İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

INVESTIGATION OF HLA DRB1 ALLELES IN THE TREATMENT OF CHRONIC HEPATITIS B PATIENTS

M.Sc. THESIS

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Department of Advanced Technologies

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KRONİK HEPATİT B HASTALARIN TEDAVİSİNDE HLA DRB1 ALLELERİN İNCELENMESİ

YÜKSEK LİSANS TEZİ

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KASIM 2011

Arta FEJZULLAHU, a **M.Sc.** student of ITU **Institute of Science and Technology** student ID **521081076**, successfully defended the **thesis** entitled "**INVESTIGATION OF HLA DRB1 ALLELES IN THE TREATMENT OF CHRONIC HEPATITIS B PATIENTS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission: 05 September 2011

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To My Dear Family and Loved Ones,

FOREWORD

I would like to express my deep graditude to my supervisor Assist. Prof. Dr. Gizem Dinler DOĞANAY for the inspiring guidance, support and the opportunities she provided me. It was a great pleasure for me to carry on this thesis under her supervision. I want to thank her for encouraging me to establish this thesis, which could not have been accomplished without her.

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ABBREVIATIONS

Adv : Adefovir
ALT : Alanine aminotransferase
cccDNA : Circular covalently closed DNA
DNA : Deoxyribonucleic acid
Etv : Entecavir
HBV : Hepatitis B virus
HCC : Hepatocellular carcinoma
HLA : Human Leukocyte Antigen
IDDM : Insulin dependent diabetes mellitus
IFN : Interferon
Lam : Lamivudin
MHC : Major Histocompatibility Complex
NK : Naturel Killer
PCR : Polymerase Chain Reaction
RD : Rheumatoid Arthritis
RNA : Ribonucleic acid
Tdf : Tenofovir
TLR : Toll like receptor
TNF : Tumor necrosis factor

INVESTIGATION OF HLA DRB1 ALLELES IN THE TREATMENT OF CHRONIC HEPATITIS B PATIENTS

SUMMARY

Chronic hepatitis B virus (HBV) infection is a global health problem with an estimated 350 million people chronically infected worldwide. Considerable evidence suggests that the progression of hepatitis B virus infection is influenced not only by the viral genotype and the level of viremia but also host genetic factors play a significant role in the pathogenesis and clinical outcome of chronic HBV infection in several ethnic groups. It is mentioned that both cellular and humoral responses are required for viral clearance. Hence, the aim of this study is to investigate the association between the polymorphisms of HLA-DRB1 alleles and viral hepatitis B in Turkish population.

In this study 324 patients were included; 63.4% male (n=204), 36.6% female (n=120) and, where 23.5% (n=76) of patients being HBe antigen positive, 23.8% (n=77) having cirrhosis. The median age was 44 years (age between 39-49), median follow up time was 86.5 months. In the control group 150 healthy cases were included (for DRB1*07 allele). HLA-DRB1 alleles in 324 patients with chronic hepatitis B and 150 controls were analyzed using the polymerase chain reaction/sequence specific primer (PCR/SSP) technique.

In our group of patients, DRB1 alleles were not related to active or inactive disease, or interferon treatment response or virologic breakthroughs observed during nucleoside analog treatments. On the other hand we have found statistically significant frequency of DRB1*07 allele (p=0.003) in our case-control study. Moreover, our data showed that, patients who had DRB1*07 allele had lower probability to have cirrhosis (likelihood ratio chi square: 4.17, p=0.04), suggesting that DRB1*07 might be associated with low risk of cirrhosis development.

In this case, DRB1*07 allele may be one of the host factors, which influences immune response to HBV, by self-limiting cirrhosis, one of the main complications of chronic HBV. Findings from this thesis are expected to acknowledge the relationship between chronic HBV infection and HLA DRB1 polymorphisms. Additional study is needed to validate these findings and to further explore the genetic pathogenesis of HBV infection.

KRONİK HEPATİT B HASTALARIN TEDAVİSİNDE HLA DRB1 ALLELERİN İNCELENMESİ

ÖZET

Kronik hepatit B virüs enfeksiyonu dünyada yaklaşık 350 milyon kişiyi enfekte eden, en yaygın bulaşıcı hastalıklardan birisi olarak global bir sağlık sorunu teşkil etmektedir. Yapılan çalışmalar, hepatit B virüs enfeksiyonun'un progresyonunda sadece viral genotip ve vireminin değil, aynı zamanda konak genetik faktorlerinin de oldukça önemli rol oynadığını vurgulamaktadır. Aynı zamanda, hem hücresel hem de humoral immün yanıtın virüsün eredike edilmesi için gerekli olduğu birçok deneysel çalışmada gösterilmektedir.

Bu nedenle, bu çalışmanın amacı, Türk toplumunda HLA-DRB1 allelleri ve viral hepatit B klinik seyri arasındaki ilişkiyi araştırmaktır. Bu çalışmaya, 324 hasta dahil edildi; 63.4% erkek (n=204), 36.4% kadın (n=120) ve 23.5% (n=76) HBe antigeni pozitif hastalar ile 23.8% (n=77) siroz olan hastalar. Ortalama yaş 44 yıl (39-49 yaş) arası, ortalama takip süresi ise 86.5 ay idi. Kontrol grubunda ise 150 sağlıklı kişi dahil edildi (DRB1*07). Kronik hepatit B olan 324 hastada ve 150 sağlıklı kişide HLA-DRB1 allelleri polimeraz zincir reaksiyonu/sekans spesifik primer (PCR/SSP) tekniği kullanılarak analiz edildi.

Bu çalışmada, aktif ya da inaktif hastalık veya interferon tedavisine yanıt veya nükleozid analog tedavisi sırasında gözlenen virolojik kırılma (*breakthrough*) olayları ile DRB1 allellerinin arasında anlamlı ilişki gözlemlenmemiştir. Diğer taraftan vaka-kontrol grubumuzda DRB1*07 alleli için istatistiksel olaraka anlmalı değerler elde edildi (p=0.003). Dahası, elde dilen veriler sonucunda, DRB1*07 alleli saptanan hastalarda siroz anlamlı olarak daha nadir gözlemlenmiştir (likelihood ratio chi square:4.17, p=0.04).

Bu durumda, DRB1*07 alleli HBV immun yanıtını etkileyen önemli konak faktörlerden biri olabilir. Ayrıca, kronik HBV ana komplikasyonlar'dan biri olan sirozun kendi kendini sınırlamasında oldukça önemli rol oynadığı düsünülmektedir. Bu çalışma, kronik HBV ve HLA DRB1 polimorfizmi arasındaki ilişkinin ortaya konmasında yardımcı bilgiler sunmaktadır. HBV'nin genetik patogenezinin anlaşılması ve bu çalışmadaki bulguların doğrulanması için daha büyük hasta gruplarında HLA polimorfizmi araştırılmalıdır.

1. INTRODUCTION

1.1 Chronic Hepatitis B

Chronic Hepatitis B virus infection is a major problem causing both acute and chronic liver disease by affecting approximately 350 million people worldwide [1]. Despite the prophylactic vaccination programs in communities, according to the data by the World Health Organisation, an estimated 600 000 people die each year due to the acute or chronic consequences of hepatitis B [2,3]. HBV infection may persist and culminate in chronic hepatitis B. In adulthood chronicity is observed approximately 15% of the cases, but still the factors that determine the HBV persistence and clearance are not very well understood [2,3]. However, various host conditions such as age of infection, gender, and immune response were observed to influence the risk of persistent HBV infection and have shown remarkable clinical and pathogenic differences among HBV genotypes. Data obtained to date mentions the importance of host immune response in determining the outcomes of HBV infection [2,3]. In this line, we aim to delineate the relation between the human leukocyte antigen class II molecule and the host immune response of chronic hepatitis B virus infection.

1.2 Genome Organization of Hepatitis B

Hepatitis B virus (HBV) is a species of the genus Orthohepadnavirus which is a prototype member of Hepadnaviridae family of viruses [2]. Partly double stranded HBV genome is a circular DNA with about 3,200 nucleotides. The minus strand of the virus is almost a complete circle and encodes the structural proteins of the virus; whereas the second strand, plus strand, overlaps with most parts of the minus strand and encodes some of the structural proteins, but it is shorter in length [4-7]. Once the virus invades the body, it binds to the cell surface and penetrates to the host cells with the help of its envelope proteins. Inside the plasma membrane of the cell, the virus is not degraded but is transported to the nucleus where the partially circular DNA is made into covalently closed circular DNA (cccDNA) and the cccDNA

functions as a template for RNA synthesis which is then reverse transcribed into an open, circular DNA.

The new formed, circular DNA is subsequently packaged into viral envelopes in the endoplasmic reticulum and then transported out of the cell (Figure 1.1) [4-7].

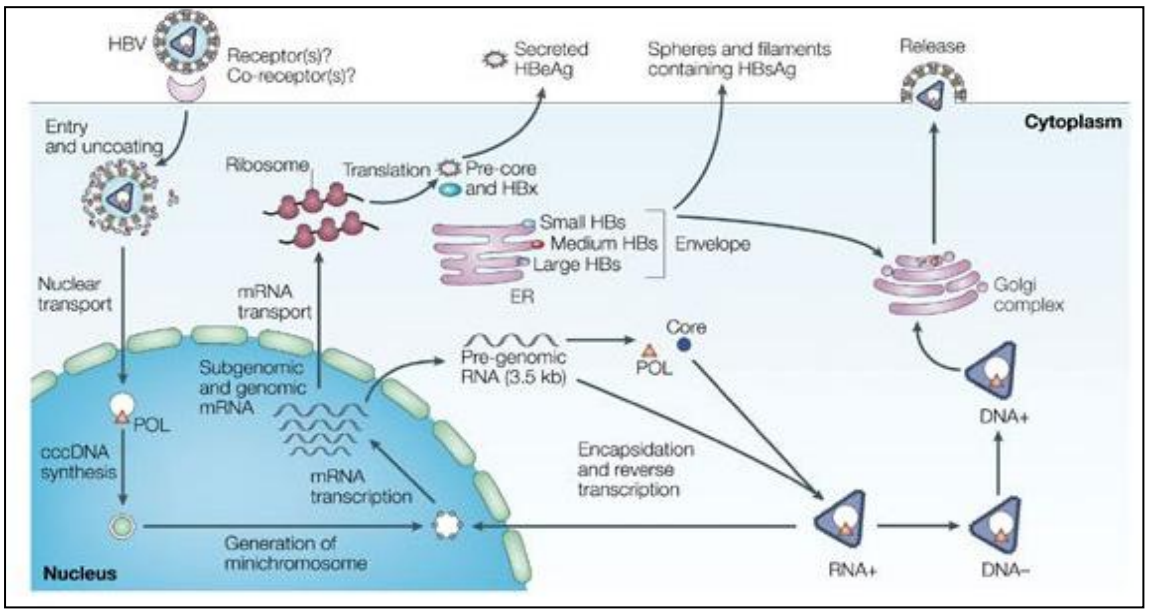


Figure 1.1 : A simplified diagram of HBV replication [8].

Unlike other viruses, HBV does not integrate itself into the host genome but retains a core of cccDNA in the nucleus of the cell by transporting some of the newly synthesized HBV DNA back to the nucleus as seen in the chronic condition of hepatitis B [4-7] causing a continues replication of itself inside the host's cells (Figure 1.1) [4-7].

There are four open reading frames (four known genes) running in one direction without any noncoding regions and these open reading frames (ORFs) code for: C, P, X, and S genes. These overlapping ORFs in the genome are responsible for the transcription and expression of seven different hepatitis B proteins [3].

The C gene is divided into a core (codes the structural protein of the nucleocapsid-HBcAg) and a precore (codes the HBeAg) region by 2 in-frame initiating AUG start codons. The function of nucleocapsid protein is to enclose the viral DNA and the DNA polymerase that is essential for HBV replication. HBeAg which is produced by the proteolytic processing of the pre-core protein, [9-11] is considered to be a marker for the viral replication; hence, seroconversion from HBeAg to anti-HBe positivity usually indicates a low level of viral production and low level of serum HBV DNA.

