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An investigation on significantly higher allele frequency of Human Leucocyte Antigen A, B, C, DR and DQ in Hepatitis B infected patients as compared to the healthy controls by Next Generation Sequencing in Nepalese population

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ABSTRACT:

— ***Objective:*** Different Human Leukocyte Antigen (HLA) types are linked with different clinical manifestations of Hepatitis B virus infection. However, the association of HLA with different stages of the Hepatitis B infected population in Kathmandu, Nepal, is not yet known.

— ***Materials and methods:*** To understand the relationship between HLA class I (A, B, C) and HLA Class II (DR, DQ) molecules, DNA and data on clinical history, serology, liver enzymes and copy number of the virus in the blood samples were collected for 90 hepatitis B infected patients (including 30 immune active, 30 immune tolerant and 30 acute patients) matched to 90 healthy control individuals for HLA. Human DNA was sequenced by next-generation sequencing.

— ***Results:*** Significantly higher frequencies of the HLA A*11, HLA A*68, HLA B*15, HLA C*04 and HLA DR*15 alleles were found in patients as compared to healthy controls. In healthy controls were more frequently found HLA A*01, A*33, B*07, B*27, B*55, C*01, C*15 and DR*04 positive ($p < 0.05$). HLA-C*04 ($p = 0.002$) was significantly higher in immune active chronic patients.

— ***Conclusions:*** This study shows the HLA allele frequency distribution and diversity in Hepatitis B infected population and healthy controls in Kathmandu, Nepal. This information could also be useful as a marker in various stages of Hepatitis B virus infection for better treatment and management of the disease on a local basis.

— ***Keywords:*** HLA class I, HLA class II, Hepatitis B, Nepal.

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INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic DNA virus that causes acute and chronic infection across the global population[1]. HBV infection has many clinical manifestations, including acute hepatitis, self-limiting recovery, chronic infection, chronic hepatitis, cirrhosis, hepatocellular carcinoma, and hepatic failure[2]. Moreover, despite the availability of effective vaccination, infection and transmission are still ongoing[3].

According to virological and clinical manifestation, hepatitis B infection can be categorized as either acute or chronic. An acute HBV infection diagnosis is made whenever a patient tests positive for HBV surface antigen (HBsAg) and IgM antibodies against HBV core antigen (anti-HBc IgM)[4]. If the infection spontaneously resolves, we will find a negative HBsAg, but positive HBV surface antibody (anti-HBsAg) and IgG antibodies against HBV core antigen (anti-HBc IgG) along with normal alanine aminotransferase (ALT) levels[4,5]. This profile has also been defined as functional healing[6]. Chronic HBV infection is characterized by a phase of immune tolerance followed by immune clearance, ending in a status of the inactive carrier with periodic reactivation phases. While most of the patients experience spontaneous HBV e antigen (HBeAg) seroconversion to the inactive carrier state, a small part of the patients may experience periodic reactivation or persistent hepatitis[7-10]. The further deleterious effects are liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC)[8].

The disease outcome and its course are related to several host factors. Variability in the host immune response is, however, linked to Human Leucocyte antigen (HLA) and its polymorphisms[11]. HLA genes are classified as HLA class I (A, B, C, E, F and G) and class II (HLA DP, DQ, DR, DM and DO)[12]. Class I HLA A, B, and C and class II HLA molecules DR and DQ are well known for their adaptive and innate response against various microbes. HLA II molecules have been associated with the CD4+ and CD8+ response against the microbes which further determines the course of the disease persistence or clearance[13].

The allele frequency in HBV positive patients and how this distribution compares to the healthy population in Nepal is still not known. The aim of this study was to detect the allele frequency of HLA class I (HLA A, B and C) and class II (HLA DR, DQ) among patients with acute HBV infection, immune tolerant chronic hepatitis B and immune active chronic Hepatitis B. We also performed a comparison with healthy controls.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Ethical Committee of the Review Board of the Nepal Health Research Council (Ref Number:138/2018). Written informed consent was signed by all the study participants at the enrollment.

Subjects

We included in this study adult individuals, older than 18 years, with clinical and serological evidence of HBV infection. Three subgroups, such as acute infection, chronic immune tolerant infection, and chronic immune active infection, were identified according to clinical history and serological tests results (HBsAg, anti-HBsAg, HBeAg, anti-HBeAg, anti-HBcAg) collected for at least 24 months. Moreover, we obtained data about ALT, aspartate aminotransferase (AST), alkaline phosphatase (AlP) and HBV plasma viral load (HBV pVL) to better categorize patients. Table 1 shows how the patients were divided into three subgroups. We also included healthy controls, matched with patients by age, ethnicity, HIV and HCV status.

