a distinct global geographical distribution (Tan *et al.,* 2008; Chang *et al.,*2007;Locarnini 2008) (Table 1.1).

All genotypes have the potential to infect individuals, leading chronic infection with various stages of progressive liver disease including cirrhosis and hepatocellular carcinoma as well as death. Disease progression and incidence of advanced liver diseases, affected by environmental, host and viral factors, may also be different according to each HBV genotype.

For instance, Genotype C and D are, generally, associated with more severe liver disease than B and A. Studies showed that HBV genotype A infected patients have a higher rate of clearance for HBsAg and HBV DNA as well as biochemical remission than either genotype D or F. HBV genotype C infected patients' biopsies showed more advanced fibrotic liver disease and a higher incidence of cirrhosis compared to genotype B pathogenic differences among HBV genotypes (Tan *et al.,* 2008).

Groups of mutations have been defined to reduce or block expression of HBeAg. According to the ORFs, gene mutations are summarized: C gene mutations may result in inability to produce HBeAg. HBeAg – negative variants are common for genotypes D as observed in Turkish population in the pre-core region. (Bertoletti *et al.,* 2005; Coash *et al.,* 2007; Chotiyaputta *et al.,* 2009).

S gene mutations in the HBV surface protein are responsible for the failure of immune prophylaxis in infants. Also these mutations enabled the escape from host immunity (Perillo *et al.,* 2009; Chotiyaputta *et al.,* 2009).

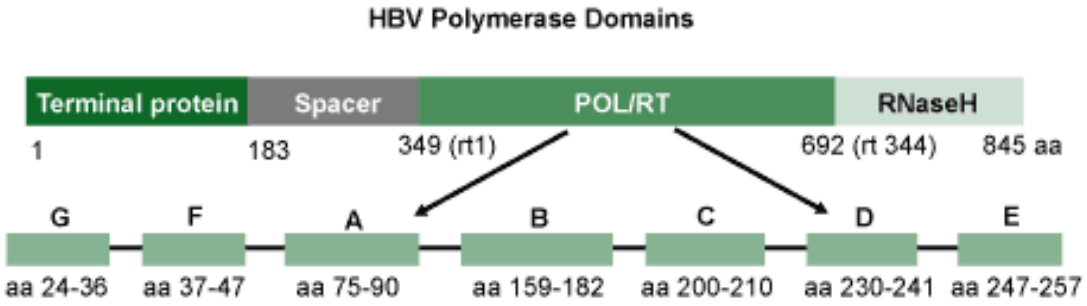


Figure: 1.4 : Characterized domains of HBV Polimerase (adopted from http://www.clinicaloptions.com)

The largest ORF in the HBV genome, which encodes for the hepatitis B polymerase protein (P gene), has four domains; a terminal protein (the N-terminus portion of the protein acts in priming (-) DNA strand synthesis and ends up covalently linked to the 5' end of the (-) DNA strand, and termed primase), a spacer region (no enzymatic function, just acts as a spacer between the first and third domains), a reverse transcriptase (encodes for the RNA and DNA dependent polymerase activity) and an RHase H (possesses its RNase H activity) domain (Figure 1.5) (Stchaefer *et al*., 2007).

Table 1.2 HBV mutations and their potential impact for HBV infection pathogenesis (Thomas *et al.*, 2007).

HBV region	Mutation	Molecular phenotype	Clinical relevance
Pre-S/S	Pre-S1 / Pre-S2/ S- Promoter	Misassembly	Fibrosing cholestatic hepatitis
	S	Alteration of B and T cell epitopes	Vaccine escape
	S splicing		Immune escape Diagnostic escape
Pre-C	Pre-C-stop	Loss of HBeAg	Severe hepatitis HBeAg- deficiency
Core	Core	Alteration of T-cell epitopes	Viral persistance Severe hepatitis
RT/Pol	Pol	Replication deficieny	Viral latency Viral persistence
	Pol	Resistance to antivirals	Therapy escape
Regulatory Elements	Core promoter	Enhanced replication and core expression	Severe hepatitis Modulation of drug resistance
		Decreased HBeAg Synthesis	HBeAg seronegativity
	Enhancer I	Decreased replication	Chronic hepatitis

X gene mutations can affect DNA repair, cell cycle control and apoptosis (Coash *et al.,* 2008) because X gene affects the control of transcription by acting as a transactivator of various viral and cellular promoter/enhancer factors. Also, mutations in this region can activate signal transduction pathways, but the clinical significance of HBx mutations are not clearly understood yet (Stchaefer *et al*., 2007; Coash *et al.,* 2008).

HBV mutations and their potential impact for HBV infection pathogenesis are summarized in Table 1.2. The naturally occurring mutations in the context of various genotypes have been identified in the structural and non-structural genes as well as regulatory elements in the virus. The best characterized mutants are the pre-core (pre-C) stop codon mutations resulting in loss of HBeAg, core promoter mutations resulting in enhanced viral replication, the mutations in the reverse transcriptase / polymerase genes resulting in resistance to antivirals, and mutations in the HBV surface gene resulting in alteration in the antigenicity of the viral surface proteins (HBsAg) and structure of the viral envelope (Thomas *et al.,* 2007).

1.4 Epidemiology of HBV

1.4.1 Transmission of HBV infection

Hepatitis B virus (HBV) is spread by contact with the infected blood, unprotected sexual activity, organ transplantation, horizontal, nosocomial injury and intravenous drug use.It can also be transmitted from mother to infant at birth and leads to acute or chronic necroinflammatory liver diseases (Mauss *et al.,* 2013; Ishikawat *et al*., 2012; Wang *et al*., 2010).

Transmission mode strongly correlates with geographic areas. For instance, Western Europe is a low prevalence areas, unprotected sexual intercourse and intravenous druguse are main cause disease for the transmission.Sub-Saharan Africa is a high prevalence area, perinatal infection is considered as the major route of transmission. In intermadiate prevalence areas, especially in early childhood, horizontal transmission is regarded the prevalent way ofdiffusion (Mauss *et al.,* 2013).

1.4.2 HBV infection worldwide

Annually, around one million people who suffer from HBV, die of HBV and related diseases. In different areas in the World, HBV has a wide range of prevalence from 0.1% up to 20%.Low prevalenceareas which is the range of 0.1-2% include Australia, United States, Canada, New Zealand and Western Europe; Mediterranean countries such as Japan, Central Asia, the Middle East and Latin and South America are the intermediate prevalence areas which are the rates of 3-5% and lastly high prevalence areas the rates of 10-20%, are mainly China, Southeast Asia and Sub-Saharan Africa (Mauss *et al.,* 2013). This diversity depends on the risk of chronicity and differences in age at infection.

This epidemiological difference is related with divergence in virtualperiodof infectious virus, primary site of viral infection and maturity of the immune system. In Asia, both host and viral factors can be effective for the initation of HBV infection (Ishikawat *et al.,* 2012;Mauss *et al.,* 2013).

1.5 Immunopathogenesis of Chronic Hepatitis B Virus Infection

Innate immunity involves nonspecific responses to pathogens. It is present before any exposure to pathogens and is effective from the time of birth (Campbell and Reece, 2005). Following HBV infection, inital stages of hepatitis may be symptomatic. The mechanism of HBV persistence is not fully understood. Succesful clearance and resolution of infection link to age and immune status of the individual. Both cellular and humoral immune responses are important for the chronic HBV infection (Campbell and Reece, 2005).

Following infection with HBV, Hepatocytes release IFN-α and IFN-β. Because hepatocytes have low expression of human leukocyte antigen (HLA) class I. IFN-α/βactivates the antigen presenting cells (APCs), especially, Kupffer cells (macrophages that reside in the liver) and dendritic cells (DCs). These APCs produce interleukin-18 (IL-18) and the chemokine CCL3which stimulates natural killer (NK) and natural killer T (NKT) cell activity (Figure 1.5) (Chang *et al.,* 2007). In HBV transgenic mice models, IFN-γ producing NKT cells can directly inhibit HBV replication. Recent studies have shown that NK cells, NKT cells and Kupffer cells play a significant role in the initial response against HBV (Glebe.,2007) (Chang *et al.,* 2007)

Adaptive immune response, involves a very specific response to pathogens. It is activated in many ways, such as through cytokines and involves the activity oflymphocytes. Humoral and cell-mediated immunity defend against different types of threats. Adaptive immunity includes two branches; the humoral immune response involves the activation and clonal selection of B cells, resulting in the production of secreted antibodies andthe cell-mediated immune response involves the activation and clonal selection of cytotoxic T cells. Both make signaling reactions (Campbell and Reece, 2005).

In patients with chronic HBV infection, the HBV-specific CD4+ and CD8+ T-cell response is decreased significantly.HBV-specific CD8+ T-cells are found in the liver where they may cause an inflammatory response because they have low responsiveness to produce IFN-γ especially in HBeAg-positive chronic carriers. They are almost undetectable. In some persistent HBV infection have known as partially tolerant (Chang *et al.,* 2007) (Glebe.,2007).

HBV-infected dendritic cells might cause CD4+ T-cell 'hyporesponsiveness'. Also, DCshave limited IFN-γ,TNF- αand IL-12 production (Chang *et al.,* 2007) (Glebe.,2007).

CD25+ CD4+ T-cells which are known regulatory T-cells, are also inhibit of proinflammatory responses to reduce immune modulated damage. They may limit clearance in other chronic viral infections by suppressing proliferation of virus-specific CD8+ T-cells (Glebe.,2007). This regulatory T-cell activity reduce production of antiviral cytokines such as IFN-γTNF- αin HBV. There is an increase or decrease in CD25+ CD4+ T-cells in peripheral blood as compared to uninfected healthy controls (Chang *et al.,* 2007).

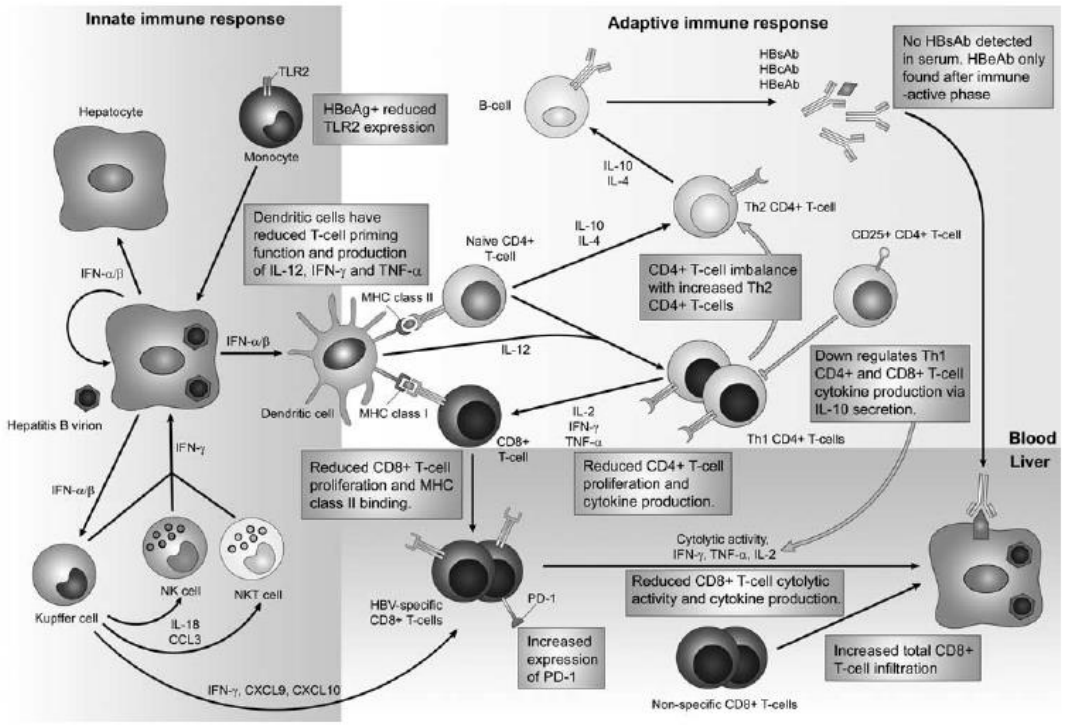


Figure 1.5 : Immunopathogenesis of Hepatitis B virus (taken from Chang *et al.*, 2007).

1.6 Treatment of HBV Infection

In generally, the aim of the treatment for chronic hepatitis B is to supress viral replication, eliminate the virus, prevent disease progression and improve patient survival (Pramoolsinsup *et al.*, 2002).

Although, many drugs have been effectively used in the treatment of chronic hepatitis B, interferon and lamivudine are the most widely used agents for the treatment of chronic hepatitis B disease (Pramoolsinsup *et al.*, 2002). Interferon (today pegylated form is used as apotent drug), which play as reverse transcriptase inhibitor of the HBV polymerase, has a direct antiviral effect. Lamivudine, a nucleoside analog, advanced HBV-induced liver disease is a potent inhibitor of HBV replication. The other nucleotide analogs adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF) are active on the priming of reverse transcription as well as on elongation of viral minus strand DNA. Entecavir (ETV) inhibits both minus and plus strand DNA synthesis. Telbivudine (LdT) inhibits all of these three enzyme activities (Chen *et al.*, 2013; Locarnini *et al.*, 2008). These medications perform

mainly by blocking reverse transcription of the pregenomic RNA to HBV DNA (Chotiyaputta *et al.*, 2009; Gerlich *et al.*, 2013).

1.7 Human Leukocyte Antigen (HLA)

The human leukocyte antigen (HLA) is the major histocompatibility complex (MHC) in humans that contains most significant region in the human genome with respect to inflammation, infection and autoimmunity (Kaufman *et al.*, 1999; Tamori *et al.*, 2013). Human leukocyte antigen (HLA) presents virus antigens to immune cells that is reliable for the clearance of virus-infected cells (Jiang *et al.*,2013). Genes in this complex fall into three groups: class I, class II, and class III where the HLA region located on the short arm of chromose 6 (6p21.1 to p21.3) (Figure 1.9) (Tamori *et al.*, 2013; Klein *et al.*, 2000). This region contains many other immune response genes and it is important in the regulation of the immune response (Taştan *et al.*, 2004; Godkin *et al*., 2005).

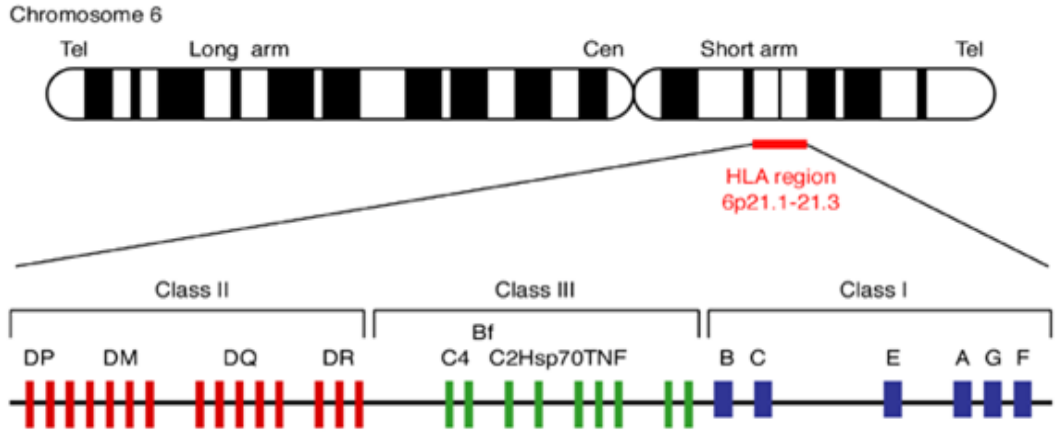


Figure 1.6 : Location and Organization of the HLA Complex on Chromosome 6 (Mehra *et al*, 2003)

The HLA class I known as HLA-A, HLA-B, and HLA-C code for the α polypeptide chain of the class I molecule (Klein *et al.*, 2000) (Figure 1.6). Class I genes are expressed by largely somatic cells (Klein *et al.*, 2000). There are 3 major MHC class II proteins encoded by the HLA known as class IIHLA-DR, HLA-DQ, and HLA-DP (Gebe*et al.*, 2002). These genes provide codes for making proteins that are present almost exclusively on the surface of certain immune system cells, specifically on antigen-presenting cells. Class II genes, specifically, are expressed in B cells, activated T cells, macrophages, dendritic cells, and thymic epithelial cells. Proteins

contain α and β chains, both of which are quite polymorphic (Toh *et al.,* 2000; Marsh *et al.,* 2010) (Figure 1.7). Each HLA subtype has a specific binding motif that dictates the specific range of peptides that can physically bind in a groove on the surface of HLA genes (Godkin *et al.,* 2005; Klein *et al.,* 2000). For instance, HLA class II genes are expressed as cell surface glycoproteins that bind and present peptide epitopes to CD4+ T cells, resulting in their activation.

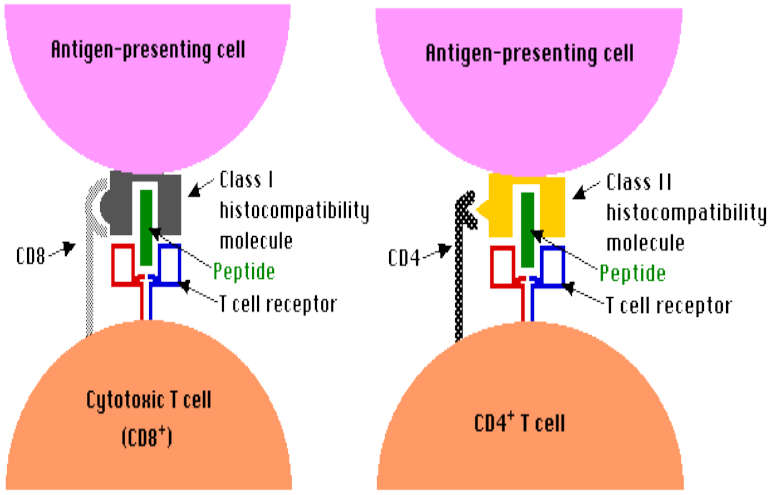


Figure 1.7 : Structure of HLA Class I and Class II Molecules (Adopted from, http://journals.cambridge.org/fulltext)

HLA loci on chromosome 6 involves three letters: the first (D) implies the class, the second (M, O, P, Q, or R) the family, and the third one (A or B) implies the chain (α or β, respectively). For instance, HLA-DPB represents class II genes of the P family coding for the β chains. Effective presentation of viral antigens to CD4+ T cells and CD8+ T cells by HLA class II and class I molecules respectively, is responsible against viral infection, and additional controls viral clearance or persistence (Jiang *et al.,* 2013; Singh *et al.,* 2007). Class III genes implicated in inflammation and other immune-system activities (Klein *et al.,* 2000).

Besides, HLA class I presents 15 peptides to the cytotoxic T cells with the receptors on the cytotoxic T cells, also binds inhibitory receptors on NK cells, hence stimulates immune responses against 'endogenous' antigens (Klein *et al.,* 2000). Whereas, HLA class II molecules stimulate the presentation of 'exogenous' antigens to T helper cells. Proteosomes degrade worn-out or defective proteins into peptides in the cytosol (Klein *et al.,* 2000).

HLA-DP is an HLA class II heterodimeric molecule expressed on antigen presenting cells (APC), including B cells, dendritic cells, and macrophages, where it functions by presenting extracellular antigens to CD4+T cells (Godkin *et al.,* 2005). Also HLA-DP contains alpha and beta chains which are encoded by the HLA-DPA1 and -DPB1 genes, respectively (Lok *et al.,* 2007; Vermehren *et al*., 2012).

