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Current trends of Hepatitis C virus genotypes and associated risk factors in hemophilia patients in Pakistan

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Abstract. Hemophilia is a rare bleeding disorder that needs plasma or clotting factor concentrate transfusion. Therefore chances of blood-borne pathogens like HCV transmission increase due to high prevalence in healthy donors. This study was aimed to determine the prevalence of HCV genotypes and associated risk factors in hemophilia patients of Khyber Pakhtunkhwa, Pakistan. Blood samples and data were collected from 672 hemophiliacs after proper consent obtained from each patient. Samples were analyzed for anti-HCV, HCV RNA and HCV genotype/s detection. Of the total, 22.32% (150) were anti-HCV positive, of which HCV RNA was detected in 18.45% (124) individuals. HCV genotype 3a was found with significantly higher prevalence ($p<0.05$) (19.35%) as compared to 2a (16.13%) and 1a (12.90%). HCV-3b and HCV-4 were found each in 3.22% samples. Dual infection of genotypes was found in 22.58% of individuals and 22.58% HCV RNA positive sampels were not typed. A total of 572 (85.12%) subjects had hemophilia A and 100 (14.88%) had hemophilia B. In hemophiliacs A the most dominant genotype was 3a (19.27%) while in hemophilia B, genotype 1a was prevalent (26.67%). Whole blood and plasma transfusion were observed as the main risk factors of HCV. It is concluded that HCV genotype 3a and 2a are prevalent in hemophilia patients of Khyber Pakhtunkhwa Pakistan and the main risk factor observed was an unscreened whole blood transfusion.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major global public health problem particularly in multi transfused patients like hemophiliacs. Hemophilia is a rare X-linked recessive bleeding disorder caused by partial or complete lack of clotting factor VIII (hemophilia A) or factor IX (hemophilia B) and the affected individuals need blood plasma or clotting factor concentrate transfusion (Srivastava *et al.*, 2013). The prevalence of hemophilia is about 1 per 5000–10000 male live births, with about 85% hemophilia A and 15% hemophilia B across the globe (Bowen, 2002). HCV transmits through intravenous drug use, hemodialysis, needle stick injuries, unprotected sex, surgery, and blood and blood product transfusion (Ghany *et al.*, 2009). Blood and blood product transfusion is common in genetically transmitted blood disorders like thalassemia and hemophilia and the chances of transfusion-transmitted infections like HCV increases due to frequent transfusion (Alter and Klein, 2008). In developing countries like Pakistan where blood screening facilities are not widely available, chances of blood-borne pathogens increase due to its high prevalence in the general public population (WHO, 2011).

HCV is an enveloped virus with a positive-sense single-stranded RNA genome. Due to poor fidelity of RNA-dependent-RNA-polymerase, the virus exhibits a high level of genetic diversity and leads to varying genotypes (1-6) and many subtypes distributed variably in different regions

across the globe (Moradpour *et al.*, 2007). Distributions of HCV genotypes are highly variable among hemophiliacs and most of the studies reflect the genotypes distribution to the general public population. HCV genotypes 1, 2 and 3, are the more dominant North America, genotype 4 in the Middle East and North Africa and genotype 5 in South Africa (Giangrande, 1998) while in European countries more than one genotype exists especially among hemophiliacs (Sereno *et al.*, 2009). HCV genotype 1a and 3a are common in hemophiliacs of Iran (Samimi-Rad *et al.*, 2012), while in India the most prevalent genotype is 3a (Rehan *et al.*, 2011). In Pakistan, HCV genotype 3 is the most prevalent followed by genotype 2 and 1 in the general population (Khan *et al.*, 2014; Waqas *et al.*, 2015). Some studies from parts of the country reported HCV genotype 1 and 3a from Punjab province (Waheed *et al.*, 2011).

The province Khyber Pakhtunkhwa of Pakistan has about 3000 hemophilia patients who have been registered in various blood transfusions providing organizations located in the capital city Peshawar (Nazir *et al.*, 2014). Data regarding the prevalence of HCV and HCV genotypes and associated risk factors in this area is scarce. This study aimed to evaluate the prevalence and risk factors of HCV genotypes in hemophilia patients of Khyber Pakhtunkhwa Pakistan.