The gene P also known as HBV polymerase is an enzyme with multiple viral life related functions. Its primary polymerase activity enables the synthesis of DNA using either RNA or DNA templates, its RNase H activity provides the degradation of the RNA strand of RNA-DNA hybrids, and the packaging of the RNA pregenome into nucleocapsids [2,3].

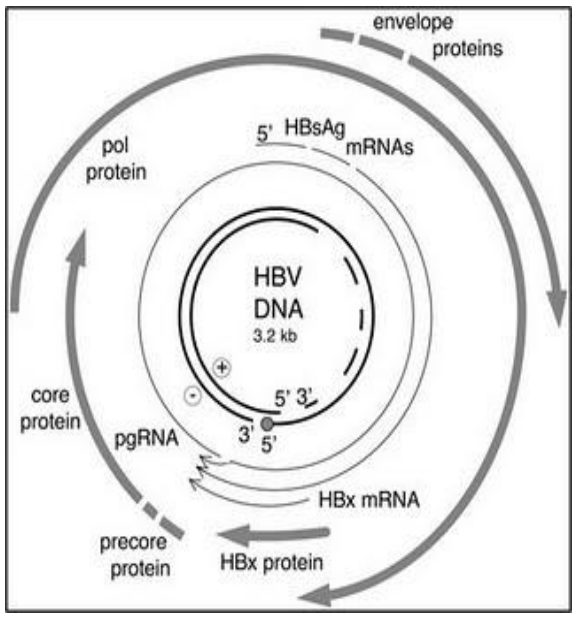


Figure 1.2 : Genomic structure of HBV [8].

X - a transactivator of viral transcription, is thought to play a major role in hepatocellular carcinoma (HCC) development [9,13]. *In vitro* studies presented that HBx is implicated as one of the causative agents in carsinogenesis by being responsible for the activation of both cellular immune response and viral promoter regions, however, its contribution to tumor invasion and metastasis in the course of HBV infection has not been established. A study done in Turkey, which successfully generated stable transmission and expression of the hepatitis B virus total genome in hybrid transgenic mice until F10 generation, showed that even in the presence of HBx protein expression in hepatocytes there was not any observation of neoplasia during the life span and HCC development [12]. The observation of distinct results on susceptibility to hepatocarcinogenesis among independent study groups was mentioned to be due to mouse strains used and other possibilities such as deletions in the sequence of HBx gene or trangene instegration sites of HBV [12].

The S gene codes for the surface antigen (HBsAg) (Figure 1.2), which is one long open reading frame that encodes 3 polypeptides of the surface antigen (preS1, preS2 and S - produced from the alternative translation start sites) [3,9-11,14]. The most significant part of HBV genome among the four ORFs (C, P, X and S genes), is the HBsAg gene, which is particularly important because the S protein product contains an epitope that generates a protective immune response against the virus, and is thus key in the current therapy of HBV infection [3,9].

1.2.1 Genotypes and mutations of HBV

According to overall nucleotide sequence variation, in the genome of HBV seven genotypes were defined [7,9]. The figure below shows the genotypes together with its serotypes with distinct geographical distribution (Figure 1.3) [7,9].

Genotypes of Hepatitis B Virus		
Type	***Subtype***	***Geographical Distribution***
A	***adw, adw2, ayw1***	***US, Northern Europe, Africa***
B	***adw2, ayw1***	***China, Indonesia, Vietnam***
C	***adr, ayr***	***China, Korea, Japan, Vietnam***
D	***ayw2, ayw3***	***Mediterranian, Middle East, India***
E	***ayw4***	***West Africa***
F	***adw4***	***Polynesia, US (rare)***
G	***adw4***	***Europe, US (rare)***

Figure 1.3 : Genotypes of Hepatitis B virus [7,9].

Amongst the genotypes, A-G, the most frequently observed genotype in our country is the genotype D [15] and in many studies it is shown that the clinical outcome of the hepatitis B disease differs in accordance with genotypes [15]. For instance, the study conducted in Turkish population including genotype D demonstrated that isolates from five AntiHBe positive patients contained specific mutations, which were reported to increase viral replication and result in a poor prognosis [15]. The correlation between HBV genotypes and clinical outcome of patients with HBV in Chinese population was shown that patients with genotype C infection have a more aggressive clinical phenotype than those with genotype B infection [16]. Clinical studies also revealed that, the rate of HBsAg clearance was higher in genotype A than genotype D patients [16].

The gene mutations are summarized according to the ORFs: S gene mutations: Mutations in the HBV surface protein that facilitate the escape from host immunity are responsible for the failure of immune prophylaxis in infants who received HBV vaccine and in liver transplant recipients who received hepatitis B immune globulin

[3,17]. The common amino acid mutations 125 (M → T) and 127 (T → P) were observed in surface antigen in Turkish population [18]. Another study have revealed four novel mutations sL97S, sL98S, sG102R, and sA159P) in the preS/S gene region. However, the biological importance of these mutations is not well determined [19]. Another clinically importat mutation was shown to be G145R present on the S region of the HBV genome. G145R mutation results not only in the loss of group-specific antigenic determinant a, which is the main target of the vaccine response, but also in impaired S secretion and decreased virion stability [14].

C gene mutations: The mutations in the pre-core region may result in inability to produce HBeAg (G>A at nt 1896), and this affects disease severity or serological and clinical manifestations [9]. HBeAg – negative variants are common for genotypes D as observed in Turkish population [5,13,17] Substitutions in the core region are shown to concentrate in B cell epitopes in progressive disease and in T helper cell epitopes during clinical remission [12]. P gene mutations can affect replicative efficiency and resistance to antiviral therapy, and these mutations are detected after the use of the reverse transcriptase inhibitor drugs [9].

The largest ORF in the HBV genome, which encodes for the hepatitis B polymerase protein (P gene) has been divided into four characterized 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 (Table 1.1) [9].

Table 1.1 : Clinically important hepatitis B virus (HBV) mutations [9].

Clinically important hepatitis B virus (HBV) mutations			
MUTATION	*LOCATION IN THE HBV GENOME*	*RISK FACTORS FOR MUTATION*	*SIGNIFICANTE*
YMDD and multiple others	*P (polymerase) gene*	*Previous treatment with nucleoside analogues*	*Development of drug resistance*
Precore or core	*C (core) gene*	*More common in Asia and Southern Europe (30%-90%) than in the United States (10%-40%)*	*Failure of HBV e antigen secretion but with a remaining risk for progressive liver disease*
Vaccine escape	*''a'' determinant of the S gene*	*HBV vaccine or immune globulin recipients*	*Possibly increased risk of hepatocellular carcinoma or prolonged HBV infection*

HBV Polymerase

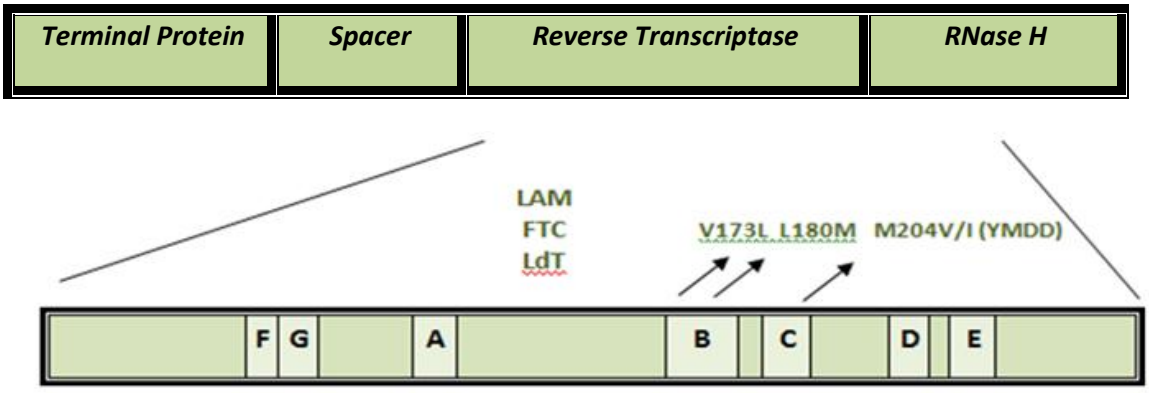


Figure 1.4 : Mutations in HBV confer resistance to antiviral drugs [20].

The most common drug-resistance mutation appears in the YMDD locus of the reverse transcriptase domain of the HBV DNA polymerase gene, and leads to the substitution of methionine by valine or isoleucine, which is denoted by reverse transcriptase M204V/I [21-24]. These mutations decrease the capacity of viral replication. On the other hand, although the presence of other compound mutations (rtL180M, rt173L, rtL80V/I, rtL82M, rtF166L, rtA200V ve rtV173L) may result in an increase of viral replication. Adefovir dipivoxil resistance mutations on the DNA polymerase are positioned in the 181st and 236th residues by the conversion of

alanine to valine (A181V) and asparagine to threonine (N236T), respectively [21-24].

X gene mutations: To our knowledge, X protein may alter host gene expression by causing the development of HCC [9,13]. X gene affects the control of transcription by acting as a transactivator of various viral and cellular promoter/enhancer factors and can mediate the activation of signal transduction pathways [9,13]. Any mutation on the X gene can therefore affect DNA repair, cell cycle control, and apoptosis [9,13]. Even though various roles of the X gene is attributed, its exact function in the cell is still unknown, so the clinical significance of HBx mutations are not clearly understood yet [9,13].

1.3 The Epidemiology of Hepatitis B

1.3.1 Transmission of HBV infection

Transmission of hepatitis B virus results from the exposure to infectious blood or body fluids containing blood, where the possible forms of transmission include sexual contact, blood transfusions, re-use of contaminated needles and syringes, and vertical transmission from mother to child during childbirth [25]. In countries with high prevalence of HBV infection, the transmission of hepatitis B infection is observed more frequently in prenatal period and most common at birth through blood and infected fluids. In this case, the rate of transmission of infection to infants from HBeAg positive and negative mother is about 70-90% and 5-20%, respectively. After 2-3 months, antigemia is observed in infants. If the blood is positive for HBV surface antigen but negative for HBeAg, the risk of HBV transmission from a single needle stick is 1% to 6% and 22% to 40% if positive for both antigens [7,26].

1.3.2 HBV infection worldwide

HBV infection is highly prevalent in Asia, sub-Saharan Africa, and other areas of the developing world, but less in United States, except in Alaskan natives and immigrants (45% and 80%) from regions of high prevalence. A study done in Turkish population with 2683 controls under age 30 (in 8 different regions of Turkey), showed that the rate of positivity of HBsAg was 5.4% and for the Anti HBcIgG, it was 17% and the rate of positivity for HBsAg in transplant cases was approximately 4.9% [7, 25,27-29].

1.4 Immunopathogenesis of Hepatitis B virus

The course of hepatitis B virus infection appears to be determined by variations in viral virulence and by the host immune response [host factors like the major histocompatibility complex [30] (MHC)]. Considerable evidence suggests that host genetic factor plays a significant role in the pathogenesis and clinical outcome of chronic HBV infection in several ethnic groups, where the damage of the liver is observed mostly associated with the host`s immune response [2-3].

Hepatitis B virus infection may either be acute or chronic. The figure below shows the clinical outcome of Hepatitis B virus infection (Figure 1.5).