In the HLA-DP region, there are two DPα and two DPβ genes, arranged in the order: DPβ2, DPα2, DPβ1, DPα1. The two pairs of α and β genes have their promoter ends adjacent: DPβ1 with DPαl; and a larger distance, DPβ2 and DPα2. The DPα2 and DP β2 genes are probably non-functional pseudogenes but in transfection experiments, DPαl and DPβ1 encode DP antigens and could function to present antigen to appropriate DP-restricted T cell clones (Kelly *et al.,* 1985).

Nomenclature of HLA alleles are updated as of July 2014 *(http://hla.alleles.org/)*(Table 1.3)

Table 1.3 : Nomenclature of HLA alleles*(http://hla.alleles.org/)*

Nomenclature	**Indicates**
HLA	the HLA gene region
HLA-DPB1	a particular HLA locus DPB1
*HLA-DPB1*15*	a group of alleles which encode the DP15 antigen or sequence homology to other DPB1*15 alleles
*HLA-DPB1*15:01*	a specific HLA allele
*HLA-DPB1*15:01:02*	an allele that differs by a synonymous mutation from *DRB1*15:01:01*
*HLA-DPB1*15:01:01:02*	an allele which contains a mutation outside the coding region from *DPB1*15:01:01:02*

Each HLA allele has a unique number and seperated by four sets of digits colons. First digits descripe the type (HLA) which related with serological antigen carried. The next set of digits are descriped to list the subtypes which DNA sequencing have been determined. If the allele numbers are different in two sets of digits, must be different at least one nucleotide substitutions. Alleles might be differ only by synonymous or non-coding substitutions that are distinguished by the use of the third set of digits. The fourth set digits are used that alleles only differ by sequence polimorphism both in the introns and in the 5’ or 3’ untranslated region (Table 1.3).

In addition to unique number of alleles, there are additional optional suffixes according to alleles expression status. If alleles have been shown that not to be expressed, suffix 'N' are described 'Null' alleles. These alleles may have the suffix 'L', 'S' and 'Q' (http://hla.alleles.org/).The suffix 'L' is used to 'Low' cell surface expression of allele's compared to normal levels. The 'S' suffix is used to denote an allele specifying a protein that is as a soluble 'secreted' molecule but it is not present on the cell surface. The lastly 'Q' suffix is used to be expression of an allele 'questionable' and affects normal levels (http://hla.alleles.org/).

1.8 Genetic Association Studies

Genetic association studies are used to find candidate genes or genome regions. Also these studies contribute a correlation between disease status and genetic variation for specific diseases. Associations with polymorphisms in candidate genes have been verified in many different diseases (Lewis C. *et al*., 2005). These studies are performed to determine whether a genetic variant is associated with a disease or trait: if association occurs, a particular allele, genotype or haplotype of a polymorphism may be shown more often than expected by chance in an individual. Hence, if a person carries one or two copies of a high-risk variant, he is at increased risk of developing the associated disease or having the associated trait. In association studies, SNPs are the most widely used test. Also, both genetic and environmental factors contribute to the susceptibility risk (Lewis C. *et al.*,2005).

Candidate gene studies generally require multiple SNPs. Significant genetic association can be interpreted as 1- direct association, (the genotyped for the disease susceptibility); 2-indirect association, in linkage disequilibrium (LD); 3- a false-positive result, (such as population stratification). Distinguishing between direct and indirect association may require the candidate region (Cathryn *et al.*,2012) (Lewis C.*et al.*,2005).

In population genetics, Linkage disequilibrium (LD) is used in the study for the non-random association of alleles at two or more loci. It may occur on the same chromosome or different chromosome than would be expected from a random formation of haplotypes from alleles based on their frequencies (Cathryn *et al.*,2012) (Lewis C. *et al.*,2005).

Linkage Disequilibrium depends on three study groups:Case–Control Study,in genetic case-control studies, genotypes or the frequency of alleles are compared between the cases and controls. With the case-control design, genotype and haplotype frequencies can vary between ethnic or geographic populations (Cathryn *et al.*,2012).

A difference in the frequency of an allele or genotype of the polymorphism may increase risk of the disease or be in linkage disequilibrium with a polymorphism. Haplotypes can also display association with a disease or trait. Family based association designs aim to avoid the potential confounding effects of population stratification. If an allele increases the risk of having a disease, in populations,that alleles can be transmitted from parent to offspring. A quantitative trait shows continuous variation, such as height or weight. Quantitative trait association can also be performed using an unrelated population sample or family (Cathryn *et al.*,2012).

Genome-wide association study (GWAS) using a inclusive approach for human genotyping showed single nucleotide polymorphisms (SNPs) that are located near HLA loci in chromosome 6p21 (Tamori *et al*., 2013;Hu *et al*., 2012). These are related with the outcomes of hepatitis virus infection (Mbarek *et al.,* 2011). Many SNP candidates associated with common diseases were detected by GWAS (Nishida *et al.,* 2012 Chan *et al.,* 2011). According to previous studies, GWAS determined candidate SNPs of the HLA loci that are related with chronic HBV or HCV infection, hepatic fibrosis and hepatocellular carcinoma(Mbarek *et al.,* 2011). Moreover, recent GWAS done on chronic hepatitis B infected patients revealed SNPs in HLA class II genes, especially HLA-DPA1 and HLA-DPB1 region (Gebe *et al.,* 2002; Tamori *et al*., 2013;Wong *et al.,* 2013).

Some studies show that genetic variants in the HLA-DP locus are effectively related with risk of persistent infection with hepatitis B virus (Gebe *et al.,* 2002). In Japannese populations, HLA analysis demonstrated that HLA-DPA1*0202-DPB1*0501 and HLA- DPA1*0202-DPB1*0301 were risk types for persistent HBV infection, while HLA-DPA1*0103, DPB1*0402 and HLA-DPA1*0103, DPB1*0401 were protective alleles for HBV infection in Japanese population.

Genome association analysis demonstrated that HLA loci are one of the most significant host determinants of the clinical characteristics of HBV and HCV infections (Wong *et al.,* 2013; Kim *et al.,* 2013).

Hardy Weinberg Equilibrium calculates the genotype frequencies from allele frequencies. This test supplies a comparison of the expected and observed genotype frequencies.For instance, for alleles G and T, the frequency of allele G is p and the frequency of allele T is q = (1 – p), the expected frequencies of genotypes GG, GT, and TT are p2, 2pq, and q2 (Cathryn *et al.*,2012).

1.9 Hypothesis

Recent genetic association studiesrevealed that HBV infection related diseases are associated with the variations observed in the HLA-DP region of the genome. Our study investigates the presence of possible variations in the HLA-DP region in Turkish population that were infected by HBV. We think that our study could determine alterations in the HLA-DPB1 site of the genome of HBV infected Turkish patients and link these possible polymorphisms to disease outcome in Turkish population.

2. MATERIAL AND METHODS

2.1. Materials and Laboratory Equipments

2.1.1 Used equipments

The laboratory equipment used for this project was listed in Appendix A.

2.1.2 Used chemicals, enzymes, markers and buffers

The chemicals, markers and enzymes used were given in Appendix B together with their suppliers.The preparation and compositions of buffers and solutions were shown in Appendix C.

2.2 Selection criteria of the chronic hepatitis B patients and control group

A total of 94 unrelated Turkish subjects were used in this study, from Department of Gastroenterology, Goztepe Teaching and Research Hospital. A case group of asymptomatic HBV carriers were recruited according to the results of serological tests (liver functional indexes, HBV virological indexes) and symptoms of hepatitis B. Chronic hepatitis B was diagnosed if serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were continuously abnormal, with HBsAg and/or HBeAg seropositive, anti-HBs antibodies (anti-HBs) seronegative. Asymptomatic HBV carriers wereidentified with the following diagnostic criteria: HBsAg seropositive last for more than 1 year, with normal liver functional indexes and without any signs and symptoms of hepatitis and HBV DNA levels were below 2000 IU.

The control group was selected from the healthy people of the Goztepe Teaching and Research Hospital. The ages of the controls change between 38 and 49. The members of the control group have no known history of any chronic inflammatory diseases, free from acute or chronic hepatitis B virus infection at the time of the study and their functional capacity was sufficient.

2.3 Collection and Storage of Blood Samples

The peripheral blood samples were collected from the patients of Department of Gastroenterology, Goztepe Teaching and Research Hospital and chosen as respect to the chronic hepatitis B criteria defined previously. The blood samples were collected in vacuum tubes with EDTA. The samples were stored at −80 C.

2.4 DNA Isolation from Human Whole Blood

Genomic DNA (gDNA) isolation was done by using Invıtrogen PureLink Genomic DNA Purification Kit (Invitrogen). For each isolation process approximately 1 mL of peripheral blood sample was used. The isolated stock DNA was stored at -20 °C.

2.5 DNA Amount, Purity and Working Solution Calculations

The concentration and purity of the isolated DNA was calculated based on the absorbance values measured at 260, 280 and 320 nm. of the spectrometer. The concentration of the DNA was calculated by equation 2.1 and the purity of the DNA samples were calculated by equation 2.2 given below.

DNA Concentration (ng / L) (A260- A320)X 50X Dilution Factor 2.1

DNA Purity= A260-A320/ A280-A320 2.2

In order to have a set of DNA samples containing same amount of DNA (50 ng/μL), dilutions from the stock DNA were prepared (working solutions). The required amount of stock DNA solutions was calculated for 500 μL of 50 ng/μL working solutions (approximately 25 μg DNA).

2.6 Polymerase Chain Reaction (PCR) Amplification

Isolated genomic DNAs of the chronic hepatitis B group were used for PCR reaction. Primers sequences and PCR product sizes are listed in Table 2.1.

2.6.1 Oligonucleotid primers

The oligonucleotide primers used in this study are given in the Table 2.1 below. The confirmation of the actually binding sites, binding efficiencies and amplicon sizes was made by Amplify 3X software.

As it was also essential to analyze the primer sets for dimer formation, the hairpin, heterodimer and self dimer analysis of the primer set were done and confirmed with the SciTools on the IDT DNA website (OligoAnalyzer 3.0). PCR Product size 299 bp.

Table 2.1 : List of primersused in the study

Gene	Sequencing (5’ to 3’)		PCR Product
DPB1 primers	5’primer	GTGAAGCTTTCCCCGCAGAGAATTAC	299
	3’primer	ACCTGCAGTCACTCACCTCGGCGCTG	

2.6.2 PCR conditions

The PCR cycle conditions are varied according to the desired amplicon. In this study, PCR cycle conditions are given in the Table 2.2 below.

Table 2.2: PCR conditions

1 X	1 X
DNA	2.5 µl
10XGreen Buffer	5 µl
MgCl2	2 µl
dNTP	4 µl
F-Primer	1 µl
R-Primer	1 µl
TagPolymerase	0.25 µl
dH2O	34.25 µl
Total reaction	50 µl

2.6.3 PCR optimization

Generally, PCR reactions were required optimization for higher performance related with the amplification of the correct target and determination of appropriate annealing temperature for primer set. In this thesis, PCR conditions were optimized via gradient PCR methods.

2.6.4 Gradient PCR

It is necessary to use gradient PCR to determine the optimum annealing temperature for a PCR reaction when appropriate annealing temperature is not known for a

primer set. In gradient PCR, the thermo cycler is set in such a way that the wells in the thermo cycler block have a gradient of temperature from high to low for the annealing phase of the cycles.Hence, gradient PCR allows the run of a set of identical PCR in the same block with different annealing temperatures, which saves time and prevents multiple usage of the thermo cycler for the same optimization. After analyzing the PCR products with agarose gel electrophoresis, annealing temperature of the PCR mixture giving the best band intensity waschosen as the optimum annealing temperature. In this thesis, the optimization of the annealing temperature of PCR was made by gradient PCR by forming a gradient from 58 to 65°C and the best band intensity was seen at 60°C, therefore this temperature was used as the annealing temperature of HLA alleles PCR for the rest of the studies.

2.6.5 PCR cycle conditions of HLA DPB1alleles

The PCR conditions for the HLA DPB1gene region is shown in the Table 2.3

Table 2.3 :PCR cycle conditions for the HLA DPB1gene region

Repeat Number	Degree	Time	Phase
1	94 °C	3 minute	Initial denaturation
38	94 °C	30 seconds	Denaturation
	60°C	30 seconds	Annealing
	72°C	50 second	Extension
1	72°C	7 minute	Final Extension

2.6.6 Agarose gel electrophoresis of PCR products

The right percentage of an agarose gel is beneficial for observing the PCR bands accurately. In this thesis, the PCR products of amplicon sizes can be seen on a 2%

agarose gel. PCR products is observed either on 2% mini gels prepared with 2.0 g agarose into 100 mL and maxi gels prepared with 2.4g agarose into 120 mL of 1X TAE buffer, which is diluted from 50X stock TAE. 5 µL of PCR product is mixed with 1 µL SyberGreen in order to load into the wells. Lengths of the PCR products are calculated according to O‟RangeGeneRuler Low Range marker (Fermentas) and O‟RangeGeneRuler Ultra Low Range marker (Fermentas). The gels are run in 1X TAE buffer, at 100V with power supplier, for at least 30 minutes. The observations of the gels under UV light are made by transilluminator and UV PhotoMW software is used in order to take the photos of the gels.

2.7 PCR-RFLP (Restriction Fragment Length Polymorphism) Method

The study depends on PCR-RFLP method using some restriction enzymes which have either a single cleavage site or, alternatively no cleavage site in the amplified DNA region, based on the HLA alleles, making reading of RFLP band patterns much easier. 9 different restriction endonucleases, Bsp12861, FokI, DdeI, BsaJI, BssHII, Cfrl31, RsaI, EcoNI and AvaII, were selected for digestion to detect allele- specific cleavage after PCR-amplification of the DPBl alleles. Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by 9 different restriction endonucleases DPB1 alleles are shown in the Table 2.5 below and after the PCR amplification RFLP reaction are given in the Table 2.6 below.

Table 2.4: Reaction Conditions of Restriction Endonucleases

Restriction Endonucleases	Degree	Incubation Time	Inactivation Degree	Inactivation Time
Bsp1286I	37°C	10h	60°C	20m
FokI		1h		
AvaII	37°C	10h		
DdeI				
BssHII				
Cfr13I				
RsaI				
EcoNI				
BsaJI	55°C	10h	80°C	20m

Table 2.5: After the PCR amplification, RFLP reaction

Add	**Amount of 1X**
PCR Product	12µl
Nuclease Free Water	6 µl
10X Buffer	2 µl
Enzymes	0.4µl
Total	20µl

2.8 Agarose Gel Electrophoresis of RFLP Analyzes

In this study, the RFLP analyzes after the PCR amplification can be seen on a different percentage of agarose in the agarose gel. RFLP analyzes is observed either on 2% mini gels prepared with 2.0g agarose into 100 mL of 1X TAE buffer, which is diluted from 50X stock TAE. 10 µL of PCR product is mixed with 1 µL 6X loading dye and with 1 µL SyberGreen in order to load into the wells.Differentpercentage of agoroseaccording to restriction endonucleases were given in theTable 2.8. Lengths of the RFLP Products were calculated according to O‟Range GeneRuler LowRange marker (Fermentas) and O‟Range GeneRuler Ultra Low Range marker (Fermentas). The gels were run in 1X TAE buffer, at 90V with power supplier, for at least 40 minutes. The observations of the gels under UV light were made bytransilluminator and UV PhotoMW software was used to take the photos of the gels.

Table 2.6 : DifferentPercentage of AgoroseGel according to Restriction Endonucleases

Restriction Enzymes	Bsp1286I	FokI	DdeI	BsaJI	BssHII	Cfr13I	RsaI	EcoNI	AvaII
% Agarose Gel	%2	%2	%2	%4	%2	%2	%4	%4	%2

2.9 Genotyping

The genotyping was done by analyzing the agarose gel photos of PCR-RFLP products for the all HLA DPB1 alleles observed in patients, respectively. O‟Range GeneRuler LowRange (Fermentas) or O‟Range GeneRuler Ultra LowRange (Fermentas) markers were also loaded to decide the band sizes along with positive

controls. Indeed, the allelic type was determined according to the presence or absence of the desired length PCR products**.**

2.10 Statistical Analysis

In this study, to analyze the association of HLA DPB1 alleles in patients with chronic hepatitis B infection, statistical analyses were performed. A 2 × 2 contingency table was used to compare allelic frequencies according to clinical statues of the patients. All statistical analyses were performed using the SPSS program, version 21 (SPSS, Chiacago, IL). Analysis of categorical data and HLA alleles, where 2 × 2 contingency table was used, statistically were performed by $\chi 2$ test. In case of expecting any data bellow in any cell in 2 × 2 contingency table, Fisher's exact test was used. Additionally, we used Hard Weinberg analysis using HWE calculator. In all analysisone-sided p was taken into account and if $p<0.05$ it was considered as significant.

3. RESULTS

3.1 Demographic Data of the Chronic Hepatitis B Group

In this study, a total of 94 hepatitis B patients (group A) were included from Department of Gastroenterology, Hepatology Clinic, Goztepe Teaching and Research Hospital.Age and gender matched 85 HBsAg negative, anti-HBs positive and anti–HBc IgG positive spontaneous seroconverted healthy subjects (group B) were also included in the study.

Group A, the patient group,consists of 58 male (61.7%) and 36 (38.3%) female patients with a mean age 47.9±14.7. Among these patients 71 (75%) of them were HBeAg negative, 16 (17%) had inactive disease. The pretreatment mean ALT,AST and log DNA were 102.1±113 U/L, 70±96 U/L and 4.9±2.8 IU/ml, respectively. Among them 54 (57.4%) had liver biopsy; the mean Ishak fibrosis score was 2±1.5 and hepatic activity index was 6.5±3.3. Twenty (21.3%) patients had cirrhosis.

Table 3.1: Demographic data of the sample population

Patient Group (Group A)			n (%)
Sex	*Male*		58 (61.7)
	Female		36 (38.3)
HBeAg		*Negative*	23 (25)
		Positive	71 (75)
Cirrhosis		*yes*	20 (21.3)
		no	74 (78.7)
Control Group (Group B)			n (%)
Sex	*Male*		46 (54,1)
	Female		39 (45,9)
HBsAg		*Negative*	85 (100)
		Positive	0 (0)
Anti HBsAg		*Negative*	0 (0)
		Positive	85 (100)
Anti HBc IgG		*Negative*	0 (0)
		Positive	85 (100)

The mean follow up time for the patients was 76±52 months.

Control group was constructed (Group B) from healthy people who had already hepatitis B infection with spontaneous HBsAg seroconversion (HBsAg negative, Anti HBs positive, Anti HBc IgG positive). Group B has 54% male, 46% female individuals and the age average of this group was 54±13 (Table 3.1).

We also used historical controls as Group C by taking disease related information directly from the published literature, which includes Turkish and German populations. Group C is used to compare HLA-DPB1 frequencies from general population with the disease population of this study.

3.2 DNA Isolation Results

In this thesis, DNA isolation was done according to the procedure provided by Invitrogen PureLink Genomic DNA Purification Kit.

3.3 Genotype Analysis

3.3.1 PCR results

In this study, we did PCR reactions to amplify the target HLA DPB1 DNA sequence. Selected primers (Table 3.2) are used in the PCR reaction, and the resulting PCR product has a mass corresponding to 299 bp (Ota *et al.*, 1991).

