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Multiplex PCR to Detect the Association between HLA DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 Alleles and Hepatitis B Viral Infection

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ABSTRACT

Background: Worldwide, Hepatitis B virus infection (HBV) leads a serious public health problem. Several genome-wide association studies revealed an association between the HLA class II gene regions and HBV chronic infection.

Aim: To determine the role of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 alleles among acute and chronic hepatitis B virus infected patients.

Methodology: This case control study was done in the department of Microbiology for a period of 3 years (August 2015 to July 2018). Blood sample (3 ml) was collected from HBsAg seropositive patients and healthy control. Serum samples were screened for other HBV seromarkers to differentiate the patient into acute and chronic hepatitis B infection by ELISA method. Multiplex PCR/SSP method was done to analyse HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 alleles in HBV patients and healthy control.

Statistical analysis: Allele frequency of the study groups were compared using Chi-square test.

Results: Among the 182 HBV infected patients, 32 and 150 were suffering from acute and chronic infection. Risk factors such as alcohol consumption (OR=1.60, P=0.05), tattooing (OR=4.37, P=0.003), history of previous surgery (OR=2.02, P=0.012), family history of hepatitis infection (OR=2.99, P=0.005) showed significant differences among the two groups. Among the 3 alleles, significant association of allele frequencies of HLA DQB1*03:01 in the CHB group (48.67%) were higher than those in the normal control group (19.78%).

Discussion and conclusion: HLA class II allele is one of the factors determining the outcome of HBV infection. DQB1*03:01 is closely related with susceptibility to CHB infection.

Keywords: Chronic hepatitis B (CHB) infection, Acute hepatitis B infection (AHB) infection, Human leukocyte antigen (HLA), DQB1, DRB1 and DPB1 alleles

INTRODUCTION

Worldwide, Hepatitis B viral infection (HBV) leads to a serious public health problem [1] and it affects more than 350 million people [2]. HBV infection is usually variable and complex that result in different clinical outcomes includes chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [3]. Several factors like virulence of the viral strains and the host immune response determines the outcome of HBV infection [4]. Host factors such as gender, age of infection, smoking, volume of alcohol intake may be closely associated with the HBV outcome [3].

Major histocompatibility (MHC) class II molecules play an important role in the protection against infections and also involved in antigen presentation to CD4+ T cells thereby enhancing antibody production and cytotoxic T cell activation. Such molecules are encoded by Human leukocyte antigen (HLA) -DR, -DQ and -DP loci [5,6]. The genes encoding HLA class II molecules are the highly polymorphic genes in the human genome and are therefore ideal candidates for the investigation of HBV infection [7]. Several genome-wide association studies revealed an

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association between the HLA class II gene region (DR, DQ and DP) and HBV chronicity [8-10].

A study from Chinese population showed that DQB1*03:01was associated with viral persistence [11]. A study from Turkey revealed that HLA DR7 and DQ3 alleles were related to susceptibility to chronic infection [12]. A Trans ethnic association study revealed that HLA DPB1*09:01 was a risk allele to chronic HBV infection [13] In the present study we have analysed the association of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 alleles in patients with chronic and acute hepatitis B infection and healthy control using multiplex polymerase chain reaction with sequence specific primers.

MATERIALS AND METHODS

The study was done in the department of Microbiology at Chettinad Hospital and Research Institute from August 2015 to July 2018 for a period of 3 years. Three study groups have been enrolled. A case group of acute hepatitis B infected patients (first group) and chronic hepatitis B patients (second group). The third group of healthy controls with age and sex matched HBsAg – negative individuals who had no history or clinical evidence of hepatitis infection. A detailed evaluation of patient history (demographic, past medical and behavioural risk) and informed consent were recorded from each patient and controls in a questionnaire form. Undergone Institutional Human Ethical Clearance and Clinical trial registry done (CTRI/2018/01/011460).

Serological testing

All serum specimens were stored at -20°C, thawed and tested according to manufacturer's specifications for HBsAg, HBeAg, total anti HBc, antiHBc IgM and antibodies to HBsAg (Dia.Pro Diagnostic Bioprobes, Italy) by Enzyme-Linked Immunosorbent Assay (ELISA). Subjects with Acute hepatitis B infection were positive for HBsAg, total antiHBc, antiHBcIgM and negative for antiHBs. Subjects with Chronic HBV were positive test for HBsAg, total antiHBc and negative for anti HBc IgM, antiHBs. Healthy control group were negative for HBsAg, total antiHBc IgM and antiHBs without evidence of HBV infection in past or present.