Both patients and controls were enrolled at the Nepal Public Health Laboratory of the Teku and Tribhuvan University in Kathmandu, Nepal, from August 2019 to April 2020. Study participants were not related.

Inclusion Criteria

Patients were included in this study if their age was over 18 years, and their clinical history included at least two time points of observation separated by at least 6 months for at least 24 months.

Controls were included in this study if they were older than 18 years with no evidence of HBV infection (defined as negative HBsAg, HBeAg and anti-HBcAg IgM and IgG, with no history of HBV vaccination).

Exclusion Criteria

Patients were excluded when they were younger than 18 years, more than one case in the same family, use of immunosuppressive drugs, coinfection with human immunodeficiency virus (HIV) or hepatitis C virus (HCV).

Controls were excluded if they were younger than 18 years, related to patients, or they used immunosuppressive drugs.

Table 1. Criteria during sample selection. IT†: Immune tolerant, IA‡: Immune Active.

	Infection		
	Criteria	**Acute**	**Chronic**
		IT†	IA‡
HBsAg	+/-	+	-
HBeAg	+/-	+	-
Anti-HBs	-	-	+
Anti-HBe	+/-	-	+
Anti-Hbc	IgM+	IgG	IgG
Liver enzyme	High	Normal	high
Viral Load	>20000 IU/ml	>20000 IU/ml	<20000 IU/ml

Serological, Biochemical and Viral Load Screening

Blood samples were tested for HBsAg, HBeAg, anti-HBsAg, anti-HBeAg and anti-HBcAg using a commercial kit produced by Tulip Diagnostics (P) Ltd (Alto Santacruz, Complex Alto Santacruz, Bambolim, Goa, India) according to the manufacturer's instructions. Only the clearly positive and negative tests underwent further investigations. The information regarding liver enzymes (ALT, AST, AlP) was collected from the participants' clinical records, available during the screening at the Nepal Public Health Laboratory, in Teku. Viral load was estimated by Real Time PCR (Corbett Research RG6000, Qiagen, MD, USA) using Real Time PCR kit (Artus R HBV RG PCR kit Handbook, Qiagen, Germany). Briefly, 25 µl of the master mix was added to each well. To this 25 µl of the sample was added followed by the positive control and negative control in respective wells. The internal control was also added during the sample preparation. PCR conditions for the copy number detection was 95˚C for 2 minutes, followed by 50 cycles of 95°C for 15 seconds, 58˚C for 20 seconds and 72˚C for 30 seconds.

DNA Extraction

The genomic DNA extraction was performed by QIAmp^{R}DNA Blood mini kit (Qiagen, MD, USA), according to the manufacturer's instructions. Briefly, 200 µl of whole blood were mixed with 200 µl of AL buffer along with 20 µl of proteinase k. The mixture was vortexed for 15 seconds and incubated at 56°C for 10 minutes. Then, 200 µl of ethanol were added to the sample and vortexed for 15 seconds. This mixture was then added to a QIAamp Mini spin column tube and centrifuged at 8000 rpm for 1 minute. The process was again repeated after adding 500 µl of AW1 Buffer followed by AW2 buffer and finally with the provided AE buffer. The filtrate obtained after the last transfer was utilized for genomic analyses.

HLA Typing

After extraction, the DNA was quantified and sent to Supratech Laboratories pvt. Limited, in Ahmadabad, India for typing of class I and class II HLA molecules by Next Generation sequencing (NGS) with Ion torrent NGS platform (Thermo Fisher Scientific, Waltham, MA, USA), using NGSgo® workflow (GenDx, Utrecht, the Netherlands).

Statistical Analysis

Patients and controls data were collected anonymously in an electronic datasheet. Statistical analysis was carried out with IBM SPSS statistic version 20 for Windows. Categorical variables were analyzed with descriptive (count, percentage) and inferential statistics. Quantitative variables were analyzed with descriptive (mean ± standard deviation, SD) and inferential statistics.

The HLA allele frequency of class I and II were calculated and then compared with each other by chi square test and/or Fisher's exact test. Results were considered significant if the *p*-value was lower than 0.05. The odd ratio (OR) and risk factor (RF) analyses were performed with a 95% confidence interval using the online contingency table analysis at stat pages info/ctab 2x2.html.