MATERIALS AND METHODS

Study area and study population

This study was performed in the capital city of Khyber Pakhtunkhwa province Pakistan. The total area of the province is 101741 km^2 having a population of 35.53 million with an average density of 350/ km^2. This area is one of the important zones located in the northwestern part of the country along the international border with Afghanistan (Fig. 1). Since major health facilities like tertiary care hospitals and blood donation centers are located in Peshawar, therefore, hemophiliacs had frequently visited the blood centers there to receive hemotherapy. Hemophiliacs belonged to various parts of the province were enrolled in the study who visited various blood transfusions providing organizations located in Peshawar, Pakistan.

Ethical approval

This study was approved by the Advanced Studies and Research Board (ASRB), and ethical committee Centre of Biotechnology and Microbiology University of Peshawar Pakistan.

Data and Samples collection

Data was collected using a predesigned questionnaire from patients or their parents after written consent including the history of hepatitis in family, whole blood or plasma transfusion, factor concentrate transfusion, type of hemophilia, the severity of the disease, etc. Blood (3 mL) was collected from patients and sera were separated and stored at -20°C at Molecular Biology and Virology Laboratory Department of Zoology University of Peshawar Khyber Pakhtunkhwa Pakistan for further process.

Anti-HCV screening

Sera samples were screened for anti-HCV using ICT device kit (Accurate Diagnostic, Canada), according to manufacturer instructions.

HCV RNA isolation and amplification

RNA was isolated from anti-HCV positive sera using a nucleic acid isolation kit (Favorgen, USA) as per manufacturer protocol. cDNA was synthesized of the 5'UTR of HCV genome using antisense primer and 200U of M-MLV RTase (Invitrogen, USA). cDNA was then amplified in nested PCR with two sets of 5'UTR specific primers (outer and inner) using 5U of *Taq* DNA polymerase (ThermoFischer, USA). A reaction mixture (20 µL) for nested PCR was consisted of 2 µl of 10X PCR buffer, 2.5 µl of 25 mmol $MgCl_2$, 1 µL of 10 mM dNTPs, 1 µL *Taq* DNA polymerase (2UI/uL), 2 µL of previous round PCR product, and 1 µL each primer (10 pmol). The reaction program was composed of 25 cycles with the following conditions: 94°C for 30 seconds, 55°C for 40 seconds, and 72°C for 40 seconds with an initial denaturation

Figure 1. The geographical location of the study area (Khyber Pakhtunkhwa).

at 94°C for 5 minutes and a final extension at 72°C for 10 minutes.

HCV genotyping

cDNA was synthesized from extracted RNA using antisense primer specific for the HCV core region and 200U of M-MLV RTase (Invitrogen, USA) according to the manufacturer protocol. cDNA was amplified with a pair of core region-specific primers and *Taq* DNA polymerase (Fermentas USA). Two aliquots of reaction mixtures were prepared, different in antisense primers (subtype-specific primers). The anti-sense primers of one mix were 1b, 2a, ID-8, 2b, 3b and the other were 1a, 3a, 4, 5a, 6a. The reaction was processed as mentioned by Ohno *et al.* (1997).

Electrophoresis

PCR amplified product was electrophoresed on 2% agarose gel along with 50 bp DNA ladder marker (Fermentas, USA) and visualized under UV-transilluminators.

Statistical analyses

Some variables of the study were presented in percentages (%). Chi-square ($\chi 2$) test was used for data analysis and measuring the association between variables and p-value of 0.05 or less was considered significant.

RESULT

Demographic characteristics of patients

The baseline characteristics of hemophiliacs of this study are shown in Table 1. Of the total 672 hemophiliacs included were of age ranged from 3-57 years. Of these, 68 patients were of severe hemophilia (clotting factor level <1%), 540 of moderate (clotting factor level 1-5%), and 64 of mild (clotting factor level 6-50%) hemophilia. 572 were suffered of hemophilia A and 100 (14.88%) of hemophilia B. Most of the patients had a history of different risk factors like whole blood, plasma and factor concentrate transfusion, surgery, circumcision, etc. Most of the patients had multiple transfusion of plasma or factors' concentrate.

HCV prevalence among hemophiliacs

Of the total, HCV antibodies were detected in 150 (22.32%) patients. The rate of anti-HCV was more in patients with hemophilia-A. A high prevalence of anti-HCV was observed in severe hemophiliacs. HCV RNA was detected in 124 (18.45%), in samples positive for anti-HCV. Higher detection of HCV RNA was recorded in patients with hemophilia-A. The prevalence of HCV RNA was found higher in patients with severe hemophilia as compared to the moderate and mild type of disease. No significant difference (P>0.05) was seen between anti-HCV and HCV RNA positive patients and disease type and severity (Table 2).

