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Post-transfusion hepatitis C virus infection among β-thalassaemic individuals with associated clinical parameters

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***Background & objectives*: Multiple transfusions in β-thalassaemia patients undergoing regular transfusion regimen are at a risk of developing transfusion transmitted infections, including hepatitis C virus (HCV). The present study was conducted to investigate the association of HCV viraemia and genotype with clinical parameters in HCV seroreactive β-thalassaemic individuals.**

***Methods*: A total of 172 HCV seroreactive β-thalassaemic individuals aged between 2-35 yr with at least 25 units of blood transfusion were catagorized into four groups (2-12 yr, group 1; 13-19 yr, group 2; 20-29 yr, group 3; 30-35 yr, group 4). Aged matched control samples (n=87; β-thalassaemics without HCV infection) were also included. HCV RNA was detected by nested reverse transcriptase polymerase chain reaction (RT-PCR) based on 5' UTR of HCV genome, viral load was determined by real-time RT-PCR. Nested RT-PCR amplified partial core region was used for DNA sequencing. Liver function parameters [serum total bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] were also determined.**

***Results*: Of the 172 HCV seroreactive individuals, 59.30 per cent (n=102) were HCV RNA positive. HCV viral load ranged from 173 to 32.04×10^5 IU/ml; 87.65 per cent were infected with HCV genotype 3. Liver enzymes, such as ALT, AST and serum total bilirubin were significantly elevated in all age groups compared to control groups. Serum ferritin levels were found to be high in all individuals, but 16.27 per cent of HCV-infected individuals with >10,000 IU/ml viral load also showed high ferritin levels (>1500 µg/l) where the majority of them were infected with HCV genotype 3.**

***Interpretation & conclusions*: HCV genotype 3 was the major circulating genotype among β-thalassaemia patients in this region. Our findings indicated an association between HCV replication and hepatic iron load and also highlighted the need for sensitive quantitative RT-PCR-based detection of HCV RNA in the high risk population.**

Key words Hepatitis C virus - serum ferritin - transfusion transmitted infections - β-thalassaemia

Beta-thalassaemia (β-thalassaemia) is a hereditary blood disorder (haemolytic anaemia) with common single gene abnormality[1]. In β-thalassaemia, the abnormalities are seen in beta-chains; result in reduced or no synthesis of the particular chain that needs to make up functional haemoglobin (Hb). Around 60-80 million people worldwide are affected by β-thalassaemia trait alone[2]. In India, various thalassaemia traits vary from 3 to 17 per cent[3] and β-thalassaemia is one of the major traits[4,5]. Treatment for thalassaemia major patients includes chronic blood transfusion, splenectomy and management of iron overload with iron chelation[6]. β-thalassaemic major individuals require to receive blood or blood products at regular intervals for their health management. Transfusion transmitted infections (TTI) such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) are major risk factors leading to chronic infection[3,7]. HCV infection is one of the most common post transfusion transmissible infections among β-thalassaemic individuals[4].

Worldwide 180 million people are infected with this virus and its seroprevalence within the general population is 0.5 to 1 per cent in India[8], but in multi-transfused cases, it varies from 4 to 20 per cent in some cases even up to 60 per cent depending on transfusion centres[9]. In India, HCV seroprevalence in thalassaemia patients varies from 11 to 30 per cent[10-12]. HCV infections are asymptomatic, rare to identify and significantly increases the rate of morbidity and mortality[8]. On a routine basis, the disease is diagnosed by immunoassay using ELISA or such rapid test[13]. Screening of blood products with new molecular tests such as nucleic acid test (NAT) and better blood transfusion protocols reduce the risk of TTI among thalassaemic individuals[7]. Treatment options in HCV patients is also limited as interferon treatment is found to control the HCV viral load but causes haemolysis and increases the transfusion requirements and iron toxicities[14,15]. In addition, elevated level of hepatic iron load interferes with interferon treatment by resisting its action[16]. The present study was undertaken to investigate HCV viraemia, HCV viral load and genotype, and association between biochemical and haematological parameters associated with HCV infection along with HCV genotype and viral load among HCV seroreactive multi-transfused β-thalassaemic individuals.

Material & Methods

A total of 172 HCV seroreactive β-thalassaemic individuals aged two years and above, up to 35 yr, with at least 25 units of blood transfusion referred from collaborating Thalassaemia Clinics from different hospitals of Kolkata, India, were included in the study and the laboratory work done at ICMR Virus Unit, Kolkata. The study was conducted during January 2013 to December 2014. This study was approved by the institutional ethical committee of ICMR-National Institute of Cholera and Enteric Diseases. Written informed consent was taken before inclusion of the individuals in this study.

