

Research Article

Molecular prevalence of hepatitis C virus genotypes in district Kohat, Khyber Pakhtunkhwa, Pakistan

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Abstract

Hepatitis C Virus is a causative agents of liver cirrhosis, having highly diversified genome. Genotype study has important role in clinical setup and prognosis of hepatitis C virus infection. The aim of this was to investigate the prevalence and risk factors associated with hepatitis C virus genotypes in district Kohat, Khyber Pakhtunkhwa, Pakistan. Hepatitis C virus pre-infected patients (n = 600) were analyzed to assess circulating HCV genotypes, using reverse transcriptase approach. Moreover serum ALT levels were determined followed by correlation with genotypes. In current study cohort, majority of patients (62%) were, having elevated ALT (> 50 U/L). similarly patients with mixed HCV genotypes, 3a/3b have relatively higher ALT levels (~62) compared to 2a/3a (~43) and others. We found significant correlation ($p = 0.002$) of genotype 2a in connection with ALT (49.48) level. Similarly 3a, 3b, mixed as well as untypeable genotypes correlations were also found highly significant ($p < 0.001$) with their mean ALT levels (47.14, 31.58, 46.69 and 43.68) respectively. In our studied population, most prevalent genotypes were 3a (25%) followed by 3b (20%) and 2a (15%). Fifteen percent of patient's infections were untypeable while in 10% patients mixed genotype were observed. Among total 30 (5%) were blood transfusion cases, 90(15%) surgical, 540 (90%) dentistry, 360 (60%) Barber, 492 (82%) Pricks, whereas 180 (30%) cases were those having early type of HCV/HBV infection in their family. In current study, genotypes 3a and 3b were more prevalent, with two potential risk factors; dentistry and barbers. Moreover, genotypes and ALT investigations were found to be more fruitful in prognosis as well as management of HCV infection.

Keywords: HCV; Genotypes; ALT; Risk factors; Kohat

Introduction

Hepatitis C Virus (HCV) is a leading causative agent of liver cirrhosis and

hepatocellular carcinoma. HCV is a single stranded positive sense RNA virus belongs to the genus *hepacivirus* of *Flaviviridae*

family [1]. It has a single open reading genome of ~9.6 kb, encoding a polypeptide of 3010 amino acids, flanked by untranslated region at both 5' and 3' terminus [2]. This polypeptide is post-translationally modified into various structural (C, E1, E2, and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins by viral and cellular proteins [3]. HCV possessed extreme genetic heterogeneity due to poor RNA-dependent RNA polymerase (NS5B-protein) activity [4]. The route of transmission of HCV shown very complexity but it is more intended on futile neutralizing immune responses including unscreened blood transfusions, unsterilized dental and surgical instruments, unhygienic barber conditions and tattooing [5]. Patients carrying HCV infection shows symptoms like acute/chronic liver disease, elevated serum ALT level that finally may lead to hepatocellular carcinoma [6]. There are ~200 million HCV patients which account for ~3.3% of the total population of the world [7]. Among them, ~10 million people having HCV infection accounting ~4.7% of the total population of Pakistan [8]. The first report about HCV was reported in 1992 by screening 45 patients from the northern part of Pakistan [9]. There are six major genotypes of HCV and more than one hundred subtypes reported throughout the world [10]. Out of these, the genotype-1, genotype-2 and genotype-3 are most popular genotypes in terms of prevalence [10]. The prevalence rate varies from region to region and have variation geographically. The prevalence of genotype 1 is a bit high (7.04%), followed by genotype 2 (3.82%). However, the prevalence rate of genotype 3 is very high 78.96%, while it subtype 3a reported 58.01%, followed by 3b (9.76%). The other genotypes such as 4, 5, 6 are very seldom reported in Pakistan. This revealed that the genotype 3a have high prevalence rate at Khyber Pakhtunkhwa, and Punjab.

