Frequency distribution and risk factors of hepatitis B virus and hepatitis C virus infections among thalassemia patients: a regional study

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Background Thalassemia is a group of inherited hematological disorders caused by mutation in globin's genes. Regular blood transfusion lengthens the life of thalassemia patients but it carries a definite risk of the infections of blood-borne diseases.
Aim/Objective The current study was carried out for the frequency distributions and risk factors of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections among thalassemia patients in Hazara regions, Pakistan.
Methods A total of 324 enrolled thalassemia major patients were diagnosed in five different centers of Hazara regions. The study participants were screened for HBV and HCV using the immunochromatographic techniques test and real-time PCR for immunochromatographic technique-positive specimens.
Results Out of the 324 major thalassemia patients, 24 (7.41%) were diagnosed with HBV and HCV infections. In total, 206 were male patients and the rate of HBV and HCV infections was 0.97% (two patients) and 3.88% (eight patients), respectively. Similarly, 118 were female patients and the rate of HBV-positive patients was 3.39% (four patients) and HCV was 8.47% (10 patients). The results also showed that 50% of HBV and HCV infections were found in the age group of 26–30 years, while 1.81% was found in the age group of 11–15 years. The positive HBV and HCV samples were also verified with the band size of 242 and 227 bp, respectively.
Conclusion Thus, to reduce the incidence of HBV and HCV in thalassemia patients, we must call for critical look on the transfusion practices as well as adoption of stricter donor selection. Eur J Gastroenterol Hepatol 31:248–252
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Introduction

Thalassemias are the inherited hematological disorders of the hemoglobin due to mutations. It is found worldwide with different mutations in the globin genes [1]. Abnormal synthesis of either α or β globin chains that form tetramers of hemoglobin ($\alpha_2\beta_2$) lead to the most common forms of inherited anemia [2]. Defects in the production of either α-like globin chain caused (α thalassemia [3]) or β-like globin chain (β-thalassemia [4]) globin chains is considered to be a major cause of inherited anemia. In a study it was found that around 1.5% of the world population is candidates for β-thalassemia, with 50 000–60 000 new born patients of thalassemia each year [5]. In the Mediterranean region, the most prevalent form is β-thalassemia. It is also found in Southeast Asia, Meddle East, and Africa [6]. In Pakistan, transfusion services are fragmented and depend on the family replacement donations in different type of blood centers [7].

In Pakistan, thalassemia is the most prevalent genetic disorder; according to a study report, there are 100 000 patients suffering from thalassemia, indicating the most severe form of thalassemia presence in Pakistan, and every year the numbers of infected patients are increasing by the rate of 5–900, with the total prevalence rate of 5–7% (8–10million population [8,9]). A thalassemia patient's life extension is dependent only on regular blood transfusion because the other medical management and chelation therapy is out of economic range of the patient's family. Therefore, thalassemia is a great challenge as well as places huge amount of financial stress on the affected patients' families, as well as on the national health system. Moreover, the gene of the thalassemia patients is not randomly distributed in Pakistan and is mainly found in the affected families [8]. The thalassemia patients' number has increased day by day because of lack of well-defined policy and preventive measurement at the national level. A recent survey presented that Pakistan has the highest prevalence of children with β-thalassemia in the world. This is because of the social and cultural systems and the choice of marriages in the same family and within same ethnic groups [10]. Transfusion-related transmitted infections such as hepatitis B [hepatitis B virus (HBV)] and hepatitis C [hepatitis C virus (HCV)] are the major risk in developing countries. Thalassemia patients are at particularly

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increased risk for HBV and HCV [6]. Thalassemia patients depend on regular transfusion of blood to sustain the life, and transfusion without proper safety standards in place and quality control can lead to infections like HBV and HCV [10].

Although several studies have been carried out on the prevalence of thalassemia patients with its association with HBV and HCV in Pakistan [8,9,11], there is no published work available about the frequency distributions and prevalence of HBV and HCV among thalassemia patients in the Hazara region.

