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Prevalence of hepatitis C virus (HCV) genotype in general population of Peshawar District Khyber Pakhtunkhwa (KP), Pakistan

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Abstract

Hepatitis C is a blood born infectious disease caused by a small (50 nm in size), enveloped, positive sense, single-stranded RNA virus known as hepatitis C virus (HCV). It belongs to genus hepacivirus in the family *Flaviviridae*. HCV has six major genotypes. HCV spreads through blood to blood contact and no vaccine against this virus has been developed until now. Most of the people unfortunately develop chronic infection and it leads HCC. In current study, the HCV prevalence and its genotype was determined in the general population of Peshawar, Khyber Pakhtunkhwa (KP) province. Blood samples were collected randomly from general public of various age groups for the prevalence of anti-HCV antibodies and HCV genotype infection employing immune chromatographic assays and multiplex-PCR techniques. Our study reports an overall prevalence of HCV as 19.48% with 1.9%, 3.33%, 7.4% and 6.36% in age groups ≤ 20, 21-40 and ≥ 41 respectively. The highest prevalence of HCV was observed in age group ranging from 21-40 (28.0%) followed by age group ≤ 20 (9.52%). The prevalence of active HCV infection in male population (23.80%) was higher compared with the females (14.2%). In the present study, we conclude that HCV genotype 3b is the most prevalence genotype circulating in this region. Regional difference do exists in HCV genotypes. Majority of the infected patients are young ages between 31-40 years old. These alarming results call for a nationwide screening for HCV infection in general population.

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Introduction

Globally hepatitis C infects about 200 million people and 3-4 million people developing new infection each year (Idress and Riazuddin, 2008). Approximately 85% of HCV infection progress to chronic infection is indolent and asymptomatic disease in human (Mengal *et al.*, 2012). Hepatitis C is caused by virus known as Hepatitis C virus (HCV) (Narendra *et al.*, 2004). Which is a blood-borne hepatotropic virus (Hoofnagle, 2002) belongs to the family Flaviviridae and genus hepacivirus (Sheikh *et al.*, 2008). International standardization of nomenclature classified HCV into 6 major genotypes (1 – 6) (Simmonds, 2004).

HCV has been observed in the recipients of multiple or repeated blood transfusions (Idress and Riazuddin, 2008). The overall observed mode of transmission in Pakistan were: multiple use of needles/syringes (61.45%) (Jafri *et al.*, 2006), major/minor surgery/dental procedures (10.62%) (Khan, 2000), blood transfusion and blood products (4.26%) (Jafri *et al.*, 2006), sharing razors during shaving or circumcision by barbers (3.90%) (khan, 2000), piercing instruments, nail clippers, tooth brushes, miswaks, less than 1% due to needle stick, from infected mother to baby and sexual transmission (Idress and Riazuddin, 2008).

The prevalence of hepatitis C infection differ between and within countries as global prevalence of HCV is 3.1% (Raza *et al.*, 2007), lower in Europe 1.03% (Posta *et al.*, 2009) and America 1.7% (Roman *et al.*, 2008) and highest in Africa 5.3% (Mujeeb *et al.*, 1997). Estimated prevalence of HCV in Pakistan is 3% (Sarwat *et al.*, 2008).

In Pakistan the observed genotypic distribution of HCV were 3 (67.46%), 2 (8.41%), 1 (11.50%), 4 (1.49%), 5a (0.18%), 6a (0.12%) and mixed infection (4.80%) (Farhana *et al.*, 2009).

Diagnosis of hepatitis C is based on serological assays (Zeuzem *et al.*, 2000) and HCV RNA detection (Simmonds *et al.*, 1994). For screening and epidemiological surveillance enzyme-linked Immunosorbant assay (ELISA) (Davis and Lau, 1997) and a confirmatory recombinant Immunoblot assays are initially used which detect HCV-specific antibodies (anti-HCV) (Reed and Rice, 2000). Qualitative polymerase chain reaction (PCR) is used to find out the presence of the viral genome in order to confirm active infection (Siddiqi *et al.*, 2002).

The purpose of the present study was to determine the prevalence of HCV genotype in the general population of the Peshawar district Khyber Pakhtunkhwa (KP), Pakistan.

Materials and methods

Study population

The experimental design is a prospective study and it included individuals from the general population of Peshawar, Khyber Pakhtunkhwa province, Pakistan.

