

## RESEARCH ARTICLE

# Genotypes of Hepatitis C Virus in Relapsed and Non-respondent Patients and their Response to Anti-Viral Therapy in District Mardan, Khyber Pakhtunkhawa, Pakistan

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### Abstract

Hepatitis C is a blood-borne infectious disease of liver, caused by a small enveloped, positive-single stranded RNA virus, called the hepatitis C virus (HCV). HCV belongs to the Flaviviridae family and has 6 genotypes and more than 100 subtypes. It is estimated that 185 million people are infected with HCV worldwide and 5% of these are in Pakistan. The study was designed to evaluate different genotypes of HCV circulating in District Mardan and to know about the behavior of these genotypes to different anti-viral regimes. In this study 3,800 patients were exposed to interferon alfa-2a plus Ribavirin treatment for 6-months and subjected to real-time PCR to check the viral response. Among these 3,677 (97%) patients showed no detectable HCV RNA while 123 (3%) patients (non-responders) remained positive for HCV RNA. Genotypes of their analyzed showed that most of them belonged to the 3a genotype. Non-responders (123) and relapsed (5) patients were subjected to PEG-interferon and Ribavirin therapy for next 6 months, which resulted into elimination of HCV RNA from 110 patients. The genotypes of the persisting resistant samples to anti-viral treatment were 3b, 2a, 1a and 1b. Furthermore, viral RNA from 6 patients remained un-typed while 4 patients showed mixed infections. HCV was found more resistant to antiviral therapy in females as compared to mals. The age group 36-45 in both females and males was found most affected by infection. In general 3a is the most prevalent genotype circulating in district Mardan and the best anti-viral therapy is PEG-interferon plus Ribavirin but it is common practice that due to the high cost patients receive interferon alfa-2a plus Ribavirin with consequent resistance in 3% patients given this treatment regime.

**Keywords:** HCV infection - anti-viral treatment - interferon - Ribavirin - resistance - PEG-interferon - Pakistan

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### Introduction

Hepatitis C is a blood-borne infectious disease of liver, caused by Hepatitis C virus (Frank et al., 2000). Hepatitis C infection causes acute and chronic hepatitis (Kanda et al., 2013). When a person first infected with hepatitis C virus, it can develop an acute infection, which may be a very mild illness with few or no symptoms or may be a very serious condition, in which the patient might be hospitalized. Acute hepatitis C is a short-term illness which occurs within the first six months after the infection with hepatitis C virus. Hepatitis C virus (HCV) is a hepacivirus of Flaviviridae family (Alter et al., 1992). It is a small (5nm in size), enveloped, positive-single stranded RNA virus (Arshad et al., 2012). Its genome is 9.6 kb in size, encodes a poly protein (about 3,000 amino acid) which is processed and release 3 structural ( core, E1, E2) and 7 nonstructural (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B ) proteins (Lin et al., 1994; Reed et al.,

2000). HCV has six genotypes (1-6) and more than 100 subtypes (Bowden et al., 2006). This extensive genetic diversity is major problem for vaccine design (Friedrich et al., 2004).

HCV infection is a serious and one of the most important global health problems (Alter et al., 2007). It is estimated that 185 million people are infected with hepatitis C virus (HCV) worldwide. Prevalence is higher in some countries as 22% in Egypt, 2 to 5 million in Europe (Rockstroh et al., 2012), 4 million in United States (Shev et al., 1995). The prevalence of hepatitis C in Pakistan is 5% (Afridi et al., 2013) and Khyber PakhtunKhwā (KPK) is 5-9% (Khan et al., 2004), contributing to liver cancer (Munaf et al., 2014). Each year 3 to 4 million new patient of hepatitis C is diagnosed (Butt, 2005; Koziel et al., 2007) and approximately 350,000 people infected with HCV are die every year. Most people are unaware of their HCV infection. Some persons (20 to 40%) with acute HCV infection cleared spontaneously and 40 to

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60% lead to chronic complication e.g. fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Levrero, 2006; Tang, 2009).

The analysis of HCV infection is carried out by detecting the anti-HCV antibodies using Immunochromatographic test (ICT) method (Afridi et al., 2009) and Enzyme Linked ImmunoSorbant Assay (ELISA). The Polymerase Chain Reaction (PCR) is the next main step which is used for the diagnosis of HCV infection (Gretch, 1997; Iancu, 2001; Castillo et al., 2010). The Real Time PCR is more reliable and more specific (more than 99.5%) than the other methods (Michelin et al., 2007; Sabato et al., 2007).