RESULTS

Characteristics of Study Subjects: Clinically Diagnosed Hepatitis B Patients and Recruited Healthy Individuals

We included 90 HBV positive subjects and 90 controls. Fifty-two patients (57.77%) were male and 38 (42.22%) were female. The patients' mean age was 29.02 years (SD ± 10.26). Sixty healthy controls (66.66%) were males and 30 (33.33%) were females. Healthy controls' mean age was 30.75 years (SD ± 8.59) (Table 2). Among 90 recruited hepatitis B infected patients, 60 (66.6%) were in a chronic phase of disease. However, the remaining 30 patients (33.3%) were in an acute phase of the infection. Table 3 shows the serological tests results, HBV pVL, and liver function enzymes.

Table 2. Demographic Details of the studied population.

Study subjects	**Patients**	**Controls**
Number of participants (N)	90	90
Age (Mean ± SD) years	29.02 ± 10.26	30.75 ±
8.59		
Range (years)	18-67	19-71
Sex (Male/Female); ratio	52/38; 1.3:1	60/30; 2:1
Type of infection		
Acute; %	30; 33.33%	
Chronic; %	60; 66.66%	
Age wise distribution of study subjects		
Age (in years)	**Hepatitis B infected patients**	**Healthy controls**
<17	0	0
18-28	30	28
29-38	20	25
39-48	19	16
49-58	10	14
59-68	11	7
Total	90	90

±Standard Deviation (SD)

Table 3. Serological and viral load investigations.

Serology	Hepatitis B infected patients
$HBsAg^+$ (%)	60:66.66%
$HBsAg^+/HBeAg^+$ (%)	60:66.6%
$HBsAg^-/HBsAb^+$ (%)	30:33.33%
$HBe\ Ab^+$ (%)	57:63.33%
HBcAb (%)	IgM:30; 33.33%, IgG:60:66.66%

Hepatitis B Surface Antigen (HBsAg), Hepatitis B Envelope Antigen (HBeAg), Hepatitis B surface AntibodyHBsAb), Hepatitis B Envelope Antibody (HBeAb), Hepatitis B core Antibody (HBcAb), Immunoglobulin M (IgM), Immunoglobulin G (IgG).

Viral Load	Copy number (IU/ml)	Number of patients%
Low	<2000	38:42.22%
Intermediate	2000-20,000	31:34.44%
High	>20,000	21:23.33%

HLA alleles: A*11 and A*68 was Significantly Higher in Hepatitis B Patients

The sequencing data showed that the HLA-A*11 (18.8%; p = 0.001) and HLA-A*68 (5%; p = 0.031) allele were significantly more frequent in HBV positive individuals compared with healthy controls (Table 3, Figure 1A and Figure 2). On the other hand, HLA-A*01 (16.67%; p =0.005) and A*33 (31.67%; p =0.001) were significantly higher in healthy individuals as compared with HBV positive individuals (Table 4 and Figure 2).

HLA alleles: B*15 was Significantly Higher in Hepatitis B Patients

The sequencing data showed that HLA-B*15 (21.11%; p= 0.001) allele frequency was significantly higher in HBV positive individuals compared with healthy controls (Table 4, Figure 1B and Figure2). On the contrary, HLA-B*07 (8.33%; p=0.015), B*27 (6.67%; p=0.057) and B*55 (8.33%; p=0.015) were significantly higher in healthy controls as compared with HBV positive individuals (Table 5, Figure 1B and Figure 2).

HLA-C*04 was Significantly Higher in Hepatitis B Patients

The sequencing data showed that HLA-C*04 (25.56%; p = 0.034) was significantly higher in patients compared to healthy controls (16.67%) (Table 6, Figure 1C and Figure 2). On the other hand, HLA-C*01 (11.67%; p=0.003) and C*15 (16.67%; p=0.009) were strongly significantly higher in healthy individuals when compared with diseased individuals.

HLA-DRB1*15 of HLA Class-II Allele Was Significantly High in Hepatitis B Patients

The sequencing data showed that HLA-DRB1*15 (38.33%; p=0.009) allele frequency was significantly higher in HBV positive individuals compared with healthy controls (Table 7, Figure 1D and Figure 2). On the contrary, HLA-DR*O4 (6.67%; p=0.003) was significant in healthy controls as compared with HBV positive individuals (Table 7, Figure 1D and Figure 2). Interestingly, we did not observe a difference between groups of HLA-DQB1 frequency.