Table 3.2 : Selected primers from the HLA-DPB1 gene region

GGGTGGGAAGATTTGGGAAGAATCGTTAATATTGAGAGAGAGAGGGAGAAAGAGGATTAGATGAGAGTGGCGCCTCCGCTCATGT
CCGCCCCCTCCCCCGCAGAGAATTACCTTTTCCAGGGACGGCAGGAATGCTACGCGTTTAATGGGACACAGCGCTTCCTGGAGAGA
TACATCTACAACCGGGAGGAGTTCGCGCGCTTCGACAGCGACGTGGGGGAGTTCCGGGCGGTGACGGAGCTGGGGCGGCCTGCTG
CGGAGTACTGGAACAGCCAGAAGGACATCCTGGAGGAGAAGCGGGCAGTGCCGGACAGGATGTGCAGACACAACTACGAGCTGGG
CGGGCCCATGACCCTGCAGCGCCGAGGTGAGTGAGGGCTTTGGGCCGGCGGTCCCAGGGCAGCCCCGCGGGCCCGTGCCCAGGGC
GCAGGAGCAGCCGGGTTGGCCTAAGGGACCTTAGTGCCGGGCGGAAAGGGGACTTTGGGTTGGGGATTCATGGGGGGAGCCCATC
TGGAGCTTGTCAGGGGAGCGAGCGCG

The genotyping was done by comparing the bands on the agarose gel. An example of a PCR gel isshown in Figure 3.1.

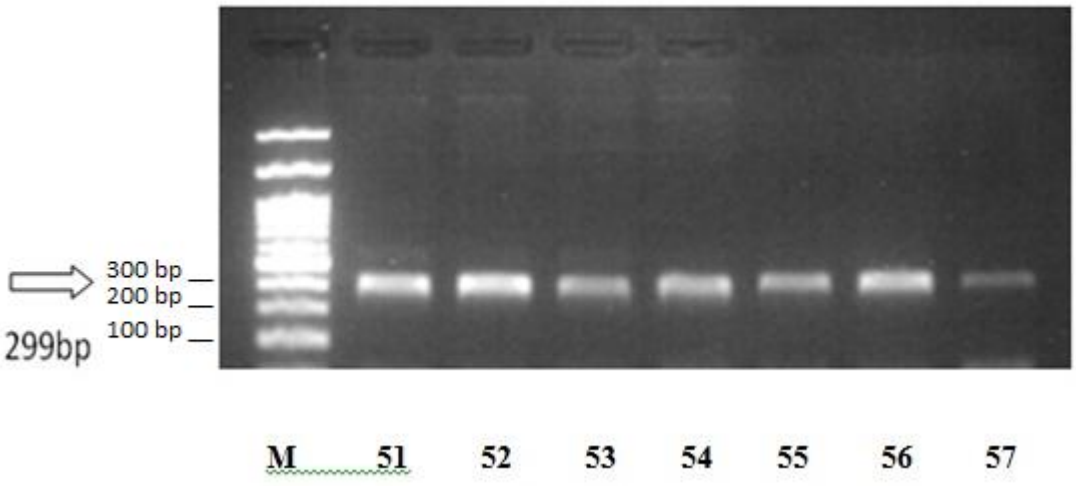


Figure 3.1 : Agarose gel electrophoresis analysis on 2.0% agarose gel for the target DNA sequences, HLA-DPB1gene, from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder), from patient groups, 51–57 lines show each number of amplified PCR reaction. The number on the left indicates the molecular size (in base pairs) of the amplified PCR products which are 299 bp.

3.3.2 PCR-RFLP results

Our study is based on a PCR-RFLP method using restriction enzymes to genotype HLA-DPB1 alleles from HBV infected patients and HBV infected healthy subjects. These restriction endonucleases have shown us that homozygotes and heterozygotes can be unequivocally discriminated in the amplified DNA region. DPB1 gene was selectively amplified from genomic DNAs of 94patients and 85 healthy Turkish people by PCR. Amplified DNAs were digested with restriction endonucleasesand then subjected to electrophoresis, assaying simply forcutting, or no cutting, of the DNAalleles of the DPBl gene. After the PCR amplification, 9 restriction enzymatic cut patterns were analyzed on agarose gel electrophoresis. These restriction endonucleases are Bsp12861, FokI, DdeI, BsaJI, BssHII, Cfrl31, RsaI, EcoNI, and AvaII enzymes (Ota *et al.,* 1991).

This modified PCR-RFLP method can be successfully applied to heterozygotes (Ota *et al.,* 1991). Examples of the PCR-RFLP results are shown in Figures3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 3.10. The rest of the PCR-RFLP results are shown in Appendix D.

In this study, 299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

Each restiriction enzyme cleavage results is shown seperately:

3.3.2.1 FokI restriction enzyme

19alleles of the DPBl gene can be discriminated with 9 different restriction enzymes. Table 3.3 shows the cleavage fragments of FokI restriction enzyme with 19 DPB1 alleles depending on the base pairs after the agarose gel electrophoresis.

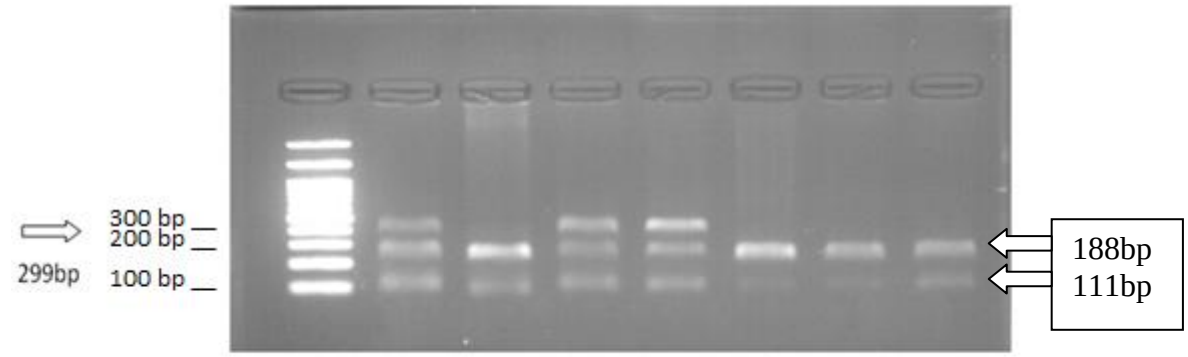
Table 3.3 : Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by FokI restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
FokI	186 57 56	183 59 57	183 59 57	183 59 57	183 59 57	183 116	183 59 57	299	242 57	242 57
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
FokI	183 116	183 116	183 116	183 116	183 116	299	242 57	183 59 57	183 59 57	

FokI restriction endonuclease's recognition sites are indicated as below:

5'...G G A T G $(N)_9$↓...3'
3'...C C T A C $(N)_{13}$↑...5'

Our RFLP experiments are shown in Figure 3.2 for the FokI restriction enzyme. This is a representative figure for the digest experiment. In Figure 3.2, the presence of heterozygotes are shown in lanes corresponding to 81, 83 and 84 due to the presence of both cleaved fragments and the uncleaved form of the PCR product. The rest of the lanes have cleavage patterns suggesting the presence of only homozygotes (Figure 3.2). The number on the left indicates the molecular size (in base pairs) of the amplified PCR products. The number on the right indicates the molecular size (in base pairs) of FokI enzyme's cleavage patterns.



Patient numbers	M	S1	S2	S3	S4	S5	S6	S7
Numerical representation of allelic distribution		0/1	1	0/1	0/1	1	1	1

Figure 3.2: Agarose gel electrophoresis analysis on 2.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: M, size marker DNA (100 bp DNA ladder); from patient groups, 81–87 lines show each number of amplified by Fok I restriction enzyme). Cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.2 AvaII restriction enzyme

Cleavage sites depending on the base pairs after the agarose gel electrophoresis for AvaII restriction endonuclease are shown in Table 3.4 with 19 HLA-DPB1 alleles.

Table 3.4: Fragments (bp) detected by digestion of the PCR-amplified DPBl genes byAvaII restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
AvaII	299	299	299	299	299	299	299	194 105	194 105	194 105
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
AvaII	299	299	299	299	299	194 105	194 105	299	299	

AvaII restriction endonuclease's recognition sites are indicated as below:

5'...G↓G W C C...3'
3'...C C W G↑G...5'

Our RFLP experiments are shown in Figure 3.3 for the AvaII restriction enzyme. In Figure 3.2 the presence of heterozygote is shown in lane corresponding to 91 due to the presence of both cleaved fragments and the uncleaved form of the PCR product.

The rest of the lanes have cleavage patterns suggesting the presence of only homozygotes (Figure 3.3). The number on the left indicates the molecular size (in base pairs) of the amplified PCR products. The number on the right indicates the molecular size (in base pairs) of AvaII enzyme's cleavage patterns.

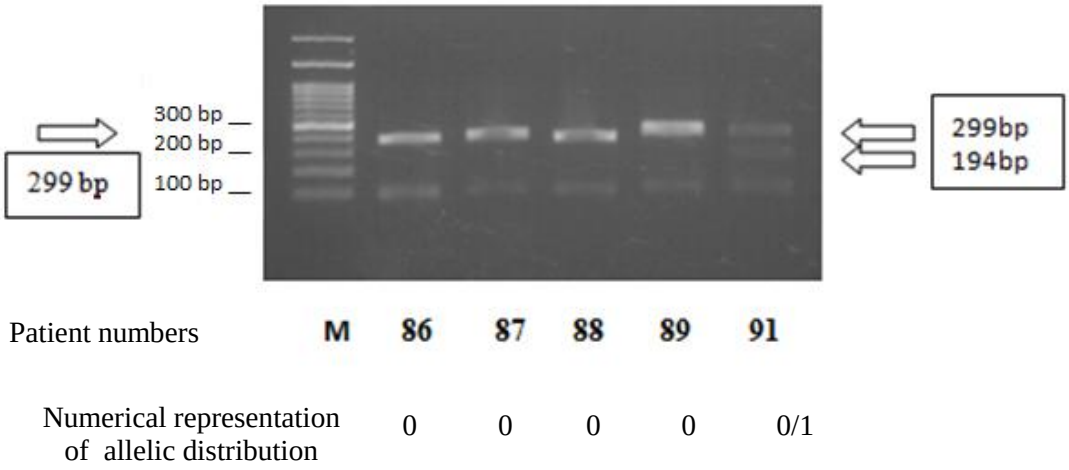


Figure 3.3: Agarose gel electrophoresis analysis on 2.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: M, size marker DNA (100 bp DNA ladder); from patient groups, 86–91 lines show each number of amplified by Ava II restriction enzyme. 299 base pair non-cleaved fragments were indicated as "0, showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.3 BsaJI restriction enzyme

Table 3.5 shows the cleavage fragments of BsaJI restriction enzyme with 19 HLA-DPB1 alleles after the agarose gel electrophoresis.

Table 3.5 : Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by BsaJI restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
BsaJI	246 32 21	246 32 21	246 32 21	246 32 21	246 32 21	246 32 21	246 32 21	278 21	278 21	278 21
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
BsaJI	246 32 21	278 21	278 21	278 21	246 32 21	278 21	246 32 21	246 32 21	278 21	

BsaJ restriction endonuclease's recognition sites are indicated as below:

5'...C↓C N N G G...3'
3'...G G N N C↑C...5'

Our RFLP experiments are shown in Figure 3.4 for the BsaJI restriction enzyme. This is a representative figure for the digest experiment. In Figure 3.4, the presence of heterozygotes are shown in lanes corresponding to 145, 146 and 147 due to the presence of both cleaved fragments and the uncleaved form of the PCR product. The rest of the lanes have cleavage patterns suggesting the presence of homozygotes (Figure 3.2). The number on the left indicates the molecular size (in base pairs) of the amplified PCR products. The number on the right indicates the molecular size (in base pairs) of BsaJI enzyme's cleavage patterns.

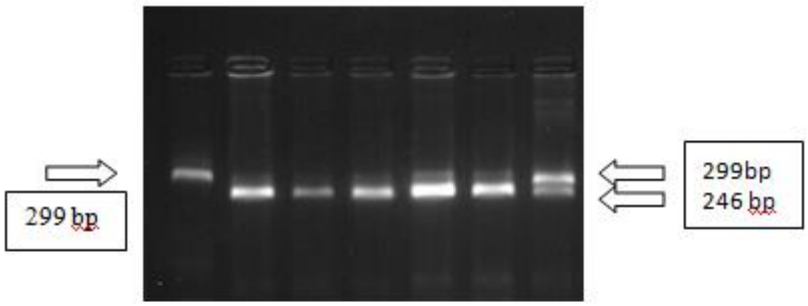


Figure 3.4: Agarose gel electrophoresis analysis on 4.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: N, Negative control;from control groups, 142–147 lines show each number of amplified by BsaJI restriction enzyme.Cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.4 Cfr13I restriction enzyme

Cleavage sites depending on the base pairs after the agarose gel electrophoresis for Cfr13I restriction endonuclease are shown in Table 3.6 with 19 HLA-DPB1 alleles.

Table 3.6 : Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by Cfr13I restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
Cfr13l	258 40	258 40	258 40	258 40	258 40	299	299	194 105	194 105	194 105
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
Cfr13l	299	299	299	299	299	194 105	194 64 40	299	299	

BsaJ restriction endonuclease's recognition sites are indicated as below:

5'...G↓G N C C...3'
3'...C C N G↑G...5'

Our RFLP experiments are shown in Figure 3.5 for the Cfr13I restriction enzyme. This is a representative figure for the digest experiment. In Figure 3.5, the presence of heterozygotes are shown in lanes corresponding to 143, 144, 36 and 64 due to the presence of both cleaved fragments and the uncleaved form of the PCR product. In lane 142 has cleavage patterns suggesting the presence of only homozygote (Figure 3.5). The number on the left indicates the molecular size (in base pairs) of the amplified PCR products. The number on the rightindicates the molecular size (in base pairs) of Cfr13I enzyme's cleavage patterns.

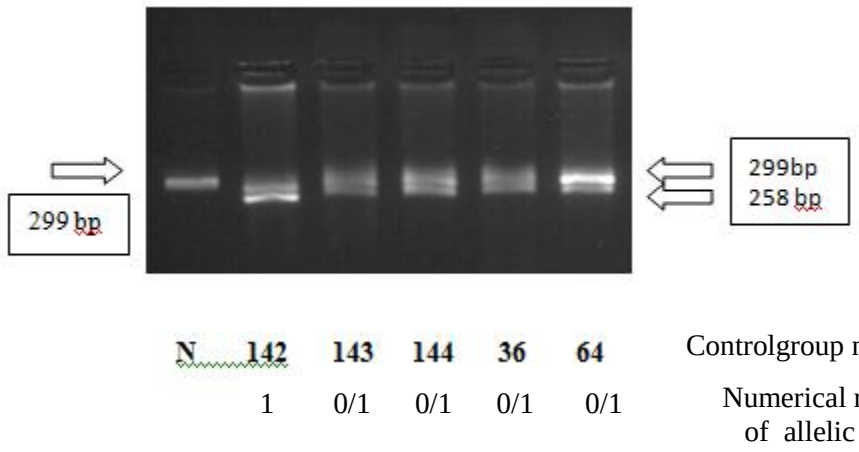


Figure 3.5: Agarose gel electrophoresis analysis on 2.0% agarose gel of RFLP-PCR methods from blood samples. Lanes N, Negative control;from control groups, 142–64 lines show each number of amplified by Cfr13I restriction enzyme. Cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.5 Bsp1286I restriction enzyme

Table 3.7 shows the cleavage fragments of Bsp1286I restriction enzyme with 19 HLA-DPB1 alleles after the agarose gel electrophoresis.

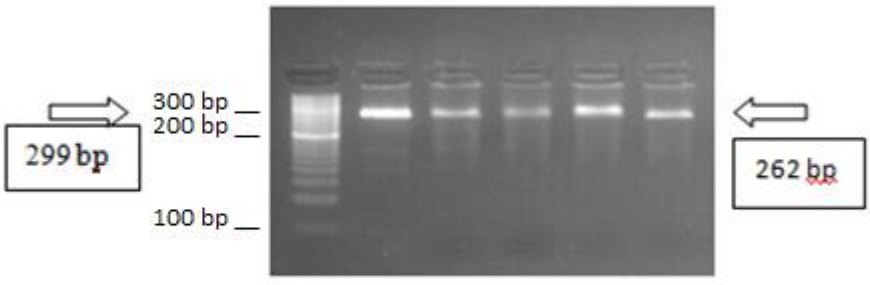
Table 3.7: Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by Bsp1286I restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
Bspl2861	262 37	262 37	262,37	262 37	153 109 37	299	190 109	299	299	299
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
Bspl2861	299	268 31	268 31	299	299	268 31	262 37	299	268 31	

Bsp1286I restriction endonuclease's recognition sites are indicated as below:

5'...G D G C H↓C...3'
3'...C↑H C G D G...5'

Our RFLP experiments are shown in Figure 3.6 for the Bsp1286I restriction enzyme. It can be observed from the figure that in lanes 27,28 and 29, there are no digestions as observed from the single band. Only the last lane 36, there is a digestion pattern suggesting the presence of homozygote. The number on the left indicates the molecular size (in base pairs) of the amplified PCR products. The number on the rightshows the molecular size (in base pairs) of Bsp1286I enzyme's cleavage patterns.



M	N	27	28	29	36	Control group numbers
		0	0	0	1	Numerical representation of allelic distribution

Figure 3.6 : Agarose gel electrophoresis analysis on 2.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: M, size marker DNA (100 bp DNA ladder); N, Negative control ;from control groups, 27–36 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes.

3.3.2.6 DdeI restriction enzyme

Cleavage sites depending on the base pairs after the agarose gel electrophoresis for DdeI restriction endonuclease with 19 HLA-DPB1 alleles are shown in Table 3.8

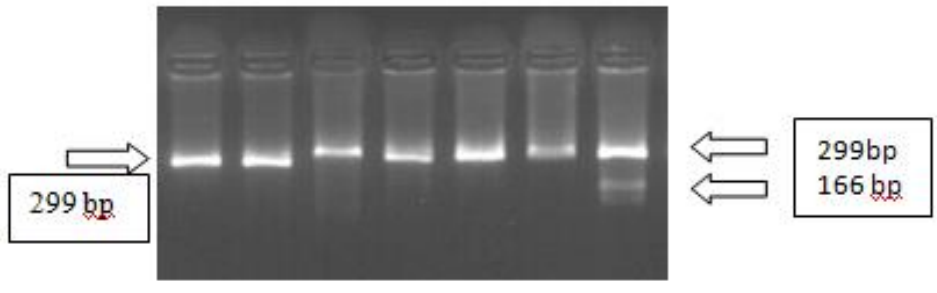
Table 3.8: Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by DdeI restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
Ddel	299	299	299	299	166 133	299	166 133	299	299	299
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
Ddel	299	299	299	299	166 133	299	299	299	299	

DdeI restriction endonuclease's recognition sites are indicated as below:

5'...C↓T N A G...3'
3'...G A N T↑C...5'

Our RFLP experiments are shown in Figure 3.7 for the DdeI restriction enzyme. In Figure 3.7, the presence of heterozygote is shown in lane corresponding to 143 due to the presence of both cleaved fragments and the uncleaved form of the PCR product. It can be observed from the figure that in lanes 138 and the rest until 143, there are no digestions as observed from the single band suggesting the presence of only homozygotes (Figure 3.7) The number on the left shows the molecular size (in base pairs) of the amplified PCR products. The number on the rightindicates the molecular size (in base pairs) of Ddel enzyme's cleavage patterns. In general all our patients revealed non-digestion patterns for the DdeI restriction enyme.