All the 172 HCV seroreactive individuals were classified into four groups (group 1 to 4) depending on their age. Children with age between 2 and 12 yr were classified in group 1 (n=108), those between 13 and 19 yr were classified in group 2 (n=40), youth between 20 and 29 yr were classified as group 3 (n=15), middle age between 30 and 35 yr were classified as group 4 (n=9). For validating the experimental setting, age-matched control samples (n=87, β-thalassaemic without HCV or any other liver diseases) were included and distributed into four control groups - Control group 1 (n=54), group 2 (n=20), group 3 (n=8) and group 4 (n=5).

Individuals who had been transfused less than 25 units of blood and more than 35 yr old were excluded. Patients undergoing interferon treatment were also excluded from this study. Participants with other co-infection such as HBV or HIV were also not included.

Viral RNA was extracted from all seroreactive individuals using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Briefly, viral RNA was extracted from 140 µl serum, eluted in 50 µl elution buffer and stored at −80°C for further usage.

To detect HCV RNA, 5' untranslated region (5'UTR) of HCV genome was amplified by nested reverse transcriptase polymerase chain reaction (RT-PCR) according to the method described elsewhere[17].

HCV viral load was determined in house. Primers and probe sequences were designed against 5' untranslated region (5' UTR) of HCV genome. Quantification was done by real-time RT-PCR using ABI quantitative real-time reverse transcription kit (Ag-path ID™ one step RT-PCR, Applied Biosystems, USA) based on TaqMan chemistry[18].

Nested RT-PCR amplified 405 bp partial core gene of HCV genome were gel purified using QIAquick

gel extraction kit (Qiagen, Hilden, Germany) and directly used for sequencing using Big-Dye terminator 3.1 kit (Applied Biosystem, USA) in an automated DNA sequencer (3130XL, Applied Biosystem, USA)[18]. HCV sequences obtained were used to determine HCV genotype using the NCBI genotyping tool[19-22].

Liver function parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total serum bilirubin were measured by kinetic rate and diazo method, respectively (Beckman Coulter, Synchron, CX5Pro, USA).

Hb and serum ferritin levels were estimated by Automated Cell Counter (Medonic CA530-16 Open, Merck, Germany) and chemiluminescence enzyme immuno assay methods (Beckman Coulter Access 2, USA), respectively.

Statistical analysis: Continuous variables were represented as mean±standard deviation while categorical variables were expressed as frequencies (%). Non-categorical data were assessed using Student's *t* test with 95 per cent confidence interval using SPSS programme (version 10.0, SPSS Inc., Chicago, IL, USA).

Results

Of the 172 HCV seroreactive β-thalassaemic individuals, 109 (63.37%) were males and 63 (36.63%) were females. Their ages ranged from 2 to 35 yr with the mean age being 12.6±10.15 years. Their body weight ranged from 9.0 to 60.0 kg with a mean was 36.86±12.0 kg, and height ranged from 89 to 174 cm with a mean of 140.59±20.37 cm (Table I).

All these patients were diagnosed as β-thalassaemic within three months to two years of age. Most of the individuals had undergone transfusion of 2-3 units of blood per month depending upon their need with a mean transfusion rate of 2.56 units per month. Of the 172 seroreactive individuals, 39 (22.68%) had taken more than 300 units of blood transfusion. Seventy four individuals (43.02%) had splenectomy as a part of their clinical management (Table I).

Hb level of all 172 individuals ranged from 7 to 10 g/dl with a mean 8.74±0.64 g/dl, and serum ferritin level ranged from 817 to 3008 µg/l with a mean 1711.48±756.19 µg/l (Table II). Analysis of liver function parameters showed that serum total bilirubin levels were significantly (P<0.001) elevated in all age groups compared to the control groups (Table II). Liver enzymes such as ALT, AST which are indicators of the health of liver were significantly elevated in all the groups than control population. Of the total 172 individuals, high ALT (>40 IU/l) and AST levels (>42 IU/l) were found in 147 (85.46%) and 152 (88.37%) individuals, respectively.

Of the 172 seroreactive individuals, 102 (59.30%) were HCV RNA positive based on nested RT-PCR of HCV 5' UTR of HCV genome whereas 126 (73.24%) individuals were HCV RNA positive by real-time RT-PCR-based detection (Table III).