HLA-C*04 was Higher in Immune Active Chronic Patients

We also studied differences between various phenotypes of clinical manifestations. We found out that HLA-C*04 was significantly higher (p = 0.002) in Immune active chronic group of patients compared with immune tolerant chronic patients.

DISCUSSION

The interplay of HLA against infectious diseases including HBV infection, in a varied range of populations, has been documented in several researches[14,15]. In this study, analysis of 2 digit allele frequency of the HLA I(A, B, C) and HLA II(DR, DQ) molecules on the HBV infection and its comparison with healthy individuals have been investigated.

Regarding allele A, the more frequent alleles in HBV infected patients were A*11 (18.89%) and A*68 (5%) as compared to A*33 (38.33%), and A*01 (16.67%), which were more frequently found in healthy controls. In a report from China[1] the frequency of 33:03:01G was increased in persistent HBV infection group patients. Further investigation revealed that the HLA locus A*29 (8.33%) was detected in high frequency only in healthy individuals and not in HBV positive patients. Similarly, HLA A*34 (3.13%) was detected only in immune active chronic patients. Furthermore, HLA A*26 (1.67%) was detected only in acute HBV infected patients. In Japan, HLA A*26 has been associated with no disease progression in HBV carriers[16]. Such data indicate that these alleles could be used as a marker for the various stage of infection in Nepalese population.

Responsiveness to HBsAg has been associated with HLA A*11 in Indians of Asian origin and A*10, A*01 and B*07 in Caucasians[17]. HLA A*02 and A*11 are protective alleles for HBV chronicity in Russians and Kazakhs respectively[18]. HLA A*1101 have been associated with viral clearance in Caucasians and in African Americans[19]. The diversity noted in A*68 was 68:01:01, 68:01:02 and 68:01:02G in infected patients as compared to the diversity of 68:01:02:02 in healthy controls.