DNA extraction

In total, three ml of blood was collected from patients and stored in EDTA tubes at -20℃ for the day of DNA extraction. Genomic DNA was extracted using 200 µl of peripheral blood using Nucleospin blood column genomic DNA purification kit according to manufacturer's instructions. (MACHEREY- NAGEL GmbH & Co. KG, Germany).

Primer synthesis and reagents

The sequence specific PCR primers were used for the detection of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 loci by Olerup et al. [14], the primers amplifying human growth hormone gene were synthesized by Biocorporals Company, Chennai. Buffer mix (2X) was purchased from Emerald Amp GT PCR Master Mix, Incell Technologies.

Multiplex PCR amplification

The alleles of HLA DRB1*07:01, DQB1*03:01 and DPB1*09:01 was detected by a multiplex polymerase chain reaction with sequence-specific primer (PCR-SSP). Primer sequence and PCR product size are listed in **Table 1**. Internal positive-control primer was a fragment of human growth hormone gene 1 (chr 17), consisting of 439 bp were included in the reaction system to eradicate false negative. PCR was performed in 25 µL reaction mixture containing 15.5 µl of 2X buffer, 100 ng genomic DNA (2 µl), 0.4 µmol/L primer, 0.2 µM of the internal control primer and 4.5 µl of deionized water. The multiplex PCR cycling parameters of HLA class II alleles were as follows: initial denaturation at 95°C for 5 min, first 10 cycles consisted of denaturation at 95°C for 30 s, combined-annealing extension step at 66°C for 30 s, following 20 cycles consisted of denaturation at 94°C for 10 s, annealing at 61°C for 60 s and extension at 72°C for 30 s in thermo cycler (Agilent Technologies Sure Cycler 8800). In each PCR reaction a primer pair was included to amplify the human growth hormone gene, which functioned as an internal positive amplification control.

Table 1. Primers used in the study.

Allele studied	Primers	PCR product (bp)
HLA DRB1*07:01	5' CCTGTGGCAGGG AAGTATA 3' 5'CCCGTAGTTGTGTCTGCACAC 3'	232
HLA DQB1*03:01	5'GAC GGA GCG CGT GCG TTA3' 5'AGT ACT CGG CGT CAG GCG3'	122
HLA DPB1*09:01	5'TCC CCG CAG AGA ATT AGG TGC3' 5'TCC TTC TGG CTG TTC CAG TAG3'	185
HGH Human Growth Hormone (Internal control)	5′ CAGTGCCTTCCCAACCATTCCCTTA 3′ 5′ ATCCACTCACGGATTTCTGTTGTGTTTC 3′	439

Detection of PCR products

After amplification, bands were visualized in 1.5% agarose gel stained with ethidium bromide electrophoresed for 100 V for 45 min. DNA marker 100 bp (Bio-Rad, USA) was used to measure the molecular size of the amplified products. The allelic types were determined according to the presence or absence of PCR products of the desired length. The DNA bands were visualized using Wealtec Dolphin view Gel Imaging System (Wealtec Bioscience Co, Ltd., USA).

STATISTICAL ANALYSIS

Allele Frequency (AF) of HLA class II alleles were calculated by direct count. To assess the association of HLA class II allele with AHB and CHB infection, Chi-square test was applied to a two-by-two contingency table based on the allele frequencies. Odds ratios (OR) were calculated based on the 2 × 2 table of allele count. Demographic characteristics of the study groups were compared using Pearson's Chi- square test. The p value <0.05 considered as statistical significant for all tests. Statistical analyses were done using SPSS 10.0 software.

RESULTS

In this case control study, a total of 150 patients (104 males and 56 females) was positive for HBsAg, anti HBc Total and negative antiHBc IgM (i.e., indicating they are suffering from chronic hepatitis B viral infection). Nearly 32 patients (15 males and 17 females) were positive for HBsAg, AntiHBc Total, antiHBc IgM (i.e., indicating they are acute hepatitis B viral infection). Healthy controls negative for all HBV seromarkers were 182 (119 males and 63 females). We found no significant difference among cases and controls in terms of gender (P=1.000).

