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ORIGINAL ARTICLE

Genotype Base Prevalence of Hepatitis C

FAWAD MAHMOOD[1], SUMERA KAZMI[2], HIRA WAKIL[3], KHALIDA KOUSAR[4], AAMER ALI KHATTAK[5], ZAHID ULLAH KHAN[6]
[1]*Lecturer, Department of Medical Laboratory Technology, Riphah International University, Islamabad, Pakistan.*
[2]*Associate Professor, Department of Medicine, Abbottabad International Medical Institute, Abbottabad, Pakistan.*
[3]*Trainee Medical Officer, Department of Ophthalmology, Hayatabad Medical Complex, Peshawar, Pakistan.*
[4]*Assisant Professor of Microbiology, Department of Pathology, Bacha Khan Medical College, Mardan, Pakistan.*
[5]*Associate Professor, Department of Medical Laboratory Technology, University of Haripur, Haripur, Pakistan.*
[6]*Assistant Professor of Microbiology, Department of Pathology, Khyber Girls Medical College, Peshawar, Pakistan.*
Corresponding author: Zahid Ullah Khan, Email: drzahidullah1978@gmail.com

ABSTRACT

Hepatitis C virus (HCV) has a positive sense single-stranded enveloped RNA virus. HCV is one of the most common blood-borne diseases causing significant morbidity and mortality globally. HCV causes acute and chronic hepatitis which can eventually lead to permanent liver damage, hepatocellular carcinoma and death. World Health Organization reported that 3 % of the world's population is suffering from HCV infection that is about 170 million people. The prevalence of chronic HCV infections in Pakistan is about 5%, with very common individuals being infected with HCV genotype 3a prevalence is in the range of 4.1 to 36% reported from various parts of Khyber Pakhtunkhwa Province of Pakistan. This study was carried out in District Mardan Khyber Pakhtunkhwa. A total 1500 HCV suspected individuals visited District Headquarter hospital Mardan during September 2014 to April 2015 were enrolled. HCV antibodies were detected in about 230 patient serums during this screening. Out of these 230 patients 217 patient were further confirmed by qualitative Real Time PCR. All 217 samples were further analyzed for HCV genotyping by PCR based molecular technique. Genotyping results showed that HCV genotype 3a was found in 156 (72%) of the HCV positive isolates, 2a genotype in 22 (10%) and 3b genotype 17 (8%) and remaining 10% were mixed genotype. HCV genotype genotype 3a was declared as the most prevalent genotype with same in both genders that is 72 %. Genotypes 2a, 2b and 3b were more prevalent among male patients while mixed genotype was mostly observed in female infected individuals. Moreover majority of the infected population were form the age group 41-50 year. In 21-30 years age group the most prevalent genotype 3a after than following in competition 3b, in 31-40 years age group 2a 5 (7%), in years age group 41-50 2a 8 (9%) more prevalent.

Keywords: Hepatitis C, Genotypes, Pakistan

INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989 through molecular techniques. HCV is very common chronic blood borne infection[1]. HCV is an enveloped positive-strand RNA virus. It related to the family Flaviviridae and genus hepacivirus. The size of viron is about 55–65 nm in diameter[2]. Each year approximately 3-4 million persons are newly infected with HCV and 170 million people are chronically infected. Furthermore 350,000 deaths occur every year due to HCV related causes[3]. HCV does not any direct cytopathic effect on host. HCV develop 20 % of cases of acute and 70 % of cases of chronically hepatitis in worldwide[4].

It is reported that 20 % of infected individuals produced cirrhosis over a span of 10-30 years. The progression of fibrosis can lead to increases morbidity and mortality in chronic HVC patients due to complications caused by cirrhosis or hepatic-cellular-carcinoma of HCV which can even lead to death[5]. HCV showed worldwide great genetic heterogeneity to increase in multiple strains. HCV is globally prevalent pathogen show high genetic variability HCV divide six major genotype and multiple subtype i.e. heterogeneous distribution of HCV worldwide[2].

HCV dominance in Khyber Pakhtunkhwa province of Pakistan is reported to be an the range of 4.1-36% in different area[2]. The prevalence of HCV in district Buner Khyber Pakhtunkhwa was 4.57%, in Abbottabad it was recorded 3.12%, while in Peshawar its prevalence 1.26%[6]. According to the Pakistan Medical and Research Council Islamabad, reported prevalence of HCV in Khyber Pakhtunkhwa; Hango 6.4%, Bannu 1.5%, Swabi 0.7%, Mansehra 0.6%, Kohat 0.6%, Mardan 2.1% and most common genotype is 3a[2].