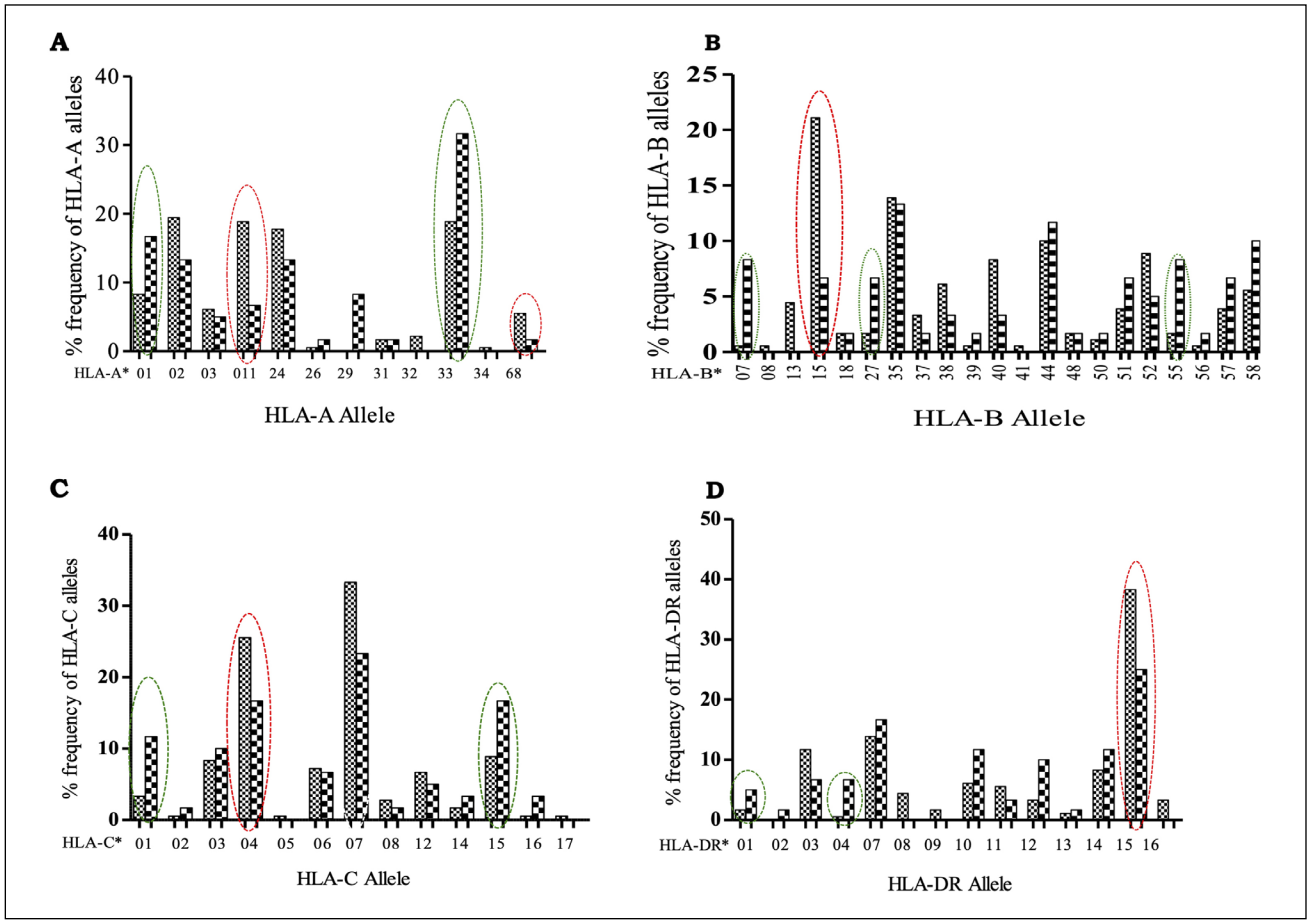

Figure 1. A, Significantly higher frequency in Hepatitis B individuals detected in HLA A*11,A*68 as compared to the alleles A*01,A*33 in healthy controls. **B**, Significantly higher frequency in Hepatitis B individuals detected in HLA B*15 as compared to the alleles B*07, B*27, B*55 in healthy individuals. **C**, higher frequency in Hepatitis B individuals was detected in C*04 as compared to the alleles C*01,C*15 in Healthy Individuals. **D**, Significantly higher frequency in Hepatitis B infected individuals were observed in DR*15 as compared to DR*04 in healthy controls.

However, in healthy population, A*01 and A*33 were significantly detected as compared to the diseased population. The diversity detected in HLA A*33, in both healthy and infected patients were A*33:03:01:01 and A*33:03:01, A*33:40:01 respectively. The presence of A*3303 is also reported to be high in Asian, Japanese and Korean population[20-22]. The diversity detected for A*01 was A*01:01:01:03 and HLA A*01:01:01:01 in healthy controls as compared to HLA A*01:01:01 in hepatitis B infected patients. HLA A*0101 have been reported as an allele of high frequency in Caucasians and Jews which is also found frequent in Nepalese population[21].

Regarding the B allele, the highest frequency observed in the Hepatitis B infected patients was B*15 (21.11%) as compared to B*35 (13.33%), B*44 (11.67%) and B*58 (10%) in healthy controls. HLA B*35 have been associated with chronic Hepatitis B virus infection and liver cirrhosis in Latin American Caucasoid patients and in Italy respectively[23,24]. Similarly, B*07, B*27, and B*55 were detected in significantly higher frequency in healthy individuals as compared to the HBV positive patients. HLA B*07 and B*58 have been associated with a protective effect against chronic HBV[2].

The diversity detected in the significantly higher allele B*15 in HBV positive patients are HLA B*15:01:01, B*15:05:01, B*15:17:01, B*15:25:01, B*15:32:01 and B*15:18: 01G as compared to 15:17:01:01 and 15:01:01:01 in healthy individuals. Non-responsiveness to HbsAg vaccination is reported to be associated with HLA A*1, B*15, and B*40 in Indians of Asian origin[17].

Furthermore, a significantly higher allele frequency B*07 with the diversity of B*07:02:01:01 and B*07:05:01:01 was observed in healthy controls. HLA B*07 has been associated with disease conditions such as cervical cancer and sarcoidosis[25].

Diversity of allele B*27 in healthy controls detected were 27:05:02:01,27:05:02:11 and 27:07:01 as compared to HLA B*27:04:01 along with 27:05:02G and 27:07:01 in Diseased patients. HLA B*57 and B*27 have been associated with lower rate of disease progression in HIV[26].