The Baluchistan province of Pakistan is reported to have high prevalence of genotype 1a and the least prevalence is for genotype 3a [11]. There are very high numbers of patients having HCV infection in Pakistan and this number is increasing on daily basis. Therefore, it is the need of today and tomorrow to investigate the risk factors, occurrence and molecular level of HCV genotypes and search for the possible effective treatment in order to stop the transmission and save the lives of the patients having this chronic disease [12]. District Kohat is a multicultural and highly populated regions of Khyber Pakhtunkhwa having different communities of the local FATA zones in addition to local people. There is still very few report from district Kohat and need updated information in order to plan and eradicate this infection from local population.

Materials and methods

Collection of samples from patients

Total 600 blood samples were collected from HCV positive patients through pre-designed questionnaire including patient details i.e. age, socioeconomic status, risk factors, duration of infection, racial origin, and liver function test. Written consents were signed from patients and the study was initiated with proper approval from ethical committee. Samples were transported to the lab immediately and stored for onward analysis.

Antibody screening and ALT analysis

Immuno- chromatographic method was used for the screening of HCV antibodies utilizing the kit (Accurate Diagnostics™ Canada) and the sera of patients were tested by ELISA using commercially available kits (Axen Diagnostic™, Germany). ALT, as hepatocytes marker was investigated according the manufacturer's protocol by using biochemical analyzer (Microlab 200, Merck USA).

Genotyping of HCV patients

Approximately 200 μ L of the blood was taken and HCV genomic RNA was extracted according the manufacturer's protocol by using RNA extraction kit of UltrascripTM (Anagen, USA). The molecular analysis was done with little modification using the Ohno *et al.*, (1997) [13] method.

Then cDNA was made from viral mRNA by using 10 μ L of RNA with 1 μ L (200U) of RevertAidTM M-MuLV reverse transcriptase enzyme (Fermentas) at 42°C for 30 min followed by 92°C for 2 min in thermocycler (Techne, USA). The cDNA was amplified in first round of PCR using forward and reverse primers, one unit of Taq polymerase and 3mM MgCl₂ at 94°C for 3 min followed by 92°C (45 Sec), 52°C (45 Sec) and 72°C (1

min) with final extension at 72°C (10 min) in PCR. Again, 4 μ L of the first PCR product was further amplified by genotype specific primers (1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a) in two mixtures. Mixture-1 contained antisense primers 1b, 1.D-8 (2a), 2b, 2a and 3b while Mixture-2 composition included the antisense primers 1a, 3a, 4, 5a and 6a. The reaction mixture of both mixtures composed of one unit of Taq polymerase and 3mM MgCl₂ having PCR conditions as mentioned for the first round of PCR. PCR products were separated by agarose gel electrophoresis on 2% agarose gel along with 100 bp DNA ladder and later on stained with ethidium bromide and checked under UV illuminator (Figure 1).