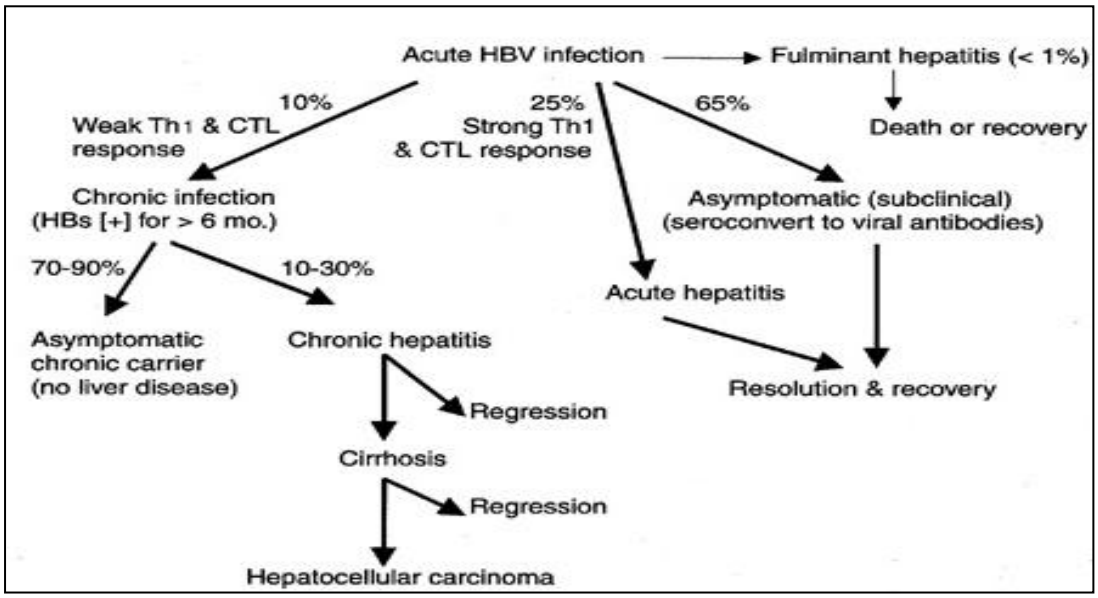


Figure 1.5 : Outcome of Hepatitis B Virus Infection [2-3].

Acute infection with hepatitis B virus is associated with acute viral hepatitis where the infection may be entirely asymptomatic and may go unrecognized and the immune response is normal (self-limiting). However, the insufficiency of immune response against the virus leads to chronic hepatitis B virus infection (long-standing). Roughly about 5-10% of chronic hepatitis B is observed in patients after they experience the infection. Chronic hepatitis B virus may be either asymptomatic or associated with a chronic inflammation of the liver, causing cirrhosis and liver cancer (hepatocellular carcinoma) [31, 32].

Both innate immunity and adaptive immunity are involved during infection invasion, where the immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during this infection. Innate immunity gets

triggered immediately after infection to limit the spread of the pathogen and initiate efficient development of the adaptive immune response. To date, there are several real animal model of HBV infection in the chimpanzee and mice. The experiments in chimpanzees with acute hepatitis B from the period of logarithmic replication of virus (5-7 weeks) showed that there was not observed any activation of innate immunity [33,34]. In several virus infections when the virus enters the host, recognition of viruses by Toll like receptors (TLR) result in secretion of interferon alpha/beta showing an anti-viral activity by triggering intracellular response pathways, where these cytokines increase the expression of cell surface MHC I related genes [4,35]. Thus, cytokines induce an efficient triggering of immunity and the fragments of viruses within the infected cells are then displayed to cytotoxic T cells by MHC Class I molecules. Cytotoxic T cells kill the infected cells and lead to elimination of them. However, in hepatitis B infection there is no sign of innate immune response even in the first 5-7 weeks of disease. Moreover, several studies show that both in chimpanzees and the woodchuck model until the logarithmic increase of HBV DNA in the liver or the peripheral circulation in silent period, there is no evidence of activation of the innate immune system and the logarithmic replication of HBV DNA to 10^{10} copy/ml result in infection of all hepatocytes (Figure 1.6) [4-6,10,13,30,34].

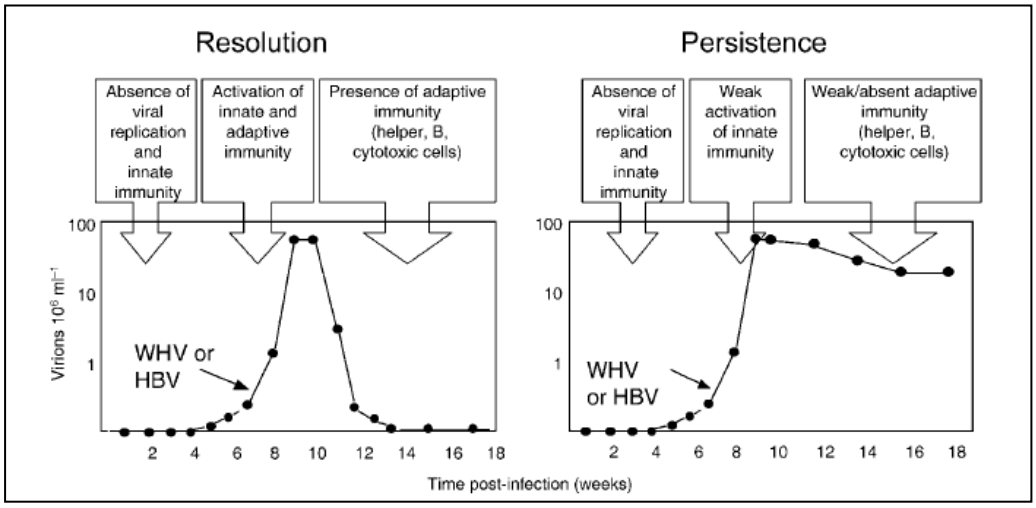


Figure 1.6 : Kinetics of innate and adaptive immune response during hepadnavirus infection. HBV, hepatitis B virus; WHBV, woodchuck HBV [4].

In the course of hepatitis B virus infection, the observation of logarithmic increase of HBV DNA in blood results in the activation of the adaptive immune response.

Subsequently, the stimulation of CD8+ T lymphocytes lead to clinical outcome of hepatitis B infection together with the increase of enzymes involved in this period. The secretion of the different IgG subclass profiles of anti-HBs, anti-HBc, and anti-HBe in HBV infection status were revealed by CD4+ T cells. CD 4+ cells cause the stimulation of anti-HBc antibody, followed by activation of anti-Hbe and anti-preS [5,34]. The antibody component of the adaptive immune response, provides a defense against virus infection, and as observed T cells are the primary mechanisms in the adaptive immune response to clear virus-infected cells. When major histocompatibility complex (MHC) restricted, T cells enter the liver and recognize antigen, they kill some of the infected cells and secrete IFN-γ, which induces the expression of a large number of genes that enhance antigen processing and presentation; recruit macrophages, NK (Natural Killer) or dendritic cells, and T cells that also produce IFN-γ; and amplify the process [4,6,10,13,30,34,35]. Moreover, several studies have shown that cytotoxic T cells response to HBV infection is polyclonal and multispecific for acute HBV infection, and these highly coordinated cellular and molecular events continue until the infection is terminated (Figure 1.7) [5,34]. It is also important to note that the studies in the HBV transgenic mouse model revealed that while the depletion of CD4+ T cells result in minor effects in the course of HBV infection, the depletion of the CD8+ T cells result in major effects as delayed and prolonged virus clearance and final virus elimination [5,34, 36,37]. After a long period of infection the reappearance of the CD8+ cells with IFN-γ rapidly clear the remaining infected cells as actually observed in acute hepatitis B infection. In contrast to the strong and multispecific T cell responses observed during self-limited HBV infection, the appearance of such an immune response in the patients with chronic HBV infection was shown to be highly weak and narrowed [5,34,36,37]. Despite of efforts in the field, still the virus specific T cell response in chronic HBV infection is poorly understood. The unresponsiveness to virus is correlated with many reasons such as T cell deletion, ignorance, T cells dysfunction, specific HLA polymorphisms, and abnormal HLA/peptide tetramer binding.

Although the presence of mutations on genes of HBV may lead to inactivation of T cell response, it was shown to occur much less when compared with hepatitis C virus [5,34,36,37]. In our study, we aim to understand the association of HLA alleles with

the course of chronic HBV infection one of the reasons of T cell failure mentioned above.

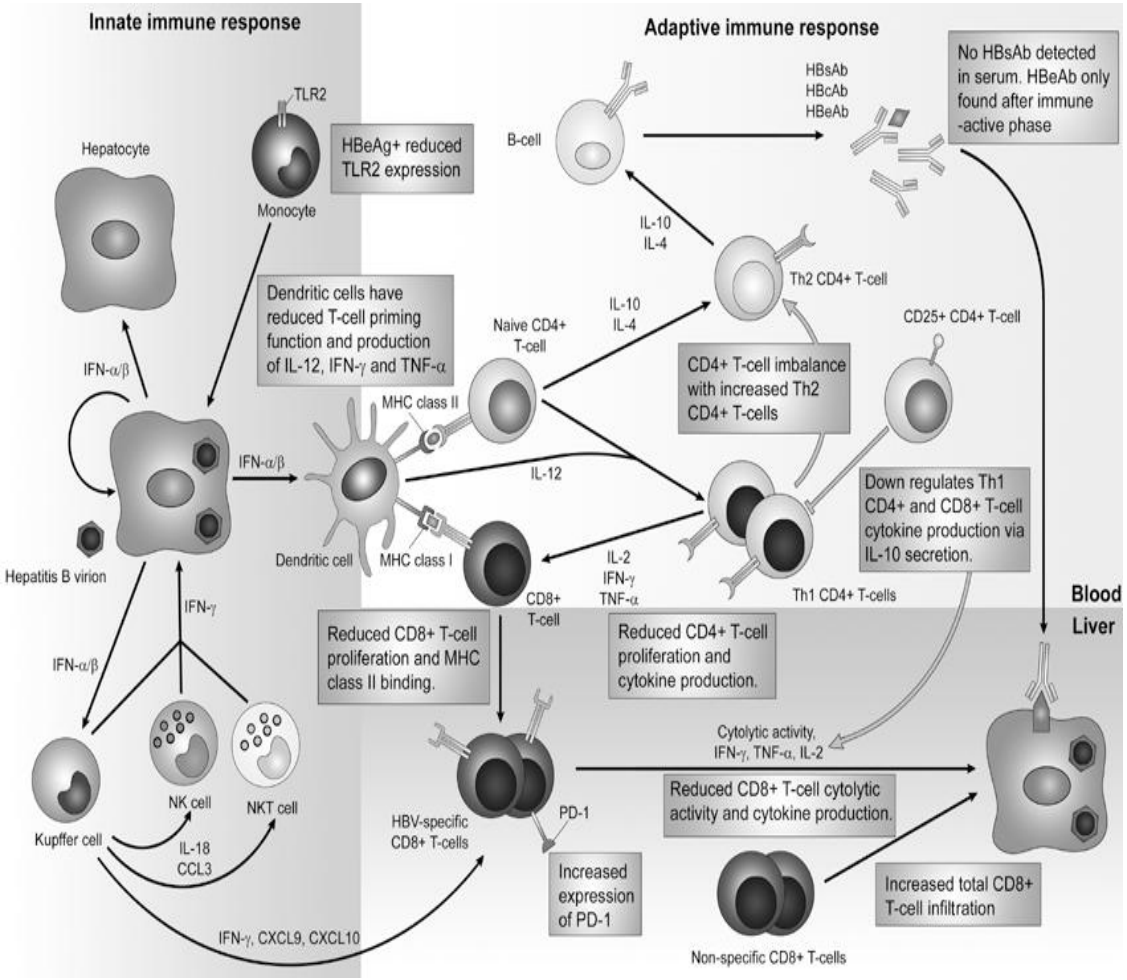


Figure 1.7 : Immune responses against HBV and effects of chronic HBV infection [34].

1.5 Clinical Outcome and Treatment of Chronic Hepatitis B Virus Infection

Based on the virus and host interactions, the natural history of perinatally acquired HBV infection in carriers who acquire the virus early in life can be divided into four dynamic phases (Figure 1.8) [38].

