

Molecular and genetic characterization of hepatitis B virus among multitransfused thalassaemia patients in Islamabad, Pakistan

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ABSTRACT

Background: Hepatitis B virus (HBV) is the aetiological agent of transfusion-transmitted hepatitis globally. Beta thalassaemia major individuals are at greater risk of contracting HBV infection due to multiple blood transfusions required for the medical management of these patients. Based on HBV genetic variability, it is divided into 10 genotypes. The determination of HBV genotypes has significant implications for clinical management and treatment regimens. **Aim:** This study was performed to assess the HBV epidemiology and circulating genotypes in multi-transfused β -thalassaemia major patients with the aim to be considered while formulating the treatment pattern taking into account particular needs of thalassaemia patients. **Materials and Methods:** This study was performed from September 2018 to June 2019, at the Department of Pathology and Transfusion Medicine, Shaheed Zulfiqar Ali Bhutto (SZAB) Medical University, Islamabad. A total of 2,260 thalassaemia patients were enrolled in the study. The study was endorsed by the Ethics Committee of the SZAB Medical University, Islamabad. The samples were serologically screened for HBsAg on the LIAISON® XL Murex HBsAg Quant assay (DiaSorin S.p.A., Italy) a chemiluminescence based immunoassay (CLIA). HBV quantitative PCR kit was used to measure the HBV DNA in serum samples. The HBV genotypes were determined using universal primers targeting the P1 and S1 region amplification. **Results:** Of 2,260 thalassaemia patients, 64.6% were males while 35.4% were females. The HBsAg was identified in 98 individuals (4.33%). The PCR analysis was done for these 98 patients and in this cohort, genotype D was 59.18% (n = 58), genotype A was 21.42% (n = 21) while genotype C was 19.38% (n = 19). **Conclusion:** The determination of HBV genotypes in the multi-transfused patients is key to the effective management of chronic HBV patients as the severity and course of the disease is dependent on a specific type of genotypes. Quality assured screening of donated blood will prevent the incidence of HBV in thalassaemia patients.

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Keywords: Epidemiology, genotype, HBV, Pakistan, polymerase chain reaction, thalassaemia

Received: 13-09-2020

Revised: 24-10-2020

Accepted: 09-12-2020

Published: 27-02-2021

Access this article online

Quick Response Code:



Website:
www.jfmpc.com

DOI:
10.4103/jfmpc.jfmpc_1880_20

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How to cite this article: Farooq A, Waheed U, Saba N, Kaleem M, Majeed N, Wazeer A, *et al.* Molecular and genetic characterization of hepatitis B virus among multitransfused thalassaemia patients in Islamabad, Pakistan. *J Family Med Prim Care* 2021;10:998-1002.

Introduction

Infection with Hepatitis B Virus (HBV) is a fatal liver disease. Despite immense efforts to eradicate the disease, Hepatitis B infection still exists as a serious global health concern. The WHO estimates that 257 million people are harboring the HBV infection while 887,000 died due to chronic HBV in a single calendar year.^[1] HBV is a member of the hepadnavirus family with a circular genome of double-stranded DNA to facilitate the infection of the liver.^[2] The virus is transmitted through transfusion of infected blood, blood products, or body fluids either percutaneous or mucosal also including vaginal, menstrual, and seminal fluids are among the common cause of HBV spread.^[3]

Thalassaemia is a manageable genetic disorders. With a population of 220 million inhabitants and high ratio of consanguineous marriages, Pakistan is among the highest thalassaemia burden countries around the globe with 100,000 transfusion-dependent thalassaemia patients in the country.^[4] Thalassaemia patients require a regular supply of blood and blood components to sustain life. These multi-transfused patients are dependent on regular transfusions, so more prone to acquire transfusion-transmitted infections most notably HBV and HCV.^[5]

