Journal of Chitwan Medical College 2024;14(48):30-34
Available online at: www.jcmc.com.np

ORIGINAL RESEARCH ARTICLE

FREQUENCY OF HUMAN LEUCOCYTE ANTIGEN (HLA) CLASS I (A,B,C) DIVERSITY COUPLED WITH IMMUNOMOLECULAR PANEL: A POTENTIAL IMMUNOGENETIC MARKER FOR HEPATITIS B INFECTION

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Received: 9 May, 2024

Accepted: 13 Jun, 2024

Published: 17 Jun, 2024

Key words: Hepatitis B; HLA; Serology.

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*DOI:*https://doi.org/10.54530/jcmc.1525

Citation

Shrestha S, Manandhar KD. Frequency of human leucocyte antigen (HLA) class I (A, B, C) diversity coupled with immunomolecular panel: a potential immunogenetic marker for hepatitis B infection. Journal of Chitwan Medical College.2024;14(48):30-34.

ABSTRACT

Background: Hepatitis B virus infection involves a complex interplay of the host pathogen interaction. The host factors involves the Human Leucocyte Antigen molecule, associated with the persistence or clearance of the infection in a population. Viral factors involves antigen, antibody and viral copy number. The aim of the study was to generate a combined information on the serology panel, copy number and HLA diversity as immunogenetic markers in an HBV infected population.

Methods: This quantitative study was conducted at the Central department of Biotechnology, Tribhuvan university , Sukraraj Tropical Hospital and National Public Health Laboratory ,Teku in the time period of December 2018 to march 2020. Serological panel was detected followed by the copy number of the virus. Diversity of class I HLA –A, B and C was detected by sequencing. Calculation were performed by SPSSv.22 and graphically represented by PrismPad.v.5.

Results: Out of the total 828 Hepatitis B positive patients, majority of 56.15% detetcted the serological panel of HBsAg+/HBeAg-/ HBsAb-/HBeAb-/HBcAb±/IgMHBcAb± . The highest detection of copy numbers in the range of 20-20,000 IU/ml(70.14%). Diversity detected was HLA A*11:01, A*68:01, B*15:01, B*15:05, B 15:18, B*15:25, B*15:32, C*04:01, C*04:03 in Hepatitis B infected individuals as compared to the healthy controls. The level of significance was detected in the HLA A*11:01(p=0.02) and A*68:01(p=0.01) in HBV infected individuals.

Conclusions: Serological panel, copy number and HLA detects immunogenetic markers in Hepatitis B infected patients which can aid in better management and treatment of the infection.

INTRODUCTION

In an era of the sustainable Goal development (SDG), introduced by United Nations General Assembly and adopted by Nepal, the elimination of viral Hepatitis by 2030 remains a commitment.[1] Globally, ample evidences indicate the importance of serological panel of markers and the copy number of the double stranded DNA virus belonging to *Hepadnaviridae* family, the Hepatitis B virus, to play an important role in the course of infection and its management.[2-4] However, given the complex interplay of the host pathogen interaction, the host as well as the viral factors play an important role in the management of the disease. Regarding the host factors, the Human Leucocyte Antigen (HLA) , present in chromosome 6, highly polymorphic, has been associated with the infection.[5,6] These molecules acts a genetic marker for the persistence of infection or protection against it in various population. Besides this, HLA has been ,since a long time, associated with the humoral and cell mediated response of the host against the infection.[7] However, in Nepal , scarce information regarding the diversity of the HLA class I: A, B and C in Hepatitis B virus infection is available. Similarly, the information on serology is mostly limited to HBsAg and HbeAg detection. Given, the resource limited setting of the county, such information makes the work less tedious for further diagnosis but might overlook some of the circulating epidemiological status of the complex disease. This infection has to be checked, not only to confirm the symptoms of viral hepatitis but in other medical conditions as well.[8,9] Given its nature of infection, the use of single marker of exposure is associated with unpredictabity and a potential for poor prognosis, given the role of host factors as well. Besides this, studies have indicated the relationship of the HBeAg seroconverts with the remission and further complications associated with it.[10,11] The serological panel combined with the information on the copy number of the virus and diversity detected in the HLA molecules can provide an important information on the prevalence of the immunogenetic markers present in the population. The detection of such markers can predict the immunological response and/ or therapeutic response of a population against a HBV infection. The research work shows the serological panel, copy number detected and the HLA I A, B and C diversity in the study population. Hence, the aim of the study was to generate a combined information on the serology panel, copy number and HLA diversity as

immunogenetic markers in an HBV infected population.