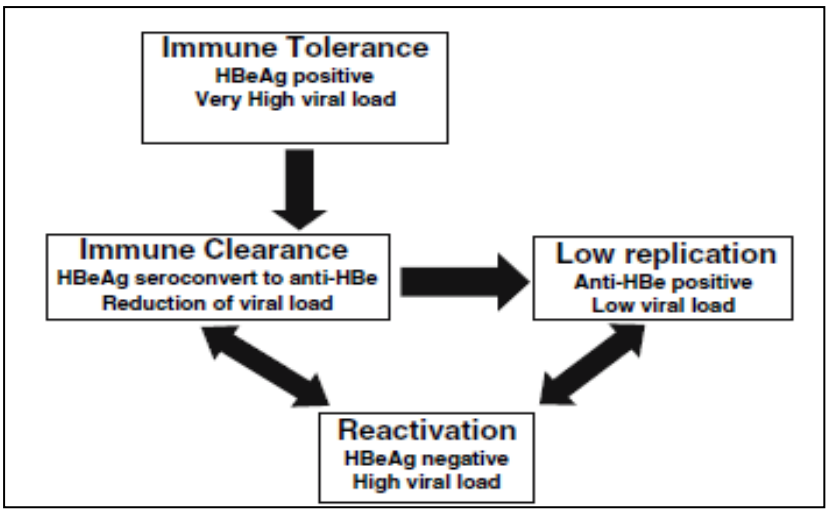
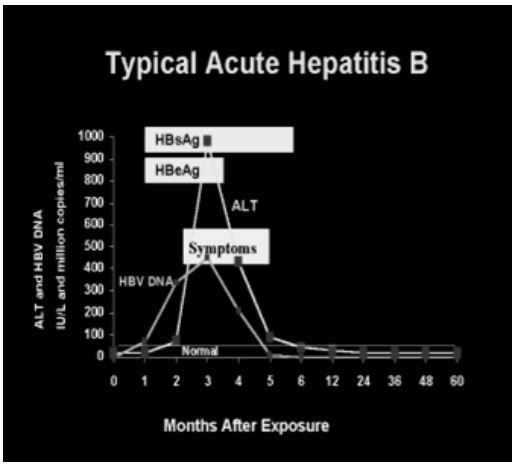


Figure 1.8 : Natural history of perinataly acquierd HBV infection [38].

The immune tolerance phase is associated with high serum levels of HBV DNA ($<10^7$ copies/ml), limited immunological reactivity to HBV, extensive viral replication, and expression of HBeAg. In immune tolerant cases with positive HBeAg, the level of alanine aminotransferase (ALT) in serum is presented normal for a long period and no symptoms are observed, although liver of some young HBV carriers who have immune tolerance, shows some abnormalities [3,9,14,25]. Chronic HBV patients show increased levels of ALT, triggering the immune clearance and the majority of HBV carriers seroconvert from HBeAg to anti-HBe [3,9,14,25]. After HBeAg seroconversion, patients usually evolve into the low replication phase that is characterized by absence of HBeAg, presence of anti-HBe, persistently normal ALT levels, low or undetectable serum HBV DNA ($<10^4$ copies/ml), and even HBsAg seroclearance with mild hepatitis and minimal fibrosis. Patients negative for HBeAg are considered to have non-replicative HBV infection, and their serum ALT levels are observed to be normal or nearly normal [3,9,14,25].

HBV could replicate in the absence of HBeAg and the reactivation of HBV may occur spontaneously or as a consequence of immunosuppression. In this phase, detectable HBV DNA ($<10^4$ copies/ml), elevated serum ALT, and progressive liver disease will develop [3,9,14,25].

In addition, the presence of high serum levels of ALT in patients with acute hepatitis B virus infection in a period after 6 month is considered as a chronic Hepatitis B infection (Figure 1.9 & Figure 1.10) [39].



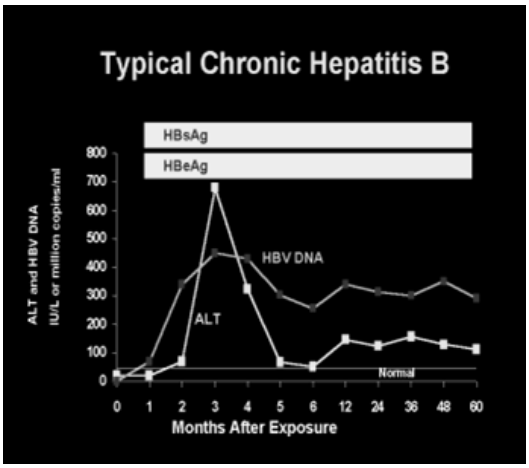


Figure 1.9: Clinical Outcome of Acute HBV [40].**Figure 1.10:**Clinical Outcome of Chronic HBV[40].

Patients with acute HBV infection does not usually require treatment because they clear the infection spontaneously, whereas, treatment of chronic HBV infection may be necessary to reduce the risk of cirrhosis and liver cancer, although, none of the available drugs are able to clear the infection, they only can stop the virus from replicating, thus minimizing cirrhosis and liver damage [41-51]. There are some medications currently in use for treatment of active hepatitis B infection, including antiviral drugs and immune system modulators Lamivudine, Adefovir dipivoxil (Hepsera), tenofovir (Viread), entecavir (Baraclude).Interferon alpha-2a and PEGylated interferon alpha-2a (Pegasys- Pegintron). Lamivudine inhibits hepatitis B viral DNA synthesis [41-51]. Pretreatment ALT is an important predictor of response, with HBeAg conversion occurring in over a third of patients when the ALT is greater than five times normal. While Lamivudine (Lam) benefits patients with HBeAg-negative chronic hepatitis B, the vast majority of patients relapse once treatment is stopped [41-51].

Adefovir (Adv) dipivoxil inhibits DNA polymerase activity and reverse transcriptase. About 50 - 60 % of HBeAg positive and negative hepatitis B (<67% HBV DNA negativity) patients respond to this medication [41-51]. The optimal duration of therapy is not yet clear. The negativity of HBV DNA in patients with HBV treated with tenofovir (Tdf) and entecavir (Etv) showed a percentage above 90. HBeAg negative patients treated with PEGylated interferon alpha-2a showed about 25% negativity of HBV DNA, 34% seroconversion, and about 3% loss of HBsAg, where after the third year 20% of patients show a HBV DNA level below 400 copies [41-51]. Additionally, according to the European Association for the Study of the

Liver (EASL), while the treatment is not recommended to patients involved in immune tolerance group, in the patients with compensated and decompensated cirrhosis, independent of DNA level the treatment is recommended. Furthermore, in patients with high value of ALT and HBV DNA level over 2000 IU biopsy is recommended followed by treatment of chronic HBV infection using antiviral drugs and immune modulators [52].

1.6 Human Leukocyte Antigen (HLA)

Human leukocyte antigen (HLA) is the human version of the major histocompatibility complex (MHC). It is a large genomic region or gene family that consists of more than 140 genes. MHCs play a significant role in the immune system and autoimmunity by distinguishing the body's own proteins from proteins made by foreign invaders such as viruses and bacteria [53-54].

Genes in this complex are categorized into three basic groups: class I, class II, and class III, where the HLA region spans 4 x 106 nucleotides on chromosome 6p21.1 to p21.3, with HLA class II, HLA class III and HLA class I genes located from the centromeric to the telomeric end (Figure 1.11) [54], a region of strong linkage disequilibrum containing many other immune response genes.

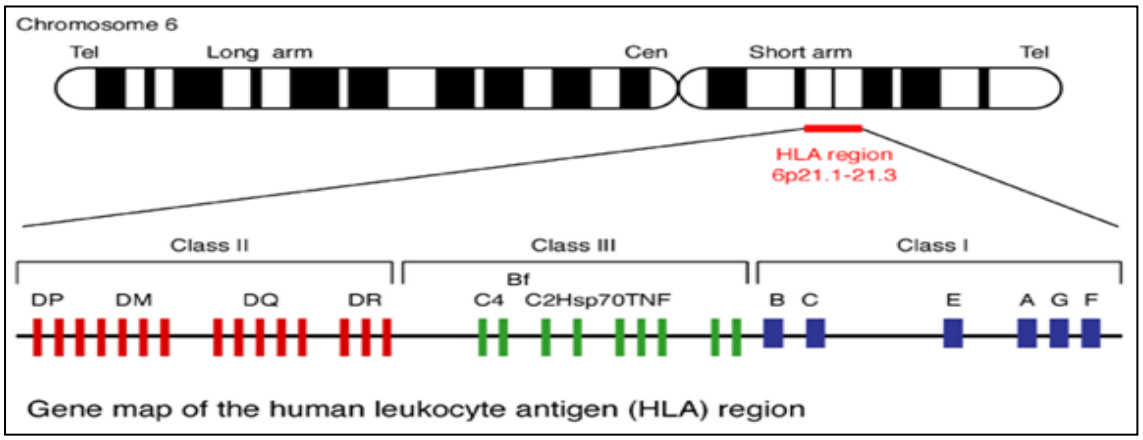


Figure 1.11 : Gene map of the human leukocyte antigen (HLA) region [55].

Humans have three major MHC class I genes, known as HLA-A, HLA-B, and HLA-C. These genes are highly polymorphic, which enables the recognition of a great diversity of virus and bacteria. MHC class I genes are expressed in all nucleated cells. These genes encode two polypeptide chains; α chain & β2-micro-globulin (Figure 1.12), where only the α chain is polymorphic [56-57]. HLA class I presents

peptides to the cytotoxic T cells via the receptors on the cytotoxic T cells and also binds inhibitory receptors on NK cells, thus stimulates immune responses against 'endogenous' antigens and virally infected targets [56,57]. Whereas HLA class II molecules are involved in the presentation of 'exogenous' antigens to T helper cells. There are 3 major MHC class II proteins encoded by the HLA known as HLA-DQ, HLA-DR, and HLA-DP. These genes provide instructions for making proteins that are present almost exclusively on the surface of certain immune system cells, specifically on antigen-presenting cells and proteins contain α & β chains both of which are polymorphic and encoded in the MHC (Figure 1.13) [56-57]. They present antigen fragments to T helper cells by binding to the CD4 receptor on the T helper cells and hence trigger an appropriate immune response to fight against foreign proteins [54,58,59]. In other words, HLA class II genes are expressed as cell surface glycoproteins that bind and present peptide epitopes to CD4 T cells, resulting in their activation. 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 the HLA genes [54,58,59]. There are various crystal structures with HLA-DR1 [69]. These structures revealed that these HLAs have allele-specific motifs [69]. The peptide binds as a straight extended chain with a pronounced twist interacting with the ά and β chains [69]. Peptide specificity of HLA class II proteins comes from the determined polymorphic pockets in the peptide-binding cleft. T-cell receptor recognition is done by both the peptide and the HLAs [69]. There are some HLA-DRB1 types that show polymorphism at the T-cell recognition sites without altering the peptide binding [69]. Due to these changes at the T-cell recognition residues, a weak immune response occurs. Especially HLA-DRB1*07 contains four substitutions at the T-cell interaction residues, which are located at beta30, beta 67, beta71 and beta86 [69].

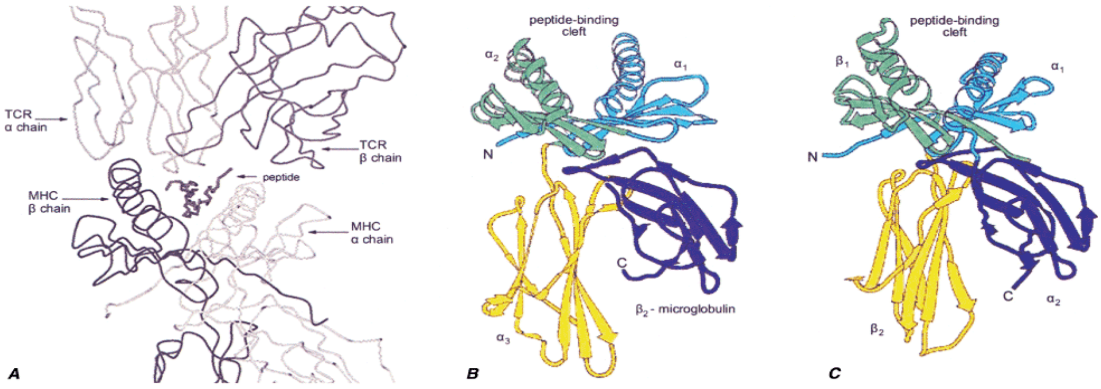


Figure 1.12 : The trimolecular complex of TCR, MHC molecule [56].