Participants and methods

In the current study, samples were collected from the different regions of the Hazara division of Khyber Pakhtunkhwa, Pakistan, which comprises Abbottabad, Haripur, Mansehra, Battagram, and Muzaffarabad. A total 324 blood samples were collected from thalassemia patients during the period of 22 October 2013 to 10 March 2014. Out of 324 blood samples, 206 were from male patients and 118 from female patients, with the mean age range of 1–30 (± SD), that is, 124 from Abbottabad, 123 from Haripur, 63 from Mansehra, 10 from Battagram and four from Muzaffarabad. History was taken from each thalassemia patient by using a detailed multiple-choice questionnaire to ensure data collection and prevent any misunderstanding. The study principles and protocols were submitted and approved by the committee of Haripur Thalassemia Care and Prevention Center, Abbottonians Medical Association, and Kids Blood Disease Organization.

Samples collection

Two blood samples from each thalassemia patient were collected under aseptic conditions in a plane tube and labeled according to the data provided by the patient, and then were kept at the room temperature for further processing. Blood samples were then centrifuged, and the serum from the first tube was tested within 2 h for HBsAg and anti-HCV antibodies. Serum from the second sample was frozen after adding DNAse and RNAse in a tightly capped tube at 70°C for the PCR analysis.

Virology

To determine the HBsAg, HBsAg Immunochromatographic techniques (ICT) 2.0 Kit (Accurate Diagnostics, Houston, Taxas, USA) was used. Nonreactive samples were declared negative HCV, while the reactive sample was retested to confirm the result. Serologically, positive HCV samples were further subjected to PCR for confirmation. Serologically, HBsAg-positive samples were tested individually through PCR. Similarly, for anti-HCV antibodies detection, HCV, version 3.0 ICT kit (Accurate Diagnostics) was used.

Nucleic acid extraction of hepatitis B virus and hepatitis C virus

HBV DNA and HCV RNA were extracted from the thalassemia patient's serum samples using Ana-gen DNA/RNA extraction kits (Duluth, Georgia, USA), according to the manufacturer's protocol.

Hepatitis B virus detection through polymerase chain reaction

For the detection of hepatitis B virus, specific portion of the surface gene was amplified through PCR using HBV genomic DNA using specific forward and reverse primers in the following way.

Polymerase chain reaction for hepatitis B virus

For the qualitative deduction of HBV DNA, the extracted DNA was subjected to PCR using the 5-U Taq DNA polymerase added in a thermal cycler (ABI PCR system 2700) (Fermentas Life Sciences, Waltham, Massachusetts, USA). 20 µl PCR reaction mixture contained DNA, PCR master mix containing buffer 10× (1.0 µl), 25 mm $MgCl_2$ (1.2 µl), 500 µmol/l deoxyribonucleotide triphosphates (dNTPs) (1.0 µl) (10 pmol/l), outer sense primer 2823–2845 (5′-TCACCATATTCTTGGAA CAAGA-3′) (1.0 µl) (10 pmol/l), outer antisense primer 685–704 (5′-CGAACCACTGAACAATGGC-3′) (1.0 µl), Taq DNA polymerase (1 U/µl) (1.0 µl) (nuclease free), and DH_2O (10.0 µl). In the first round, the samples were incubated at 94°C for 2 min for thermo cycler (ABI PCR system 2700), and then in the second step of first round, they were incubated by 35 cycles consisting of 94°C for 30 s, then 58°C for 45 s only and 72°C for 1 min before the last, and at the last stage, for 7 min at 72°C.

Second round polymerase chain reaction

Each first-round product in the second round of PCR was subjected into two distinct PCR reaction tubes using the above-mentioned master mix in which the first-step tube had 8 µl of mix and 10 pmol/l of each primer for genotype A–E (5′-GGTCMAGTTCMGACAGT-3′), A (5′-CTCGC GAGATTGACGGATGT-3′), B (5′-CAGTTGGTGAGTG ACTGAGA-3′), and C (5′-GGTCTAGGAATCTGATGT TG-3′); $MgCl_2$ in 1× PCR buffer 2.4 mmol/l, dNTPs of 500 µmol/l and a unit of DNA Taq polymerase. While in the second PCR reaction, the tube contained 8 µl of mix B having 10 pmol/l of each primer for genotype D–F (5′-GGAGGCGATYTGCTGGCAA-3′), D (5′-GCCAACA GGTAGGAGCT-3′), E (5′CACCAGAATCCAGATTGG ACCA3′), and F (5′-GYTACGTCCAGGTTACCA-3′) [12] for additional 35 cycles.