METHODS

This descriptive study was conducted at Central Department of Biotechnology, Tribhuvan University, Sukraraj Tropical hospital, and National public health laboratory, Teku, Kathmandu. Ethical approval was taken from the Ethical Committee of review Board, Nepal Health Research Council (Ref Number: 138/2018). Totally, 828 HBV infected samples were included in the study in a time period of December 2018 to April 2020, for a period of 1.5 years.

Patients over the age of 19 years with symptoms of HBV along with the clinical history and observed for at least 6 months were included in the study. However, Patients less than 18 years of age and co infection with Human Immunodeficiency virus (HIV) and Hepatitis C virus (HCV) were excluded from the work. Further, for controls less than 19 years of age were excluded.

Determination of serological panel by ELISA

The serological panel which included detection of Hepatitis B antigen (HBsAg, HBeAg) and antibody(HBsAb, Total HBcAb, IgM HBcAb) was performed by ELISA according to manufacturer's procedure using diagnostic kits (Autobio diagnostics Co.Ltd., Zhengzhou, China).

Briefly, 50µl of the sample was added to an equal volume of conjugate mixture and then incubated at 37^{0}C for 3 minutes. This was followed by washing with the wash solution. After removal of the residual wash solution, 50µl of the chromogen solution was added. To this, 50µl of the substrate solution was added and mixed for 15 seconds. The plate was incubated in dark for 10 minutes and further the reaction was stopped by adding 50µl of the stop solution. The absorbance was measured at 450nm.

The procedure for the antibody determination was similar to the antigen and was performed according to the manufacturer protocol with slight modifications.

However, the calculation of the cut- off value was calculated as follows:
For Antigen
Cut off value= 2X Negative control (Mean of Negative control)
For Antibody
Cut of value= 2.1X Negative Control (Mean of Negative control)
The samples with known serological panel was then further processed for the estimation of the copy number of the virus.

Viral DNA extraction and load detection

Viral genomic extraction and its copy number detection was performed according to the manufacturer's protocols by QI amp DNA Mini kits (Qiagen, Hilden, Germany). Briefly, 200µl of the whole blood was mixed with equal volume of buffer containing Proteinase K. The whole mixture was vortexed for 15 seconds and incubated at 56^{0} C for 10 minutes. This mixture was then briefly centrifuged at 8000rpm to collect the eluent using the eluent buffer. The final eluent contained the viral DNA which was then further quantified by Real Time PCR.

Further, for the load detection, 25µl of the sample was mixed with 25ul of the master mix along with the standard. Besides this positive and negative controls were also added in the respective wells. The PCR conditions were 95^{0}C for 2 minutes followed by 15 seconds and then 58^{0}C for 20 minutes and then 72^{0}C for 30 minutes.

The samples with known viral DNA copy number were then processed for class I HLA- A, B and C subtyping

Identification of Class I HLA- A alleles by Sequencing

The identification of the class I HLA- A alleles were done by Next generation sequencing and were sent at Supratech Laboratories pvt Limited, Ahmadabad, India. The instrument had ion torrent platform (Ion S5) and used GenDx NGSgo workflow.

RESULTS

A total of 828 HBV infected patients were included in the study population. Age of the patients ranged from 19-70 years with a mean and standard deviation of 32.25±8.2. Out of them 53.74%(n=445)showed a major serological panel for Group I as HBsAg+/HBeAg-/ HBsAb-/HBeAb-/HBcAb±/IgMHBcAb± followed by 46.25%(n=383) which showed a serological panel for group II as HBsAg+/HBeAg+/HBsAb-/HBeAb-/HBcAb±/IgMHBcAb± respectively. This shows the presence of Hepatitis B virus Surface antigen along with the presence/absence of antibodies circulating in HBV infected population. However, following the detection of surface antigen, the other major group of panels detected the presence of Hbe Ag along with presence or absence of the antibodies. This group of serological profile formed the basis of further work as the seroconverts detected in these groups, given their clinical importance have to be further monitored for management of the infection.