Many autoimmune diseases show strong correlations with particular HLA alleles. Studies conducted by many authors have proposed the differential binding of pathogenic peptides to allele specific HLA class I glycoproteins as a mechanism of disease protection [60-62]. However, the mechanism underlying the correlation of particular HLA class II alleles and infectious disease is still unknown. Also the details such as whether specific HLA class II glycoproteins are involved, their specific binding motifs are present or another immune response is involved in the immune response against the infection are not clarified [60-62]. X-ray crystallographic structures revealing the interaction between HLA and antigenic peptides and studies investigating the biophysical properties of MHC relation with its peptides have correlated a potential role for HLA in various autoimmune diseases [36]. For instance, a study in Caucasian population showed that DRB1*0401 and DRB1*0404; DRB1*0101, DRB1*1001, and DRB1*1402 disease susceptibility genes are the major HLA alleles associated with rheumatoid arthritis (RD) [36]. Another study, presented that DRB1*1501, which is in linkage disequilibrium with DRB1*0101 and DQB1*0602 is strongly linked to multiple sclerosis, an inflammatory autoimmune disease in which T cells are reactive towards the myelin sheath [36]. Celiac disease that is one of the most studied HLA class II associated diseases, is observed to be related with the DQA1*0501/DQB1*0201 being in linkage disequilibrium with DRB1*03 [36].

Furthermore, various HLA class II molecules were presented to be linked to an increase in the insulin dependent diabetes mellitus (IDDM) susceptibility [36].

The immune system uses the HLA complex to differentiate self cells and non-self cells, thus protecting the body from several diseases. Table 1.2 shows some disorders related to HLA Class I and HLA Class II genes (polymorphisms).

Table 1.2 : HLA Class I and HLA Class II Related Diseases [36].

	Serological marker	Gene	
Spondilartropatie			
Ankylosing spondylitis	B27	B*2702, -04, 05	++++
Reiter's Syndrome	B27		++++
Acute anterior uveitis	B27		+++
Reactive arthritis	B27		+++
Psoriatic arthritis	B27		+++
Connective Tissue Diseases			
Juvenile arthritis	DR8		++
	DR5		++
Rheumatoid arthritis	DR4	DRB1*0401, -04, -05	+++
Sjögren's syndrome	DR3		++
Systemic lupus erythematosus			
Whites	DR3		+
Japanese	DR2		++
Autoimmune diseases			
Coeliac disease	DQ2	DQA1*0501	+++
		DQB1*0201	
Chronic active hepatitis	DR3		++
Dermatitis herpetiformis	DR3		+++
Psoriasis vulgaris	Cw6		++
Pemphigus vulgaris	DR4	DRB1*0402	+++
	DQ1	DQB1*0503	
Bullous pemphigoid variant	DQ7	DQB1*0301	+
Autoimmune Endocrine Disorders			
Diabetes mellitus type 1	DR4	DQB1*0302	+++
	DQ8	DRB1*0401, -04	
	DR3		++
	DR2	DQB1*0602	__a
Hyperthyroidism **(Graves')**	B8		+
	DR3		+
Hypertiroidism (Japanese)	B35		+
Adrenal insufficiency	DR3		++

1.7 Hepatitis B Virus Infection and HLA

The observation of different immune responses in patients with hepatitis B virus infection has led the scientists to search the MHC (human leukocyte antigen presenting cells) complex in HBV infection. In literature, it was mentioned that

although the mechanism of susceptibility to chronic HBV infection is not well clarified, the course of HBV infection does not appear to be determined only by variations in viral virulence but also may be influenced by the host immune response. Several studies shows that host genetic factors and environmental factors involving HBV genotype are widely viewed as common basis for the distinct outcomes of HBV infection [36,37,60]. In addition, HLA plays a significant role in immunological reaction to HBV infection. Although the pathogenesis of chronic HBV infection is poorly understood, several experimental evidences have reported that successful clearance of HBV has been shown to be associated with vigorous CD4+ T cell response to the nucleocapsid protein, where the kinetics of this response is parallel to the CD8+ T response to both the envelope and nucleocapsid proteins [37]. There are many studies which use HBV as a model to analyze the physical interaction of the HLA system with the pathogen derived peptides, assess the possible mechanism underlying HLA class II associations by measuring the binding affinities in vitro with the purified HLA-DR1, HLA-DR3, HLA-DR7 and HLA-DR13 molecules [37]. The peptide binding data validate the obtained results in case if there is other important immune response gene in linkage of associate allele related to HBV [37].

Hence, the analysis of physical binding characteristics of overlapping peptides derived from HBV proteins to various HLA class II genes offers a mechanistic explanation for the dominant CD4+ T cell response during infection [37].

Several studies analyzed in healthy patients who had HBV (HBsAg negative, Anti HBc Ig G positive, Anti HBs positive) and chronic HBV infection presented that specific HLA haplotypes were found to be meaningful, and these were shown to increase the risk of chronicity in HBV infection (Table 1.3) [63].

Table 1.3 : HLA Class I Alleles and HBV Persistence [63].

HLA I Allele	Clinical Situation	Country
A*0206	Chronicity	Taiwan
B-35	Chronicity	Taiwan (Han Chinese)
A*2	Chronicity	Japan
B18, B35, B40, Cw3	Chronicity	Russia
A3, B18	Chronicity	Kazakhs
B8-Cw7 Halpotype	HCC	Senegal
B*8	Chronicity	U.S. (White)
A*01-B*08-DRB1*03B*44-Cw*1601, B*44-Cw*0501	Chronicity	U.S. (White)

The relationship of HLA antigen with the outcome of HBV infection has been demonstrated for several ethnic groups; Gambia, Caucasians, Koreans, and Africans. A study in Gambia population (children and adults), showed that the HLA class II allele DRB1*1302 was associated with the protection against persistent HBV infection [63]. Other data indicate that HLA-DR6, especially HLA-DR13, is one of the host factors that influences the immune response to HBV, and may be associated with self-elimination of HBV in Koreans [63].

In a Chinese Han population, HLA-DQB1*0502, HLA-DQB1*0503 and HLA-DQB1*0303 allele were shown to be independently related with the outcome of HBV infection and mentioned as one host genetic factor affecting the HBV infection outcome [63]. Class II gene related studies in many diverse populations displayed that HLA-DRB1*13 is associated with viral clearance of HBV, whereas HLA-DRB1*11/*12 with viral persistence.

Experiments on HBV vaccine response indicated that especially polymorphisms observed in genes of HLA-DR and HLA-DQ are associated with host immune response against HBV infection [63]. Furthermore, the polymorphisms in HLA genes that affect the immune response are a significant topic in chronic persistence HBV infection (Table 1.4-1.6).

Table 1.4 : HLA Class II Alleles and HBV Persistence [63].

HLA II Allele	Clinical Situation	Country
DRB1*1202	Chronicity	Taiwan (Han Chinese)
DQA1*0302	Chronicity	China
DRB1*0301, DQA1*0501, DQB1*0301	Chronicity	China
DR9, DQ9	Chronicity	China
DRB1*06,DRB1*08,DRB1*16	Chronicity	China
DR9	Chronicity	Korea
DQA1*0501, DQB1*0301	Chronicity	U.S. (African Americans)
DRB1*1201/1202	Cirrhosis	China
B13,B8, DR7,DR13,DQ3	Chronicity	Turkey
DQA1*0501,DQB1*0301, DRB1*1102 haplotype	Chronicity	U.S. (African Americans)

Table 1.5 : HLA Class II Alleles and HBV Clearance [63].

HLA II Allele	Clinical Situation	Country
DRB1*4001	Viral clearance	Taiwan
DRB1*0406, DRB1*0701	Viral clearance	Taiwan (Han Chinese)
DQA1*0301, DQA1*0102	Viral clearance, Inactive Group	China
DRB1*1101/*1104, DQA1*0301	Viral clearance	China
DR6	Viral clearance	Korea
DRB1*1301,DRB1*1302	Viral clearance	Gambia
DQB1*0503	Early e seroconversion	Taiwan (Children)
DRB1*1301/1302	Viral clearance	Spain
DRB1*1301/1302	Viral clearance	Germany
DR13	Viral clearance	Germany
DRB1*1501/1502	Cirrhosis protection	China
HLA DR5	Without progression disease	Japan

Table 1.6 : Class II Alleles and Vaccine-Interferon Response [63].

HLA II Allele	Clinical Situation	Country
DR7	Vaccine – No response	Turkey
DRB1*1302, DQB1*0604	Vaccine – No response	Sweden
DRB1*0701, DQB1*0202	Vaccine – No response	England
DRB1*0101, DRB1*0832, DQA1, DQB1, DPA1	Vaccine response	Japan
DQB1*0301, DQB1*0501	Vaccine response	Belgium
DRB1*1301, DR*15, DQB1*0603	Vaccine response	Sweden
DRB1*11, DQB1*0301	Vaccine response	Italy
DQA1*0501, DQB1*0301	Interferon response	China
DRB1*14	Interferon response	China
DQB1*07	Interferon – No response	China

1.8 Purpose of the Thesis

In literature, it is presented that HBV infection lead to viral persistence and the development of chronic hepatitis B in approximately 15% of adult cases, but still the factors that determine the HBV persistence and clearance are not very well understood. However, as variety of host conditions such as age of infection, gender, and immune response were observed to influence the risk of persistent HBV infection and have shown remarkable clinical and pathogenic differences among HBV genotypes. The data obtained to date mentions the importance of host immune response in determining the outcomes of HBV infection. Moreover, the studies have shown the degree of concordance for hepatitis B surface antigen (HBsAg) status differs even between monozygotic twins and that most available experimental evidence speculated that immune response genes; human leukocyte antigen (HLA) class II molecules and cytokine molecules with susceptibility of chronic HBV infection are the genetic components of chronic HBV infection [64].

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 (HLADRB1 Alleles) with the clinical outcome of chronic HBV infection in Turkish population.

For this purpose such chronic hepatitis B patients (N=324) with a control group (N=150) were selected and DNA was extracted from peripheral blood, genotyping was done via SSP-PCR and agarose gel electrophoresis followed by statistical analysis. The obtained genotyping results were compared with Turkish controls. Findings from this thesis, in general, aim to understand the relationship between immunopathogenesis of chronic hepatitis B infection and HLADRB1 Alleles.

2. MATERIAL AND METHODS

2.1. Materials and Laboratory Equipments

2.1.1. Used equipments

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

2.1.2. Used chemicals, enzymes, markers and buffers

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

2.2. Selection Criteria of the Chronic Hepatitis B Patients and Control Group

A total of 324 unrelated Turkish subjects are enrolled in this study, from Department of Gastroenterology, Goztepe Teaching and Research Hospital. A case group of asymptomatic HBV carriers are recruited according to the results of serological tests (HBV virological indexes, liver functional indexes) and symptoms of hepatitis B. Chronic hepatitis B is diagnosed if serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are continuously abnormal, with HBsAg and/or HBeAg seropositive, anti-HBs antibodies (anti-HBs) seronegative. Asymptomatic HBV carriers are identified 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 are below 2000 IU.

The control group is selected from the healthy people of the Goztepe Teaching and Research Hospital. The ages of the controls change between 38 and 49 and the average is 43,5. 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 is sufficient.

2.3. Collection and Storage of Blood Samples

The peripheral blood samples are 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 are stored at −80 °C.