Blood sampling

Informed consent was taken from the patients. After initial information, 3 cc of blood sample was collected from each individual in a disposable syringe. Serum was extracted and dispensed in sterile tubes and was transported to laboratory of KUST, Kohat University of Science and Technology for analysis.

Lab procedure and tests

Immune-chromatographic tests (ICT)

The collected samples were screened by using ICT test through a test device (Acucheck USA). 10µL serum was applied on to the sample well and two drops of sample diluents was added immediately.

The mixture was allowed to migrate along the test strip. After 15 minutes two distinct lines appeared, one line on the control (C) region and another on test region (T) indicates positive results. One line on control (C) region with no other line in the test (T) region was taken as negative result, while control line which failed to appear was considered invalid result, so negative samples did not produce a test line. Anti-HCV antibodies positive blood samples were used for further analysis.

HCV RNA Detection

RNA extraction

The HCV RNA was extracted by using the protocol of Favor Prep viral nucleic acid kit (Favorgen Taiwan).

Qualitative RNA Detection

Reverse Transcription PCR

After extraction of RNA, cDNA was synthesized from 10µL of extracted RNA with 200U/µL of molony murine leukemia virus reverse transcriptase (MMuLV RTase) (Fermentas Germany). The reverse transcription was carried out in thermal cycler (NyxTechnik USA) for about 35 minutes at 42℃. The mixture for the preparation of cDNA for a single reaction containing the following parameters:

Regular PCR for HCV (5 UTR)

After amplification of cDNA, the next step was preceded by using a sense and antisense primer specific for 5` UTR in thermal cycler (NyxTechnik USA). The reaction mixture for a single reaction consisted of:

Nested PCR (5 UTR)

The nested PCR was carried out with the same condition as the regular PCR, but in this case inner primers C3 and C4 were used. Apart from this, annealing temperature used which was 62℃ for the inner primers. The reaction mixture for a single reaction consisted of:

Gel Electrophoresis

Analysis of PCR products were carried out by using 2% Agarose gel prepared in 0.5% TBE buffer (boiled for 2 minutes in a microwave oven and cooled up to 50℃), ethidium bromide (10 µg/ml) was added and evaluated under UV light. DNA ladder of 50 bp (Fermentas USA) was used as DNA size marker.

HCV Genotyping

Reverse Transcription PCR

After extraction of RNA, cDNA was synthesized from 10µL of extracted RNA with 200U of molony murine leukemia virus reverse transcriptase (MMuLV RTase) (Fermentas Germany). The reverse transcription was carried out in thermal cycler (NyxTechnik USA) for about 35 minutes at 42℃. The mixture for the preparation of cDNA for a single reaction containing the following parameters:

Regular PCR (core region)

After synthesis of cDNA, the next step was preceded by PCR amplification by using two primers (one sense and one antisense) that is specific for core region of HCV in a polymerase chain reaction (Nyxtech Inc. USA). PCR mix proportions:

Multiplex PCR (Core region)

Genotype with type specific primers for the core region of HCV genome was performed for the nine most common subtype and types of HCV (1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a). For determination of the different HCV genotypes amplified product, the type specific PCR mix was divided into mix A and mix B. For mix B all the reagents were the same except antisense primers which were replaced by the following:

Gel Electrophoresis

Analysis of PCR products were carried out by using 2% Agarose gel prepared in 0.5% TBE buffer (boiled for 2 minutes in a microwave oven and cooled up to 50℃), ethidium bromide (10 µg/ml) was added and evaluated under UV light. DNA ladder of 50bp (Fermentas USA) was used as DNA size marker. HCV genotypes were determined by comparing the amplified product of specific genotypes mention below:

Results

A total of 154 samples were collected from the general population of district Peshawar, of which 30 (19.48%) were found positive for the anti-HCV (Table 8).

Gender wise out of 154 samples (14.2%) female were found to be positive for the anti HCV while 23.80% male were found to be HCV positive (Table 9).

Age wise all the subjects were distributed into three groups according to their age ranges (Table 10).

Table 1. Mixture for the preparation of cDNA for a single reaction.

Items	5X FS buffer	dNTPs (10mM)	Anti-sense primer (10pM)	Distilled water (DEPC treated)	RTase (200U/µL)	Extracted RNA
Amount	4.0µL	2.0µL	1.0µL	2.5µL	0.5µL	10.0µL

Table 2. Reaction mixture for a single reaction for regular PCR.