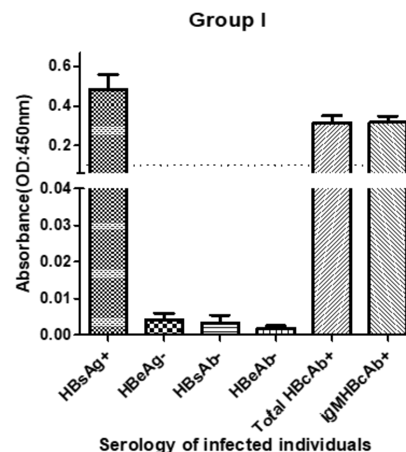

Figure 1: Serological panel for Group 1 obtained for the Hepatitis B infected Individuals. Cut off value >0.1.Positive to HBsAg, HBcAb and IgMHBcAb. Negative to HBeAg, HBsAb and HBeAb

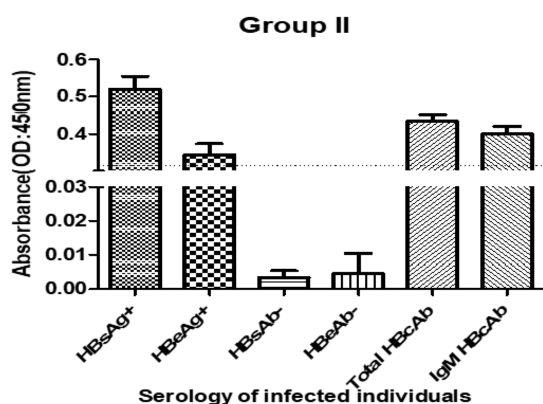

Figure 2: Serological characteristic of Group II HBV infected patients indicating a positive HBsAg, HBeAg and HBcAb. Further, the figure depicts a negative HBsAb and HBeAb. Dotted lines indicates the cut -off value

Further, based on the serological panel, the classification of the copy number of the detected virus was done. This was performed according to the WHO classification system, which is shown below in Table 1

Table 1: Viral Load concentration in infected patients

Viral DNA(IU/ml)	Count	Percentage
<200	97	11.71%
200-20,000	583	70.14%
>20,000	148	17.87%

Out of 828 HBV infected cases, based on history, serology and copy number of virus detected, 127 samples were sequenced for the HLA detection. However, out of 127 samples, HLA could be detected in 96 samples. The diversity detected in the samples as compared with the controls is shown in the table below.(Table 2). The statistical difference of $p<0.05$ between the HBV infected patients and controls was found only in the A*11 and A*68. Further, B*15:05, B*15:18, B*15:25, B*15:32 and C* 4:03 were detected only in the HBV infected patients and could be further studied for their role in the disease progression.

Table 2: Frequency of HLA class I (A, B, C) diversity in HBV infected individuals

HLA Allele	Diversity detected	Frequency of HBV infected patients (%)	Frequency of Healthy controls (%)
A*11	11:01	19.79*	4.16*
A*68	68:01	6.25*	1.04*
B*15	15:01	9.375	3.12
	15:05	2.08	-
	15:18	5.20	-
	15:25	2.08	-
	15:32	4.16	-
C*04	04:01	17.7	9.37
	04:03	8.33	-

Legend: * indicates $p<0.05$

DISCUSSION

The transmission of HBV infection is dependent on both the viral as well as the host factors. Among host factors, Human Leucocyte antigen have shown to play an important role in determining the course and severity of the infection. [5, 12] The molecule is known to be polymorphic and prevalent in different allelic forms. It is considered as tremendously important in terms of pathological and physiological aspects of the population. Further, the diversity of the HLA molecule can detect the strength of response of the population against the infection. Among HLA alleles, HLA A* 11 showed the genetic diversity of A 11:01:01 in both acute and chronic group of HBV infected individuals. In works previously done, in Indian and Caucasians, HLA A*11 and A * 01, A *10 and B*07 have been associated with responsiveness to HBsAg .[13] Further, HLA A*11 have been associated as one of the alleles with protection against the infection in Russians and Kazaks population.[14] Further, the diversity of HLA A*1101 have been associated with clearance of the infection in Caucasians and African Americans.[15] Determination of the type of molecule and its diversity in the populations will help in better understanding the role of host in the HBV infection.

Regarding allele B, the B*15 have been detected with its diversity. The diversity noted is associated with the non-responsiveness to the HBs vaccination along with the HLA A*1 and B*40 in Indian population of Asian origin, B*07 in Caucasians and B*54 in Chinese. [13] HBs vaccination is one of the crucial tool in the prevention of the HBV infection and the non-responsiveness to this measure could hold a clinical significance to the population.