The HBV whole genome sequence was first reported in 1979.^[6] The HBV exhibits very high genetic variability with a rate of $1.4 - 3.2 \times 10^{-5}$ nucleotide substitution each year per site.^[7] Based on these genetic variations, the hepatitis B infection is further divided into designated genotypes and sub-genotypes. Overall 10 genotypes of HBV (A-J) have been identified based on discovered variations in the nucleotide sequence of the whole genome. The frequency of specific genotypes varies geographically.^[8]

The determination of HBV genotypes has significant implications to understand HBV pathogenesis and also response to respective therapy.^[9] Moreover, HBV genotyping in thalassaemia has proven beneficial for the clinical management of these patients and for assisting decisions on therapy.^[10] Published evidence suggests that both HBV DNA level and genotype may predict response to interferon therapy.^[11,12] Besides, the HBV genotypes are also reportedly linked to the seroconversion of HBeAg, seroclearance of HBsAg, the stage of liver disease, cirrhosis, and hepatocellular carcinoma. Individuals with genotypes A, B, D, and F exhibit regular spontaneous HBeAg seroconversion as compared to genotype C.^[13]

The information regarding the prevalence of HBV genotypes in thalassaemia patients will also enable the physicians to accurately educate affected families about the risks linked with the unsafe blood transfusions and care needed to deal thalassaemia patients coupled with the HBV infection.

There is scanty data exist on the epidemiology of HBV genotypes in multi-transfused thalassaemia patients in Pakistan. Therefore, this study was undertaken to assess the HBV epidemiology and circulating genotypes in multi-transfused β -thalassemia major

patients with the aim to be considered while formulating the treatment pattern taking into account the particular needs of thalassaemia patients.

Materials and Methods

This cross-sectional single centre study was performed from September 2018 to June 2019, at the Department of Pathology and Transfusion Medicine, Shaheed Zulfiqar Ali Bhutto (SZAB) Medical University, Islamabad. A total of 2,260 thalassaemia patients were enrolled in the study. The samples were collected from Thalassaemia Centre of SZAB Medical University and the Pakistan Bait-ul-Mal Thalassaemia Centre, both located in the federal capital, Islamabad. These patients receive regular red cell transfusions every 3-4 weeks to keep a minimum haemoglobin level of 10 g/dl. Moreover, they are provided with iron chelation therapy by the respective centres.

The study was endorsed by the Ethics Committee of the SZAB Medical University, Islamabad, via letter no. F-21/10-1720. All the measures and efforts were done to maintain the confidentiality of the obtained information. Every sample was given a distinctive identity number which served to mask the name of that particular subject. The study participants or their parent/guardian filled an informed consent form before the collection of data and blood samples. The study was endorsed by the Ethics Committee of the Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad, via letter no. F-21/10-1720, dated January 20, 2018.

Blood was collected from patients aseptically followed by centrifugation at 5,000 rpm for 10 minutes to separate the serum.

The serological screening was performed by using LIAISON® XL Murex HBsAg Quant assay (DiaSorin S.p.A., Saluggia, Italy) to determine the presence of HBsAg. ExiPrep™ Dx Viral DNA kit (BIONEER, Daejeon, Korea) was used to extract viral DNA from serum positive samples according to the manufacturer's instructions. AccuPower® HBV Quantitative PCR kit was used to find out the HBV-DNA in plasma on the fully automated ExiStation™ MDx System (real-time PCR) according to standard protocol.

The HBV genotypes were determined by using universal primers targeting the P1 and S1 region amplification.^[14] The first amplification was followed by other rounds of PCR containing a specific mixture and each primer was highly specific for a particular genotype of the hepatitis B virus. Then each sample was amplified for two more rounds of PCR. The primers used for this step included universal forward primer (B2) and reverse primers including the primer sets labeled as BA1R, BB1R, and BC1R which are specific for genotype A, B, and C. Then universal reverse primer B2R and forward BD1, BE1 and BF1 were used that are specific for genotypes D, E and F. 2% agarose gel was used to analyze identified products. The 50-bp DNA ladder (Invitrogen, USA) was run as a marker.