N	138	139	140	141	142	143	Patient numbers
	0	0	0	0	0	0/1	Numerical representation of allelic distribution

Figure 3.7 : Agarose gel electrophoresis analysis on 2.0% agarose gel of RFLP-PCR methods from blood samples. Lanes N, Negative control;from control groups, 138–143 lines show each number of amplified by Ddel restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.7 BssHII restriction enzyme

The cleavage fragments of BssHII restriction enzyme with 19 HLA-DPB1 alleles are shown in Table 3.9Cleavage sites depending on the base pairs after the agarose gel electrophoresis.

Table 3.9 : Fragments (bp) detected by digestion of the PCR-amplified DPBl genes byBssHII restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
BssHll	188 111	299	299	299	299	299	299	299	299	181 111
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
BssHll	181 111	299	299	181 111	299	299	181 111	299	299	

BssHII restriction endonuclease's recognition sites are indicated as below:

5'...G↓C G C G C...3'
3'...C G C G C↑G...5'

Our RFLP experiments are shown in Figure 3.8 for the BsHII restriction enzyme. This is a representative figure for the digest experiment and this figure could observe that in lanes 123 and 124, there are no digestions as observed from the single bandsuggesting the presence of only homozygotes. In Figure 3.8 the presence of heterozygotes are shown in lanes corresponding to 121, 125, 126, 127 and 128 due to the presence of both cleaved fragments and the uncleaved form of the PCR product. The number on the left shows the molecular size (in base pairs) of the amplified PCR products. The number on the right indicates the molecular size (in base pairs) of BssHll enzyme's cleavage patterns.

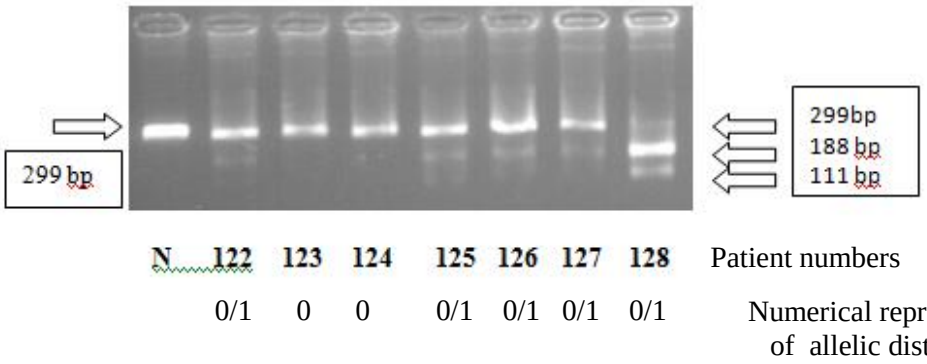


Figure 3.8 : Agarose gel electrophoresis analysis on 2.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: N, Negative control;from control groups, 122–128 lines show each number of amplified by BssHll restriction enzyme.Cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.8 RsaI restriction enzyme

Cleavage sites depending on the base pairs after the agarose gel electrophoresis for RsaI restriction endonuclease are shown in Table 3.10

Table 3.10: Fragments (bp) detected by digestion of the PCR-amplified DPBl genes byRsaI restriction endonuclease

	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
RsaI	177 122	177 122	177 122	147 122 30	177 122	177 122	177 122	269 30	269 30	147 122 30
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
RsaI	122 78 69 30	299	177 122	122 78 69 30	177 122	299	122 78 69 30	177 122	299	

RsaI restriction endonuclease's recognition sites are indicated as below:

5'...G T↓A C...3'
3'...C A↑T G...5'

Our RFLP experiments are shown in Figure 3.9 for the RsaI restriction enzyme. This is a representative figure for the digest experiment. In Figure 3.9, the presence of heterozygotes are shown in lanes corresponding to 35, 53, 81, 82 and 83 due to the presence of both cleaved fragments and the uncleaved form of the PCR product. The rest of the lanes have cleavage patterns suggesting the presence of only homozygotes (Figure 3.9). The number on the left indicates the molecular size (in base pairs) of the

amplified PCR products. The number on the right shows the molecular size (in base pairs) of RsaI enzyme's cleavage patterns.

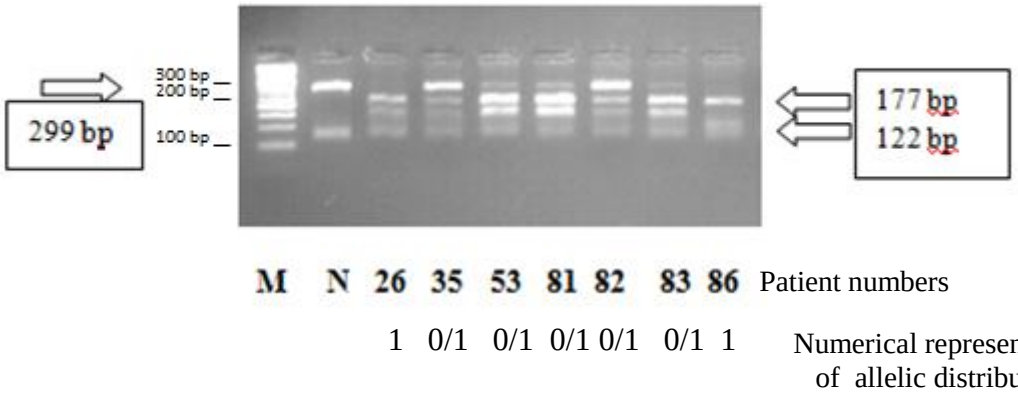


Figure 3.9 : Agarose gel electrophoresis analysis on 4.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: M, size marker DNA (100 bp DNA ladder); N, Negative control;from control groups, 26–86 lines show each number of amplified by RsaI restriction enzyme.Cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

3.3.2.8 EcoNI restriction enzyme

Table 3.11 shows the cleavage fragments of RsaI restriction enzyme with 19 HLA-DPB1 alleles after the agarose gel electrophoresis.

Table 3.11: Fragments (bp) detected by digestion of the PCR-amplified DPBl genes by EcoNI restriction endonuclease.

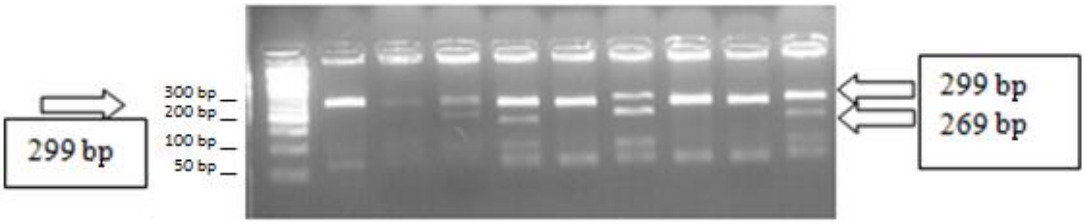
	DPB1 Alleles									
	0401	**0402**	**0201**	**1801**	**0202**	**0801**	**0501**	**0301**	**0601**	**1101**
EcoNI	269 30	269 30	174 95 30	299	174 95 30	174 95 30	269 30	201 98	201 95 30	201 98
	0101	**0901**	**1001**	**1301**	**1901**	**1401**	**1501**	**1601**	**1701**	
EcoNI	299	204 95	204 95	201 98	174 95 30	201 98	201 98	174 95 30	204 95	

RsaI restriction endonuclease's recognition sites are indicated as below:

5'...C C T N N↓N N N A G G...3'
3'...G G A N N N↑N N T C C...5'

Our RFLP experiments are shown in Figure 3.10 for the EcoNI restriction enzyme. This is a representative figure for the digest experiment. In Figure 3.10, the presence of heterozygotes are shown in lanes corresponding to 99, 100, 103 and 106 due to the

presence of both cleaved fragments and the uncleaved form of the PCR product. In lanes 98, 101, 104 and 105, there are no digestion as observed from the single band suggesting the presence of only homozygotes (Figure 3.10). The number on the left shows the molecular size (in base pairs) of the amplified PCR products. The number on the right indicates the molecular size (in base pairs) of EcoNI enzyme's cleavage patterns.



	M	N	98	99	100	101	103	104	105	106	Patient numbers
Numerical representation of allelic distribution			0	-	0/1	0/1	0	0/1	0	0	0/1	

Figure 3.10 : Agarose gel electrophoresis analysis on 4.0% agarose gel of RFLP-PCR methods from blood samples. Lanes: M, size marker DNA (50 bp DNA ladder); N, Negative control;from patient groups, 98–106 lines show each number of amplified by EcoNl restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

To detect which alleles patients have, Table 3.12 is used as a matrix. Since, 299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1" in Table 3.12. The total restriction cleavage results compile to generate the allelic frequencies of the patients and controls.

Table 3.12: Correlation between cleavage patterns obtained by 9 restriction endonucleases and DPB1 alleles (Ota *et al.*, 1991)

		Restriction Endonucleases								
DPB1 Allele	**Group**	**BspI2861**	**FokI**	**DdeI**	**BsaJI**	**BssHII**	**Cfr13I**	**RsaI**	**EcoNI**	**AvaII**
0401	A	1	1	0	1	1	1	1	0	0
1801=0402*	B	1	1	0	1	0	1	1	0	0
0201	C	1	1	0	1	0	1	1	1	0
0202	D	1	1	1	1	0	1	1	1	0
0801=1601*	E	0	1	0	1	0	0	1	1	0
0501	F	1	1	1	1	0	0	1	0	0
0301	G	0	0	0	0	0	1	1	1	1
0601	H	0	1	0	0	0	1	1	1	1
1101	I	0	1	0	0	1	1	1	1	1
0101	J	0	1	0	1	1	0	1	0	0
0901=1701*	K	1	1	0	0	0	0	0	1	0
1001	L	1	1	0	0	0	0	1	1	0
1301	M	0	1	0	0	1	0	1	1	0
1901	N	0	1	1	1	0	0	1	1	0
1401	O	1	0	0	0	0	1	0	1	1
1501	P	1	1	0	1	1	1	1	1	1

0: not cleaved, 1: cleaved

*: DPB1*1801= 0402 can be discriminated after digestion with RsaI. A 147bp fragment is positive for DPB1*1801, while a l77bp fragment positive for DPB1*0402 and heterozygote DPB1*1801/0402 has both 147 and 177 bp fragments. DPB1*0801 and 1601 can be discriminated by detecting RFLP bands digested with FokI (116bp positive for 0801 and 59bp positive for 1601). DPB1*0901 and 1701 can be discriminated by detecting RFLP bands after digestion with FokI (116bp positive for 0901 and 59bp positive for 1701) (Ota *et al.*, 1991).

3.4 HLA Results

Among the 19 analyzed DPB1 alleles in this study, DPB1*02:01 was the most prevalent (genotyped in 30.2% of patient and seroconverted group).On the other hand, HLA-DPB1*1801, HLA-DPB1*0202 and HLA-DPB1*0901 were rare alleles. The frequencies of the other alleles were given in Table 3.13.

In German populations which are the historical control groups from our study, DPB1*0202was the most prevalent alleles. On the other hand, DPB1*0601, DPB1*1101, DPB1*0201, DPB1*1901 and DPB1*1601 were rare alleles from German populations.

When we analyze the relation of the alleles according to clinical status of the patients; our data demonstrate that host HLA class II genotype determines the course of hepatitis B infection.

Table 3.13 : Frequencies of HLA-DPB1 alleles in our study groups

HLA alleles	Total		Group A Hepatitis B Patients		Group B Seroconverters		Group C Historical Controls German	
	N=179	AF[1]	N=94	AF[2]	N=85	AF[3]	AF[4] (sample size: 47)	AF[5] (sample size: 100)
DPB1*0401	29	0,162	18	0,191	11	0,129	0,09	0,06
DPB1*0402	38	0,212	25	0,266	13	0,152	0,26	0,1
DPB1*0201	54	0,3	21	0,223	33	0,388	0	0,01
DPB1*1801	0	0	0	0	0	0	0,13	0,14
DPB1*0202	0	0	0	0	0	0	0,72	0,47
DPB1*0801	9	0,05	2	0,021	7	0,082	0,19	0,13
DPB1*0501	2	0,011	0	0	2	0,023	0,06	0,01
DPB1*0301	8	0,045	3	0,032	5	0,058	0,02	0,01
DPB1*0601	2	0,011	1	0,011	1	0,011	0	0
DPB1*1101	6	0,034	1	0,011	5	0,058	0	0
DPB1*0101	30	0,167	19	0,2	11	0,129	0,02	0,03
DPB1*0901	0	0	0	0	0	0	0,02	0,02
DPB1*1001	19	0,1	5	0,053	14	0,164	0,06	0
DPB1*1301	12	0,067	7	0,074	5	0,058	0,04	0
DPB1*1901	1	0,006	0	0	1	0,011	0	0,01
DPB1*1401	13	0,073	6	0,064	7	0,082	0,02	0,01
DPB1*1501	14	0,078	1	0,011	13	0,152	0,04	0,01
DPB1*1601	30	0,168	20	0,212	10	0,117	0	0
DPB1*1701	17	0,09	10	0,106	7	0,082	0,04	0,01

[1]: AF, frequency of all alleles.

[2]: AF, frequency of Hepatitis B patients (Group A) alleles.

[3]: AF, frequency of Control Groups (Group B) alleles.

[4,5]: AF, frequency of Historical Control (Group C) alleles (Mella *et al.*, 1995; Jagiello *et al.*, 2004).

Additionaly, we have found statistically significant frequency of DPB1*15:01 allele was more frequent in the spontaneous seroconverted control group compared to chronic hepatitis B patients (15.3% vs. 1.1%, χ^2=12.5, OR = 0.06, 95% CI = 0.08-0.046 $p < 0.001$). DPB1*02:01 and DPB1*10:01 were the other alleles genotyped

more frequently found in the control group (38.8% vs. 22.3% p = 0.02 and 16.5% vs. 5.3% p = 0.02, respectively) (Table 3.14). However these two alleles' associations lost their significance after Bonferoni's correction (pc=0.4 for all).HBsAg seroconversion is the ultimate target in chronic hepatitis B. This study revealed an association of HLA DPB1*15:01 allele with spontaneous HBsAg seroconversion.

Table 3.14 : Comparison of frequency of HLA-DPB1 alleles between Hepatitis B patients group and healthy control group

HLA Alleles	Group A Hepatitis Patients(%)	Group B Seroconverters (%)	p	X^2	OR	95% CI
DPB1*0401	18(19,1)	11(12,9)	0,3	1,3	1,6	0,7-3,6
DPB1*0402	25(26,6)	13(15,3)	0,1	3,4	2,0	0,95-4,24
DPB1*0201	21(22,3)	33(38,8)	0,0	5,8	0,5	0,24-0,87
DPB1*1801	0	0	0	0	0	-
DPB1*0202	0	0	0	0	0	-
DPB1*0801	2(2,1)	7(8,2)	0,1	3,5	0,2	0,05-1,2
DPB1*0501	0	2(2,4)	0,1	2,2	0,0	-
DPB1*0301	3(3,2)	5(5,9)	0,4	0,8	0,5	0,12-2,28
DPB1*0601	1(1,1	1(1,2)	0,9	0,0	0,9	0,56-14,67
DPB1*1101	1(1,1)	5(5,9)	0,1	3,2	0,2	0,02-1,50
DPB1*0101	19(20,2)	11(12,9)	0,2	1,7	1,7	0,76-3,83
DPB1*0901	0	0	0	0	0	-
DPB1*1001	5(5,3)	14(16,5)	0,0	5,9	0,3	0,098-0,829
DPB1*1301	7(7,4)	5(5,9)	0,7	0,2	1,3	0,39-4,22
DPB1*1901	0	1(1,2)	0,3	1,1	0,0	-
DPB1*1401	6(6,4)	7(8,2)	0,6	0,2	0,8	0,25-2,36
DPB1*1501	1(1,1)	13(15,3)	**<0,001**	12,5	0,1	0,01-0,47
DPB1*1601	20(21,3)	10(11,8)	0,1	2,9	2,0	0,89-4,62
DPB1*1701	10(10,6)	7(8,2)	0,6	0,3	1,3	0,48-3,66

[1]: Based on χ^2 test. $P \leq 0,05$ are shown in bold; OR, odds ratio; 95% CI, confidence interval

4. CONCLUSIONS

Chronic Hepatitis B Infection is a global health problem that affects more than 2 billion people all around the world that can lead to chronic hepatitis, liver cirrhosisand hepatocellular carcinoma. Studies have shown that HLA class II molecules, which show high polymorphism, are the susceptibility factors of chronic HBV infection and related diseases.

Recent genetic association studies have shown that HBV infection and the progression of HBV infection related diseases are associated with the variations observed in the HLA-DP region of the genome especially HLA-DPA1 and HLA-DPB1 region. According to previous studies, GWAS determined candidate SNPs of the HLA loci that are related with chronic HBV or HCV infection, hepatic fibrosis and hepatocellular carcinomaregion (Gebe *et al.*, 2002; Tamori *et al*., 2013).

Our study investigates the presence of possible variations in the HLA-DP region in Turkish population who were infected by HBV. We analysed two groups of HBV infected patients. A total of 94 hepatitis B patients (group A) were included and 85 HBsAg negative, anti-HBs positive and anti–HBc IgG positive spontaneous seroconverted healthy subjects (group B) were also included in the study. In this line, our work aims to determine alterations in the HLA-DPB1 site of the genome of HBV infected Turkish patients and link these possible polymorphisms to disease outcome in Turkish population. In our study, frequencies of the 19 specific HLA-DPB1 allele polymorphisms were analyzed in patients with HBV infection using PCR-RFLP method.

Among the 19 analyzed DPB1 alleles in this study, DPB1*0201 was the most prevalent (genotyped in 30.2% of all group) allele. On the other hand, HLA-DPB1*1801, HLA-DPB1*0202 and HLA-DPB1*0901 were the rare alleles in this study.

In Turkish population, our preliminary date indicated that DPB1*1501 allele was more frequent allele in the spontaneous seroconverted control group compared to

chronic hepatitis B patients (15.3% vs. 1.1%, χ^2=12.5, OR=0.06, 95% CI=0.08-0.046 p<0.001,pc<0.001). This indicates an association of HLA DPB1*1501 allele with spontaneous HBsAg seroconversion. It can be speculated that people carrying HLA-DPB1*1501 allele may develop a resistance to Hepatitis B disease. In this study, no significant association between DPB1alleles of Hepatitis B infection spectrum was observed.

HLA-DPB1* 1001 allele also appeared as another protective allele for HBV infection in our study in the begining of our analysis.On the other hand, HLA-DPB1*1801, HLA-DPB1*0202, HLA-DPB1*0901 alleles do not appear to correlate with Hepatisis B Disease.

DPB1*02:01 and DPB1*10:01 were the other alleles genotyped more frequently found in the control group (38.8% vs. 22.3% p = 0.02 and 16.5% vs. 5.3% p=0.02, respectively). However, after Bonferroni correction, only DPB1*1501 maintained its significance. It can be said that, presence of DPB1*1501 was the protective allele at Turkish population just looking at the above data without HWE analysis.