Table 1. Characteristics of patients with hemophilia (N=672)

Variables	Values N (%)
Age range	3-57 years
Hemophilia type	
Hem-A	572 (85.12)
Hem-B	100 (14.88)
Severity of Hemophilia	
Sever	68 (10.12)
Moderate	540 (80.36)
Mild	64 (9.52)
Main risk factors	
Blood and plasma transfusion	160 (23.8)
Plasma and factor concentrate transfusion	115 (17.11)
Plasma transfusion	245 (36.45)
Factor concentrate transfusion	60 (8.9)
Dental and general surgery	45 (6.7)
Others	47 (6.9)

Table 2. Distribution of Anti-HCV and HCV RNA among hemophilia patients

Hemophilia types (N)	Anti HCV		HCV RNA		P-value
	+ve N (%)	-ve N (%)	+ve N (%)	-ve N (%)	
Hem A (572)	134 (23.43)	438 (76.57)	109 (19.06)	463 (80.94)	0.709
Hem B (100)	16 (16)	84 (84)	15 (15)	85 (85)	
Disease severity (N)					
Severe (68)	37 (54.4)	31 (45.6)	28 (41.18)	40 (58.82)	0.718
Moderate (540)	95 (17.6)	445 (82.4)	84 (15.6)	456 (84.4)	
Mild (64)	18 (28.12)	46 (71.88)	12 (18.75)	52 (81.25)	

HCV and associated risk factors

Different risk factors were analyzed for HCV infection in hemophiliacs shown in Table 3. In both types of hemophilia, local transfusion of unscreened blood and plasma were the main risk factors of HCV infection. Statistically, no significant associations were found (P>0.05).

HCV Genotypes in the Study Population

Of the total 124 HCV RNA positive samples, five different genotypes were detected. Overall, genotype 3a (24/124) was significantly higher (P<0.05) followed by 2a (20/124) and 1a (16/124). Each HCV 3b and 4 genotype were found in 4/124 samples. Dual HCV infection 2a/3a (8/28), 3b/3a (16/28), 2a/4 (4/28) were also observed. Of the total, 28 samples were found untypable (Fig. 2).

HCV genotypes distribution among hemophiliacs

The distribution of HCV genotypes in both types of hemophilia and disease severity was shown in Table 4. In hemophilia A the most dominant genotype was 3a (21/109) followed by 2a and 1a (20/109) and (12/109) respectively. Among patients with hemophilia B, genotype 1a was the most prevalent (4/15) followed by 3a (3/15). In severe hemophilia 3a (16/28) while in that of moderate 2a (12/84) and mild hemophilia the most dominant genotype was 1a (8/12).

Table 3. HCV prevalence and associated risk factors

Risk factors	Hem A			Hem B			P-value
	N	+ve N (%)	-ve N (%)	N	+ve N (%)	-ve N (%)	
Blood+plasma	120	46 (38.33)	74 (61.67)	40	7 (17.5)	33 (82.5)	0.839
Plasma+factor concentrate	103	16 (15.53)	87 (84.47)	12	3 (25)	9 (75)	
Only plasma	225	37 (16.44)	188 (83.56)	20	4 (20)	16 (80)	
Only factor	42	4 (9.52)	38 (90.48)	18	–	18 (100)	
Dental+general surgery	42	–	42 (100)	3	–	3 (100)	
Others	40	6 (15)	34 (85)	7	1 (14.28)	6 (85.72)	

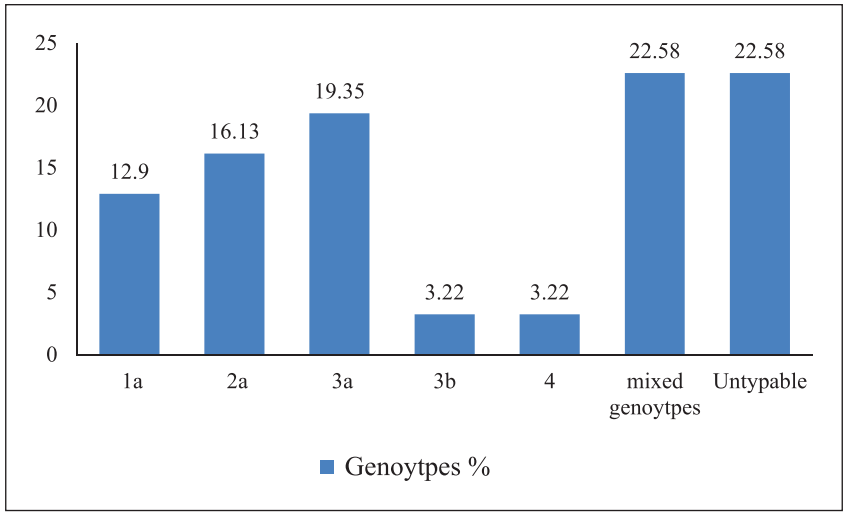

Figure 2. Distribution of HCV genotypes among the patients.