2.4 DNA Isolation from Human Whole Blood

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

2.5 DNA Amount, Purity and Working Solution Calculations

The concentration and purity of the isolated DNA is calculated due to the absorbance values measured at 260, 280 and 320 nm. of the spectrometer. The concentration of the DNA is calculated by equation 2.1 and the purity of the DNA samples are calculated by equation 2.2 given below:

$$DNA\ Concentration\ (ng/\mu L) = (A_{260} - A_{320}) \times 50 \times Dilution\ Factor \quad \textbf{(2.1)}$$

$$DNA\ Purity = \frac{(A_{260} - A_{320})}{(A_{280} - A_{320})} \quad \textbf{(2.2)}$$

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

2.6. Single Specific Primer - Polymerase Chain Reaction (SSP-PCR)

Isolated genomic DNAs of the chronic hepatitis B group are used for PCR reaction. The target DNA sequences of the alleles of HLA-DRB1 containing the related SNP regions are detected by sequence-specific primers–polymerase chain reaction (SSP–PCR) (Olerup et al., 1993). Primers sequences and PCR product sizes are listed in

Table 2.1. Positive internal control primers with HLA DRB1 specific primers are pooled into reaction system in order to eradicate pseudonegative possibilities. The internal control is a fragment of human growth hormone gene 1 (chr 17) with 439 bp. The sequences of internal control primers C5 and C3 are also shown in Table 2.1. PCR is performed in 25 μL reaction mixture containing 50 ng genomic DNA, 1 U DNA Dream Taq Green PCR Master containing, DreamTaq DNA polymerase, optimized DreamTaq Green buffer, MgCl2 and dNTPs (Fermentas), 0.2 μm primers, 0.04 μm internal control primers C5 and C3. The performance of the PCR reaction varies due to the purity of the template DNA as well as the amplicon size (length) and content (GC%) so PCR conditions are optimized in order to obtain high yield and fidelity.

2.6.1 Oligonucleotide primers

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

As it is also essential to analyze the primer sets for dimer formation, the hairpin, heterodimer and self dimer analysis of the primer sets are done and confirmed with the SciTools on the IDT DNA website (OligoAnalyzer 3.0)

Table 2.1 : List of primers used in the study.

DRB1 alleles	Primers	PCR Product
DRB1*01	5’ TTGTGGCAGCTTAAGTTTGAAT 3’ 5’ GCTGTTCCAGTACTCGGCAT 3’	168
DRB1*15/16	5’ TCCTGTGGCAGCCTAAGA G 3’ 5’ CGCTGCACTGTGAAGCTCTC 3’	310
DRB1*03	5’ GTTTCTTGGAGTACTCTAGGTC 3’ 5’ TGCAGTAGTTGTCCACCCG 3’	222
DRB1*04	5’ GTTTCTTGGAGCAGGTTAAACA 3’ 5’ CGCTGCACTGTGAAGCTCTC 3’	262
DRB1*11	5’ CACGTTTCTTGGAGTACTCTAC 3’ 5’ CTGGCTGTTCCAGTACTCCT 3’	179
DRB1*12	5’ AGTACTCTACGGGTGAGTGTT 3’ 5’ CTGTTCCAGGACTCGGCGA 3’	198
DRB1*13/14	5’ GTTTCTTGGAGTACTCTACGTC 3’ 5’ CGTAGTTGTGTCTGCA(GA)TAGG 3’	234
DRB1*07	5’ CCTGTGGCAGGG AAGTATA 3’ 5’ CCCGTAGTTGTGTCTGCACAC 3’	232
DRB1*08	5’ AGTACTCTACGGGTGAGTGTT 3’ 5’ CCCGTATTGTGTCTGCAG 3’	227
DRB1*09	5’ GTTTCTTGAAGCAGGATAAGTTT3’ 5’ CCCGTAGTTGTGTCTGCACAC 3’	236
DRB1*10	5’ CGGTTGCTGGAAAGACGCG 3’ 5’ CTGCACTGTGAAGCT CTCAC 3’	204
DRB1-Exon2	5’ TTCGTGTCCCCACAGCACGTTTC 3’ 5’ GCCGCTGCACTGTGAAGCTCTC 3’	295
Internal Control (HGH)	5′ CAGTGCCTTCCCAACCATTCCCTTA 3′ 5’ATCCACTCACGGATTTCTGTTGTGTTTC 3′	439

2.6.2 PCR cycle conditions

The PCR cycle conditions are varied according to the desired amplicon. The only variable in the PCR cycle conditions is the annealing temperatures, caused by the usage of different primer sets (Table 2.2).

Table 2.2 : General PCR cycle conditions.

Repeat Number	Degree	Time	Phase
1	94 °C	5 minutes	Initial Denaturation
35	94 °C	1 minutes	Denaturation
	Variable	60 seconds	Annealing
	72 °C	4 minutes	Extension
1	72 °C	8 minutes	Final Extension

2.6.3 PCR optimization

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

2.6.4 Gradient PCR

It might be 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. So, 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 that gives the best band intensity is chosen as the optimum annealing temperature. In this thesis, the optimization of the annealing temperature of specific SNP PCR is made by gradient PCR by forming a gradient from 65 to 72 and the best band intensity is seen for 65°C, so this temperature is used as the annealing temperature of HLA alleles PCR for the rest of the studies.

2.7 PCR Conditions of HLA DR Alleles

The PCR conditions for the HLA DR polymorphism is shown in the table 2.2 below.

Table 2.3 : PCR conditions for the HLA DR polymorphisms.

Repeat Number	**Degree**	**Time**	**Phase**
1	94 °C	5 minutes	Initial Denaturation
35	94 °C	1 minutes	Denaturation
	65 °C	60 seconds	Annealing
	72 °C	4 minutes	Extension
1	72 °C	8 minutes	Final Extension

2.8 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 of 1X TAE buffer, which is diluted from 50X stock TAE . 6 µL of PCR product is mixed with 1 µL 6X loading dye and with 1 µL SyberGreen in order to load into the wells. Lengths of the PCR products are calculated according to O'Range GeneRuler Low Range marker (Fermentas) and O'Range GeneRuler Ultra Low Range marker (Fermentas). The gels are run in 1X TAE buffer, at 80V with power supplier, for at least 45 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.

2.9 Genotyping

The genotyping is done by analyzing the agarose gel photos of SSP PCR products for the all HLA DRB1 alleles observed in patients, respectively. O'Range GeneRuler LowRange (Fermentas) or O'Range GeneRuler Ultra LowRange (Fermentas) markers are 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, in order to analyze the effect of HLA DRB1 alleles in patients with chronic hepatitis B infection statistical analysis were performed. A 2 × 2 contingency table was used to compare alleles' frequencies according to clinical statues of the patients. All statistical analyses were performed using the SPSS program, version 17 (SPSS, Chiacago, IL). Distribution of quantitative data was evaluated by Kolmogrof-Smirnov test in order to understand if the data conform to or not Gaussien distribution. Quantitative data that conform to Gaussien distribution was evaluated by Student t test, whereas the data that does not match to Gaussien distribution or do not fulfill parametric conditions was appraised by Mann Whitney U test. 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 5 in any cell in 2 × 2 contingency table, Fisher's exact test was used . In all analysis two-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 324 patients were included; 63.4% male (n=204), 36.4% female and, where 23.5% (n=76) of patients being HBe antigen positive, 23.8% (n=77) having cirrhosis (Table 3.1). The median age was 44.4 years, median follow up time was 86.5 months. In the control group 150 healthy cases were included (DRB1*07).

Table 3.1 : Demographic data of all patients.

			n (%)
Sex	***Male***		**204 (63.4)**
	Female		**120 (36.6)**
HBeAg		***Negative***	**248 (76.5)**
		Positive	**76 (23.5)**
Cirrhosis		***yes***	**77 (23.8)**
		no	**247 (76.2)**

From the total of 324 patients 198 had a course of lamivudine or adefovir treatment. Of those 96 patients had virologic breakthrough during these treatments. The rest of the patients responded well to the given treatment without any virologic resistance.

In our group of patients, 115 had interferon experience. Only 19 of them responded to interferon treatment.

Fifty-nine patients had inactive disease with persistently normal liver tests and all HBV DNA measurements during follow up for these patients were below 2000 IU.

3.2 DNA Isolation Results

In this thesis, DNA isolation is done according to the procedure provided by Invitrogen PureLink Genomic DNA Purification Kit. The average DNA concentration obtained from kit is 251 ng/µL and average A260/A280 value is 1.8 (N=324).

3.3 Genotype Analysis

The genotyping is done by comparing the bands on the agarose gel. Some samples of PCR results for SNP analysis are shown in Figures 3.1, and 3.2.

Figure 3.1 shows internal control (HGH) together with DRB1*08 and DRB1*09 from blood samples. M shows size marker DNA (100-bp DNA ladder); N, negative control; 1–5 lines show each number of amplified HGH products (Internal control >450bp). As observed from the figure all of the internal controls gave a band at the appropriate DNA size (Figure 3.1) worked. There was not seen an observation of DRB1*08 and DRB1*09 alleles on the lines 1-5 in the patients of chronic HBV infection which were loaded together with internal control. The number on the right indicates the molecular size (in base pairs) of the amplified PCR products.

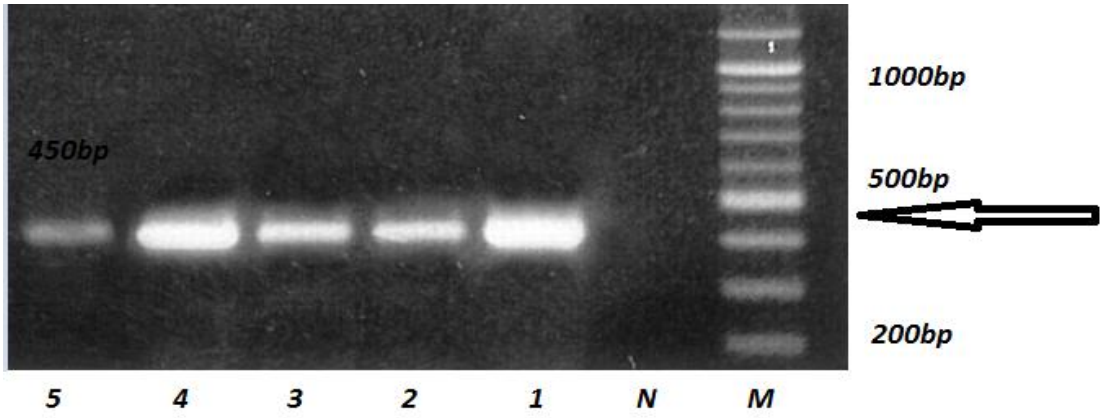


Figure 3.1 : Agarose gel electrophoresis analysis on 2.0% agarose gel for the target DNA sequences such as targeted internal control (HGH), DRB1*08 and DRB1*09 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); N, negative control; 1–5, each number of amplified HGH products (Internal control >450bp). The number on the right indicates the molecular size (in base pairs) of the amplified PCR products.

At the same time all other 8 HLA-DRB1 alleles (DRB1*01, DRB1*15/16, DRB1*03, DRB1*04, DRB1*11, DRB1*12, DRB1*13/14/11, DRB1*10) were analyzed on agarose gel electrophoresis.

From our genotyping analysis, we found the most significant relation of chronic HBV infection with DRB1*07 allele. In Figure 3.2, some of the patients DNAs were scanned using the primers specific for DRB1*07 allele. It can be observed from the figure that there are two bands for the internal control HGH (450bp) and DRB1*07 allele (232bp) (Figure 3.2).

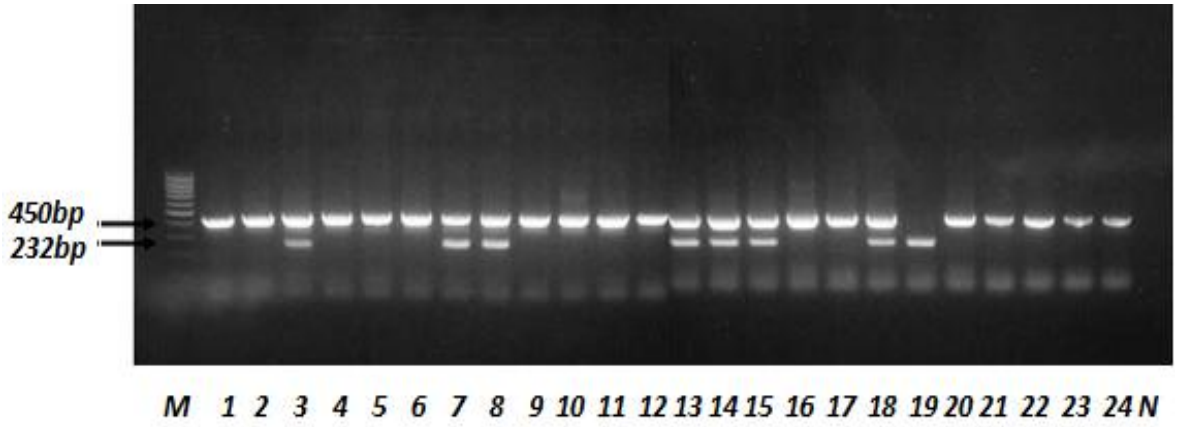


Figure 3.2 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*07 from blood samples. Lanes: M, size marker DNA (100 bp DNA ladder); 1–24 lines show each number of amplified DRB1*07 (232bp DNA fragment), N shows negative control; 450bp bands show internal control.