As we didn't have healthy control group in our study we wanted to compare results using historical control groups from literature. Two studies from Germany were selected as they belong to the same (caucasion) ethnic group with Turkish population. Comparisons of historical controls with our study groups showed that the patient group allele frequencies are more similar to historical controls and the seroconvertors were quite different. This can be seen in DPB1*0201, DPB1*1001 and DPB1*1501 alleles (which were statistically significant in chi-square test). DPB1* 0201 has the observation frequency of 39% in our study, where it has the observation frequencies of 0% and 1% for German population historical control groups. DPB1*1001 has the observation frequency of 16% in our study, where it has the observation frequencies of 6% and 0% for German population historical control groups. DPB1*1501 has the observation frequency of 15% in our study, where it has the observation frequencies of 4% and 1% for German population historical control group.

On the other hand, between our study and historical control group of German population, these alleles (which are not statistically significant in chi-square test) have similar frequencies; DPB1*0501, DPB1*0301, DPB1*0601, DPB1*0901,

DPB1*1901. Mentioned alleles have the following frequencies, in the respective order of patient group allele frequencies, seroconverted control group frequencies and historical group frequencies; DPB1*0501: 2%, 6%, 1%; DPB1*0301: 6%, 2%, 1%; DPB1*0601: 1%, 0%, 0%; DPB1*0901: 0%, 2%, 2%; DPB1*1901: 1%, 0%, 1%.

However, between our study and historical control group of German population, these alleles (which are not statistically significant in chi-square test) have relatively different frequencies; DPB1*1801, DPB1*0202, DPB1*0101, DPB1*1601. Mentioned alleles have the following frequencies, in the respective order of patient group allele frequencies, seroconverted control group frequencies and historical group frequencies; DPB1*1801: 0%, 13%, 14%; DPB1*0202: 0%, 72%, 47%; DPB1*0101: 13%, 2%, 3%; DPB1*1601: 12%, 0%, 0%.

Although an OR of 12,5 predicts a statistically significant frequency of HLA DPB1*1501 in Hepatitis B disease patients, the wide confidence interval indicates that more data should be collected to reach to a more definite conclusion.

Unfortunately most of the alleles tested did not follow Hardy-Weinberg Equation, especially seeming significant ones including 1501 allele. Expected number of heterozygotes in Hardy-Weinberg analysis are two times of number of homozygotes, which could not be observed in our study for many genotypes. It is possible that homozygotes could not be distinguished in agarose gel imaging due to too small distance between cutting points. The only way to distinguish is sequencing method and this method can reveal clearly existence of both homozygotes and heterozygotes.

For future studies, to obtain more significant results, number of subjects in the patient group and control group should be increased. The study is evaluated for Turkish population and results show that, in other populations findings can be different. As a result, the study should be evaluated with other populations rather than Turkish population. In conclusion, individuals with HLA types may differ in susceptibilty or resistance to disease and large, multi ethnic confirmatory study is needed to validate these findings and further explore the genetic pathogenesis of HBV infection.

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APPENDICES

APPENDIX A : Laboratory Equipment
APPENDIX B : Chemicals
APPENDIX C : Composition of Buffers and Solutions
APPENDIX D : Representative Gel Images of Results
APPENDIX E : Lists of patient and control groups with DPB1 alleles
APPENDIX F : Enzymes
APPENDIX G : Markers

APPENDIX A

LABORATORY EQUIPMENT

Balances	Precisa 620C SCS
	Precisa BJ 610C
Centrifuges	Sigma 1-13 B. Braun International
	Allegra 25R Centrifuge Beckman Coulter
Electrophoresis equipments	E – C mini cell primo EC320
Gel Documentation System	UVI PhotoMW Version 99.05 for Windows
Pipettes	Thermo Finnpipette 10 µL, 100 µl, 1000 µl
pH meter	Mettler Toledo MP220
Spectrophotometer	PerkinElmer Lambda25 UV/VIS Spectrometer
Thermo cycler	Applied Biosystems GeneAmp PCR
System 2700	
Corbett PalmCycler	
Techne FTGENE 5D	
Transilluminator	Biorad UV Transilluminator 2000
Vortex	Herdolph Reax top

APPENDIX B

CHEMICALS

Agarose	Appli Chem
Boric acid	Amresco
dNTP	Fermentas
EDTA	Appli Chem
Ethanol	Riedel-de Haën
SYBR	Fermentas
$MgCl_2$	Fermentas
NaOH	Riedel-de Haën
Primers	IDT DNA
NaCl	Carlo Erba
Tris Base	Amresco
10X PCR Buffer	Fermentas

APPENDIX C

BUFFERS

TE Buffer

Tris base	10 mM
EDTA	1 mM

Add ddH2O to 1 liter and adjust the pH to 8.0

TBE (Tris-Borate-EDTA) Buffer (10X)

Tris base	108 g
Boric Acid	55 g
EDTA	40 ml (0.5 M, pH 8.0)

Add ddH2O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)

Agarose	0.5 g
TBE buffer (1X)	50 mL

Midi Agarose Gel (1%)

Agarose	1.5 g
TBE buffer (1X)	150 mL

Mini Agarose Gel (2%)

Agarose	1.0 g
TBE buffer (1X)	50 mL

Midi Agarose Gel (2%)

Agarose	3.0 g
TBE buffer (1X)	150 mL

Mini Agarose Gel (4%)

Agarose	2 g
TBE buffer (1X)	50 mL

Midi Agarose Gel (4%)

Agarose	6 g
TBE buffer (1X)	150 mL

APPENDIX D

Results of PCR-RFLP gels are shown according to the patients/ controls and the endonuclease cleavage reactions, respectively.

Patients and control numbers are indicated below each figure.

FROM PATIENT GROUP

FOKI ENZYME

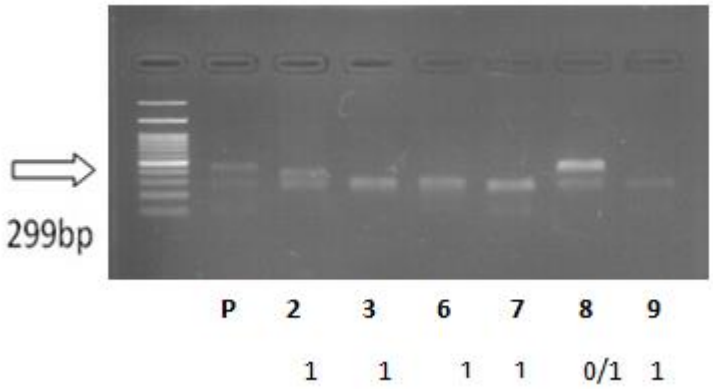


Figure D.1 Fragments detected after digestion by FokI restriction enzyme. Lanes: P: Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:2–9 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

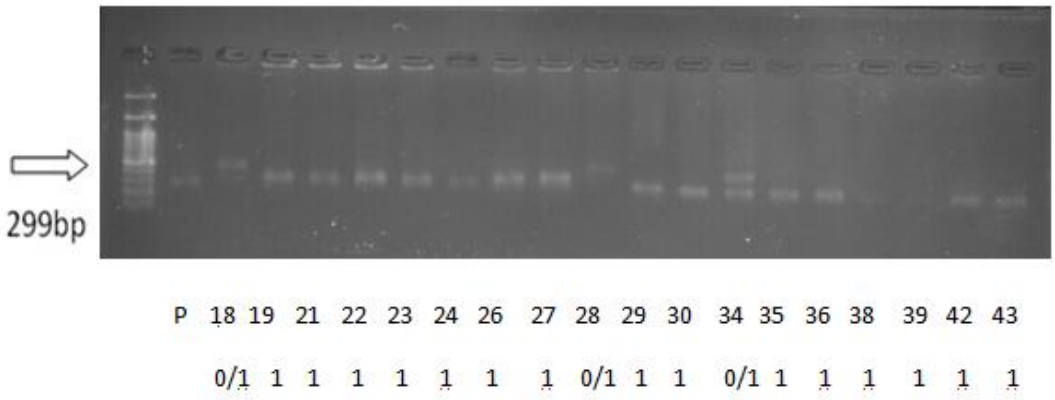


Figure D.2 Fragments detected after digestion by FokI restriction enzyme. Lanes: P: Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:18–43 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

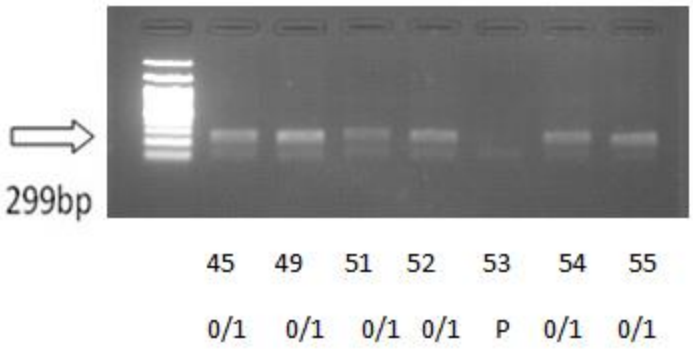


Figure D.3 Fragments detected after digestion by FokI restriction enzyme. Lanes: M: size marker DNA (100-bp DNA ladder). From patient groups:45–55 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

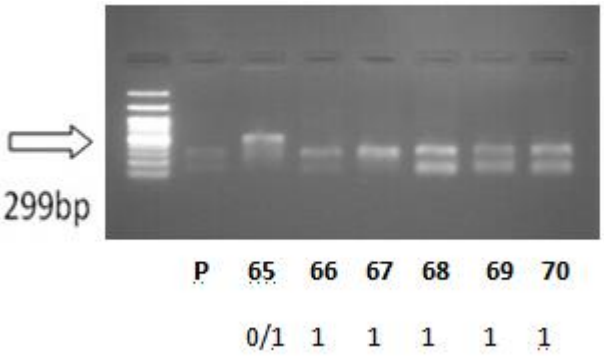


Figure D.4 Fragments detected after digestion by FokI restriction enzyme. Lanes: P:Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:65–70 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

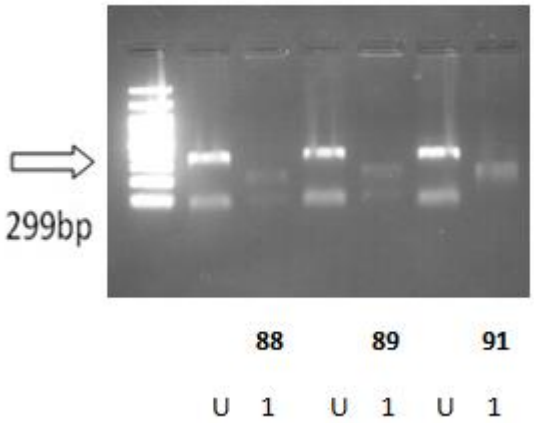


Figure D.5 Fragments detected after digestion by FokI restriction enzyme.Lanes: U:Uncut. M: size marker DNA (100-bp DNA ladder). From patient groups:65–70 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

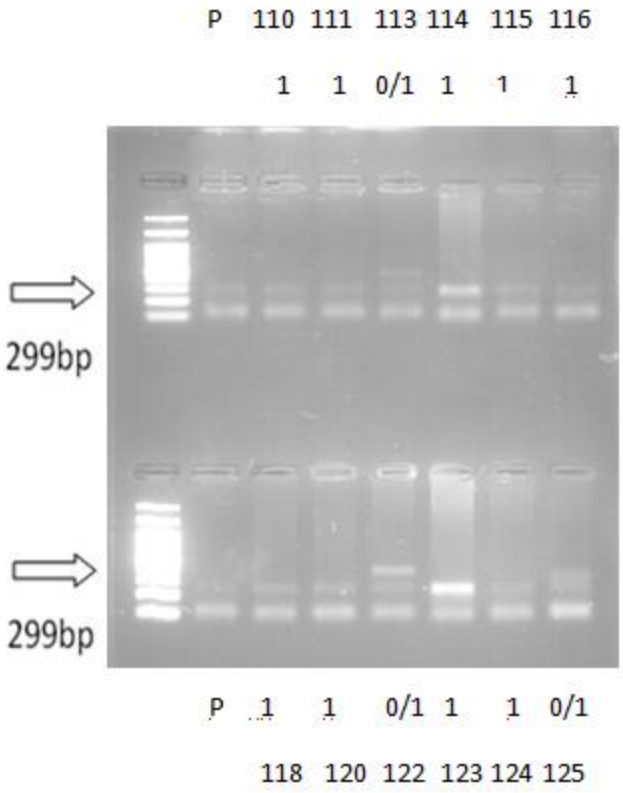


Figure D.6 Fragments detected after digestion by FokI restriction enzyme. Lanes: P:Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:110–125 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

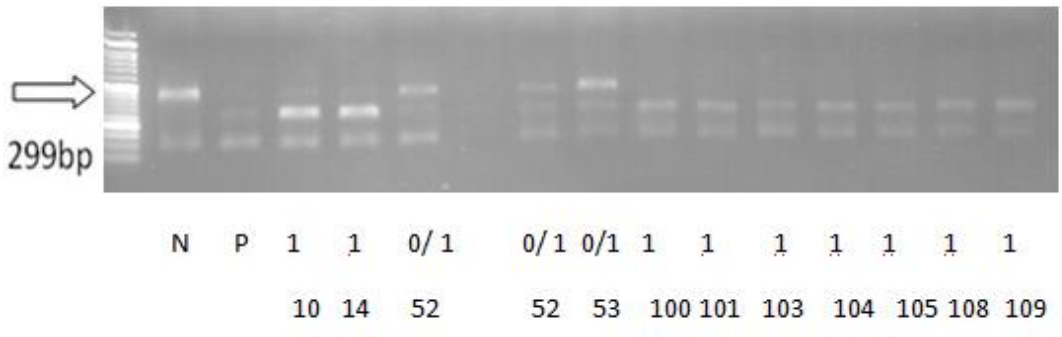


Figure D.7 Fragments detected after digestion by FokI restriction enzyme. Lanes: N: Negative control. P:Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:10–109 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number

consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

AVAII ENZYME

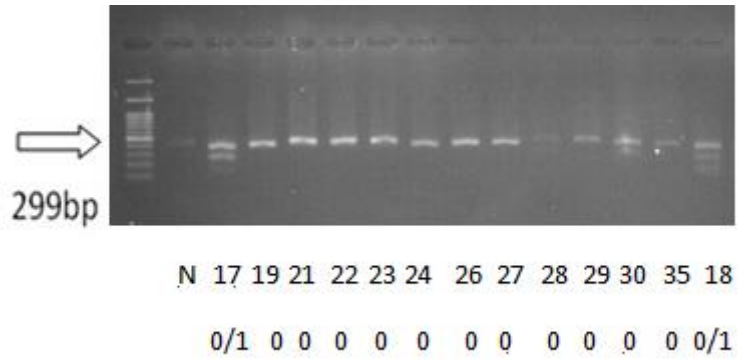


Figure D.8 Fragments detected after digestion by AvaII restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:17–18 lines show each number of amplified by AvaII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

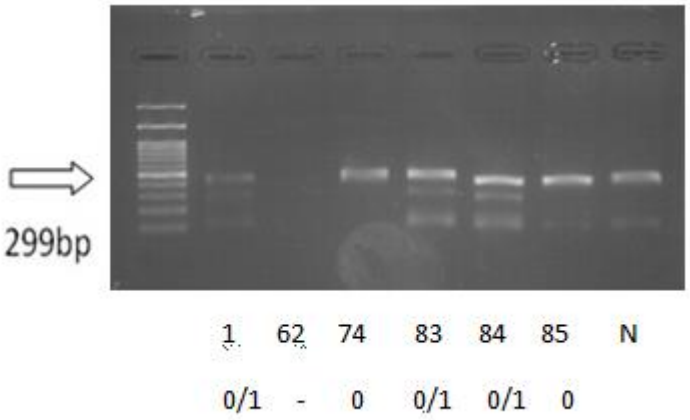


Figure D.9 Fragments detected after digestion by AvaII restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:1–85 lines show each number of amplified by AvaII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

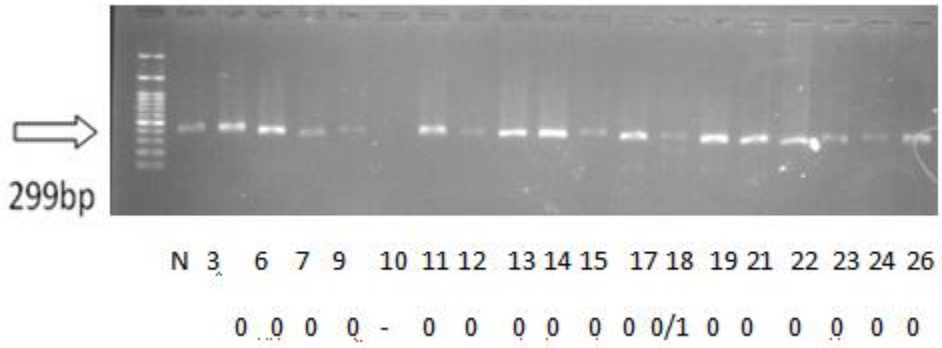


Figure D.10 Fragments detected after digestion by AvaII restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:3–26 lines show each number of amplified by AvaII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

BSaJ ENZYME

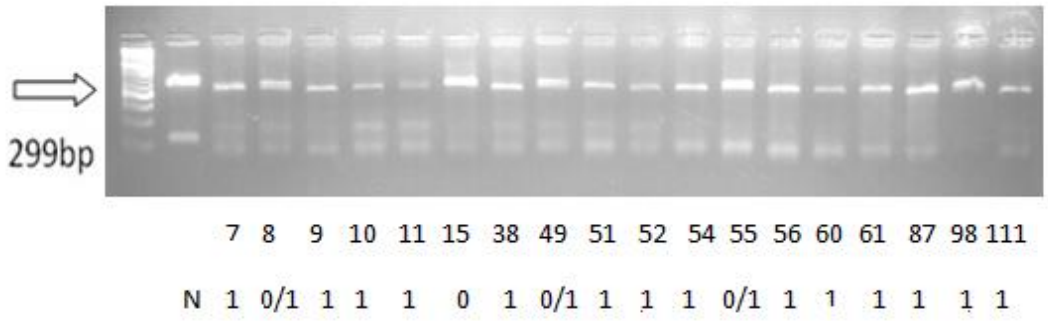


Figure D.11 Fragments detected after digestion by BsaJ restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:7–111 lines show each number of amplified by AvaII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

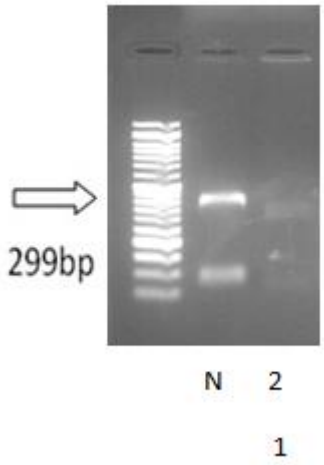


Figure D.12 Fragments detected after digestion by BsaJ restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient group:2 line shows each number of amplified by AvaII restriction enzyme.Cleaved fragments were marked as "1", showing these alleles as homozygotes.