Molecular techniques play a very vital role in detecting and prognosis of treatment for HCV as by these techniques we can exactly count viral loads before and after treatment, as Culturing of HCV is very hard. So in molecular diagnostics it is one of the first pathogens to be identified by purely molecular methods[7].

The treatment of acute Hepatitis is very limited, because the acute phase is very rarely diagnosed. In past, the golden standard treatment was interferon alone or combination with Ribavirin (RBV). INF is basically glycoprotein and Alpha interferon showed good response against HCV infection. INF therapy induces cytokine response[8]. INF and RBV became a gold standard treatment but new treatments like Peg-Interferons. This therapy shows approximately 50 % response against genotype 1 and 80 % for genotype 2 and 3[9]. The current standard therapy has change to pegylated interferon a (Peg-IFN-a) and RBV. This therapy is useful for the infected individuals which is suffering from HCV genotypes 2 and 3 achieving rate approximately 75 % to 90 %. Endoscopies are also recommended in chronic cases. Herbal treatment is not effective against HCV. There is still no effective vaccine against virus[9].

Globally prevalent HCV pathogen show high genetic variability as it has six major genotype and multiple subtypes. Genotypes 1, 2 and 3 are worldwide prevalent[10] in South Africa, Egypt and Southeast Asia have only genotypes 4, 5 and 6 while rest of the world is endemic with genotypes 1-3[2]. Genotype 2 of HCV is predominant in Western Africa while the Central Africa, Congo, Cameroon and Gabon are endemic by genotypes 1 and 4. HCV subtype 1a, 1b subtypes are the most dominant genotypes circulating in Europe and America[11]. HCV Genotype 3 and 6 are predominately found in the Indian sub-continent and South East Asia[12]. China is endemic with genotype 4 of HCV[13]. In Pakistan the most predominant genotype is 3a and this genotype is also most common in different area of Khyber Pakhtunkhwa[14, 15]. The aim of this study was to determine the prevalence of HCV in general pollution and to find different HCV genotypes of District Mardan.

MATERIAL AND METHODS

This study was carried out in district Mardan and patients visiting to District Headquarter (DHQ) hospital Mardan for Hepatitis screening were enrolled for this study after taking informed consent. A total 1500 HCV suspected individuals visited DHQ hospital Mardan form September, 2014 to April, 2015. They were tested for HCV specific antibodies using available (SD BIOLINE HCV test) devices coated with recombinant HCV antigen to detected anti-HCV antibodies in patient's serum[2]. Informed consent was taken from each enrolled patient and in case of child and mental disorder consent was obtained from parents /guardian. This study proposal was approved by department of Medical Lab Technology and DHQ Ethical

Committee, after complete discussion with pathologist and concern lab staff.

A total of 1500 patients visited DHQ hospital Mardan for HCV screening form September, 2014 to April, 2015 regardless of age and genders were included. All patients negative for HCV by Immunochromatographic technique (ICT) screening test and those not willing to be the part of this study was excluded in this study. About 5 mL of whole blood was collected aseptically in gel tubes (BD Vacutainer®) aseptically by venepuncture technique after taking proper informed consent from the patient. Serum was separated after proper clotting of blood by centrifugation at 3000 rpm for 5 min within one hour of collection and serum was stored at -20 ^{o}C for further analysis. HCV positive confirmed by qualitative PCR were genotype on HCV 1/2/3 Real-TM thermalcycler by using (Space biotechnology Italy) genotyping PCR kit.

Serum sample of patients with hepatitis screening test request form was applied on HCV serological screening device (SD BIOLINE anti-HCV ICT devices rum/plasma/whole blood sample). The test pouch was opened and the patient's name on the test device was written. Micropipette was used to collect sample 10 µL. Sample was poured into the sample well ("S" marked). Then 4 drops of assay diluents were added into the sample well. After 5-20 minutes result was noted. Reading too late can give false results. The presence of two-color bands ("T" band and "C" band) within the result window, no matter which band appears first, indicates a positive result. The presence of only one band ("C" band) within the result window indicates a negative result. No "C" line in the result window should be retested using a new test kit[16].