Items	10X PCR/*Taq* buffer	MgCl2 (25mM)	dNTPs (500µM)	Primer-1 (10pM)	Primer-2 (10pM)	dH_2O	*Taq* DNA polymerase (5Uµ/µL)	cDNA (RT-PCR)
Amount	2.0µL	2.4µL	1.0µL	1.0µL	1.0µL	8.1µL	0.5µL	4.0µL

Group 1st: It was based on the age range from ≥ 20 years in which the percentage of the anti HCV was 9.52%.

Group 2nd: It was range from 21-40 years. Anti-HCV prevalence percentage was 25%.

Group 3rd and 4th: They were based on the age greater than or equal to 41 (≤ 41). The anti HCV percentage was 5.88%.

When this anti-HCV was tested with the PCR, than only 15 samples were HCV RNA positive (Table 11).

Table 3. Reaction mixture for a single reaction for Nested PCR.

Items	10X PCR/*Taq buffer*	$MgCl_2$	dNTPs (500µM)	Inner Sense primer (10pM)	Inner Antisense primer (10pM)	Distilled water	*Taq* DNA	Regular- PCR product
Amount	2.0µL	2.4µL	1.0µL	1.0µL	1.0µL	8.2µL	0.5µL	4.0µL

Table 4. Mixture for the preparation of cDNA for a single reaction for Reverse Transcription PCR.

Items	5X FS buffer	dNTPs (10mM)	Anti-sense primer (10pM)	Distilled water (DEPC treated)	RTase (200U/µL)	Extracted RNA
Products	4.0µL	2.0µL	1.0µL	2.5µL	0.5µL	10.0µL

The HCV RNA positive subjects were taken further for the HCV genotyping (Table 12). Among which 2a was found to be 18.75%, 3a was 12.5% and 3b was 31.25% and some of the samples were positive for both 3a and 3b so having combination of these two and percentage of both were 25%. The 12.5% of the samples were unidentified which may be some subtype of n +qHCV genotype.

Table 5. Regular PCR Mixture for Core region.

Items	10X buffer	MgCl2 (25mM)	dNTPs (500M)	Outer Primer-1 (10pM)	Outer Primer-2 (10pM)	dH_2O	*Taq* DNA polymerase	cDNA
Amount	2.0µL	2.4µL	1.0µL	1.0µL	1.0µL	8.2µL	0.5µL	4.0µL

Discussion

In Peshawar, the city of Khyber Pakhtunkhwa (KP), Pakistan, we investigate the active infection of the HCV by applying the standard protocol for the HCV screening and the detection of the HCV RNA in the patient serum. My study established the anti HCV to be 19.48%. Some other study in Peshawar established the seroprevalence of HCV to 0.89% (Khan *et al.*, 2011), 2.2% (Ahmed *et al.*, 2004) and 1.57% (Mukhtar *et al.*, 2008) which is lower than my study. The reason for that was the total sampling and the environment they selected was hygienic environment. But this does not truly indicate active infection as individual anti-HCV therapy would also have antibodies in their serum and would make them positive, if detected by ELISA or ICT.

Table 6. Multiplex PCR mixture for Core region.

Mix A reagents		Mix B reagents	
Items	Amount	Items	Amount
10x buffer	2.0µL	10x buffer	2.0µL
$MgCl_2$ (25Mm)	2.4µL	$MgCl_2$ (25Mm)	2.4µL
dNTPs (500Mm)	1.0µL	dNTPs (500Mm)	1.0µL
Sense primer 10pM	1.0µL	Sense primer 10pM	1.0µL
Antisense primer (1b) (10pM)	1.0µL	Antisense primer (1a) (10pM)	1.0 µL
Antisense primer (2a) (10pM)	1.0µL	Antisense primer (3a) (10pM)	1.0 µL
Antisense primer (2b) (10pM)	1.0µL	Antisense primer (4) (10pM)	1.0 µL
Antisense primer (2a) (10pM)	1.0µL	Antisense primer (5a) (10pM)	1.0 µL
Antisense primer (3b) (10pM)	1.0µL	Antisense primer (6a) (10pM)	1.0 µL
dH_2O	4.1µL	dH_2O	4.1µL
Taq DNA	0.5µL	Taq DNA	0.5µL
PCR product	4.0µL	PCR product	4.0µL

Table 7. HCV Genotypes (Mix A and Mix B). Mix 'A' product Genotypes.