CFR13I ENZYME

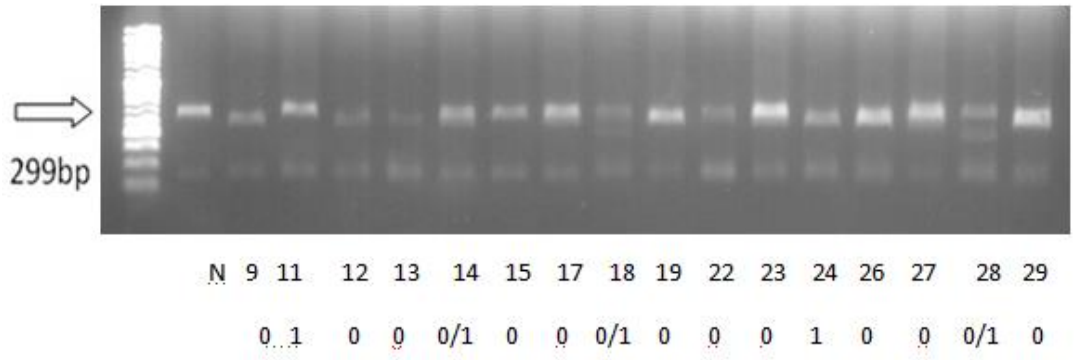


Figure D.13 Fragments detected after digestion by Cfr13I restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:9–29 lines show each number of amplified by Cfr13I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

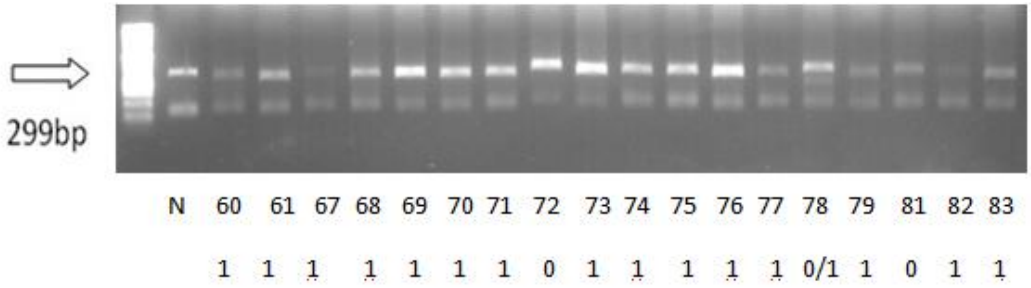


Figure D.14 Fragments detected after digestion by Cfr13I restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:60–83 lines show each number of amplified by Cfr13I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

BSP1286I ENZYME

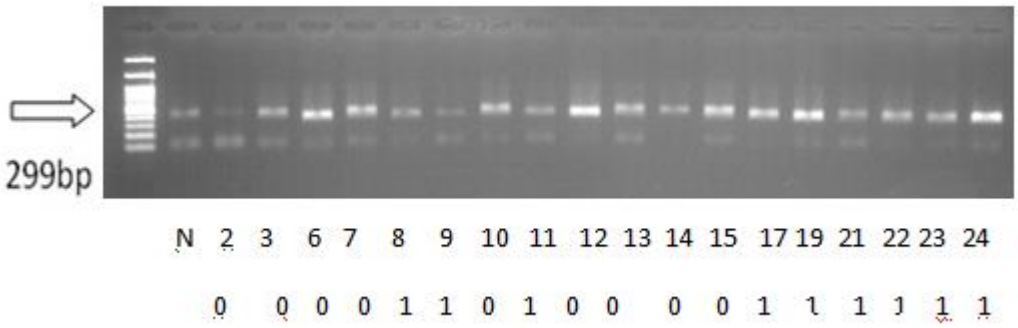


Figure D.15 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:2–24 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes.

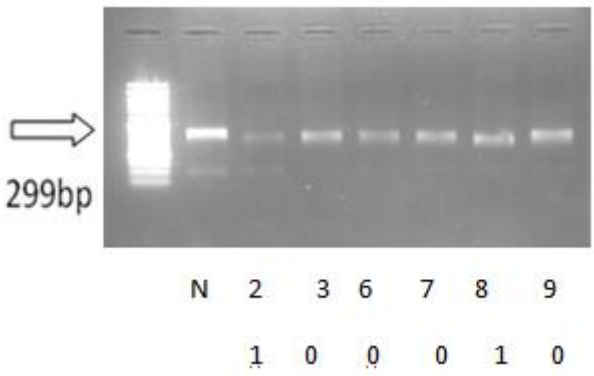


Figure D.16 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient

groups:2–9 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes.

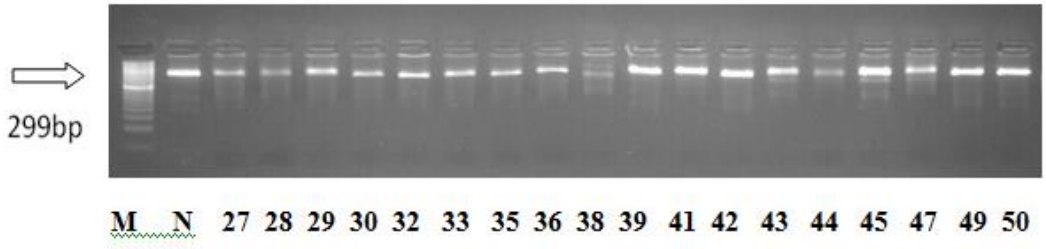


Figure D.17 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: N: Negative control. M, size marker DNA (100-bp DNA ladder).

DdEI ENZYME

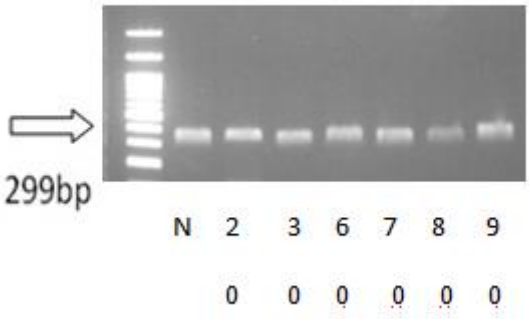


Figure D.18 Fragments detected after digestion by DdeI restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:2–9 lines show each number of amplified by DdeI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes.

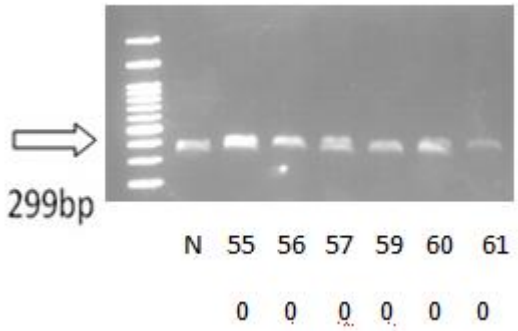


Figure D.19 Fragments detected after digestion by DdeI restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient

groups:55–61 lines show each number of amplified by DdeI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes.

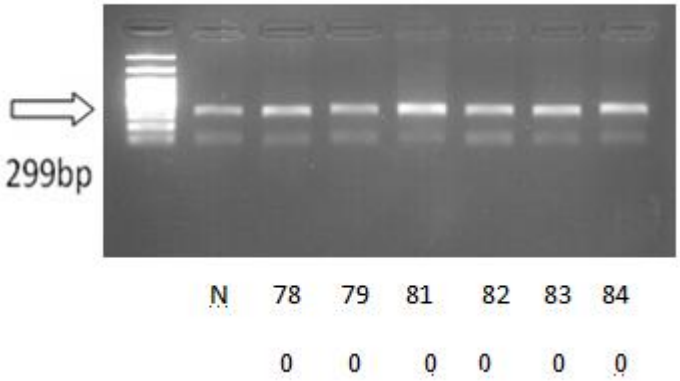


Figure D.20 Fragments detected after digestion by DdeI restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:78–84 lines show each number of amplified by DdeI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes.

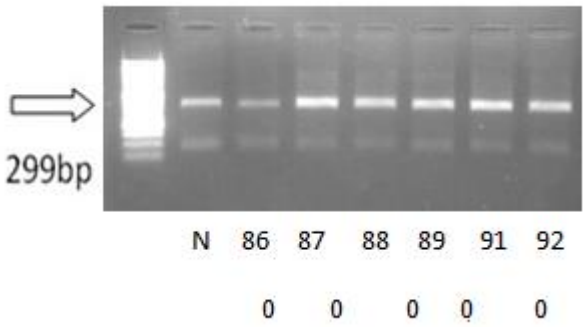


Figure D.21 Fragments detected after digestion by DdeI restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:86–92 lines show each number of amplified by DdeI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes.

BssHII ENZYME

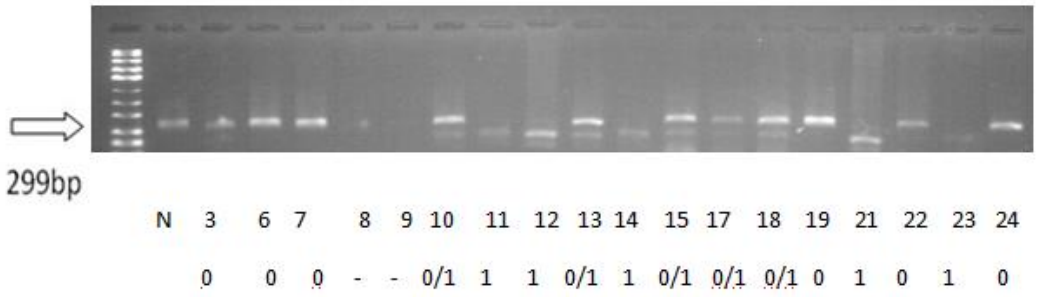


Figure D.22 Fragments detected after digestion by BssHII restriction enzyme. Lanes: N: Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:3–24 lines show each number of amplified by BssHII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

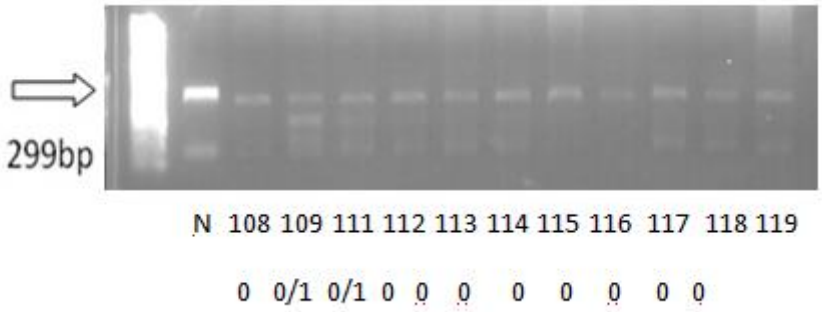


Figure D.23 Fragments detected after digestion by BssHII restriction enzyme. Lanes: N: Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:108–119 lines show each number of amplified by BssHII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

RSA ENZYME

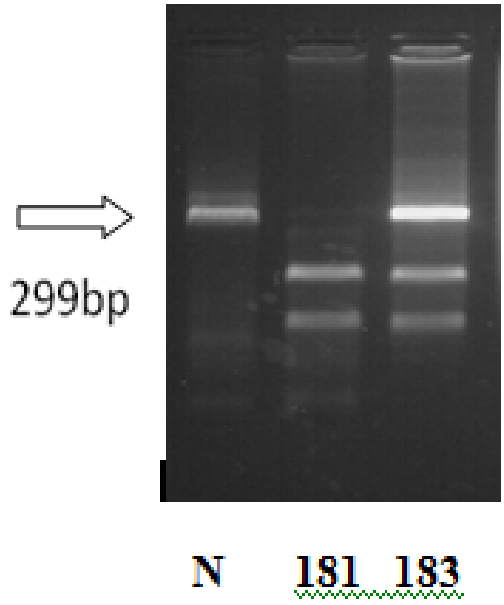


Figure D.24 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control, from patient groups lines 181-183.

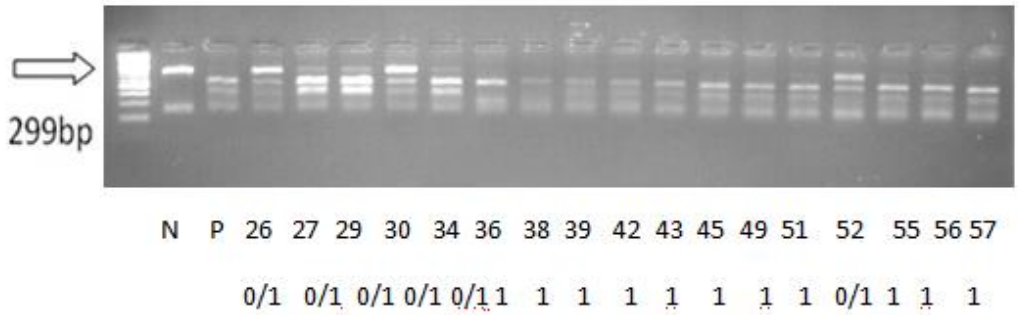


Figure D.25 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. P: Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:26–57 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

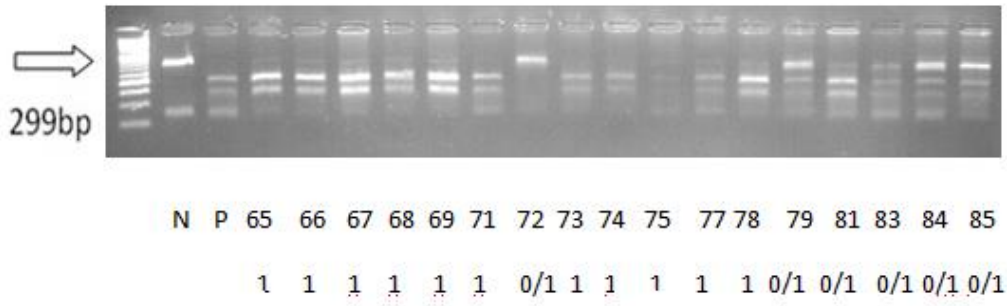


Figure D.26 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. P: Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:65–85 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

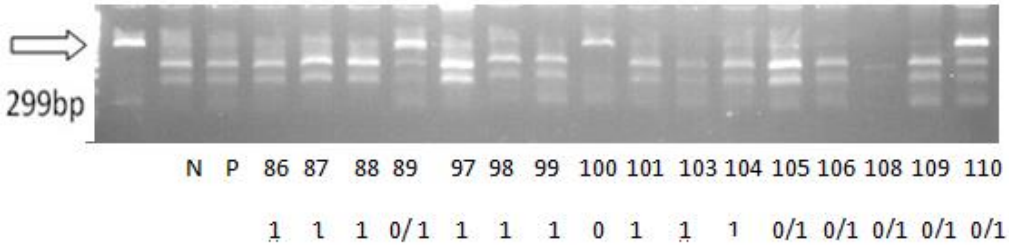


Figure D.27 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. P: Pozitive control. M: size marker DNA (100-bp DNA ladder). From patient groups:86–110 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

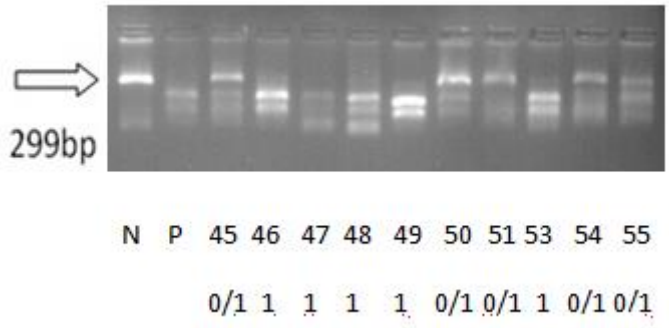


Figure D.28 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. P: Pozitive control. From patient groups:45–55 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these

alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

EcoNI ENZYME

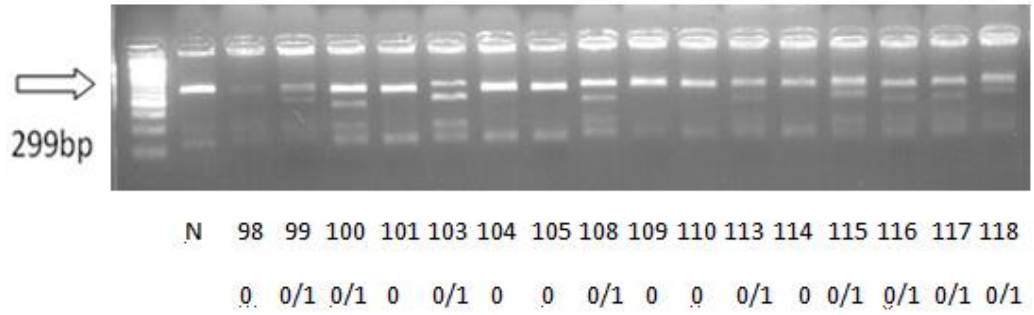


Figure D.29 Fragments detected after digestion by EcoNI restriction enzyme. Lanes: N: Negative control. M: size marker DNA (100-bp DNA ladder). From patient groups:98–118 lines show each number of amplified by EcoNI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

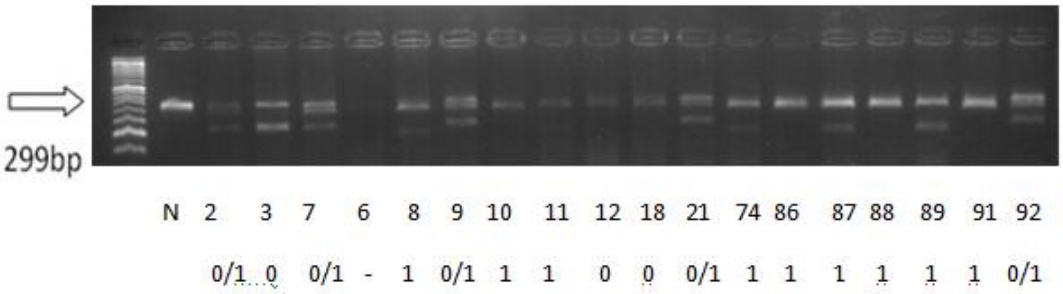


Figure D.30 Fragments detected after digestion by EcoNI restriction enzyme. Lanes: N: Negative control. From patient groups:2–92 lines show each number of amplified by EcoNI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

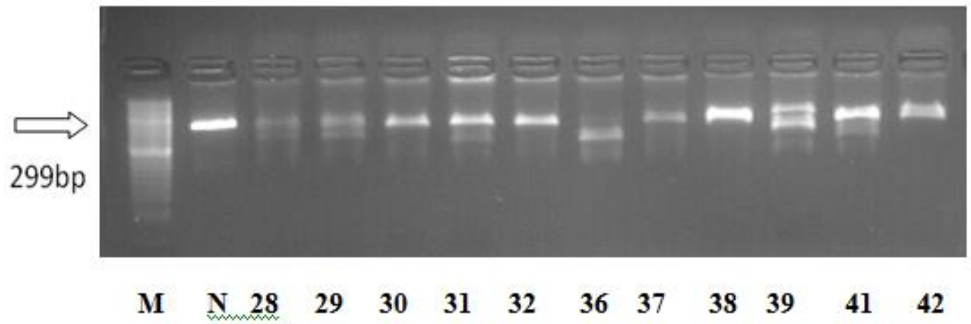


Figure D.31 Fragments detected after digestion by EcoNI restriction enzyme Lanes: N: Negative control. M, size marker DNA (100-bp DNA ladder).