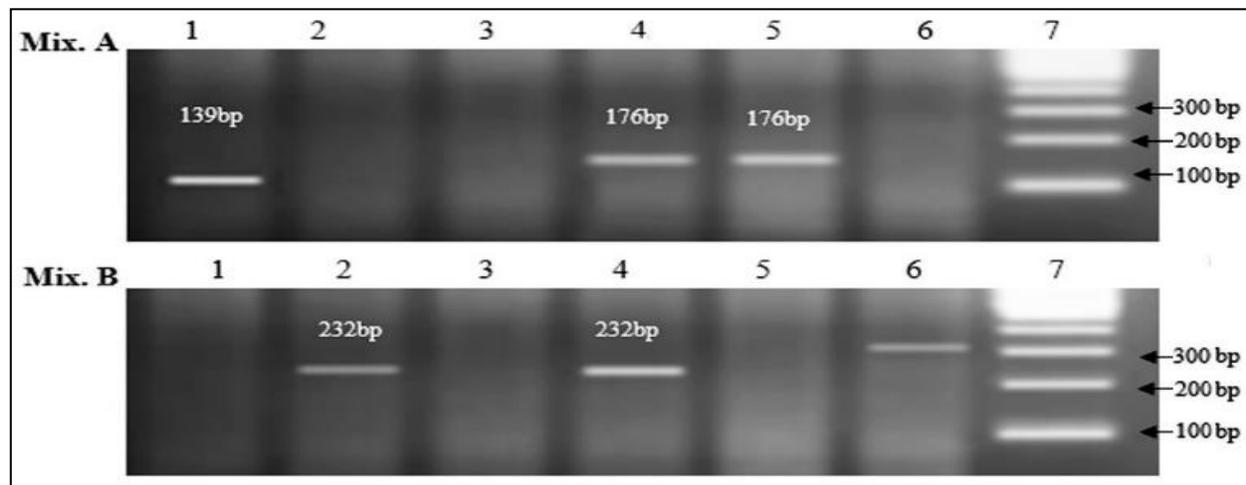


Figure 1. Gel electrophoresis of the amplified core regions of HCV genome showing different genotypes. Mix. A and Mix. B gel pictures represent mixtures of PCR constituted for HCV primers related to group A & B respectively for based on genotypes identifications

	Base pairs	Genotypes
Lane 1:	139bp (Mix A)	2a
Lane 2	232bp (Mix B)	3a
Lane 3	Nonspecific	Untypeable
Lane 4:	176bp (Mix A) 232bp (Mix B)	3a & 3b (Mixed Genotypes)
Lane 5:	176bp (Mix A)	3b
Lane 6:	Nonspecific	Untypeable
Lane 7:	100bp Ladder Marker	

Statistical analysis

Statistical analysis was performed using Origin Pro 2016 (Origin Lab, Northampton, USA) statistic software. The correlation among HCV genotypes and ALT was analyzed at 95 % confidence intervals (CIs) using simple t-test, while specific comparison were made by standard descriptive analysis. The p-values <0.05 were considered, significant for a particular test.

Results

Clinico-pathological characteristics of patients

In total, 600 patients were studied gender-wise in equal ratio ranging the age groups between 20-80 years. The patients were also reported in terms of marriage, income class and on educational background. The married patients were reported 438 (73%), unmarried

162 (27%), lower income class (\geq 100 US\$/month) 462 (77%) and middle income class (500 US\$/month) 138 (23%). In addition, 375 (~62%) were illiterate, 225 (~38%) were of middle class. However, no patient among all was found having high income per month (<500 US\$/month).

Potential risk factors in HCV transmission

Data reported from patients revealed high prevalence rate of HCV infection among patients experienced dental procedures 540 (90%), followed by unhygienic pricks 492 (82%), barber intervenes 360 (60%), early HBV/HCV running in family 180 (30%), surgical operations 90 (15%). However, the blood transfusion was reported with the least HCV transmission rates 30 (5%) as per information collected through questionnaires from the patients (Table 1).

Table 1. Frequency of HCV related risk factors among recruited patients

Risk Factors	Status	Frequency of Patients	Percentage
Pricks (needle type)	Yes	492	82
	No	108	18
Visit to barber	Yes	360	60
	No	240	40
Visit to dentist	Yes	540	90
	No	60	10
HCV/HBV in family	Yes	180	30
	No	420	70
Surgery (major/minor)	Yes	90	15
	No	510	85
Blood transfusion	Yes	30	5
	No	570	95

Frequencies of ALT levels

All the patients under study were followed for measuring their ALT levels up to six months with specific interval. Significant ALT fluctuations were reported. Moreover, 62% of patients had high ALT levels (\geq 50 U/L) and 38% patients were reported with normal ALT level (\leq 50 U/L). In this study, we have found significant increased (61.79 U/L; mean value) in ALT level. Moreover,

we analyzed the ALT level in patients circulating mixed HCV genotypes in their sera, and were found that patients infected with 3a/3b genotypes have significantly high ALT level (~62%), followed by 2a/3a (~43%) compared to other co-infected HCV patients. These elevated ALT level signify a drastic effect of HCV among individuals (Figure 2).