However, regarding serology, screening of HBV infected individuals on the basis of antigen and antibody have always been an important factor for proper monitoring and maintenance of the infection. The research shows that on the basis of the detection of Antigen and antibody, the panel containing HBsAg+/HBeAg-/ HBsAb-/HBeAb-/HBcAb±/IgMHBcAb± had the highest number of patients(53.74%,n=445) detected followed by HBsAg+/HBeAg+/HBsAb-/HBeAb/HBcAb±/IgMHBcAb±(34.50% , n=383). According to the Centers for Disease Control and prevention, mortality and morbidity report, the triple panel screening which includes HBs Ag, Anti- HBs and total anti- HBc is recommended for the initial screening and providing proper care and management for this complex disease.[16] Nucleotide analogues such as entacavir and tenofovir are the of drugs mostly used in the treatment of HBV infections. However, in a meta-analysis study, it has been shown that the serology panel can help in choosing the effective drugs for its associated complications.[17] This also indicates the usefulness of serology panel in understanding

the efficacy of different drugs exhibiting various mechanism of action for the treatment of the infection. Further, Nepal shares an open border with the neighboring country India where the prevalence of the HBV infection have been reported to be 3% as compared to 1 % in Nepal.[18] This suggests the need for the serological testing in an open boundary settings which might also help in control of the infection.

There is a paucity of data regarding the serological panel and HLA diversity of the HBV infected patients in Nepal. Our work has addressed the gap and has provided a baseline data for further work. The additional information on the status of the HBeAg, HBeAb gives us an information regarding the seroconverts who can then be monitored for any further relapses.

Further, the study detected the range of 200-20,000IU/ml as the highest category for the number of HBV patients that were recorded in the study. Previous studies have shown higher proportion of HBV infected patients were detected in the above-mentioned range. Further, the population had more males as compared to females. [19] This is of importance in terms of the infection because in one of the surveys done by WHO, the percent of HBsAg detection is very low in population below 15yrs of age [20] but still it is comparatively high in adults. This shows the presence and importance of horizontal transmission in Nepalese population. The pattern is similar to India where there are ample evidence of the role of horizontal transmission as compared to the perinatal transmission for the HBV infection to spread. [21,22]

The population under study indicated an array of serological, molecular and Immunological response against the infection. Detection of the above-mentioned serological markers along with the viral DNA shows the dynamics of the infection. Further, the detection of the diversity in HLA class I- HLA A, B and C shows the host dynamics associated with infection. Further, despite the availability of vaccine, the population still suffers from infection.[23] There are studies done in India where the average carrier rate of HBsAg was found to be 5% [24] and in pregnant women 1 to 9% [25] with 18%or less HBeAg positivity in HBsAg positive pregnant women in families.[21] However, there is a dearth of such panel in Nepal and further stringency in studies where the viral factors have been studied in association with the host genetic factors.

Besides this, there is a need for the mapping of viral hepatitis and further maintaining the HBV database. Nepal do not have the baseline data at national level regarding the serological pattern, molecular and Immunological nature of the infection prevalent in the nation. Such conditions act as a liability and burdens the infected patients with financial stress and a systematic Information in this regard hold importance particularly in developing countries like Nepal. Further, history of the patient, serology, biochemical analysis, molecular study of the infection, involving both the host and the pathogen can be a potent predictive tool for the infection outcome especially with the high-risk group of HBV infected patients.

However, more sample size is needed for conducting the serology, molecular and immunological aspect of the infection at same time. Besides this, the time span for the sample and monitoring of the disease for a longer duration was not possible. Besides, its limitation, the work has provided a baseline data regarding the serological pattern and the role of host genetics that acts as an important aspect in the severity of the infection.

CONCLUSION

Prevention, early detection and better management is the burgeoning issue of HBV infection. HLA frequency and diversity detection along with the information on history, serology and viral copy number can play an important role in the detection of the immunogenetic markers associated with the infection. The immunogenetic markers can be used for the detection of protective alleles and / or alleles which make a population susceptible to the infection, hence, providing better management for the condition.

CONFLICT OF INTEREST: None

FINANCIAL DISCLOSURE: The work was partially supported by the University Grants (Ref No.72/73-S&T-6).

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