The demographic, behavioral and past medical history of the recruited subjects is shown in **Tables 2 and 3**. We observed significant differences in marital status and education between cases and control with a P value of <0.05. Other factors such as alcohol consumption (OR=1.60, P=0.05), tattooing (OR=4.37, P=0.003), a history of previous surgery (OR=2.02, P=0.012), family history of hepatitis infection (OR=2.99, P=0.005) showed significant differences among the two groups. Even though the frequency of other risk factors, such as history of blood transfusion (P=0.132) and sharing nail clippers (P=0.880) was higher in the patient group, no significant difference was observed in the control.

Table 2. Study groups and their demographic characteristics.

Demographic characteristics		Study group		X^2	P value
		Case=182 (%)	Control=182 (%)		
Gender	Male Female	119 (65.38) 63 (34.61)	119 (65.38) 63 (34.61)	0.000	1.000
Marital status	Married Unmarried	131 (71.9) 51 (28.2)	98 (53.8) 84 (46.2)	12.92	0.000
Education	Illiterate Up to school University	80 (46.7) 65 (30) 37 (23.3)	96 (50.7) 41 (27.3) 45 (22)	7.72	0.021
Place of living	Urban Semi urban Rural	53 (29.12) 91 (50) 38 (20.9)	67 (36.8) 84 (46.2) 31 (17.03)	2.63	0.268

Table 3. Risks factors association with the case and control group.

Behavioral and Past medical history		Case=182 (%)	Control=182 (%)	X^2	OR (95% CI)	P value
Sharing nail clippers	Yes No	27 (14.8) 155 (85.2)	25 (13.7) 157 (86.2)	0.09	1.09 (0.60,1.96)	0.880
Alcohol consumption	Yes No	64 (35.2) 118 (64.8)	46 (25.3) 136 (74.7)	4.22	1.60 (1.02,2.52)	0.052
Tattooing	Yes No	20 (10.9) 162 (89.1)	5 (2.7) 177 (97.2)	9.66	4.37 (1.60,11.9)	0.003
History of previous surgery	Yes No	46 (25.2) 136 (74.7)	26 (14.2) 156 (85.7)	6.92	2.02 (1.19,3.45)	0.012
History of blood transfusion	Yes No	16 (8.8) 166 (91.2)	8 (4.3) 176 (96.7)	2.94	2.12 (0.88,5.08)	0.132
Family history of hepatitis	Yes No	27 (14.8) 155 (85.2)	10 (5.5) 172 (94.5)	8.69	2.99 (1.40,6.38)	0.005

Note: OR=Odds Ratio; X^2=Chi-square value; P=P value

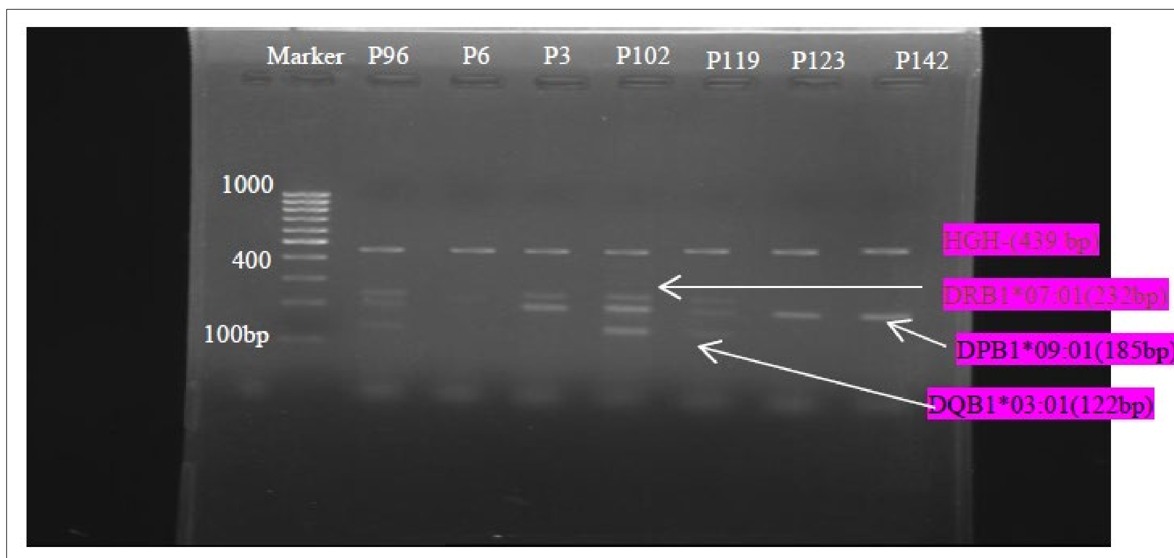
The distribution of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 alleles in patients with chronic hepatitis B and healthy control is shown in **Table 4**. The positivity of HLA-DQB1*03:01 allele in patients with chronic hepatitis B group was markedly higher than that in normal control, there was a significant difference between them. The positivity of HLA-DRB1*07:01 and -DPB1*09:01 alleles in patients with chronic hepatitis B were not different from that in normal control. The gel electrophoresis of HLA-DRB1*07:01,-DQB1*03:01 and DPB1*09:01 alleles by multiplex PCR are shown in **Figures 1a-1c**.