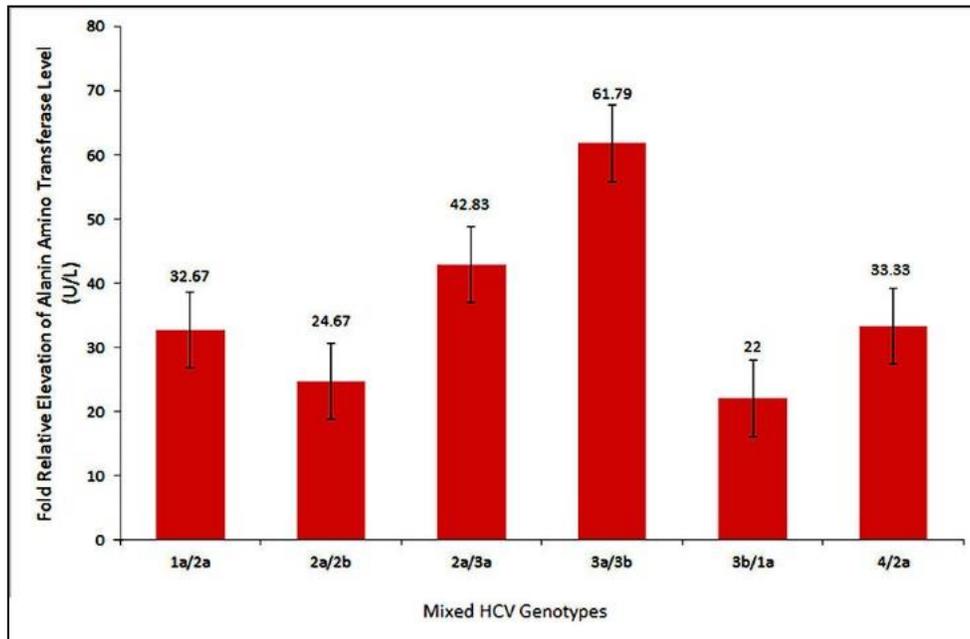


Figure 2. Alanine amino transferase (ALT) level (mean) in patients infected with HCV mixed genotypes

General prevalence of HCV genotype

The high prevalence rate among different HCV genotypes was reported for 3a (25%), followed by 3b (20%), 2a (15%), 1a (5%) and 4 (5%). However, the genotypes such as 1b, 6a, and 5a were not reported among the patients under investigation. There was 15% patients which indicated no genotype and 10% of the patients showed mixed infection of HCV genotypes. The mixed genotypes were reported in decreasing order as followed; 2a/3a (45%), 3a/3b (35%) and 5% in cases of 2a/2b, 3b/1a, 1a/2a and 4/2a (Table 2). The genotypes prevalence in terms of age groups is reported as: genotype 1a was with high prevalence rate in age group of ≤ 20 years followed by 41-60 years; genotype 2a had high prevalence in age groups 21- 40 years, followed by age group of ≤ 20 years, then 41-60 years with lowest

rate in 61-80 years; genotype 2b was only reported in age group of 61-80 years; genotype 3a was found in all age groups with relatively equal frequency among all age groups (Table 3); genotype 3b was also high in age groups of 41-60 years followed by 21- 40 years and 61-80 years with prevalence rate of 30% each; genotype 4 was only reported in age group of 61-80 years; The occurrence of mixed genotype was reported high in age groups 41-60 years and 61-80 years with 30% prevalence rate in each cases, whereas 6% rates were observed from the cohort of age 21- 40 years and ≤ 20 years; Moreover, the prevalence rate of untypeable genotype was high in the age groups of 21-40 years followed by 61-80 years and 41-60 years with least frequency rate (9%) in ≤ 20 years among the cohort (Table 3 and Figure 3).