Anti HCV therapy is available and quite successful. Once weekly subcutaneous injection of Pegylated interferon alpha (peg interferon alpha-2a or peg interferon alpha-2b) plus daily ribavirin (RBV) for 24 or 48 weeks, are used for the treatment of chronic hepatitis infection in most of the world (Ghany et al., 2009) and remained unchanged for the last ten years (Fried et al., 2002). The primary goal of interferon therapies are the achievement of sustained virological response (SVR), which is undetectable viral load in six months after the end of therapy (Martinot-Peignoux et al., 2010). Patient infected with genotype 2 and or 3 provides more favorable response to the treatment with peg interferon and ribavirin than those infected with genotype 1 and or 4 (Munir et al., 2010; Mangia, 2012).

Pegylated interferon is expensive, therefore in under developed and developing countries including Pakistan, most of the patient try herbal medicine e.g. laccase (extracted from oyster mushroom), proanthocyanidin (extracted from blue berry leaves) and Rhodiola kirilowii, but there is no single significant report documented (Zuo et al., 2007; Ahmad et al., 2013). Due to high cost of pegylated interferon and ribavirin in Pakistan it is preferred to use Interferon alfa-2a plus ribavirin therapy. Pegylated interferon plus Ribavirin therapy is 10-15 times more expensive than interferon plus ribavirin therapy.

This situation clearly demands for in depth knowledge about the ground situation regarding different Hepatitis C Virus genotypes and their response to different anti-viral regimes in local population. In same connection the present study was designed to check the overall situation of Hepatitis C in District Mardan including the number of patients detected with HCV, their treatment regime, the non-respondents to treatment, the relapsed patients, the genotype of virus in non-respondents and relapsed patients and any probable effect of age and sex of these patients on resistance to anti-viral therapy.

## Materials and Methods

### Patients and samples

The study was conducted from January 2011 to December 2013 in D.H.Q Hospital Mardan (Hepatitis Prevention and Control Program) and 3800 patients were included in the study. These patients had already completed 6-months treatment of Interferon alfa-2a (3MIU) plus Ribavirin. Blood samples from these patients were taken aseptically and sera from these samples were separated and subjected to HCV RNA-isolation and

amplification through Real-Time PCR. Furthermore, detail history of these patients was taken through a Performa.

### RNA isolation Amplification

RNA was isolated by using Magnesia™ 16 Nucleic Acid Extraction System/Magnesia™ Viral Nucleic Acid Extraction Kit (Anatolia Geneworks) with Bosphore™ HCV Quantification Kit v.1. The RNA isolated was subjected to Real Time PCR for diagnosis of HCV Genome. The following procedures were adopted.

The master mix was prepared for RT-PCR 24µl of master mix and 16µl of sample RNA was transferred to PCR tubes, similarly standard, positive and negative control were made. These PCR tubes were transferred to PCR machine. The different parameters were inserted in Real Time PCR machine as per protocol. After completion of 50 cycles of Real Time PCR results were obtained by taking reading from threshold cycles (Tc). Further genotypes of all non-respondents and relapsed patients identified were determined. The Genotyping was performed as per following procedure in Genome Center for Molecular Based Diagnostic and Research, Johar Town, Lahore.

### Genotyping of Non-Responders and Relapsed Patients

All HCV genotypes were identified by using HCV Genotyping Kit from AnaGen (USA). HCV RNA was isolated from 150µl serum of the patient and was reverse transcribed into cDNA with Molony-murine leukemia virus reverse transcriptase enzyme. The cDNA was subjected to two rounds of PCR amplification. The 1st round utilized the outer primers specific for the core region. The 2nd round was performed with one universal inner-sense and 12 genotype-specific anti-sense primers. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and evaluated on UV trans-illuminator. The HCV genotype for the sample was determined by identifying the genotype-specific cDNA band.

Non-Responders and Relapsed patients were again subjected to 6-months of anti-HCV treatment by replacing Interferon alfa-2a (3MIU) plus Ribavirin with PEG-Interferon alfa-2a (180µg) and Ribavirin therapy. After 6-months therapy these all patients were again checked for presence of HCV-RNA through real-time PCR.