Table 4. Distribution of HLA-DRB1*07:01, DQB1*03:01 and DPB1*09:01 alleles in patients with chronic hepatitis B and healthy control.

HLA alleles	Chronic hepatitis B (n=150)		Normal control(n=182)		X^2	OR	P value
	PN	AF	PN	AF			
DRB1*07:01	56	37.3	69	37.9	0.012	0.97	1.000
DQB1*03:01	73	48.7	36	19.9	31.11	3.84	0.000
DPB1*09:01	8	5.3	16	8.8	1.466	0.58	0.318

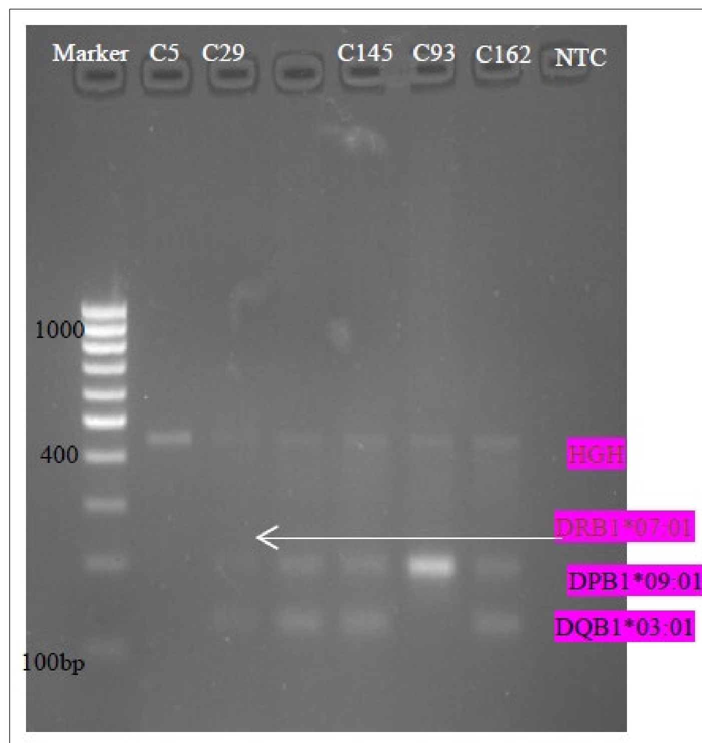
Note: PN: Positive Number; AF: Allele Frequency; OR: Odds Ratio

Figure 1a. Gel documentation of HLA class II alleles (DRB1*07:01, -DQB1*03:01 and DPB1*09:01) by multiplex PCR.