Anti-HCV positive samples by ICT method were reconfirmed by Qualitative PCR. RNA was extracted from the serum sample and following protocol was followed. RTA Viral Nucleic Acid Isolation Kit (was used for viral RNA extraction from clinical followed samples. Followed the manufacturer's instructions as stated in the kit manual were followed[17]. At third step HCV positive confirmed by qualitative PCR were genotype by using HCV 1/2/3 Real-TM. Genotyping PCR kit (Space biotechnology Italy).

To determine the upper limit, a dilution series of Acro metrix Opti Quant-S HCV Quantification Panel (Cat. No: 950350) ranging from 1 x 10^3 IU/mL to 1 x 10^6 IU/mL were prepared. Starting sample volumes were 500 µL and elution. High concentration samples was made by taking 50 µL and (1 x 10^7, 1 x 10^8 and 1 x 10^9 IU/mL) was prepared by using calibrated in vitro transcribed RNA bearing HCV quantification standard. Within this range, the relationship between log of target RNA and Ct values is linear.For INCEPTRA Cycler, Ct value = -3.29(log of target RNA) + 43.07; with a correlation coefficient (R^2) of 0.995. Upper limit is at least 1 x 10^9 IU/mL for INCEPTRA Cycler. Lower limit was calculated by profit analysis done by PASW Statistics 18 program according to the quantification results of HCV Analytical Sensitivity Studies. 95 % lower confidence limit was 9.5 IU/mL for INCEPTRA.

HCV genotyping was done by using HCV 1/2/3 Real-TM thermal cycler by using genotyping PCR kit.

Table 1: Programming of Thermal Cycler

Program Name	Cycles	Program for Smart, Cycler
cDNA Synthesis	1	30 minutes for 45 ^{o}C
Hot Start	1	95 ^{o}C for 10 minutes
Amplification	45	95 ^{o}C for 30 seconds
Cooling	1	40 ^{o}C for 30 seconds

Table 2: Shows typical Ct values of Quantification Standards for RT— PCR system.

Instrument	Quantification Standard 1 (107 IU/ml	Quantification Standard2 (106 IU/ml)	Quantification Standard 3 (105 IU/ml)	Quantification Standard 4 (104 IU/ml)
Light Cycler 1.5/2.0	21.00±2	24.00±2	27.20±2	30.20±2

Following results was interpreted: Concentration of the Original Sample (IU/mL)

Concen from Software (IU/mL) xElution (µL)volume
Original Sample Volume (µL)

If no signal was detected in FAM channel (Ct≥40) and a signal was detected in HEX channel (Ct= 33 ± 5) it is a result.

Table 3: Programming of Thermal Cycle

NO	Temperature,^{o}C	Step duration	Fluorescence measurement	Round repeats
1	50	30 min	_	1
2	95	15 min	_	_
3	95	5 s	_	5
	60	20 s	_	
	72	15 s	_	
4	95	5 s	_	40
	60	30 s	FAM, HEX/JOE/Cy3, ROX/Texas Red, Cy5	
	72	15 s	_	

RESULTS

A total 1500 HCV suspected individuals visited DHQ hospital Mardan form September, 2014 to April, 2015. The patients referred by clinicians for HCV screening, were tested for anti-HCV antibodies by available RDT devices coated with recombinant HCV antigen to detected anti-HCV antibodies. About 230 patients were positive for HCV by ICT methods. These 230 samples were further confirmed for HCV RNA by qualitative PCR out of which 217 patients were positive results, which mean HCV prevalence is about 14 % (230/1500) in Mardan. These 217 HCV positive serum samples were subjected to HCV genotypes by RT- PCR.

In these 217 individuals, female population revealed 117 (53.9 %) of HCV prevalence whereas in male 100 (46.1 %) in Mardan, Khyber Pakhtunkhwa. HCV is more prevalent in female individuals as compare to male patients as depicted in Table 3.1. In this study, we are divided HCV RNA positive infected individuals according to their age year's groups in four main categories. The high prevalence was observed in 41-50 years age group 84 (38 %) and followed by in 31-40 years age group total 65 (30 %), in 21-30 years age group total 30 (13 %), in 51-60 years age group total 31(14 %) 51-60 and above 60 years age group 6 (2 %) as shown in Tables 3.2.

In our study we found that the most prevalent HCV genotype is 3a which was observed in 156 (72 %) of the HCV infected individuals. Followed by genotypes 2a

Table 4: Gender wise prevalence of HCV RNA positive patients by PCR (n= 217).