In majority of the cases, HCV viral load was found to be low and ranged from 173 to 32.04×10^5 IU/ml. Highest viral load (>100,000 IU/ml) was found in individuals within 20-29 yr age group (Table III). Individuals having

Table I. Demographic parameters of hepatitis C virus seroreactive β-thalassaemic individuals (n=172)

Test	Group 1 (n=108)	Group 2 (n=40)	Group 3 (n=15)	Group 4 (n=9)
Sex (%)				
Male	70 (64.81)	20 (50)	13 (86.66)	6 (66.66)
Female	38 (35.18)	20 (50)	2 (13.33)	3 (33.33)
Weight (mean±SD)	36.78±11.86	36.90±11.95	37.14±12.02	40.91±12.11
Height (mean±SD)	140.52±20.15	140.73±20.29	141.04±20.31	148.01±20.42
Blood transfusion/month (mean±SD)	2.19±0.57	2.19±0.57	2.20±0.57	2.16±0.56
Total transfusion till date (%)				
>300 transfusions	4 (3.70)	11 (27.5)	15 (100)	9 (100)
<300 transfusions	104 (96.29)	29 (72.5)	0	0
History of splenectomy (%)				
Yes	35 (32.40)	18 (45)	12 (80)	9 (100)
No	73 (76.59)	22 (55)	3 (20)	0

Group 1, children between 2 and 12 yr; Group 2, 13 and 19 yr; Group 3, 20 and 29 yr; Group 4, 30 and 35 yr

Table II. Clinical parameters of hepatitis C virus seroreactive β-thalassaemic individuals

Groups	Hb (g/dl)	Serum ferritin (ng/ml)	Serum total bilirubin (mg/dl)	ALT (IU/l)	AST (IU/l)
Group 1 (n=108)	8.75±0.64*	1713.11±755.89	1.60±0.73***	78.12±30.91***	81.99±38.31***
Control Group 1 (n=54)	8.84±0.61	1711.5±572.28	0.77±0.08	52.1±7.81	54.4±13.4
Group 2 (n=40)	8.75±0.64	1713.26±758.10	1.59±0.20***	92.26±45.54**	102±51***
Control Group 2 (n=20)	8.75±0.66	1708.11±785.83	0.77±0.11	53.44±8.31	54.6±14.4
Group 3 (n=15)	8.72±0.64	1713.11±755.89	1.56±0.12***	104.78±81.58	78.06±34.50
Control Group 3 (n=8)	8.74±0.64	1703.11±855.89	0.77±0.09	53.46±8.81	54.4±14.8
Group 4 (n=9)	8.63±0.65	1707.96±762.19	1.73±0.30***	69.44±28.34	86±42.06
Control Group 4 (n=5)	8.70±0.64	1683.11±865.89	0.77±0.12	54.34±7.41	55.4±15.2

P *<0.05, **<0.01, ***<0.001 compared to respective control group. Values are mean±SD. Group 1, children between 2 and 12 yr; Group 2, 13 and 19 yr; Group 3, 20 and 29 yr; Group 4, 30 and 35 yr; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SD, standard deviation; Hb, haemoglobin

Table III. Hepatitis C virus RNA detection and distribution of viral load among hepatitis C virus seroreactive β-thalassaemic individuals (n=172)

Test	Group 1 (n=108)	Group 2 (n=40)	Group 3 (n=15)	Group 4 (n=9)
HCV RNA qualitative (detection) at 5' UTR (%)				
Positive	67 (62.01)	22 (55)	9 (60)	4 (44.4)
Negative	41 (37.99)	18 (45)	6 (40)	5 (55.6)
HCV RNA quantitative (viral load) (%)				
Below detection level (up to 50 IU/ml)	25 (23.14)	13 (32.5)	5 (33.33)	3 (33.33)
50-5000 IU/ml	48 (44.44)	14 (35)	4 (26.66)	5 (55.55)
>5000-10,000 IU/ml	5 (4.64)	4 (10)	0	0
>10,000-1,00,000 IU/ml	22 (20.38)	6 (15)	3 (20)	0
>100,000 IU/ml	8 (7.40)	3 (7.5)	3 (20)	1 (11.12)

Group 1, children between 2 and 12 yr; Group 2, 13 and 19 yr; Group 3, 20 and 29 yr; Group 4, 30 and 35 yr; UTR, untranslated region

a viral load >5000-10,000 IU/ml showed slightly higher ALT, AST levels than other groups (Table IV).

Partial core region of HCV genome was amplified in 81 samples (out of 102 5' UTR positive samples) for DNA sequencing. HCV genotype 3 was found in 71 (87.65%) individuals followed by genotype 1 (n=10, 12.35%). Among the genotype 3, subtype 3a was present in 69.01 per cent (n=49) and 3b in 30.98 per cent (n=22) and in case of genotype 1, subtype 1a and 1b both were in equal proportion (Fig. 1).