Complementary DNA synthesis and molecular screening for hepatitis C virus RNA

HCV RNA was extracted and both cDNA synthesis and PCR amplification of the target sequences were performed in a single tube using the one-step RT-PCR kit according to the manufacturer's recommendation (Puregene, Minnesota, USA). The primers were designed to specifically bind the 5′-untranslated region of the HCV genome that is highly conserved among different genotypes. Primer sequences were as follows: outer sense primer (5′-CCCT GTGAGGAACTWCTGTCTTCACGC-3′); antisense outer primer (5′-GGTGCACGGTCTACGAGACCT-3′); inner sense primer (5′-TCTAGCCATGGCGTTAG TRYGAGT GT-3′); and inner antisense primer (5′-CACTCGCAAGC ACCCTATCAGGCAGT-3′, W = A or T, R = A or G, Y = T or C) [13].

The reactions were carried out in 25 µl volumes using 10 µl RNA in the presence of 0.6 µmol/l of each HCV outer

primer, 400 µmol/l dNTP and five unit's RNase inhibitor. The reaction conditions were as follows: one cycle at 50°C for 30 min, one cycle at 95°C for 15 min followed by 40 cycles at 95°C for one min, 55°C for 1 min and 72°C for 1 min. The reactions cycles were complete at 72°C for 10 min. The second nested PCR was also performed to improve the detection capacity of the PCR test. The second PCR reactions were carried out in 25 µl volumes using 5 µl DNA templates from first PCR with 1× PCR master mix (Promega, USA) and 0.4 µmol/l of HCV inner primers. The reaction cycling conditions were as follows: one cycle at 94°C for 2 min, followed by 35 cycles of incubation at 94° C for 30 s, 58°C for 45 s, and 72°C for 1 min, and the final extension was done at 72°C for 7 min. The products were analyzed on 2% agarose gel with 100-bp ladder and stained with ethidium bromide. The appearance of a 298-bp and 235-bp bands was considered positive for the first and second PCR result.

Finally, the PCR products were electrophoresized on a 2% agarose gel prepared in 1× Tris-borate-EDTA buffer, stained with ethidium bromide, and evaluated under ultraviolet transilluminator. The sizes of PCR products were estimated according to the passage pattern of a 50-bp DNA ladder (Fermentas Life Sciences).

Statistical analysis

The present research data were collected, summarized, tabulated, and analyzed statistically by using statistical package, SPSS, version 10.0 (SPSS Inc., Armonk, New York, USA) and Graphpad Prism, version 5.0 (Graphpad Software, San Diego, California, USA). Percentages and ratios results for all variables were calculated.

Results

Patients disposition

A total of 324 blood samples of thalassemia patients were collected in the current research study for the identification of HCV and HBV viral infection. Out of total 324 thalassemia patients 206 were male patients and 118 were female patients.

Results showed that out of total 324 blood samples collected from different regions of the Hazara region for HCV and HBV screening, 206 were from male patients, in which the rate for HBV was 0.97% (two patients) and the rate for HCV was 3.88% (eight patients). Similarly, 118 samples were taken from female patients and the rate of HBV was 3.39% (four patients) and HCV it was 8.47% (10 patient) (Fig. 1).

Regions wise distribution of hepatitis B virus and hepatitis C virus in thalassemia patients

The current research study consisted of thalassemia patients belonging to five well-known regions of Hazara Division, Khyber Pakhtunkhwa, Pakistan. Out of the total thalassemia patients, 124 were from Abbottabad, 123 from Haripur, 63 from Mansehra, 10 from Battagram and four from Muzaffarabad. The rates of HCV and HBV viral infection in thalassemia patients were 6.45% (eight patients), 10.57% (13 patients), 3.17% (two patients), 10.00% (one patient), and 0.00% (none) (Table 1).