3.4 HLA Results

HLA-DRB1 alleles in 324 patients with chronic hepatitis B and 150 healthy cases were analyzed using the polymerase chain reaction/sequence specific primer (PCR/SSP) technique.

The table bellow shows the distribution of allele frequencies used in our study.

Table 3.2 : Allele frequencies.

	n	%
DRB1*01	**0**	**0**
DRB1*15/16	**63**	**19.4**
DRB1*03	**24**	**7.4**
DRB1*04	**0**	**0**
DRB1*11	**0**	**0**
DRB1*12	**23**	**7.1**
DRB1*13/14	**177**	**54.6**
DRB1*07	**24**	**7.4**
DRB1*08	**0**	**0**
DRB1*09	**0**	**0**
DRB1*10	**12**	**3.7**

In our group of patients B1*13/14 allele was the most frequent (54.6%). The frequencies of the other alleles were given in the table (Table 3.2).

When we analyze the relation of the alleles according to clinical status of the patients; our data demonstrate that DRB1 alleles were not related to the active or inactive disease, or interferon treatment response or virologic breakthroughs observed during nucleoside analog treatments.

On the other hand we have found statistically significant frequency of DRB1*07 allele (p=0.003) in our case-control study (Table 3.3). Moreover, our data showed that, patients who had DRB1*07 allele had lower probability to have cirrhosis (likelihood ratio chi square: 4.17, p=0.04), suggesting that DRB1*07 might be associated with low risk of cirrhosis development (Table 3.4).

Table 3.3 : Statistical analysis of HLA-DRB1*07 allele frequencies of chronic HBV infected patients and control group.

	Patients (n=324)	Controls (145)	χ^{2} †	p	OR	95% CI
DRB1*07	24 (7.4)	2 (1.4)	8.6	0.003*	0.17	0.04-0.75

Table 3.4 : Statistical analysis of HLA-DRB1 allele frequencies of chronic HBV infected patients.

	Cirrhosis present n=77(%)	No cirrhosis n=247(%)	χ^{2} †	p	OR	95% CI
DRB1*15/16	12 (15)	51(20.6)	0.99	0.32	0.71	0.36-1.41
DRB1*03	3 (3.9)	21 (8.5)	2.1	0.15	0.43	0.13-1.5
DRB1*12	3(3.9)	20(8.1)	1.78	0.18	0.46	0.13-1.59
DRB1*13/14	42 (54.5)	135(54.7)	0	0.99	0.99	0.59-1.66
DRB1*07	2 (2.6)	22 (8.9)	4.17	0.04 *	0.27	0.06-1.19
DRB1*10	3(3.9)	9(3.6)	0.01	0.9	1.07	0.28-4.06

In this case, our results demonstrate that DRB1*07 allele may be one of the host factors, which influences immune response to HBV, by self-limiting cirrhosis, one of the main complications of chronic HBV.

4. DISCUSSION

About 50-60% of HBeAg positive and negative hepatitis B patients respond to medication by several drugs such as Lamivudine, Adefovir dipivoxil, Interferon. Experimental evidence in many articles for the negativity of HBV DNA in patients with HBV treated with tenofovir (Tdf) and entecavir (Etv) showed a percentage above 90. HBeAg negative patients treated with PEGylated interferon alpha-2a showed about 25% negativity of HBV DNA, 34% seroconversion, and about 3% loss of HBsAg, where after the third year 20% of patients show a HBV DNA level below 400 copies. The optimal duration of therapy is not yet clear, moreover, the usage of drugs during the treatment of HBV infection is observed to lead to resistance, that is one of the main handicaps of the clearance of virus. The reasons for defective viral clearance are not completely understood. Treatment of chronic hepatitis B virus infection, even in the presence of approved drugs for adults and many promising new drugs in development, is still a serious issue due to the absence of a complete cure for hepatitis B infection

On the other hand, host and viral factors (HLA polymorphisms & viral genotypes) undoubtedly influencing the outcomes of HBV infections, respectively, have shown their importance in the progression of HBV infection and available experimental evidence speculated that immune response genes; human leukocyte antigen (HLA) class II molecules and cytokine molecules with susceptibility of chronic HBV infection are the genetic components of chronic HBV infection.[64] Hence, the aim of the present work was to ascertain whether specific HLA-DRB1 alleles are associated with the outcomes of HBV infection in the Turkish population. In our study, frequencies of the 12 specific HLA-DRB1 (Table 2.1) allele polymorphisms were analyzed in patients with HBV infection. The obtained data from the present study show a statistically significant association between DRB1*07 allele and presence of cirrhosis in patients with chronic HBV infection.

Patients who had DRB1*07 allele had lower probability to have cirrhosis (likelihood ratio chi square: 4.17, p=0.04). Moreover, we have found statistically significant

frequency of DRB1*07 allele (p=0.003) in our case-control study supporting the role of DRB1*07 allele in the development of cirrhosis.

In this case, we suggest that DRB1*07 allele may be one of the host factors, which influences immune response to HBV, by self-limiting cirrhosis, one of the main complications of chronic HBV.

In several studies HLA-DRB1*07 allele was shown to be associated with many disorders. For instance a study done in Turkish population have shown the association of HLA-DRB1*07 allele with vitiligo, a pigmentary disorder affecting approximetaly 0.38-1.13% people in distinct areas of the world. This study strongly suggested that HLA-DRB1*07 may be one of the genetic markers for general susceptibility to vitiligo in a Turkish population (p=0.0004) [66]. Another study, in Pakistan presented that HLA-DRB1*07 (p=0.008) individually or in combination with HLA-DQB1*02 was found to be associated with viral persistence of HCV infection, following IFN therapy, where the long term viral persistance of this virus lead to liver cirhossis [67]. HLA-DRB1*07 was also emphasized as an susceptible allele for the occurrence of autoimmune hepatitis type 2 by a study done in children with autoimmune hepatitis [68]. A meta-analysis done by Xuan, S.Y. and his friends showed that among the 3 HLA-DRB1 alleles studied, DRB1*07 and DRB1*12 were significantly associated with the risk of HCC in the Chinese whole populations. This study emphasizes the significante of DRB1*07 in HBV infection.

As we know in the patients with chronic hepatitis B virus infection, there is an observation of decreased level of CD4+ and CD8+ immune response, where it is thought that increased level of HBeAg and HBsAg antigens may be the reason of this immune response. Our hypothesis contrasts to the previously suggested hypothesis based on antigen type. Parallel to the literature ideas, we think that the presence of substitutions on the HLADRB1*07 T-cell recognition residues causes weak interactions to the T-cells in patients with HBV infection. This type of HLA class II molecules show highly polymorphic character in the T-cell recognition as explained in part of introduction 1.6 [69]. Also crystallographic studies reveal that T-cell recognition is associated with the HLA class II molecules rather than the antigens [36].

Therefore weak immune response of the patients carrying HLA-DRB1*07 allele is based basically on the type of allele present.

HLA class II glycoproteins, encoded by HLA-DRB1 molecules such as DRB1*07 present viral peptides to CD4+ T cells and thus influence the immune response. So we believe that HLA-DRB1*07 allele may be the key host factor to determine the development of diseases from HBV infection to cirrhosis in Turkish population. This is mainly based on the results obtained from our patients who had DRB1*07 allele showing lower probability for cirrhosis. Proposed role of DRB1*07 should be validated with a further molecular approaches. Analysing of all variants of HLA-DRB1 molecules together with screening of T cell condition not only in patients with HBV infection but also in patients who had recovered from HBV infection conducted by a control group will help us to illuminate the mechanism of disease.

On the other hand, the results of analyzed frequencies of other specific HLA-DRB1 alleles were not related to active or inactive disease, or interferon treatment response or virologic breakthroughs observed during nucleoside analog treatments.

Whereas, recently our study group demonstrated a relation between DQB1*0501 allele and chronic active disease; DQB1*0301/4 allele and interferon response; DQB1*0503 and virologic breakthrough during antiviral treatments [65].

Another study done in Gambia population (children&adults), showed that the HLA class II allele DRB1*1302 was associated with the protection against persistent HBV infection. Other data indicate that HLA-DR6, especially HLA-DR13, is one of the host factors that influences the immune response to HBV, and may be associated with self-elimination of HBV in Koreans.

In a Chinese Han population, HLA-DQB1*0502, HLA-DQB1*0503 and HLA-DQB1*0303 allele were showed to be independently related with the outcome of HBV infection and mentioned as one host genetic factor affecting HBV infection outcome. Class II HLA gene related studies in many diverse populations displayed that HLA-DRB1*13 is associated with viral clearance, whereas HLA-DRB1*11/*12 with viral persistence. A study in northwestern China showed that the allele frequencies of HLA-DRB1*03 and HLA-DRB1*07 were markedly higher in the HBV-infected group but the allele frequencies of HLA-DRB1*15 were obviously lower than that of the spontaneously cleared controls. Although, previous reports

mentioned above found that specific HLA-DRB1 alleles were significantly more frequent in hepatitis B carriers and patients of chronic hepatitis B infection, this was not the case in the present study. A primary cause for the difference in results between data by different authors and our results may be related to the great variability of the frequency of HLA alleles in different populations, where it is quite possible that one ethnic group may have some specific alleles in development/protection of chronic hepatitis B, respectively cirrhosis compared to other ethnic groups.

Furthermore, studies conducted on relatively small scale may affect the results by decreasing the power of detection of difference in the distribution of specific HLA-DRB1 alleles between chronic hepatitis B patients and controls, even a true difference exists. To highlight the association of HLA class I and II genes in the progression of HBV infection, not only the alleles that are thought to influence the T cells response but also a linked polymorphism in a neighboring immunoregulatory gene has to be taken into account. Additionally, other genetic factors in linkage disequilibrum with HLA class I and II might influence the clearance of chronic HBV infection in Turkish population, so lack of an association may not mean that associations do not exist.

In conclusion, individuals with distinct HLA types may differ in susceptibility or resistance to disease and large, multi-ethnic confirmatory study is needed to validate these findings and to 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 : Enzymes
APPENDIX F : 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	AppliChem
Boric acid	Amresco
dNTP	Fermentas
EDTA	AppliChem
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

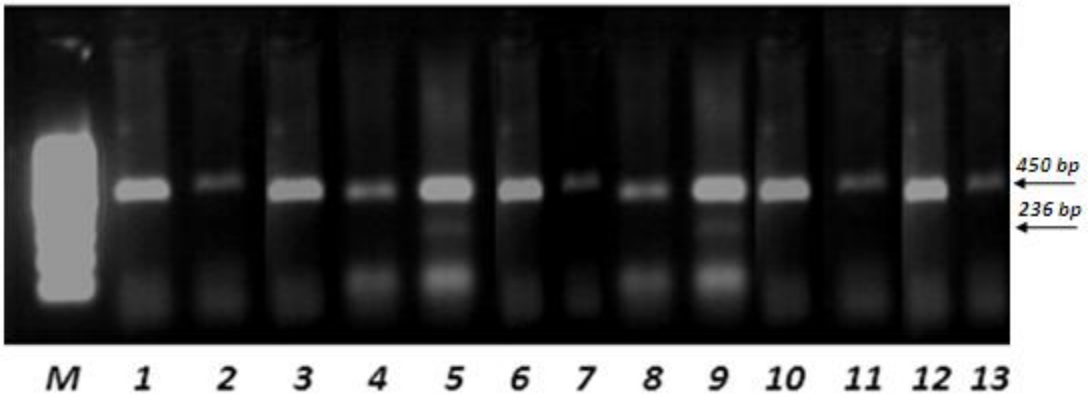


Figure D.1 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*09 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); 5 and 9 lines show each number of amplified DRB1*09 (236bp DNA fragment), 1-13 lines shows each number of amplified HGH internal control (450bp).