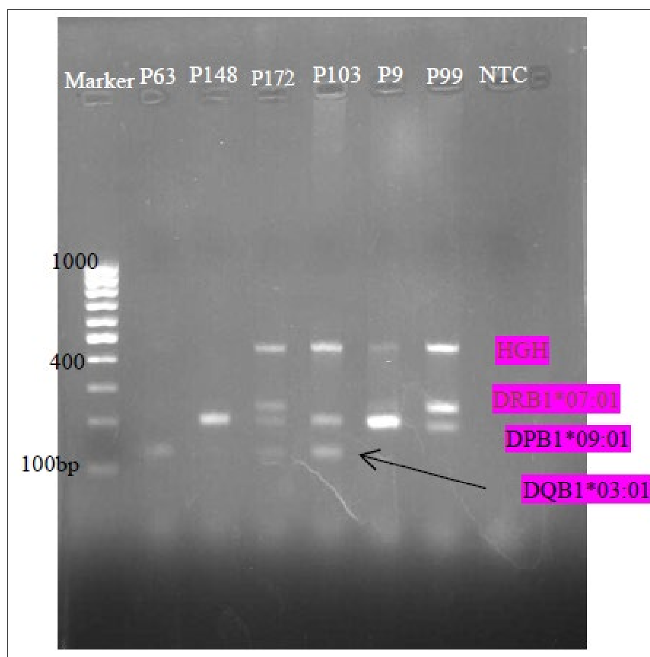
Note: Marker: 100 bp ladder DNA; P: HBV Positive patients samples; NTC: Non-Template Control; HGH: Human Growth Hormone
*Wells P96, P102, P119: Positive for 3 alleles; P6: Positive for DRB1*07:01; P3: Positive for DRB1*07:01 and DPB1*09:01; P123 and P142: Positive for DPB1*09:01*

Figure 1b. Gel documentation of HLA class II alleles (DRB1*07:01, -DQB1*03:01 and DPB1*09:01) by multiplex PCR.



Note: Marker: 100 bp ladder DNA; C: Healthy control samples; NTC: Non-Template Control; HGH: Human Growth Hormone
*Wells C5: Positive for DRB1*07:01; C29, C79, C145, C162: Positive for DPB1*09:01 AND DQB1*03:01; C93: Positive for DPB1*09:01*

Figure 1c. Gel documentation of HLA class II alleles (DRB1*07:01, -DQB1*03:01 and DPB1*09:01) by multiplex PCR.



Note: Marker: 100 bp ladder DNA; P: HBV Positive patients samples; NTC: Non-Template Control; HGH: Human Growth Hormone

*Wells P63: Positive for DQB1*03:01; P148: Positive for DPB1*09:01; P172: Positive for 3 alleles; P103: Positive for DPB1*09:01 and DQB1*03:01; P99, P9: Positive for DRB1*07:01 and DPB1*09:01*

The positivity of HLA-DRB1*07:01 and DPB1*09:01 alleles in patients with acute hepatitis B group were markedly higher than that in chronic hepatitis B, there was a significant difference between them. The positivity of HLA-DQB1*03:01 allele in patients were similar in both AHB and CHB. The distribution of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 alleles in patients with acute and chronic hepatitis B is shown in **Table 5**.

Table 5. Distribution of HLA-DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 alleles in patients with acute and chronic hepatitis B.

HLA alleles	Acute hepatitis B (n=32)		Chronic hepatitis B (n=150)		Chi square value	OR	P value
	PN	AF	PN	AF			
DRB1*07:01	21	65.6	56	37.3	8.649	3.20	0.006
DQB1*03:01	13	40.6	73	48.7	0.684	0.72	0.527
DPB1*09:01	15	15.6	8	5.3	41.22	15.7	0.000

Note: PN: Positive Number; AF: Allele Frequency; OR: Odds Ratio

HLA analysis stratified by gender revealed that the DQB1*03:01 (OR: 4.11; 95% CI: 1.85-9.16; p=0.000) allele were associated with the female gender **(Table 6)**. HLA analysis stratified by age revealed that DQB1*03:01 were more frequent in HBV infected patients aged ≥ 36 years (OR: 3.46; 95% CI: 1.71-7.01; p=0.000) **(Table 7)**. The mean age of the HBV patients was 36 years, for age ranging from 18 to 65 years.

Table 6. Association between HLA alleles (DRB1*07:01, DQB1*03:01 and DPB1*09:01) and HBV infection stratified by gender.

Gender		Cases	Controls	OR (95% CI)	P value
Male (n=119)					
DRB1*07:01	Presence Absence	45 (37.8%) 74 (62.2%)	49 (41.2%) 70 (58.8%)	0.86 (0.51-1.46)	0.690
DQB1*03:01	Presence Absence	55 (46.2%) 64 (53.8%)	24 (20.2%) 95 (79.8%)	3.40 (1.91-6.04)	4.0E-5
DPB1*09:01	Presence Absence	6 (5.0%) 113 (95%)	10 (8.4%) 109(91.6%)	0.58 (0.20-1.64)	0.437
Female (n=63)					
DRB1*07:01	Presence Absence	31 (49.2%) 32 (50.8%)	20 (31.7%) 43 (68.3%)	2.08 (1.00-4.3)	0.069
DQB1*03:01	Presence Absence	31(49.2%) 32 (50.8%)	12 (19%) 51 (81%)	4.18 (1.85-9.16)	0.000
DPB1*09:01	Presence Absence	8 (12.7%) 55 (87.3%)	7 (11.1%) 56 (88.9%)	1.16 (0.39-3.42)	1.000

Table 7. Association between HLA alleles (DRB1*07:01, DQB1*03:01 and DPB1*09:01) and HBV infection, stratified by age.