Table 2. Prevalence of HCV genotypes running among recruited patients

Variables	No, of Patients	Prevalence (%)
Genotype (typable)		
1a	30	5
2a	90	15
2b	30	5
3a	150	25
3b	120	20
4	30	5
Mixed	60	10
Total	510	85
Genotype (Untypeable)		
1b	Not detected	0
5a	Not detected	0
6a	Not detected	0
Total	90	15
Mixed Genotypes		
2a/3a	54	45
3a/3b	42	35
2a/2b	6	5
3b/1a	6	5
1a/2a	6	5
4/2a	6	5

Table 3. Age-wise correlation of HCV genotypes and ALT among studied patients

Genotypes	Prevalence of HCV genotypes among different age groups (%)				ALT (U/L) (mean values)	95% CI		Skewness	P-Values
	≤20 years	21-40 years	41-60 years	61-80 years		Lower	Upper		
1a	18	--	12	--	25.52	--	--	--	--
2a	27	36	18	9	49.48	28.22	87.23	1.53	0.002
2b	--	--	--	30	35.25	--	--	--	--
3a	30	45	45	30	51.33	42.76	57.86	0.39	<0.001
3b	--	30	60	30	47.14	38.39	51.67	0.51	<0.001
4	--	--	--	30	31.58	--	--	--	--
Mixed	6	6	30	30	46.69	33.34	60.04	0.42	<0.001
Untypeable	9	45	18	30	43.68	38.55	48.81	0.54	<0.001

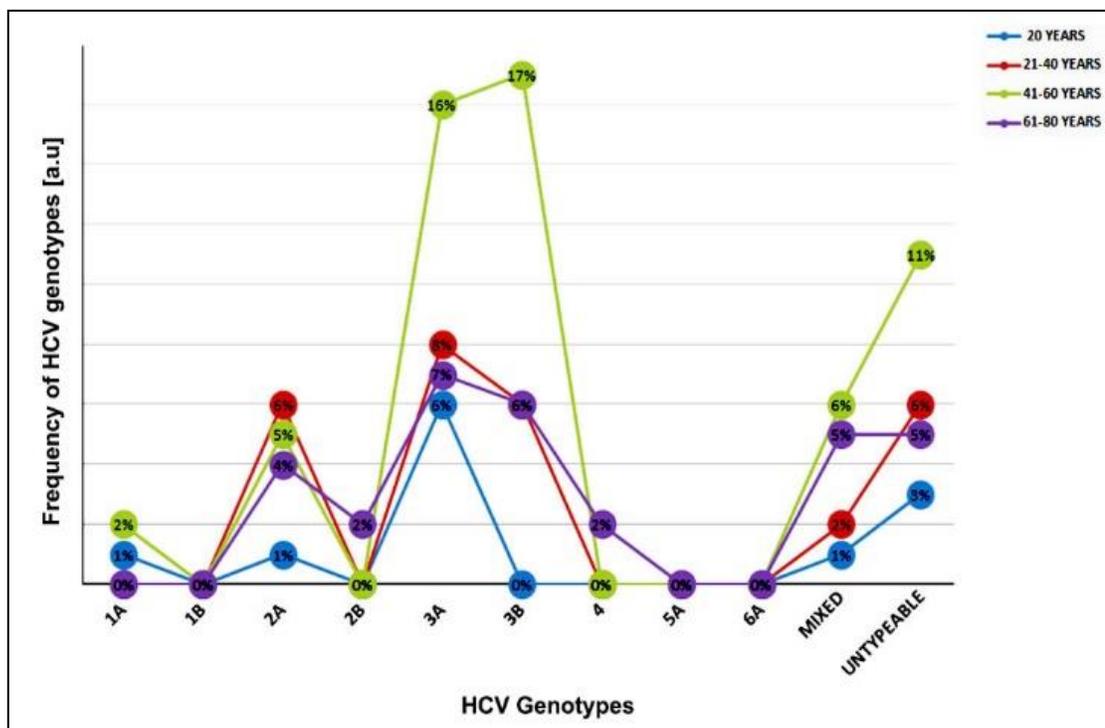


Figure 3. Age-wise distribution of HCV genotypes among recruited HCV patients

HCV genotypes and alanine amino transferase correlation

Alanine amino transferase (ALT) is the most potent hepatocytes marker to detect the degree of liver cirrhosis as a result of ongoing HCV infection. We analyzed serum ALT level for a period of six months and correlate with the respective HCV genotypes. A significant correlation ($p = 0.002$) of genotype 2a with ALT (49.48) level was reported. Similarly 3a, 3b, mixed as well as untypeable genotypes were found highly significant ($p < 0.001$) with their mean ALT levels (51.33, 47.14, 46.69 and 43.68), respectively. However genotypes 1a, 2b and 4 were observed with lower prevalence to meet statistics criteria (Table 3).