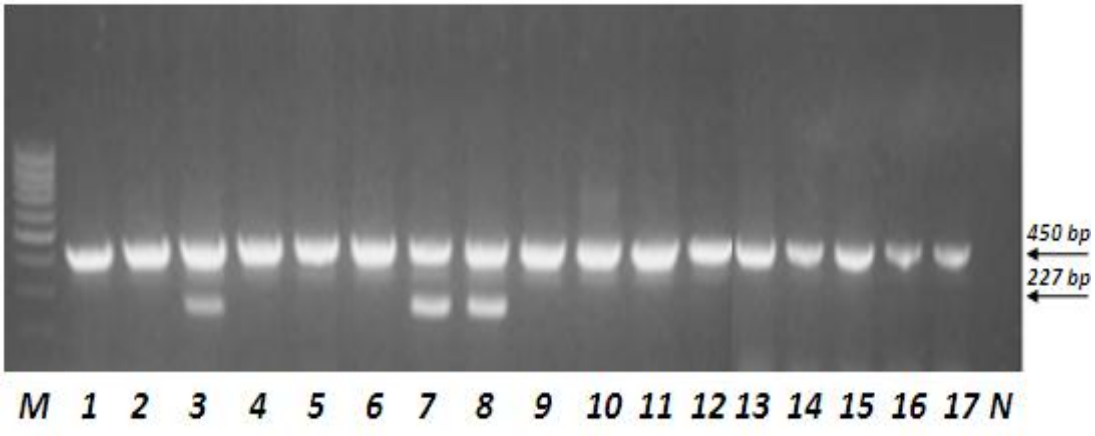


Figure D.2 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*08 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); 3, 7 and 8 lines show each number of amplified DRB1*08 (227 bp DNA fragment), 1-17 lines shows each number of amplified HGH internal control (450bp); N shows negative control.

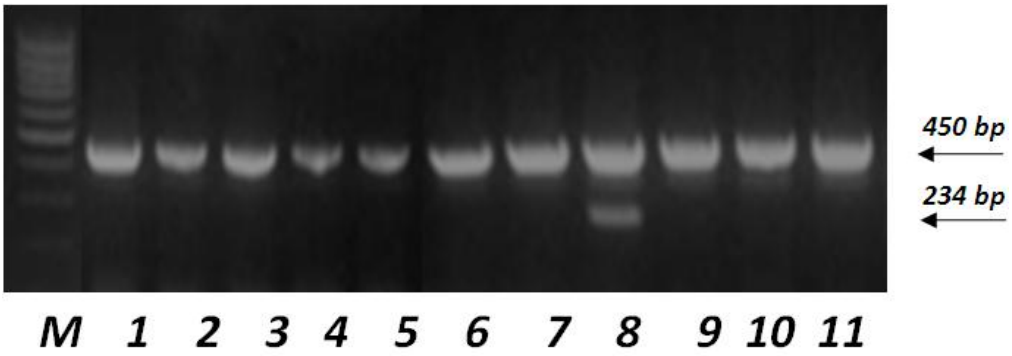


Figure D.3 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*13/14 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); The 8 line shows number of amplified DRB1*13/14 (234 bp DNA fragment), 1-11 lines shows each number of amplified HGH internal control (450bp).

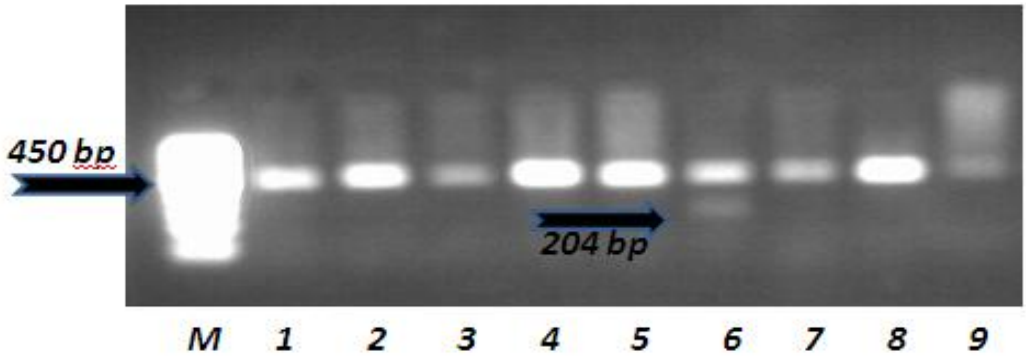


Figure D.4 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*10 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); The 6 line shows number of amplified DRB1*10 (204 bp DNA fragment), 1-9 lines shows each number of amplified HGH internal control (450bp).

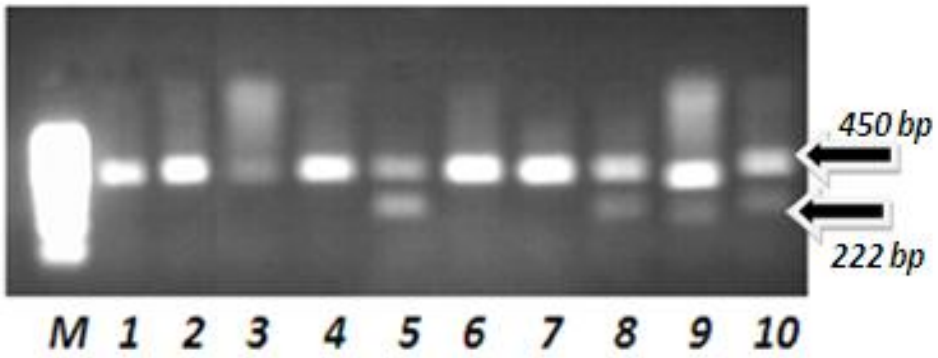


Figure D.5 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*10 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); The 5, 8-9 line show number of amplified DRB1*03 (222 bp DNA fragment), 1-10 lines shows each number of amplified HGH internal control (450bp).

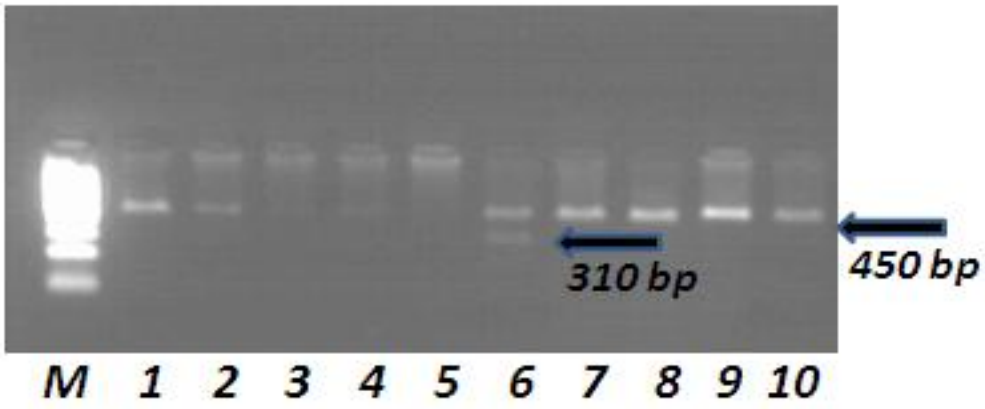


Figure D.6 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*15/16 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); The 6 line shows number of amplified DRB1*15/16 (310 bp DNA fragment), 1-10 lines shows each number of amplified HGH internal control (450bp).

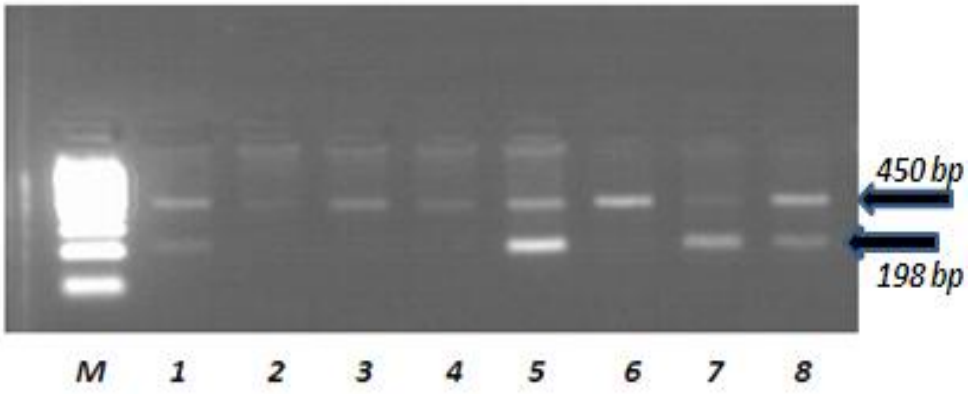


Figure D.7 : Agarose gel electrophoresis analysis on 2.0% agarose gel of DNA sequences amplified by polymerase chain reaction (PCR) assay by using outer and inner primer sets targeting DRB1*12 from blood samples. Lanes: M, size marker DNA (100-bp DNA ladder); The 1, 5, 7, 8 lines show number of amplified DRB1*12 (198 bp DNA fragment), 1-10 lines shows each number of amplified HGH internal control (450bp).

APPENDIX E

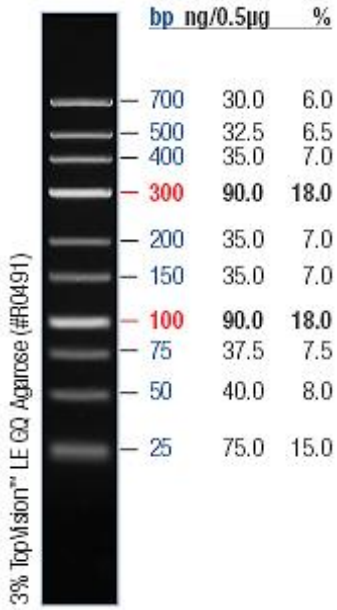
ENZYMES

DreamTaq DNA Polymerase	Fermentas
	Invitrogen

APPENDIX F

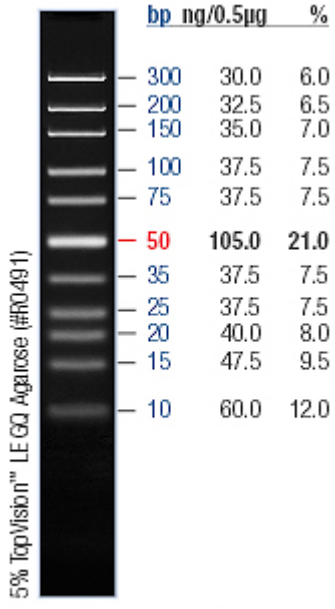
MARKERS

O'Gene RulerTM DNA Ladder Low Range Fermentas



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

O'Gene RulerTM DNA Ladder Ultra Low Range Fermentas



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

CURRICULUM VITAE

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- Fejzullahu, A., Doganay, L., Tuncer, I., Enc, F., Ozturk, O., Colak., Y., and Dinler, G. (2011). Association of Human Leukocyte Antigen Polymorphisms and Chronic Hepatitis B Infection Outcome. 12th International Congress of Human Genetics, Montreal, Canada.

- Ozgon, G., Fejzullahu, A., Senvardar, K., Biricik, A., and Fiorentino, F. (2011). Preimplantation Genetic Diagnosis Based Detection of Uniparental Disomy on Embryos Undergoing IVF. 12th International Congress of Human Genetics, Montreal, Canada.

- Fejzullahu, A.,Tekiner, T.A., Erdem, M., Anak, S., Özbek, U., and Atalar, F. (2009). Crosstalk between T-cadherin and Wnt/ β-catenin pathways in cancer, Europian Human Genetics Conference, Vienna, Austria.

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