Age category		Cases	Controls	OR (95% CI)	P value
≤ 36 years (n=103)					
DRB1*07:01	Presence Absence	44 (42.7%) 59 (57.3%)	39 (37.9%) 64 (62.1%)	1.22(0.70-2.13)	0.569
DQB1*03:01	Presence Absence	49 (47.6%) 54 (52.4%)	20 (19.4%) 83 (80.6%)	3.76(2.02-7.02)	4.0E-5
DPB1*09:01	Presence Absence	5 (4.9%) 98 (95.1%)	7 (6.8%) 96 (93.2%)	0.69(0.21-2.28)	0.766
≥ 37 (n=79)					
DRB1*07:01	Presence Absence	33 (41.8%) 46 (58.2%)	30 (38%) 49 (62%)	1.17(0.61-2.21)	0.745
DQB1*03:01	Presence Absence	37 (46.8%) 42 (53.2%)	16 (20.3%) 63 (79.7%)	3.46(1.71-7.01)	0.000
DPB1*09:01	Presence Absence	8 (10.1%) 71 (89.9%)	9 (11.4%) 70 (88.6%)	0.87(0.32-2.40)	1.000

DISCUSSION

In the present study, we examined the association of three HLA class II alleles on susceptibility of HBV infection. HBV infection is a global health problem, frequently leads to acute and chronic hepatitis. A complex combination of environmental, genetic and viral components such as gender, age and variation in the HBV genome influences persistent HBV infection [15-18]. Several association studies revealed that HLA class II alleles involved in the clearance or persistent of hepatitis B virus infection [19].

In acute hepatitis B infection specific CD8+ and CD4+ T-cell play a vital role in viral clearance and self-limited process of the disease. These responses are diminished in

persons with chronic HBV infection [20-22]. Development of antibodies to HBsAg and HBeAg becomes failure in patients with chronic HBV infection and lack of Th cell response by class II molecules with poor antigen presenting capacity [23].

A study showed that allele frequency of HLA-DRB1*07:01 in CHB patients (57.83%) was markedly higher than the spontaneously recovered group (24.71%) with an odds ratio of 3.76 was strongly associated with HBV chronicity [24]. In another study [25], the frequency of DRB1*07:01 allele was significantly higher in chronic carriers vs. natural convalescent group (10.8% vs. 4.7%; OR=2.58) indicates that this allele is associated with Hepatitis B chronicity among Koreans. Meta-analysis showed that DRB1*07:01 (OR=1.59) allele had a significantly increased risk of chronic HBV persistence in the Chinese Han group [26]. In the present study, the allele frequency of HLA-DRB1*07:01 in the acute hepatitis B group was markedly higher than those in the chronic hepatitis B group; there was a significant correlation between them **(Table 5)**. This findings suggest that DRB1*07:01 allele may be a susceptible gene for acute hepatitis B and a resistance gene for chronic hepatitis B.

The study by Jiang et al. [11] from China in the year 2003, found that the allele frequency of DQB1*03:01 in CHB group was higher than the normal control group. (35.58% vs. 18.87%; RR=4.07). Thio et al. [27] reported that allele frequency of DQB1*03:01 in patients with HBV persistence was higher than the control (32% vs. 12%; OR=3.9), suggesting that HBV persistence was significantly associated with DQB1*03:01 in American Caucasians. In another Chinese study, the meta-analysis showed that DQB1*03:01 (OR=1.37) allele was significantly associated with an increased risk of CHB [28]. In the present study, we found that the allele frequency of HLA-DQB1*03:01 in the chronic hepatitis B group was markedly higher than those in the healthy control and there was a significant correlation between them **(Table 4)**. This findings suggest that DQB1*03:01 allele may be a susceptible gene for chronic hepatitis B