Genotypes	1b	2a	2b	2a	3b
Product	230bp	139bp	340bp	190bp	176bp
Mix 'B' product Genotypes					
Genotypes	1a	3a	4	5a	6a
Product	208bp	232bp	99bp	320bp	341bp

Similarly the disease is self-limiting and asymptomatic in some individuals rendering them positive for Anti-HCV antibodies.

In the current study seroprevalence of hepatitis C is relatively higher than in other studies done in Pakistan. Investigators have reported seroprevalence rates of 5.14% from Islamabad (Asif *et al.*, 2004), 6.21% from Rawalpindi (Khattak *et al.*, 2002), 2.89% to 4.97% from Lahore (Alam and Ahmad, 2001), 6.8% from Karachi (Ahmad, 2001) and 1.87% from healthy blood donors screened in Quetta (Ali *et al.*, 2003).

Table 8. Prevalence of HCV in the general population of Peshawar.

HCV	No. of the subjects	Percentage
Positive	30	19.48%
Negative	124	80.5%
Total	154	100%

Table 9. Gender wise percentage of Anti-HCV.

Gender	No. of individual	Anti-HCV	Percentage
Male	84	20	23.80%
Female	70	10	14.2%

In this study the individuals ≤ 40 years (28.0%) were more affected with HCV as compared to older age group (> 40 years, 5.88%). From Lahore the same result was obtained of high prevalence in the age of ≤ 40 years (68.3%) (Ahmad *et al.*, 2009). The reason of high percentage might be that they are more expose to HCV risk factor i.e. barber shop, reuse of syringes etc.

Table 10. Age wise distribution of the anti-HCV in the general population.

Age group (in years)	No. of the subjects	No. of HCV positive subjects	Percentage
≤ 20	42	4	9.52
21-40	89	25	28.0
≥ 41	17	1	5.88

The prevalence of anti-HCV in this study was found to be higher in male (23.80%) as compared to female (14.2%). Low prevalence of anti-HCV and active HCV infection among the females could be attributed to the minimum exposure of female population to various risk factors for HCV infection such as barbers, tattooing, drug usage etc. and also the estrogen hormone in females is considered to play a role in the spontaneous clearance of HCV infection (Alter, 2007).

Table 11. Percentage of HCV RNA positive subjects and anti-HCV positive.

HCV	NO. of the subjects	Percentage
Anti-HCV positive	14	46%
RNA positive	16	54%
Total	30	100%

The study shows a greater frequency of 3b (31.25%) and a lower distribution of 3a as compared to the previous studies. However this difference is non-significant and can be regarded as consistent with all studies conducted previously.

The 3b was followed by the co infection 3a/3b (25%) and then 2a (18.75%) and at last the 3a and unidentified genotypes (12.5%). In 1997 it was reported in a small study that 87% of the individuals in Pakistan had genotype 3 (Azam *et al.*, 2007).

In 2004 a panel of 30 top gasteroenterologists of the country at a conference reported that 75-90% of HCV patients in Pakistan had genotype 3a (Umar *et al.*, 2010).

Table 12. Genotype prevalence percentage.

Genotypes	Percentage	Total
2a	18.75%	3/16
3a	12.5%	2/16
3b	31.25%	5/16
Mix 3a and 3b	25%	4/16
Unidentified	12.5%	2/16

In 2007 it was reported that 81% of individuals had genotype 3, while only 9.5% had genotype 1 (Ahmad *et al.*, 2007).

The most detailed study was conducted in 2008 (Idress and Riazuddin, 2008) which performed genotyping of 3351 patients and reported that genotype 3a was the most prevalent genotype in Pakistan.

Conclusion

In the present study, it is concluded that HCV genotype 3b is the most prevalent genotype circulating in this region of the world. Regional difference do exists in HCV genotypes. Majority of the infected patients are young ages between 31-40 years. Male having higher frequency of HCV infection than female, due to more exposer of male to the risk factor of HCV than female. More care is recommended

during blood transfusion, body fluids and use sterile equipment's. Also to educate the local community about the prevalence of HCV.

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