FROM CONTROL GROUPS

FOKI ENZYME

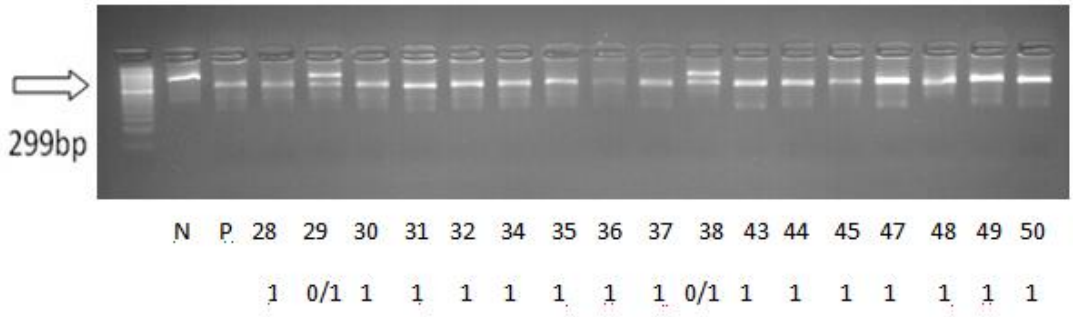


Figure D. Fragments detected after digestion by FokI restriction enzyme. Lanes: N: Negative control. P: Pozitive control. M: size marker DNA (100-bp DNA ladder). From control groups:28–50 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

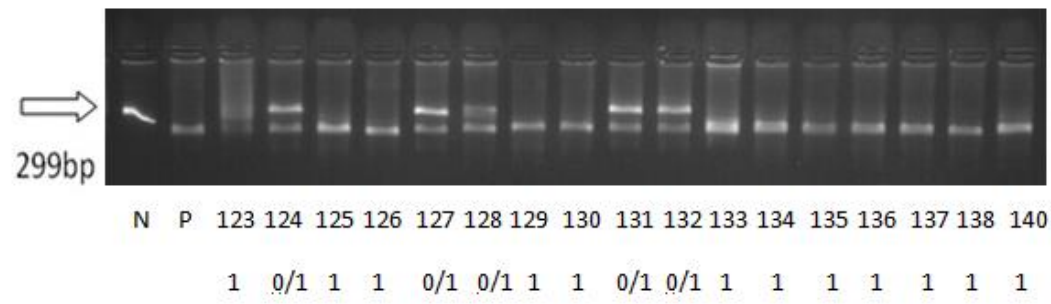


Figure D.33 Fragments detected after digestion by FokI restriction enzyme. Lanes: N: Negative control. P: Pozitive control. M:size marker DNA (100-bp DNA ladder). From control groups:123–140 lines show each number of amplified by FokI

restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

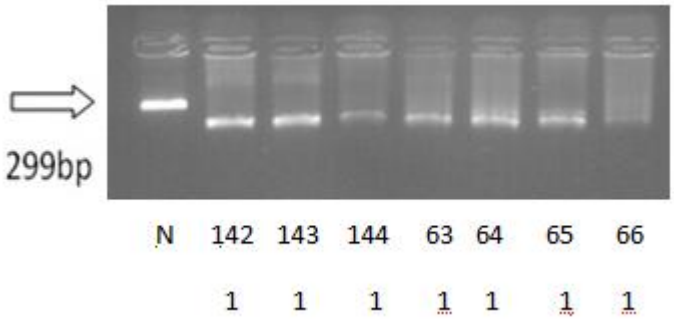


Figure D.34 Fragments detected after digestion by FokI restriction enzyme. Lanes: N: Negative control. From control groups:142-–66 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

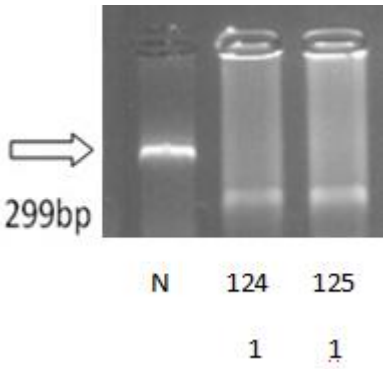


Figure D.35 Fragments detected after digestion by FokI restriction enzyme. Lanes: N: Negative control. From control groups:124–125 lines show each number of amplified by FokI restriction enzyme.Cleaved fragments were marked as "1", showing these alleles as homozygotes.

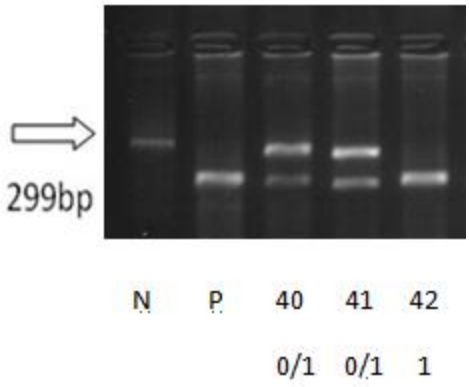


Figure D.36 Fragments detected after digestion by FokI restriction enzyme. Lanes: N: Negative control. P: Pozitive control.. From control groups:40-42 lines show each number of amplified by FokI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

AVAII ENZYME

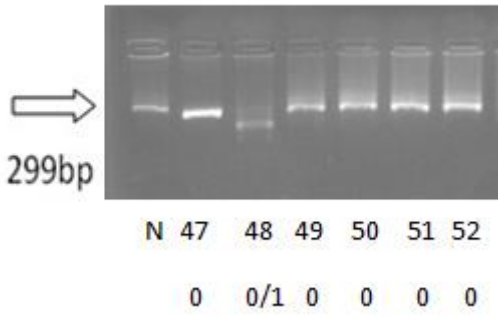


Figure D.37 Fragments detected after digestion by AvaII restriction enzyme. Lanes: N:Negative control. From control groups:47–52 lines show each number of amplified by AvaII restriction enzyme.299 base pair, cleaved fragments were marked as "1"showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

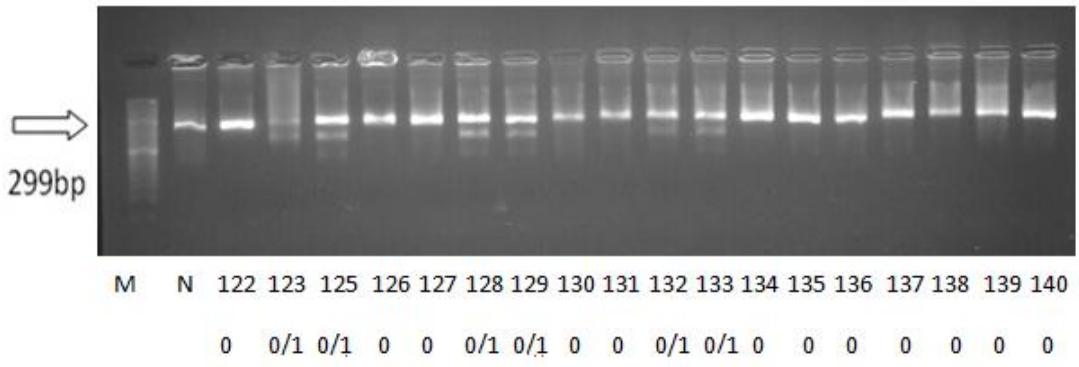


Figure D.38 Fragments detected after digestion by AvaII restriction enzyme.. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From control groups:122–140 lines show each number of amplified by AvaII restriction enzyme.299 base pair, showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

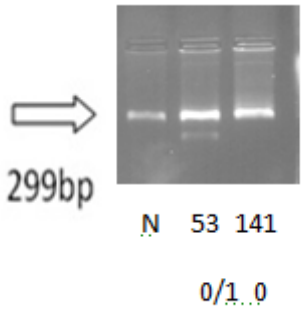


Figure D.39 Fragments detected after digestion by AvaII restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From control groups:53–141 lines show each number of amplified by AvaII restriction enzyme.299 base pair, cleaved fragments were marked as "1"showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

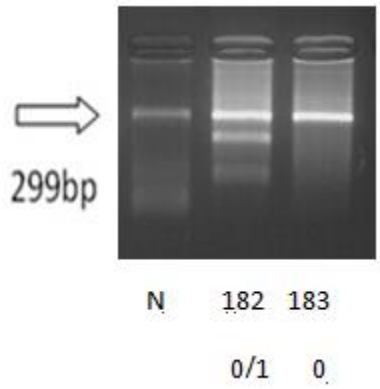


Figure D.40 Fragments detected after digestion by AvaII restriction enzyme. Lanes: N:Negative control. M: From control groups:182–183 lines show each number of amplified by AvaII restriction enzyme.299 base pair, cleaved fragments were marked as "1"showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

BSAJ ENZYME

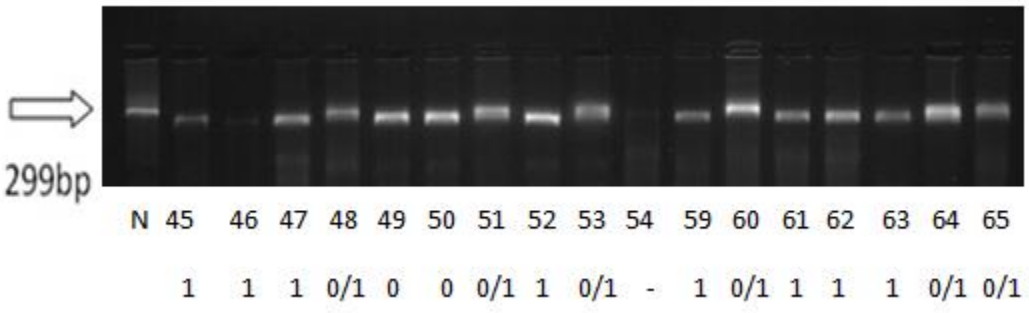


Figure D.41 Fragments detected after digestion by BsaJI restriction enzyme. Lanes: N:Negative control. From comtrol groups:45–65 lines show each number of amplified by BsaJ restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

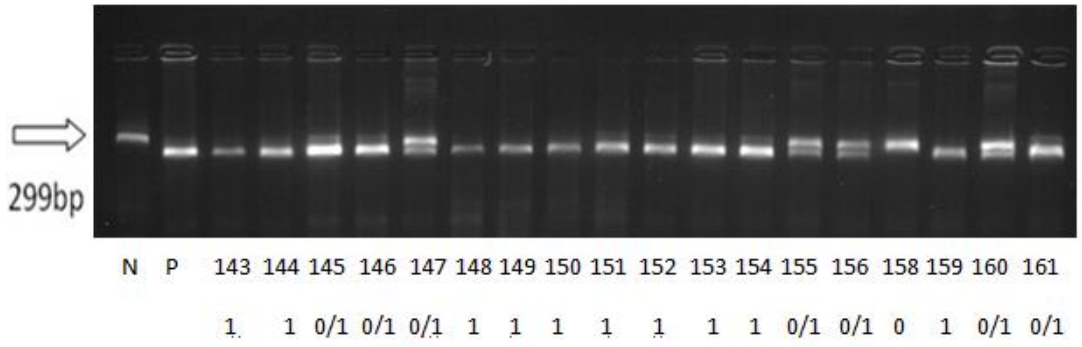


Figure D.42 Fragments detected after digestion by BsaJI restriction enzyme. Lanes: N:Negative control. P:Pozitive control. From control groups:143–161 lines show each number of amplified by BsaJ restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

CFR13I ENZYME

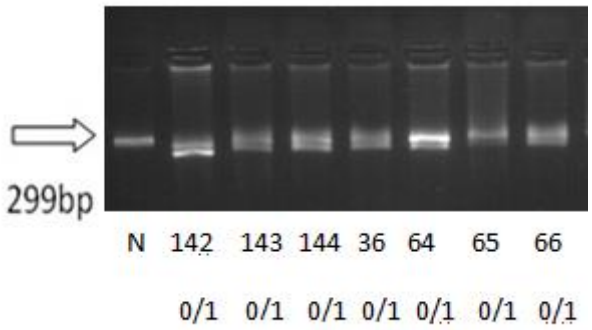


Figure D.43 Agarose gel electrophoresis analysis on 2.0% agarose gel of PCR-RFLP methods from blood samples. Lanes: N:Negative control. From control groups:142–66 lines show each number of amplified by Cfr13I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

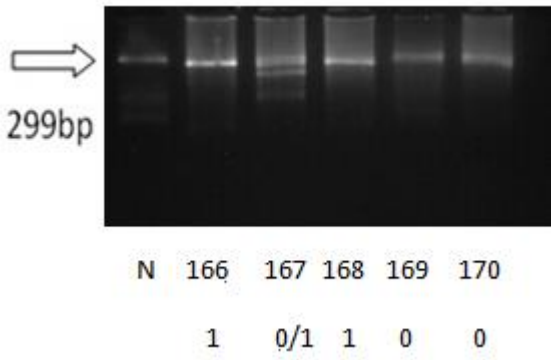


Figure D.44 Fragments detected after digestion by BsaJI restriction enzyme. Lanes: N:Negative control. From control groups:166–170 lines show each number of amplified by Cfr13I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

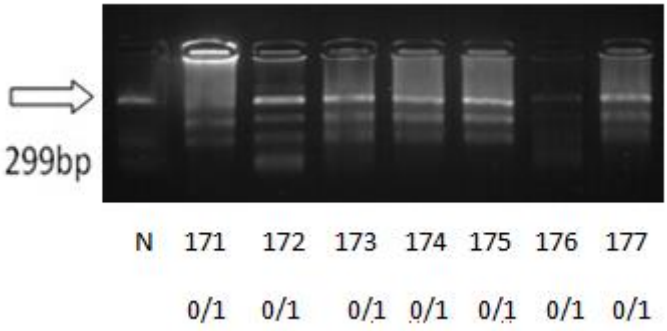


Figure D.45 Fragments detected after digestion by BsaJI restriction enzyme. Lanes: N:Negative control. From control groups:171–177 lines show each number of amplified by Cfr13I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

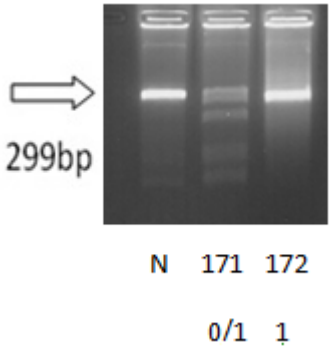


Figure D.46 Fragments detected after digestion by BsaJI restriction enzyme. Lanes: N:Negative control. From control groups:171–172 lines show each number of amplified by Cfr13I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

BSP1286I ENZYME

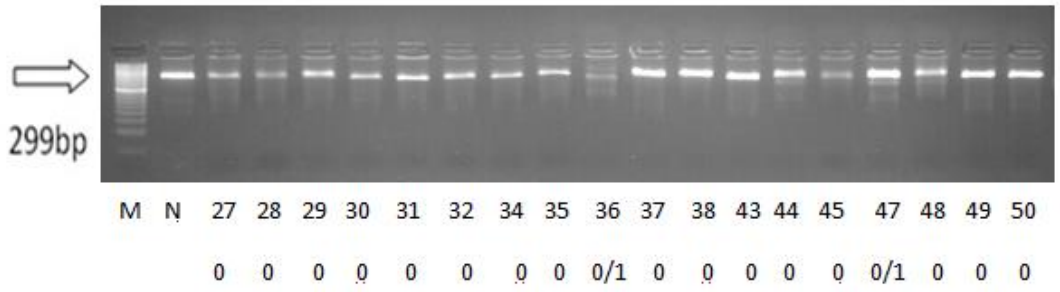


Figure D.47 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: N:Negative control. M: size marker DNA (100-bp DNA ladder). From control groups:27–50 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes.If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

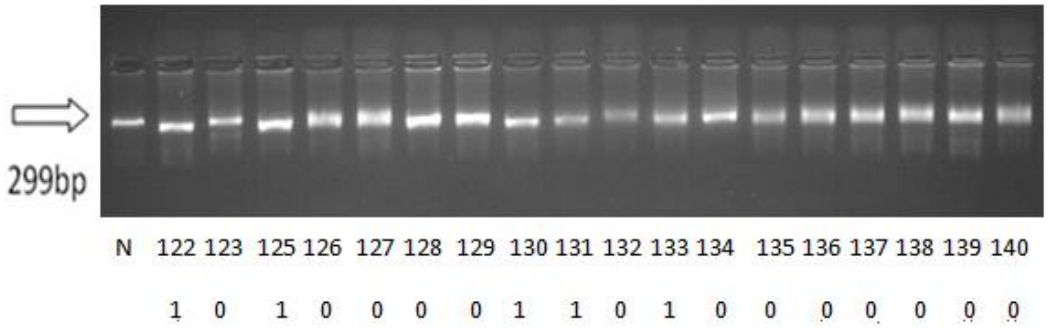


Figure D.48 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: N:Negative control. From control groups:122–140 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes.If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

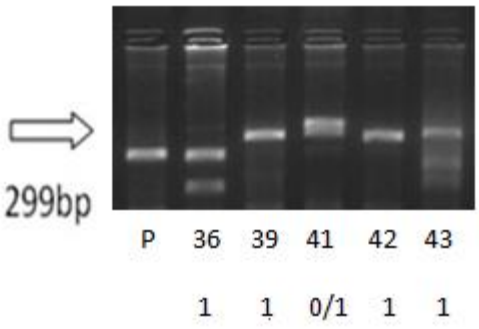


Figure D.49 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: P:Pozitive control. From control groups:36–43 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

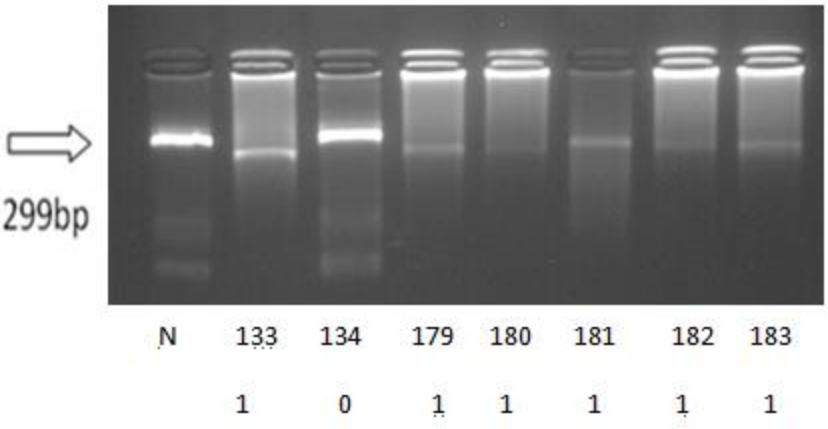


Figure D.50 Fragments detected after digestion by Bsp1286I restriction enzyme. Lanes: N:Negative control. From control groups:133–183 lines show each number of amplified by Bsp1286I restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes.