Of the 71 genotype 3 infected individuals, 88.9 per cent (n=63, 66.7% of genotype 3a and 22.2% of genotype 3b) infected individuals showed >1500 µg/l serum ferritin level and >10,000 IU/ml viral load compared to genotype 1 (3.7% of genotype 1a and 7.4% of genotype 1b, Fig. 2).

Discussion

Thalassaemia is a group of genetic disorder which occurs mainly due to defective formation of the globin chain of the Hb moiety of the red blood cell (RBC). Excessive RBC breakdown occurs at an early stage due to abnormal globin chain, which is unable to protect RBC in oxidative stress, thus, these patients require blood transfusions regularly[23]. TTI is a great concern among β-thalassaemia individuals who receive transfusion on a regular basis.

Our results showed that serum total bilirubin values were significantly elevated in HCV-infected thalassaemic individuals in all the age groups whereas, ALT and AST values were significantly elevated in HCV-infected thalassaemic individuals within age group 2 to 12 as compared to control groups. Another

Table IV. Hepatitis C viral load and its correlation with clinical parameters among β-thalassaemic individuals

Group	Viral load				
	Below detection level (up to 50 IU/ml)	50-5000 IU/ml	>5000-10,000 IU/ml	>10,000-100,000 IU/ml	>100,000 IU/ml
Hb (g/dl)	8.75±0.64	8.74±0.64	8.66±0.65	8.75±0.65	8.69±0.64
Serum ferritin (ng/ml)	1699.32±753.99	1713.01±760.31	1679.35±743.29	1698.64±759.50	1703.11±762.37
Serum total bilirubin (mg/dl)	1.60±0.14	1.61±0.12	1.6±0.01	1.60±0.04	1.64±0.09
ALT (IU/l)	83.52±41.69	84.04±41.71	88.14±41.94	84.32±41.06	83.41±41.16
AST (IU/l)	86.68±42.41	87.27±42.34	93.38±42.05	87.46±41.03	87.65±41.24

Group 1, children between 2 and 12 yr; Group 2, 13 and 19 yr; Group 3, 20 and 29 yr; Group 4, 30 and 35 yr
Hb, haemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase

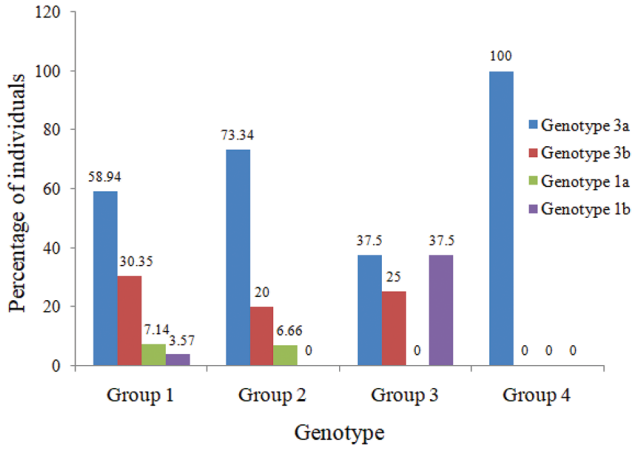

Fig. 1. Distribution of hepatitis C virus genotype among hepatitis C virus infected β-thalassaemia individuals.

point of concern is that thalassaemic individuals have an increased tendency of deposition of iron in the liver. In this study, most of the participants were children, hence, liver biopsy was avoided and serum ferritin level was used as an alternative biomarker (which is indicative of the hepatic iron load) to monitor hepatic iron content[24]. Iron overload is an independent cause of the liver dysfunction, but the relation between liver disease and iron concentration has been poorly investigated in thalassaemic patients[25]. Deposition of iron in liver (high serum ferritin) is known to have a role in damaging the liver tissue by altering liver function enzymes[14,26]. Therefore, it is imperative to monitor serum ferritin levels as excessive ferritin levels can also cause cardiac complications in thalassaemia patients[27]. In our study, increased serum ferritin levels were found in all HCV seroreactive thalassaemic patients, but an interesting observation was that HCV-infected individuals with viral load greater than 10,000 IU/ml also showed high ferritin levels (>1500 µg/l). In addition, 88.9 per cent of HCV genotype 3 infected individuals showed >1500 µg/l serum ferritin level and >10,000 IU/ml viral load compared to genotype 1. Thus, it could be presumed that there was an association between HCV replication and liver iron deposition, but more number of samples would be needed to determine the association.