Regarding allele C, Hepatitis B infected patients detected diversity of allele C*07 as C*07:01:01, C*07:01:02, C*07:02:01, C*07:04:01, C*07:06:01, C*07:18:01, C*07:26:01 as compared to C*07:01:01:01, C*07:01:01:04, C*07:01:02:01, C*07:02:01:01, C*07:02:01:03 and C*07:06:01:01 in healthy individuals. Similarly, the diversity detected in C*04 were C* 04:01:01, C* 04:03:01, which were more frequent in HBV

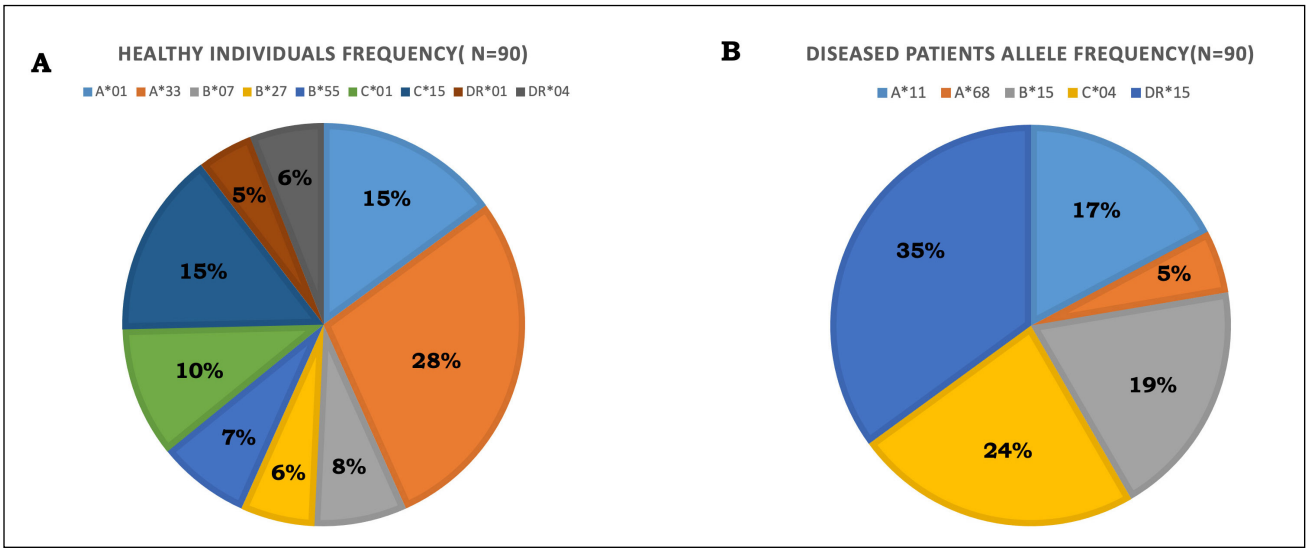

Figure 2. A comparison between the distribution of High Allele frequencies of Diseased patients and Healthy Individuals.

positive individuals, compared to healthy controls, where C*04:01:01:01 and C*04:01:01:06 were more frequently found. Regarding allele C*01 the diversity detected was C*01:02:01:01.

These data show the diversity of the Hepatitis B infected Nepalese patients in response to the healthy controls.

Regarding allele DR, the comparison between the healthy controls and HBV infected patients revealed a weakly significant higher incidence of DR*15 in HBV positive patients. Also, in healthy controls, DR*01 and DR*04 were detected in a significantly higher frequency as compared to the infected patients. DR*15 diversity detected in infected patients were DR*15:01:01, 15:02:01,15:02:02 and 15:06:01 as compared to 15:01:01:01 and 15:02:01:01 in healthy controls respectively. There are reports in which DRB1*15 is associated with significantly increased antibody response to HBV vaccines. DRB1*15 is also protective for HBV infection in a Chinese population but associated with chronic infection in Turkish and Indian populations respectively[27,28]. A meta-analysis[29] also revealed the association of DRB1*15 with the antibody response to the HBV vaccine.

Similarly, DR*04 detected the diversity of 04:01:01:01 in healthy population and 4:01:01,4:03:01, 04:06:01 in the

Table 4. Class I HLA A frequency. Significantly higher frequency in Hepatitis B individuals detected in HLA A*11,A*68 as compared to the alleles A* 01,A*33 with $p<0.005$in healthy individuals.