Gender	Positive	Percentages
Male	100	46 %
Female	117	56 %

Table 5: Age Wise Prevalence of HCV (n= 217).

	Genotypes								
Age Group (Years)	1a	1b	2a	2b	3a	3b	Mixed	Total	Percentage
21 – 30	1	0	1	0	22	6	1	30	13%
31 – 40	1	1	1	5	53	3	3	65	30%
41 -50	0	0	8	5	66	5	0	84	38%
51 -60	0	0	2	2	27	0	0	31	14%
61 - >	0	0	0	0	5	0	1	6	2%

22 (10 %) and 3b 17 (8 %), numbers and percentages of HCV genotypes was distinctly showed in Table 3.3. Genotype 3a was significantly more prevalent than 1a, 3b and 4 in district Mardan. Gender wise distribution of HCV genotype was shown Table .3.3. In this study, we have also compared the prevalence of HCV genotype according gender wise. Distribution of HCV genotype genotypes 3a was found to be the most prevalent genotype 72 (72 %) male and female 85 (72 %) positive and 2a, 2b and 3b was more prevalent among male patients while mixed genotype was mostly observed in female infected individuals, these variation of HCV genotype among male and female was distinctly shown in Table 3.4.

Predominance of HCV genotype according to different years age was observed the genotype 3a was predominant in all years age group, after their dominance the most prevalent genotype are following.

In age group of 21-30 years, after 3a genotype the second most prevalent genotype was 3b, whereas in 31-40 years of age group genotype 2b was 5 (7 %) while mixed genotype and 3b was of same ratio in both groups. While genotype 2a was 8 (9 %) prevalent among 41-50 years of group age. Genotype 3a was predominant in all year's age groups. All the detailed was shown Figure 3.2.

Table 6: Numbers and Percentages of HCV genotype (n= 217)

Genotypes	Number	Percentage
1a	2	1%
1b	1	0.5%
2a	22	10%
2b	12	5.5%
3a	156	72%
3b	17	8%
Mixed	4	2%
Untypable	3	1%
Total	217	100%

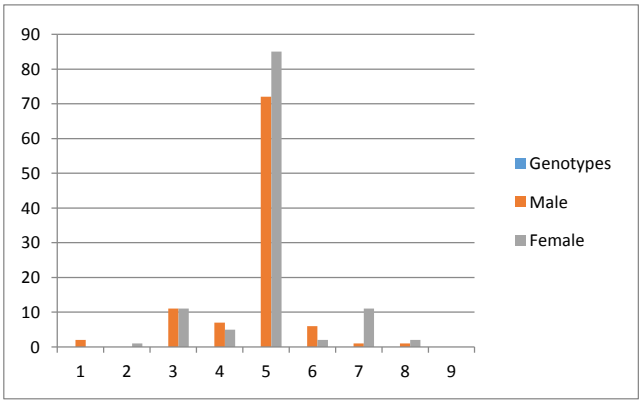

Figure 1: Gender wise distribution of HCV genotypes (n= 217).

DISCUSSION

Hepatitis C infection has a major disease burden on the global public health. HCV has significant role in the causation of chronic viral hepatitis[18]. HCV induces acute and chronic hepatitis which can finally lead to permanent hepatic damage, fibrosis, HCC and in severe case death as many deaths has been reported[8]. Each year about 0.35 million people die as a result of HCV infection[19]. It has been estimated that about 27 % of cirrhosis and 25 % of HCC cases worldwide is due to HCV infection[20].

The present study was aimed at determining genotype base prevalence of HCV in district Mardan of Khyber Pakhtunkhwa, Pakistan. Total 1500 HCV suspected individuals visited DHQ hospital Mardan form September, 2014 to April, 2015 for screening were test serologically. After serological based ICT test method 230 patients were declared anti-HCV positive which means that about 15 % (230/1500) of district Mardan's population. Another study carried out during 2011 in Mardan reported 12 % of HCV antibodies in this area[21]. But this prevalence is much higher (4.6 %) as compared to study conducted in district Buner[22]. In our study HCV genotype 3a bas was mostly observed in infected individuals (156/ 217 = 72 %), followed by genotypes 2a (22/ 217 = 10 %) and 3b (17/ 217 = (8 %). The diversity of HCV genotypes varies substantially around the countries. Prevalence of HCV genotype 3a (72 %) in this study while genotype 1 is most prevalent genotype of HCV worldwide. West Africa is endemic with genotype 1 and 2, 3 in South Asia, 4 in Central Africa and the Middle East, 5 in Southern Africa, 6 in South East Asia nwhile in India the predominant HCV genotype is 3a[14, 23].