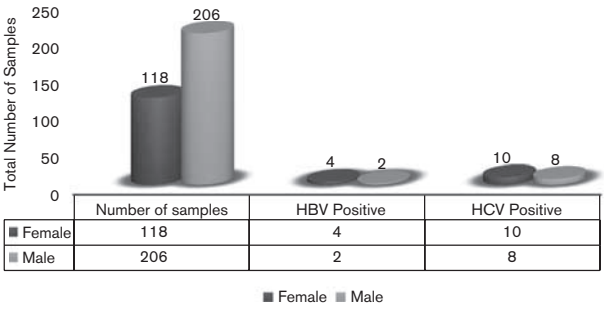

Fig. 1. Gander wise rate of HBV and HCV in thalassemia patients. HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 1. Regions wise rate of hepatitis B virus and hepatitis C virus in thalassemia patients

Districts	Total samples	HBV positive [*n* (%)]	HCV positive [*n* (%)]	Overall [*n* (%)]
Abbottabad	124	1 (0.80)	7 (5.64)	8 (6.45)
Haripur	123	5 (4.06)	8 (6.50)	13 (10.57)
Mansehra	63	0 (0.00)	2 (3.17)	2 (3.17)
Battagram	10	0 (0.00)	1 (10.00)	1 (10.00)
Muzaffarabad	4	0 (0.00)	0 (0.00)	0 (0.00)

HBV, hepatitis B virus; HCV, hepatitis C virus.

The results of the current study (Table 1) also revealed that the maximum prevalence of the HBV and HCV-positive patients among thalassemia patients were found in the Haripur region, 10.57% (13 individuals) (five HBV-positive and eight HCV-positive patients); similarly the second largest prevalence among thalassemia patients were found in Abbottabad region, 6.45% (eight individuals) (one HBV-positive and seven HCV-positive thalassemia patients). In Muzaffarabad region no one was positive for HBV and HCV among thalassemia patients, thus a 0.0% prevalence rate.

Age-wise distribution of hepatitis B virus and hepatitis C virus in thalassemia patients

The current study results showed that out of the total 324 thalassemia patients' blood samples, 105 were in age limit 1–5 years, the rate of HCV and HBV infection was 4.76% (five patients); 113 in age limit 6–10 years, the rate of HCV and HBV infection was 8.85% (10 patients); 55 in age limit 11–15 years, the rate of infection were 1.81% (one patient); 34 in age limit 16–20 years, the rate of HCV and HBV infection was 5.88% (two patients); 11 in age limit 21–25 years, the rate of HCV and HBV infection was 27.27% (three patients); and six patients were of age limit 26–30 years, the rate of HCV and HBV infection were 50.00% (three patients) which were shown in Fig. 2.

Age-wise distribution of the current study results also presented that maximum prevalence of the HBV-positive and HCV-positive patients among thalassemia patients were found in the age group 26–30 years (50.00%); in total, three individuals (no one was HBV positive and three were HCV positive among thalassemia patients). Similarly, the second leading age group was 21–25 years (27.27%); in total, three individuals (one was HBV positive and two were HCV positive among thalassemia patients).

The minimum prevalence of 1.18% was found in the age group 11–15 years.

Molecular screening of the positive samples

Out of the samples collected, ICT-positive smears of the both HBV and HCV were subjected to PCR-based detection for viral DNA in case of HBV and RNA in case of the HCV. The results revealed that both ICT-positive smears of HBV and HCV were also confirmed by gel electrophoresis, that is, the positive HBV and HCV samples were verified with the band size of ~242 and 227 bp [11], respectively (Fig. 3a and b).

Discussion

Thalassemia is a commonest monogenic syndrome [14] that shows decreased hemoglobin level called hypochromia, which may be due to decrease synthesis of one or the other polypeptide chains, and red cells small size (microcytosis). Because of continued normal production of unaffected globin chain, the formation improper globin chains leads to the accumulation of imbalanced aggregates of the unmatched globin chain, which may lead to the oxidative membrane damage and destruction of premature erythrocytes in the blood vessels circulation in the initial stage of maturation in the bone marrow. This decreases the hemoglobin level in the blood and oxygen-carrying capability of the red blood cells [15].

In patients of β-thalassemia major the initial and regular blood transfusion therapy with care decreases the complications of severe anemia and prolongs survival – especially in those patients who receive an adequate, regular iron chelation treatment, and therefore secure from organ damage by iron excess [16]. But those patients who do not receive blood transfusion in a safe manner face new clinical challenges, mainly HCV, HBV, and HIV infections [16,17]. HIV and HBV infections are not main problems in Pakistan because the HIV ratio is very less and HBV can be prevented by pretransfusion immunization; but HCV infection is highly dangerous and complicated in those patients who gain multitransfusion therapy. HCV is more prevalent in general population among blood donors in those countries, as with the number of transfusions, the prevalence rate of seropositivity increases [18]. Therefore, the post-transfusion hepatitis is much more important in the morbidity rate of thalassemia. And that's why the HCV hepatitis is more threatening than HBV hepatitis, which has a great risk for chronic liver disease [19].