The result of Japan study revealed that DPB1*09:01 allele frequency was higher in HBV patients than the healthy control (14.4% vs. 9.8%; OR=1.56), suggesting that it is a susceptible allele for CHB [9]. Study conducted by Nishida et al. [13], in the year 2014, reported that DPB1*09:01 (OR=1.97) is a new risk allele to chronic HB infection among Asian populations comprising Japanese, Korean, Hong Kong and Thai subjects. In the present study, we found that the allele frequency of HLA-DPB1*09:01 in the acute hepatitis B group was markedly higher than those in the chronic hepatitis B group; there was a significant correlation between them **(Table 5)**. This findings suggest that DPB1*09:01 allele may be a susceptible gene for acute hepatitis B and a resistance gene for chronic hepatitis B.

Bruno et al. [29] analysed HLA-DRB1 alleles among the HBV infected patients, this confirmed that the DRB1*09 allele was significantly associated with HBV infected male patients and the DRB1*08 allele was significantly associated with HBV infected patients aged ≤ 39 years. In this study, HBV infection in female and patients aged ≥ 36 years were associated with the HLA-DQB1*03:01 allele. This finding suggest that preventive measures should be taken for elder individuals and female gender with DQB1*03:01 allele.

In our study the frequency of Hepatitis B viral infection was higher in male than in females and in concordance with other study [30]. Majority of the cases were illiterate (46.7%). This might be due to the lack of knowledge about HBV disease and its transmission. Educational programs should be provided to stop the spread of HBV infection. About 71.9% of the cases were married, showed statistical significance compared to controls. This indicates the lack of awareness and recommends the need of premarital screening before marriage.

Role of risk factors analysis showed that in private hospitals and clinics outbreaks of blood borne viral infections occurs due to unsterilized surgical instruments as documented by Ott et al. [31]. In our study, 25.2% of patients infected with HBV gave a history of previous surgery and our study was in concordance with a study conducted in Iraq, that documented 20.1% of the HBV infected patients had (previous history of) undergone previous surgery [32].

In our study, family history of hepatitis infection was obtained in 14.8% of the cases and 5.5% of control. Possibility of transmission occurs within the family members due to sharing of razors, tooth brushes and other house- holding instruments [33]. In a study conducted in Iran, 8% of the HBV positive cases gave a family history of hepatitis infection [32].

History of blood transfusion, sharing nail clippers and place of domicile did not contribute significantly to HBV transmission in the study. The similar finding was documented in previous study [34]. A study Tandon et al. [35] reported blood transfusion is a major route of transmission in adults. Sharing nail clippers among friends and household members were common among the cases (14.8%) compared to the control (13.7%). Transmission of virus through this route may occur over prolonged period.

We found, cases were more likely to drink alcohol than the control group. Male gender was significantly associated with alcohol use. Too much of alcohol consumption is an independent risk-factor for HBV infection [36].

Several studies reported that tattooing is a risk factor for HBV infection [37]. In our study tattooing were found to be more prevalent among the cases (10.9%) compared to the control (2.7%).

The present study proposed that HLA DQB1*03:01 may be the susceptible gene and HLA DRB1*07:01 and -DPB1*09:01 may be the resistant genes to chronic hepatitis B. This will give some evidences to study the pathogenesis of HBV infection. Finally in this study, it was found that along with genetic factors, family history of hepatitis B and history of previous surgery play an important role in chronic hepatitis B viral infection.

CONCLUSION

Findings of this study support that HLA DQB1*03:01 may be the susceptible allele and play an important role in patients with chronic HBV infection. The association of HLA DQB1*03:01 allele in CHB patient could be either because of the inability of this allele to present viral epitopes effectively. HLA DRB1*07:01 and DPB1*09:01 alleles showed significant association with acute hepatitis B infection. Although an OR of 15.7 predicts a statistically significant frequency of HLA DPB1*09: 01 allele in AHB patients, the wide confidence interval indicates that number of acute HBV patients should be increased to reach to a more definite conclusion. So, individuals with HLA types may differ in resistance or susceptibility to disease. A large and multi ethnic confirmatory study is needed to validate these findings and to further explore the genetic pathogenesis of HBV infection. Awareness of the blood borne infection among the people, regular or a periodic screening programme among the young adult and empowering the Anti-HBsAg immunization may grossly reduce the HBV infection.

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DECLARATION

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Conflicts of interest

Nil.

Permissions

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