Discussion

Hepatitis C virus become asymptomatic at the beginning of infection, called as silent killer. HCV has no clear clinical feature and varies depending on the age, sex, genotype

and viral load of the one individual to another individual [14]. The main objectives of the study were to investigate the risk factors and prediction for early diagnosis in different age, sex and genotypes based on the serum marker evaluation. Most of the patients were reported as illiterate (~62%) and of lower income class (77%). This revealed that mostly poor and uneducated people have high exposure to HCV virus due to their unawareness of the risk factors and transmission routes of HCV infection. In similar fashion, different risk factors studied has shown high prevalence 90%, 82%, 60%, 30%, 15% and 5% in case of dental procedure, unhygienic pricks, barbers intervenes, family history, surgical cases and blood transfusion, respectively. The unsterilized instruments at barber shop, operational theater, and dental surgery are the main causes of HCV infection. In addition, the unscreened blood transfusion and family history have a major role in HCV

infection. This study is supported by previous literature where blood transfusion, un-sterilized dental & surgical instrument, multiple sex, contaminated syringes; needles, unsafe intravenous drugs users, and community barber shops were reported the major sources of HCV transmission [15]. Similarly blood transfusion as well as sedentary life style has been found to be associated positively with HCV infection among Pakistani population [16]. Other aspect of this study was to investigate the role of ALT in the early diagnosis of HCV patients. ALT has been reported as a hepatocyte marker to evaluate the treatment efficiency as well as physiological condition in liver cirrhosis [17].

The ALT levels of patients under study within repeated interval for six months duration revealed significant fluctuation in the sera of each patient. Moreover, the ALT values were also correlated with HCV genotypes of the patients with different age groups and the severity of infection was evaluated statistically. The results showed significant correlation, and concluded that ALT might be directly related to age of patient as well as with the degree of liver cirrhosis. These results are in parallel with the previously reported work [16].

Moreover, the prevalence of different HCV genotypes were also investigated. The genotypes 3a, 3b, and 2a were found more prevalent in the patients of the Kohat district of Khyber Pakhtunkhwa. These genotypes are also reported in the previous literature from the patients of other areas of Pakistan as the most common prevailing HCV genotypes [18]. In 10% of the patients under study, mixed genotypes were identified and among mixed genotypes the 2a/3a were in higher percentage (45%) followed by 3a/3b genotypes. These combined infections are also reported in previous literature and the reason could be the inter-individual mutations and transfusions of mixed viral

strains [19]. These mixed genotypes exposure might be very dangerous in future in comparison to single genotypes and may results certain highly pathogenic variants and causing severe HCV infection for which treatment may not be effective.

The HCV infection was also reported to be more prevalent among the lower class and uneducated people in comparison to middle/upper class and educated people. This could be due to frequent exposure and unawareness of poor and uneducated people to the unhygienic local barber, syringes, blood transfusions and contaminated dental instruments [20-22]. This study suggested the importance of using sterilized syringes, surgical and dental instruments, and transfusion of HCV and HIV screened bloods. Therefore, long term attention of the competent authorities/health department is required in order to control the transmission of the hepatitis C virus.

Conclusion

The utmost conclusion of this work is that the most prevalent genotypes of HCV in the area of Kohat were 2a, 3a and 3b, which might be fruitful in order to prepare the community-based vaccines for controlling HCV infections in certain areas.

Authors' contributions

Conceived and designed and performed the experiments: F Ullah, S Ahmad & M Jamil, Analyzed the data: S Saleha, N Ali, M Jamil & M Saeed, Contributed the reagents: IS Ali, STA Shah & H Rahman, Wrote and corrected the manuscript F Ullah & N Ali.

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