Our study showed that based on qualitative nested RT-PCR detection, only 59.30 per cent of HCV seroreactive individuals were RNA positive, whereas in case of detection by sensitive TaqMan quantitative real time assay 73.24 per cent individuals were RNA positive. Majority β-thalassaemic individuals with HCV in all age groups presented lower viral load (within 5000 IU/ml). This may be due to low age in most of the β-thalassaemic patients, modulation of immune system by repeated transfusions and iron chelation therapy which may indirectly or partially prevent the replication of the HCV virus. However, this should be investigated further in a large cohort of population before drawing any conclusion. Our results indicated that in β-thalassaemia patients, it was imperative to employ sensitive quantitative real-time PCR detection to accurately determine the presence of HCV RNA. HCV genotyping was also performed in RNA-positive individuals as it provided information about strain variation and was potentially associated with disease severity and treatment strategy[28]. HCV genotype 3 was major circulating genotype (87.65%) followed by genotype 1 in our study. Our results were similar to the data obtained in other HCV populations (such as haemophilia and haemodialysis) where HCV genotype 3 was the dominant infecting strain[29].

In conclusion, our results showed HCV genotype 3 as major genotype among β-thalassaemic patients and also an association between HCV replication and

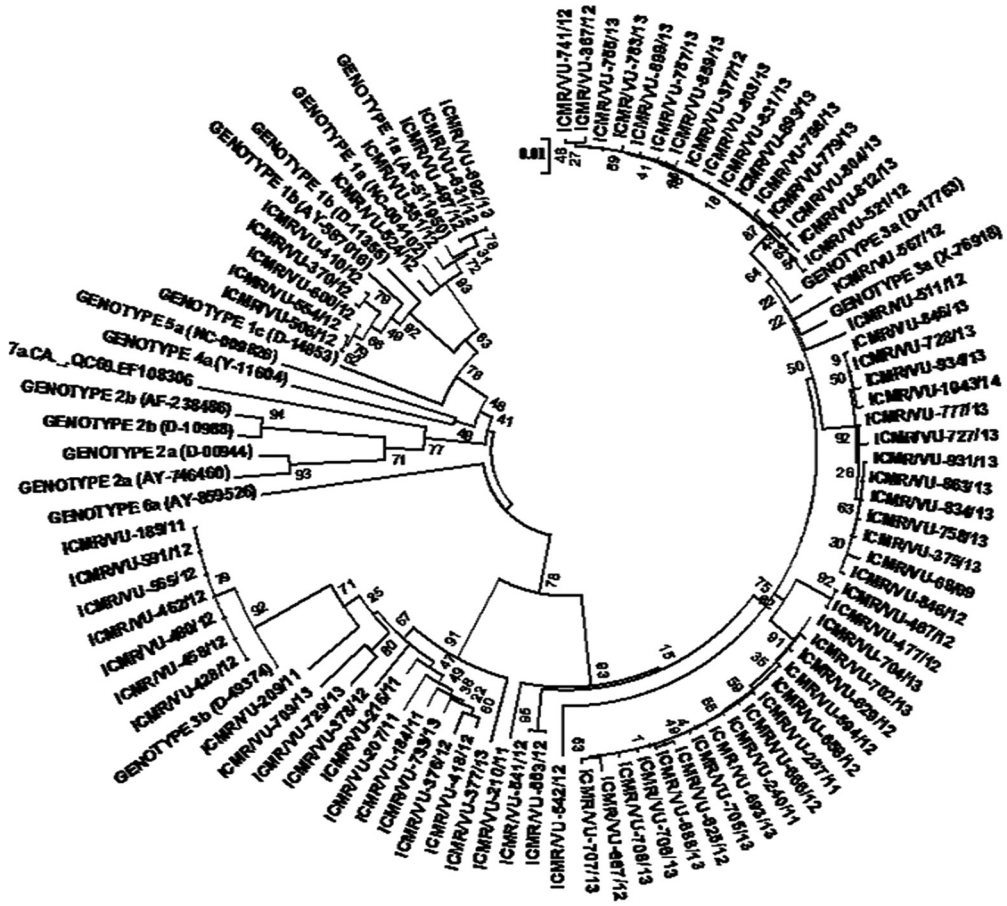

Fig. 2. Phylogenetic tree obtained from the 81 partial core sequences. The reference sequences used are Genotype-1a (NC004102, AF511950); Genotype-1b (AY587016, D11355); Genotype-1c (D14853); Genotype-2b (D10988, AF238486); Genotype-3a (X76918, D17763); Genotype-3b (D49374); Genotype-4a (Y11604); Genotype-5a (Y13184); Genotype-6a (NC008828); Genotype 7a (EF108308).

hepatic iron load. Further, sensitive molecular tests should be used for proper screening of blood or blood products before transfusion.

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***Conflicts of Interest*:** None.

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