In the current research study it was observed that, out of 324 enrolled thalassemics blood samples, 7.41% ICT-positive patients were also positive by using HCV and HBV real-time PCR. This study shows that the prevalence of HCV and HBV in thalassemia patients is more in female patients compared with male patients. In female patients, HBV infection was present in four (3.39%) patients and HCV in 10 (8.47%), whereas in male patients, HBV infection was present in two (0.97%) patients and HCV in eight (3.88%) patients (Fig. 1). The current study correlates with the previous investigational reports [20] mentioning the awareness regarding thalassemia prevalence in children.

In the current research study all the thalassemia patients belonged to five well-known regions of Hazara Division, Khyber Pakhtoonkhaw, for HCV and HBV viral screening. It was found that the HCV and HBV infections were more prevalent in district Haripur 13 (10.57%) patients. While the lowest rate was found in Muzaffarabad. The results are supported by Ansari *et al.* [8], who showed that the prevalence of thalassemia was more in a cast called as pashtoon in Pakistan as compared with the people from other different casts, which may be due to the family marriages. The prevalence of thalassemia patients in the age group of 26–30 years were at a high risk with having a high number of 50.00% as compared with other groups of patients 11–15 years having number of 1.81% (Fig. 2). Our results are supported by Qurat-ul-Ain *et al.* [21].

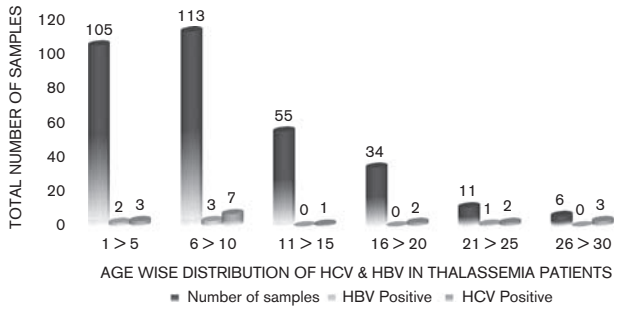

Fig. 2. Age wise rate of HBV and HCV in thalassemia patients. HBV, hepatitis B virus; HCV, hepatitis C virus.

Conclusion

In the light of study findings, transfusion-related transmitted infections had given us an alarmingly high rate for HBV-positive and HCV-positive sign among thalassemia patients. The focus of the study was on policy makers to

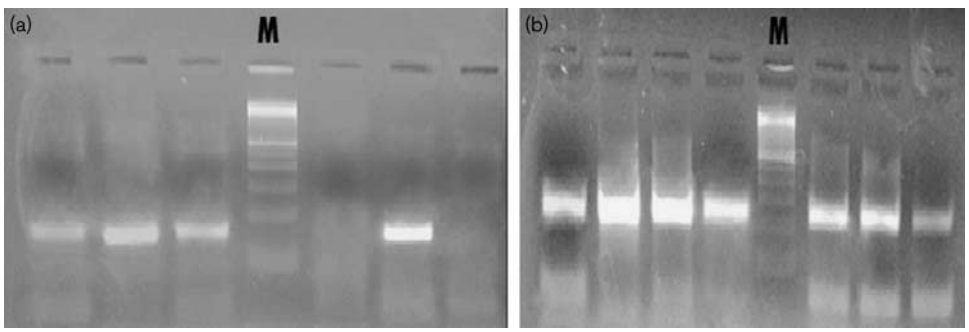

Fig. 3. A representative of the 2% of agarose gel of PCR products for the detection of HCV and HBV. Lane M: DNA marker; (a) lanes: HCV-positive samples (227 bp); (b) lanes: HBV-positive samples (247 bp). HBV, hepatitis B virus; HCV, hepatitis C virus.

deeply observe and evaluate the current practices in the blood banks to change and make quality measurements about blood safety, especially those that are related to thalassemia patients. Before any type of blood transfusion, the donor must be tested for the transmitted infections such as HBV and HCV, and people should be made aware of the disadvantages of entering family marriages.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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