Our study results are also in line with other study in which it was reported that HCV genotypes 3a was the predominant genotype (39 %) followed by genotype 2a (25 %) and no regional difference was observed in provinces of Pakistan[15]. Few other studies in Khyber Pakhtunkhwa such as a study conducted in Northern areas of Pakistan revealed 3a (66 %) genotype of HCV and followed by 2a (7.5 %). According to Ali et al. (2010) in Khyber Pakhtunkhwa HCV genotype 3a is about 80 % and 3b 6 %[14]. Another study carried out in Mardan has reported that in 90 % of cases of HCV infection the genotype was 3a, whereas 1a was 5.6 %, 3b 0.6% and genotype 4 was 0.6 %[2]. Similarly another study in Mardan genotypes 1a (11 %,) 2a (13 %), 7 %), and most prevalent was 3a[21]. When a comparative study was made among different cities and districts of Pakistan including district Mardan genotype 3a was found to be the most common genotype.

In this study, out of 217 HCV positive patients by qualitative PCR, females infectivity rate are higher (117/ 217 = 54 %) as compare to male (100/ 217 = 46 %) this gender wise HCV distribution was different from the results reported by another study conducted in Southern part of Khyber Pakhtunkhwa where they revealed that 54 % of males were HCV infected and 46 % female[6]. But this study was supported by a previous study conducted in Mardan which also reported that HCV is prevalent in females as compared to males[2]. It will be interesting to find out the factors involved in this gender specification.

The prevalence of different HCV genotypes was also Stratified according to the gender wise HCV genotype 3a most prevalent genotype in both genders (72/ 100 = 72 %) and female (85/117 = 72 %). Genotype 2a in males was about 11 % and female 9 %, 2b 7(7 %) while 3b (6 %) was more prevalent among male patients. However mixed genotype was observed in (9 %) of genotype was mostly predominant in female infected individuals. The results of this study were comparable with a previous study on the global distribution and prevalence of HCV genotypes. It was observed that the predominant genotypes in males were 1b, 1c, 3a, 3b, 5a and 6a versus 1a, 2a, 2b and 4 in females[15]. Our result were supported by in a study in Mardan that the genotype 3a was found to infected males more frequently followed by 3b and 2a, respectively whereas genotypes 2b and 1a were found to be the major causes of infections in females[14].

Another study in Mardan, genotype 3a males (59%), female 3a (63%), in male 3b (6%), in females (7%) and 5% in male are mixed genotype and 7% in female[24]. Another study reported carried out in Mardan found genotype 3a about 59 % among males and 58 % in females 2a (3%) in male while in female (2 %) and a mixed genotype in male (6 %) while in female (8 %)[15].

According to their age wise divided into four main categories. The high prevalence was observed in 41-50 years age group 84 (38 %) and followed by in 31-40 years age group total 65(30 %), in 21-30 years age group total 30 (13%), in 51-60 years age group total 31 (14%) 51-60 and above 60 years age group 6 (2 %). In 21-30 years age group the most prevalent genotype 3b, in 31-40 years age group 2a 5 (7 %), mixed genotype and 2b was a same ratio 41-50 group age. A previous study accounted that genotype 1a was found more usually in younger, while 1b, 2a and 2b were more commonly found in older patient[15]. In Mardan, the male were more affected in age group 31-40 years while female 41-50 years and genotype 3a was predominant in all ages[2] (Afridi et al., 2013). Furthermore another study in Mardan reported genotype 3a (58.2 %) is dominant, in years age group 9.2%[24].

CONCLUSION

In the current study we concluded that HCV genotype 3a was found in 156 (72 %), followed by genotypes 2a 22 (10 %) and 3b 17 (8 %). HCV genotype genotypes 3a was found to be the most prevalent genotype 72 (72 %) male and female 85 (72 %) positive and 2a, 2b and 3b was more prevalent among male patients while mixed genotype was mostly observed in female infected individuals. Moreover majority of the infected population belong age years group 41-50 years age group. In 21-30 years age group the most prevalent genotype 3a after than following in competition 3b, in 31-40 years age group 2a 5 (7 %), in years age group 41-50 2a 8

(9 %) more prevalent. Our study will help to strengthen and revise preventive as well as therapeutic strategies throughout the country.

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