HLA-A allele	% Frequency	
	Hepatitis B infected Patients	Healthy Individual
A*01	8.33	16.67
A*02	19.44	13.33
A*03	6.11	5.0
A*11	**18.89**	**6.67**
A*24	17.78	13.33
A*26	0.56	1.67
A*29	—	8.33
A*31	1.67	1.67
A*32	2.22	—
A*33	18.89	31.67
A*34	0.56	—
A*68	5.56	**1.67**

Table 5. Class I HLA B frequency. Significantly higher frequency in Hepatitis B individuals detected in HLA B*15 as compared to the alleles B*07, B*27, B*55 with $p<0.005$ in healthy individuals.

HLA-B allele	% Frequency	
	Hepatitis B infected Patients	Healthy Individual
B*07	0.56	8.33
B*08	0.56	-
B*13	4.44	-
B*15	**21.11**	**6.67**
B*18	1.67	1.67
B*27	1.67	6.67
B*35	13.89	13.33
B*37	3.33	1.67
B*38	6.11	3.33
B*39	0.56	1.67
B*40	8.33	3.33
B*41	0.56	—
B*44	10.0	11.67
B*48	1.67	1.67
B*50	1.11	1.67
B*51	3.89	6.67
B*52	8.89	5.0
B*55	1.67	8.33
B*56	0.56	1.67
B*57	3.89	6.67
B*58	5.56	10.0

Table 6. Class I HLA C frequency. Significantly higher frequency in Hepatitis B individuals was detected in C*04 as compared to the alleles C*01,C*15,with $p<0.005$ in healthy individuals.

HLA-C allele	% Frequency	
	Diseased Individual	Healthy Individual
C*01	3.33	11.67
C*02	0.56	1.67
C*03	8.33	10.0
C*04	**25.56**	**16.67**
C*05	0.56	—
C*06	7.22	6.67
C*07	33.33	23.33
C*08	2.78	1.67
C*12	6.67	5.0
C*14	1.67	3.33
C*15	8.89	16.67
C*16	0.56	3.33
C*17	0.56	—

Table 7. HLA Class II HLA*DR frequency. Significantly higher frequency detected in Hepatitis B infected individuals were detected in DR*15 as compared to DR*04 in healthy controls.

HLA-DR allele	% Frequency	
	Diseased Individual	Healthy Individual
DR*01	1.67	5.0
DR*02	—	1.67
DR*03	11.67	6.67
DR*04	0.56	6.67
DR*07	13.89	16.67
DR*08	4.44	—
DR*09	1.67	—
DR*10	6.11	11.67
DR*11	5.56	3.33
DR*12	3.33	10
DR*13	1.11	1.67
DR*14	8.33	11.67
DR*15	**38.33**	**25.0**
DR*16	3.33	—
HLA-DQ allele	**% Frequency**	
	Diseased Individual	**Healthy Individual**
DQ*02	23.33	18.33
DQ*03	16.67	25.0
DQ*04	1.11	-
DQ*05	37.78	38.33
DQ*06	21.11	18.33

infected patients. Meta-analysis on association of HLA class II molecules in Hepatitis B infection has shown that HLA DRB1*04 and HLA DRB1*13 as protective factors in HBV clearance[30].

Regarding DQ, the highest DQ allele frequency in healthy population, as well as diseased population was detected to be DQ*05 (38.33% and 37.78%) respectively. The diversity observed was DQ*05:01:01:01, DQ*05:01:01:02, DQ*05:01:01:05, DQ*05:02:01:02 and DQ*05:03:01:01 in healthy population and 05:01:01G,05:02:01G and 05:03:01, respectively.

CONCLUSIONS

From the investigation we performed, we can conclude that the above-discussed alleles can be used as a marker for the various stages of Hepatitis B infected patients of Nepal. Further, we also report the diversity observed in HLA alleles in Nepal. Such information could also be used as an aid for the further development of antiviral drugs and novel Immunotherapeutic strategies for the control, treatment, and management of Hepatitis B virus infection.

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Author Contribution Statement:
Smita Shrestha (SS): contributed to the work by conceptualizing the research work, in performing the Serological investigation, DNA extraction and data collection of the selected samples. Sweety Upadhyay (SU), Jagat Baniya (JB) and Dr. Mukunda Sharma Bhattarai (MB): contributed to the sample collection and then selection of the samples. Dr. Manish Maurya (MM): contributed to the data analysis and manuscript preparation. Dr. Krishna Das Manandhar (KDM): contributed to conceptualizing the work and in manuscript preparation.

Conflict of Interest:
The authors declare that they have no conflict of interest.

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