DdeI ENZYME

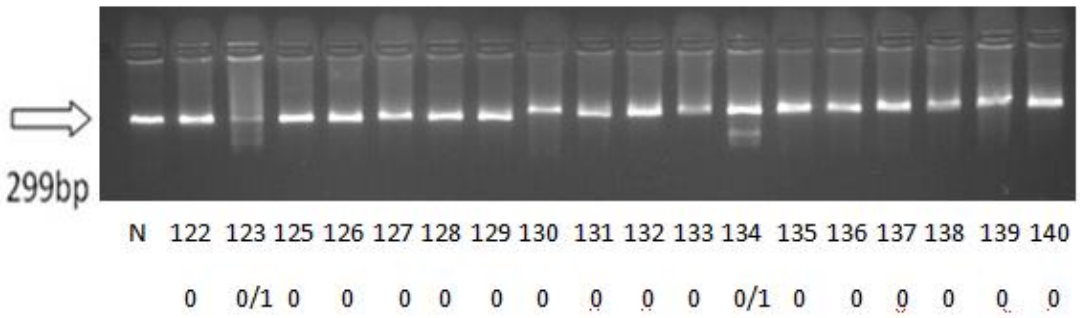


Figure D.51 Fragments detected after digestion by DdeI restriction enzyme. Lanes: N:Negative control. From control groups:122–140 lines show each number of amplified by DdeI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

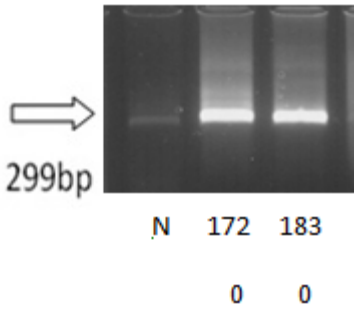


Figure D.52 Fragments detected after digestion by DdeI restriction enzyme. Lanes: N:Negative control. From control groups:172–183 lines show each number of amplified by DdeI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0", showing these alleles as homozygotes.

BSSHII ENZYME

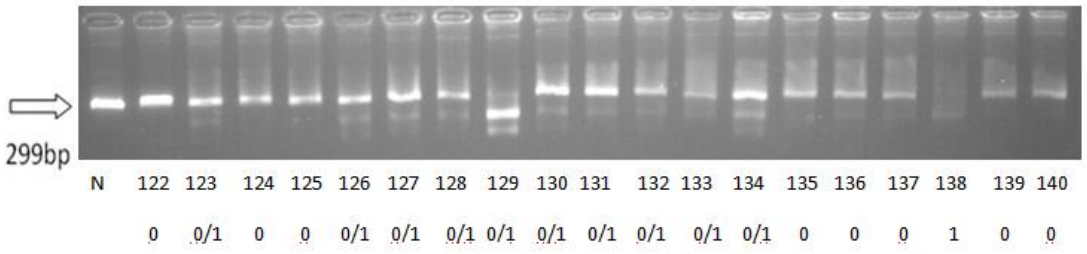


Figure D.53 Fragments detected after digestion by BssHII restriction enzyme. Lanes: N: Negative control. From control groups:122–140 lines show each number of amplified by BssHII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

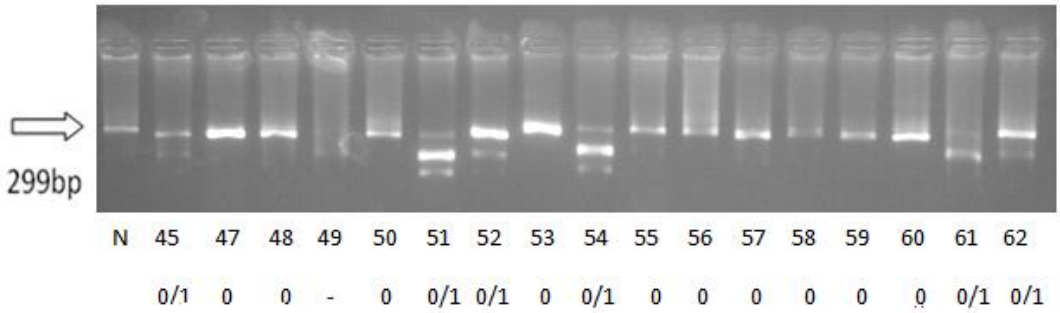


Figure D.54 Fragments detected after digestion by BssHII restriction enzyme. Lanes: N: Negative control. From control groups:45–62 lines show each number of amplified by BssHII restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

RSA ENZYME

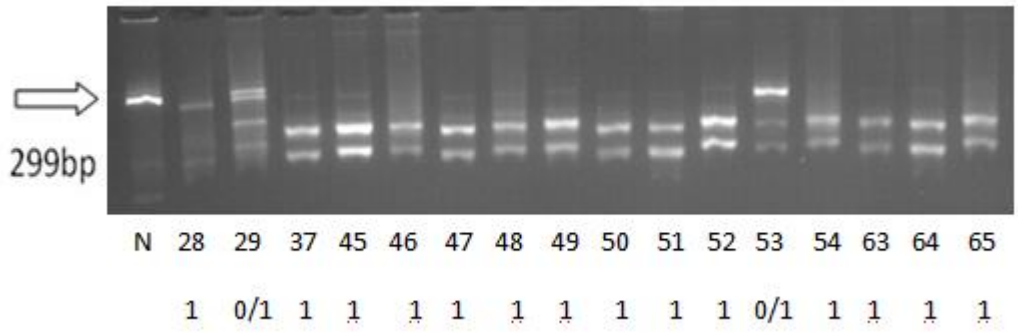


Figure D.55 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. From control groups:28–65 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

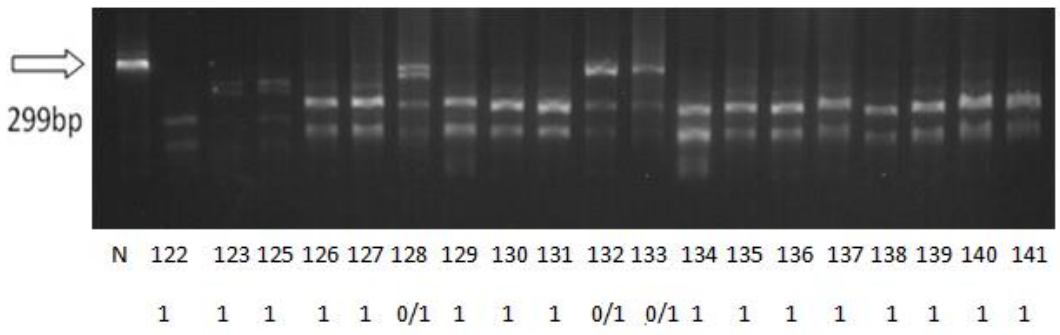


Figure D.56 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. From control groups:122–141 lines show each number of

amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

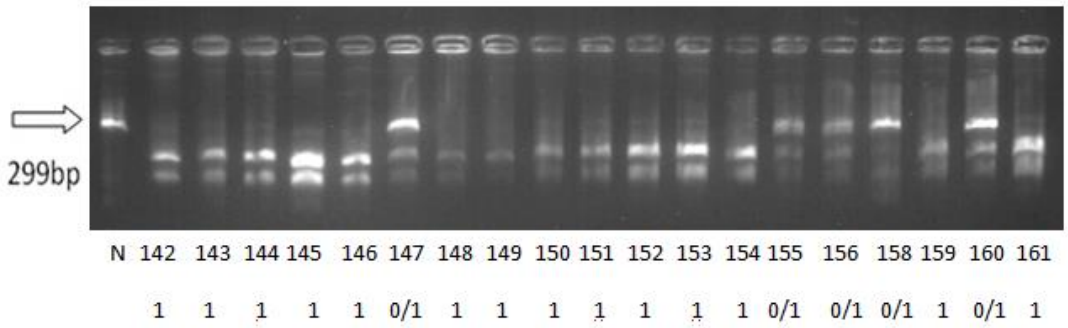


Figure D.57 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. From control groups:142–161 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

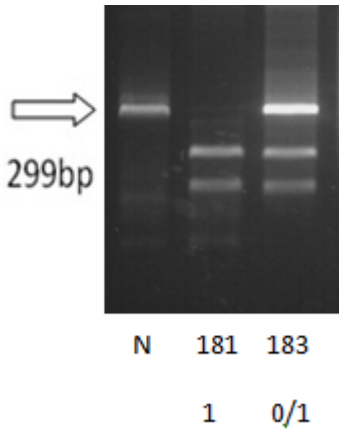


Figure D.58 Fragments detected after digestion by RsaI restriction enzyme. Lanes: N: Negative control. From control groups:181–183 lines show each number of amplified by Rsa restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

EcoNI ENZYME

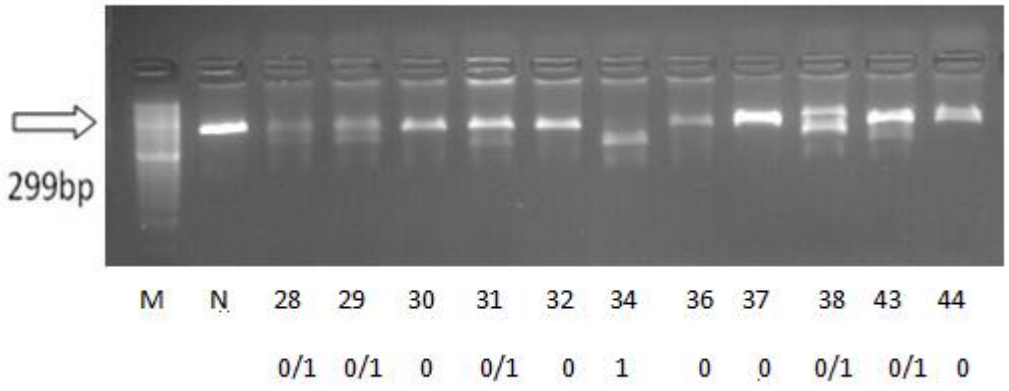


Figure D.59 Fragments detected after digestion by EcoNI restriction enzyme. Lanes: N: Negative control. M: size marker DNA (100-bp DNA ladder). From control groups:28–44 lines show each number of amplified by EcoNI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

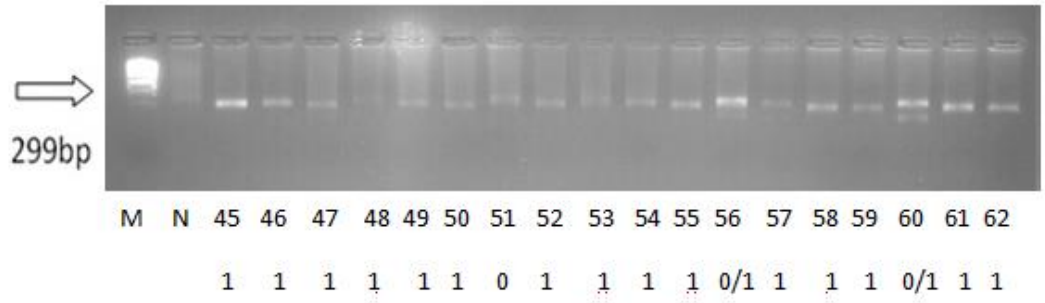


Figure D.60 Fragments detected after digestion by EcoNI restriction enzyme. Lanes: N: Negative control. M: size marker DNA (100-bp DNA ladder). From control groups:45–62 lines show each number of amplified by EcoNI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

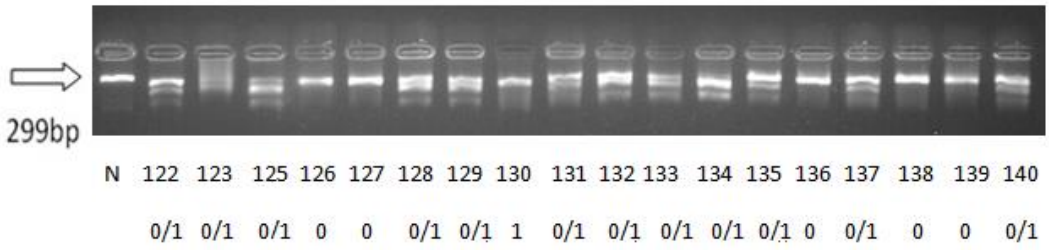


Figure D.61 Fragments detected after digestion by EcoNI restriction enzyme. Lanes: N: Negative control. From control groups:122–140 lines show each number of amplified by EcoNI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

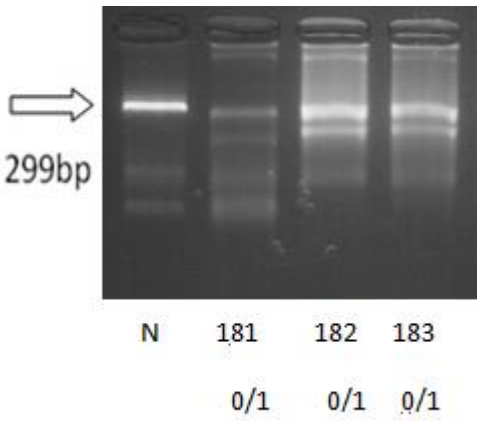


Figure D.62 Fragments detected after digestion by EcoNI restriction enzyme. Lanes: N: Negative control. From control groups:181–183 lines show each number of amplified by EcoNI restriction enzyme.299 base pair non-cleaved fragments were indicated as "0" and cleaved fragments were marked as "1", showing these alleles as homozygotes. If the number consists of both celaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1".

APPENDIX E

E.1 Lists of PatientGroup with DPB1 Alleles

Patient No	DPB1 Allels	Patient No	DPB1 Allels	Patient No	DPB1 Allels	Patient No	DPB1 Allels
2	0402/1501	**36**	0401/0401	**69**	0402/0402	**98**	1701/0402
3	0402/0201	**38**	0401/0201	**70**	0201	**100**	0402/0201
6	1601/1601	**39**	0401/0201	**71**	0401/0201	**101**	0402
7	1601	**42**	1601/0101	**72**	0401/0401	**103**	1701/0402
8	0601/1401	**43**	1001/1301	**73**	1701/0301	**104**	0402/0402
9	0402/0402	**45**	0402/0201	**74**	0201/0201	**105**	0402
10	0402/0402	**49**	1601/1601	**75**	0402/0201	**108**	1601/0402
11	0101	**51**	0402/0201	**77**	0401	**109**	0101/0101
12	0401/0401	**52**	0201/0201	**78**	0101/0101	**110**	0101
13	0401	**53**	1001/1401	**79**	0101	**111**	1601/1601
14	0201/0101	**54**	0402/0201	**81**	1401/0101	**113**	1601/1601
15	0101/1301	**55**	1601/0301	**82**	0101/0101	**114**	0402
17	1701/0401	**56**	0201/0201	**83**	1401/0101	**115**	1601/1601
18	0101/1401	**57**	0301/1001	**84**	1401/0101	**116**	1601/1601
19	0101/0101	**59**	0201/0201	**85**	1701/0402	**117**	1601/1601
21	0201/0201	**60**	0401/0401	**86**	1601/1601	**118**	0402/0201
22	0101/1301	**61**	1601/1301	**87**	1601/1601	**120**	0101/0101
23	0402/1701	**62**	1701/0401	**88**	1601/1601	**121**	1601/1301
24	0401/0401	**63**	0401/0401	**89**	1601/1601	**122**	1701/0402
26	0402/0201	**64**	0402/0201	**91**	0801/0801	**123**	1701/0401
27	0801/0801	**65**	0401/0201	**92**	1701/1401	**124**	0401/1001
29	0101	**66**	1001/1301	**93**	1601/1601	**125**	1101/0101
30	0401/0401	**67**	1601/1601	**95**	1601/0301		
35	0402/0201	**68**	0402/0402	**97**	0101/0101		

E.2 Lists of control group with DPB1 alleles

Control No	DPB1 Allels	Control No	DPB1 Allels	Control No	DPB1 Allels	Control No	DPB1 Allels
27	1101	**53**	0402/0301	**137**	1001/0101	**171**	1601/1401
28	1601/1701	**54**	0201/1501	**138**	0101	**172**	0801
30	0401/0402	**55**	1001	**139**	1001	**173**	0402/1701
31	0201/0402	**56**	0101/1001	**140**	1001	**174**	0201/1501
32	0402	**58**	0201	**141**	1601	**175**	0401/1701
34	0201	**59**	0201	**143**	0201/0801	**176**	0201/1501
35	0201	**60**	0301/1001	**145**	1601/1301	**177**	0401/0201
36	0501	**61**	0201/1501	**147**	1401/0402	**178**	1701/0401
37	0101/0402	**62**	0201/1501	**148**	1001/0201	**179**	0201/1501
38	0301/1301	**63**	1001/1501	**149**	0201	**180**	0201/1101
39	0201/1501	**64**	1601/1101	**150**	0201	**181**	0401/0201
40	0201/1501	**65**	1301/1601	**153**	0201	**182**	0402/1401
41	0201	**66**	1601/1101	**154**	1001	**183**	0401/1701
42	0201	**122**	0801/0101	**157**	1601/0201		
43	0201/0402	**123**	0501/0601	**159**	1601/1001		
44	0402/0801	**125**	0201/1001	**161**	0101		
45	0201/1501	**126**	0101/0801	**162**	1401/1101		
46	0201	**127**	0101/0801	**163**	1401/0401		
47	0201	**128**	1401/0401	**164**	0201/1501		
48	0402/1001	**129**	0301/0101	**165**	1001/1501		
49	0401/0402	**130**	1301/1601	**166**	0201/1501		
50	0201	**131**	1001/0101	**167**	0201		
51	0401/1301	**134**	1301/1901	**168**	0402/1401		
52	0401/0201	**135**	1601/0101	**169**	0201		

APPENDIX F

ENZYMES

DreamTaq DNA Polymerase Fermentas

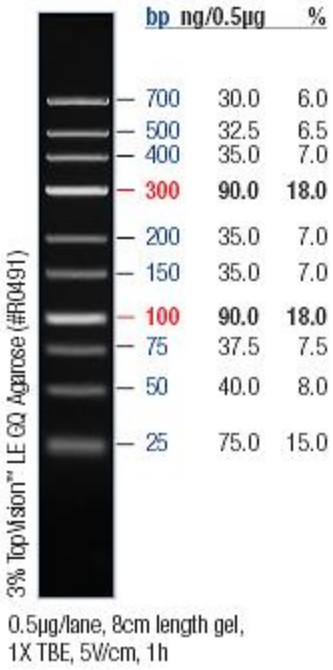
Invitrogen

Bsp12861, FokI, DdeI , BsaJI Fermantas
BssHII, Cfrl31RsaI, EcoNI Sacem
AvaII

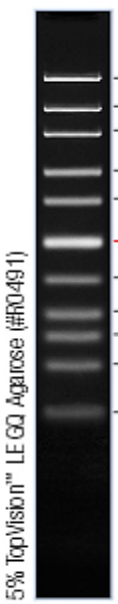
APPENDIX G

MARKERS

O'Gene RulerTM DNA Ladder Low Range Fermentas



O'Gene RulerTM DNA Ladder Ultra Low Range Fermentas



bp	ng/0.5µg	%
300	30.0	6.0
200	32.5	6.5
150	35.0	7.0
100	37.5	7.5
75	37.5	7.5
50	**105.0**	**21.0**
35	37.5	7.5
25	37.5	7.5
20	40.0	8.0
15	47.5	9.5
10	60.0	12.0

0.5µg/lane, 8cm length gel,
1X TBE, 5V/cm, 1h

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

- Katrinli S., Doganay L., Colak Y., Senates E., Ozturk O., Ulasoglu C., **Karatas G. N.,**Tuncer I., Dinler-Doganay G., 2013: The of HLA-DQB1 Alleles with Nonalcoholic Fatty Liver Disease in Turkish Population. *2nd Internatinal Congress of the Molecular Biology Association of Turkey* 22-23 November 2013 ITU Istanbul- TURKEY
- Katrinli S., Doganay L., Colak Y., Senates E., Ozturk O., Ulasoglu C., **Karatas G. N.,**Tuncer I., Dinler-Doganay G., 2013: The association of HLA-DQB1 Alleles with Nonalcoholic Fatty Liver Disease in Turkish Population. *American Society of Human Genetics Congress* 22-26 October 2013 Boston-USA