Table 4. Prevalence of HCV genotypes in HCV RNA positive samples

Parameter	1a N (%)	2a N (%)	3a N (%)	3b N (%)	4 N (%)	Mixed N (%)	Untypable N (%)
Hem-type (N)							
Hem-A (109)	12 (11.01)	20 (18.35)	21 (19.27)	4 (3.67)	4 (3.67)	20 18.35)	28 (25.69)
Hem-B (15)	4 (26.67)	–	3 (20)	–	–	8 (53.33)	–
Severity of disease							
Sever (28)	–	4 (14.29)	16 (57.14)	–	–	–	8 (28.57)
Moderate (84)	8 (9.52)	12 (14.28)	8 (9.52)	4 (4.76)	4 (4.76)	28 (33.33)	20 (23.81)
Mild (12)	8 (66.7)	4 (33.33)	–	–	–	–	–

DISCUSSION

Transfusion transmitted diseases are the major public health problems particularly in multi transfused patients like hemophiliacs and are the most important complications which lead to chronic conditions cause high morbidity and mortality across the world. Of this hep-C is the most important due to high prevalence in the general public population (Karimi *et al.*, 2002) and was frequently observed in hemophilia patients treated with clotting factor concentrates before the 1980s (Choi, 2014). Patients with hemophilia have a high risk of acquiring HCV and other pathogens like HBV and HIV due to frequent blood or blood products transfusion (Rezvan *et al.*, 2007) mainly in regions with poor blood screening practices and high prevalence among blood donors (De Paula *et al.*, 2005).

In the study population five different HCV genotypes 1a, 2a, 3a, 3b, and 4 were detected. Highest prevalence of genotype 3a was found in study population (19.35%) followed by 2a (16.13%), 1a (12.90%), 3b and 4 (3.22% each) and (22.58%) respectively with some mixed genotypes as 2a/3a 8 (6.25%), 3b/3a 16 (12.5%), 2a/4 4 (3.12%) while (22.58%) were untypable. Prevalence of HCV genotypes in hemophilia patients of Khyber Pakhtunkwa province also determine their distribution in the general public. This study is not in line with studies conducted in Latin America (Martins *et al.*, 2000) and the USA, Japan, and Brazil where HCV genotype 1 was prevalent (Sereno *et al.*, 2009). In most of the European countries like Italy reported the distribution of genotype 1 and 2 there (Oliveira *et al.*, 1999). Our study has similar findings to that reported from Iran (Amini *et al.*, 2009; Samimi-Rad *et al.*, 2012). Similar findings were found by Rehan *et al.* (2011) in India and Alfaresi, (2011) in the UAE.

In our study genotype, 3a is more common among the study population reflecting the overall distribution of HCV genotype 3a in the general population of Pakistan (Ali *et al.*, 2010). Studies conducted in various regions of Pakistan in hemophilia patients showed that HCV genotype 3a is common (Khokhar *et al.*, 2003).

The presence of untyped samples indicates that some novel genotypes may be present in this population not detected by the genotyping method used in this study or maybe with low viral load. The prevalence of mixed genotypes of the current findings are similar to the study of Zoulim (1999), showed that hemophilia patients are more susceptible to reinfected several times with different HCV genotypes and subtypes due to frequent plasma and factor concentrate transfusion derived from donors blood. The present study suggested that the distribution of HCV genotypes in the hemophilia population of Khyber Pakhtunkhwa reflected the prevalence of HCV genotypes in the general public.

It is obvious from the findings of the present study that whole blood, plasma and factor concentrate transfusion were the main risks of HCV transmission in hemophilia patients. Similar findings of Asif *et al.* (2009) mentioned that plasma transfusion was the main risk of HCV in hemophilia population. A study conducted in Iran by Hajiani *et al.* (2006) was related to current study suggested that plasma transfusion was the leading risk

factor for HCV transmission in hemophilia patients treated with regular plasma transfusion.

CONCLUSIONS

A high prevalence of HCV was detected in hemophilia patients of Khyber Pakhtunkhwa province and genotype 2a and 3a were widely distributed in these patients. Whole blood and poorly screened plasma transfusions are the important risk factors of HCV.

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