Then 5 µl of PCR samples were added with a loading dye (0.25% bromophenol blue with 40% sucrose) and this mixture was added in each well. The electrophoresis conditions were 100 volts (80 mA) for 90 minutes using 1 X TBE buffer. The products along with the ladder were then visualized on the UV Transilluminator (Biometra Germany).

Results

Thalassaemia patients screening

Among the 2,260 thalassaemia patients, 62.83% (n = 1,420) were males while 37.17% (n = 840) were females. Patients had a mean age of 7.9 (range 1 to 30 years). The mean age at first transfusion was 8.5 months. About 55% patients (n = 1,243) were between age 1 to 12 years while 45% patients (n = 1,017) were above 12 years of age. Out of 2,260 patients, 98 individuals (4.33%) were serologically positive for HBsAg. Among HBV positive patients, 55 (56.12%) were males and 43 (43.88%) were females. Among hepatitis B positive 58 (59.1%) were less than the age of 12 while 40 (40.9%) patients were more than the age of 12. The results are shown in Table 1.

Out of 2,260 thalassaemia patients, 98 (4.33%) were positive for HBsAg. Regarding the genotypes distribution, genotype D was 59.18% (n = 58), genotype A was 21.42% (n = 21) while genotype C was 19.38% (n = 19).

The representative gel electropherograms for detection of genotypes are shown below in Figures 1 and 2:

Discussion

HBV is among the major causes of post-transfusion hepatitis in thalassaemia major patients.^[15] The infection is considered a

global burden especially on the developing economies with low HDI (human development index). The viral infection is further classified on the basis of circulating genotype. Studies on the interaction of HBV genotypes, their pathogenesis in chronic liver disease, particularly hepatocellular carcinoma, and their role in therapy are of great interest, as this permits to understand the transmission and risk dynamics of HBV infection around the globe.^[16,17] This is much-needed information for physicians while dealing and treating HBV patients especially those also suffering from thalassaemia.

In the current study, HBsAg was identified in 4.33% of the thalassaemia patients, which is significantly high compared to earlier reports in the general population of Pakistan (1.65%).^[18] Earlier studies on the prevalence of HBV in thalassaemia patients report a prevalence of 8.40%^[19] in the Balochistan province, 3.0%^[20] in Islamabad, 5.1%^[21] in Karachi, 5%^[22] in Rawalpindi, and 12.2%^[23] in Bahawalpur. Although screening for HBsAg is mandatory under the Islamabad Blood Safety Ordinance,^[24] the finding of HBsAg in thalassaemia patients indicates poor screening techniques^[25] and low voluntary blood donations. The reporting of HBV infection incident in such patients urges quick response to monitor any uncontrolled transfusion in these patients and demands to maintain quality transfusion services. Screening methods for TTTs are not unanimously implemented in different provinces as well as public and private sector blood transfusion centers of Pakistan. There is a vital need to revise and implement standard blood transfusion policy across the country. Before the adaptation/usage of any diagnostic method in Pakistan, it must be strictly validated under standard quality defined parameters.

HBV genotypes exhibit a diverse geographical distribution around the globe.^[17] Our study reported a high frequency of genotype D. When compared with genotype distribution data from Northern Europe, North America, and Central Africa, genotype A is the most prevalent genotype.^[26] Genotype B and C are predominantly reported from Asia.^[27] Western Africa has reported genotype E while Northern Latin America is the region from where the genotype H has been reported.^[28] The studies have also reported genotype “I” in Vietnam, Eastern India, Laos, and North-Western China,^[29-31] and genotype J in Japan.^[32,33] In the Eastern Mediterranean region where Pakistan is located, D genotype is predominantly present in countries like Bahrain (61%),^[12] Saudi Arabia (81%),^[34] Iran (100%),^[35]

Table 1: Features of Thalassaemia Patients

Factors	Number of patients n=2,260	Hepatitis B positive patients n=98
Males n (%)	1,420 (62.83%)	55 (56.12%)
Females n (%)	840 (37.17%)	43 (43.88%)
Age of all patients (Mean±SD)	7.9±SD 4.5	10.6±6.4
Patients <12 year of age	1,243 (55%)	58 (59.1%)
Patients >12 year of age	1,017 (45%)	40 (40.9%)
Age on first transfusion	9.56 months±SD 8.0 months	

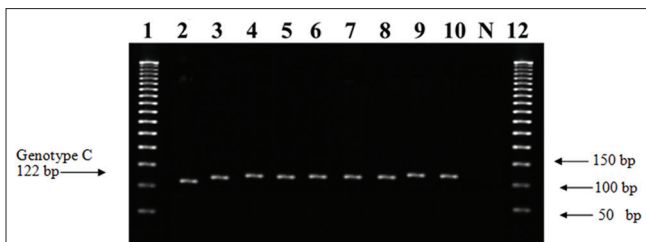


Figure 1: Gel electrophoresis of the ethidium bromide-stained agarose gel displaying genotype C. The Arabic numerals indicate wells containing the samples. The “N” represents the negative sample

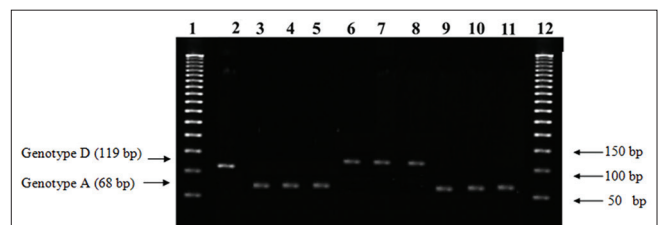


Figure 2: Gel electrophoresis of the ethidium bromide-stained agarose gel displaying genotypes A and D. The Arabic numerals indicate wells containing the samples

UAE (79.5%),^[36] and Jordan (100%).^[37] The high incidence of genotype D in some developed Eastern Mediterranean countries is due to the presence of a high percentage of migrant workers from endemic countries who may become the main source of HBV transmission.

When compared with previous studies conducted within the country, our study findings were comparable. A study conducted in three big cities of Pakistan including Karachi, Rawalpindi, and Islamabad, reported that genotype D was the most prevalent (58.5%) genotype followed by mixed genotype A and D (31.5%) while genotype A was only reported in 10% samples.^[38] Some studies from Karachi showed the predominance of genotype D.^[39-41] Reports from Khyber Pakhtunkhwa province also showed a higher incidence of genotype D.^[42] Two studies from Rawalpindi also reported a high incidence of genotype D, i.e., 96%^[43] and 90.2%.^[44] However, in the province of Punjab, the genotype C was found most commonly.^[45] In Islamabad, Masood *et al.* reported a 52.17% prevalence of genotype D followed by genotype C 16.30%.^[46] A study in all four provinces of Pakistan, genotype C (28%) was most common followed by genotype B (18%), genotype A (14%), genotype D (13%), genotype E (0.6%) and genotype F (1.3%).^[47]

The high prevalence of genotype D among thalassaemia cohort of patients in our study was in agreement with other reports on HBV-infected patients from different groups in Pakistan. Our findings will assist the physicians to choose the right protocol of treatment for HBV positive thalassaemia patients. The study will also provide guidelines for medical practitioners to formulate treatment regimens particularly considering the needs of thalassaemia patients. The results will allow the primary care physicians to provide awareness and counseling to the affected families about the existing threats of unsafe blood transfusion and the subsequent transmission of viral infections.

Also, quality-assured screening of donated blood will prevent the incidence of hepatitis B in thalassaemics. Epidemiological data concerning hepatitis B will genotypes will provide important data to health managers to curtail and manage the disease concerning its aetiological spectrum.

Acknowledgements

The authors would like to acknowledge the support extended by Mr. Shahan Irshad, Mr. Junaid Shokat, and Mr. Mahmood Awan from Sind Medical Stores.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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