## Results

**Table 1. Genotypes of Non-responders and Relapsed Patients and their Status after Taking PEG-Interferon plus Ribavirin for 6 Months**

S.NO	Genotype	Negative*	Positive*	Total
1	3a	82	12	94
2	3b	3	1	4
3	2a	12	2	14
4	1a	2	1	3
5	1b	3	1	4
6	Mixed	4	0	4
7	Un-typed	4	1	5
Total		110	18	128

\*HCV RNA after PEG-Interferon Plus Ribavirin Therapy

**Table 2. Age and Sex wise Distribution of Genotypes of Non-responder Patients (Male)**

S.NO	Age(years)	Genotype							Become Negative*	Remain Positive*	Total
		3a	3b	2a	1a	1b	Mixed	Un-typed			
1	15-35	08	1	2	0	0	0	1	10	02	12
2	36-45	16	-	2	1	1	1	1	20	02	22
3	46-55	08	1	1	1	2	1	2	09	07	16
4	>55	04	1	1	-	-	-	0	04	03	06
Total		36	3	6	2	3	2	4	43	14	56

\*PEG-Interferon Plus Ribavirin Therapy

**Table 3. Age and Sex wise Distribution of Genotypes of Non-responder Patients (Female)**

S.NO	Age(years)	Genotype							Become Negative*	Remain Positive*	Total
		3a	3b	2a	1a	1b	Mixed	Un-typed			
1	15-35	12	1	3	0	1	0	0	16	1	17
2	36-45	31	0	2	0	1	0	0	34	0	34
3	46-55	9	0	3	0	0	1	1	11	3	11
4	>55	02	0	0	0	0	0	0	02	0	02
Total		54	1	8	0	2	1	1	63	04	67

\*PEG-Interferon Plus Ribavirin Therapy

In this study 3800 patients that have already taken Interferon alfa-2a (3MIU) plus Ribavirin treatment for 6-months were selected and subjected to Real Time PCR to check the viral response to anti-viral therapy. Among these 3677 (97%) patients showed no detectable HCV RNA on PCR while 123 (3%) patients (Non-respondents) were still remained positive for HCV RNA.

The Genotype of 128 samples (including 123 samples of non-responder patients and five from relapsed patients) were determined and subjected to PEG-Interferon alfa-2a (180µg) and Ribavirin therapy for next 6 months. It resulted into elimination of HCV RNA from 110 patients but yet 18 patients had clues of HCV RNA. The Genotype of these all samples analyzed showed that most of them belong to 3a genotype while the samples resistant to anti-viral treatment were gone to other genotypes. Furthermore, viral RNA from 06 patients couldn't be recognized and remained un-typed while 04 patients showed mixed infection (Table 1).

The genotypes and age wise distribution of Male non-responders and Female non-responders patients are mentioned in Table 2 and 3 respectively. HCV was found more resistant to antiviral therapy in female as compared to male. Age group 36-45 in both female and male was found most effected with infection (Table 2 and 3). Relapsed patients responded well to PEG-Interferon alfa-2a (180µg) and Ribavirin treatment and four out of five patients sera showed no detectable virus while one female patient having 3a genotype didn't respond to treatment.

## Discussion

In this study the End of Treatment Response (ETR), (Defined as the absence of HCV RNA at the end of six months Interferon (INF) therapy) of standard INF-alfa-2a(3MIU)and Ribavirin combination therapy was 97% while 0.3% did not respond to therapy. Resistance to INF alfa-2a (3MIU) plus Ribavirin therapy is common and similar results demonstrated by Ahmad et al. (2013) and Bashir et al. (2012) that 19% and 25.28% of patients in their studies respectively, didn't respond to the INF alfa-

2a (3MIU) plus Ribavirin therapy. In addition to routine resistance of different viral genotypes and immunodeficiencies of the host few other observations were made that may add in failure to respond positively to antiviral therapy. It has been noted that some patients take their medicines to their homes rather injecting on spot in front of health officer of the Hepatitis Control Program just to avoid schedule revisits to health center. They keep these medicines and injectable in their home as per their will and not following the instructions of the manufacturer. They keep these medicines and injectable in refrigerator and freezers with the knowledge that daily electricity failure in Pakistan extends sometimes up to 8-10 hours and in some cases the patients stored these medicines in water pots. This mismanagement and not following the cold chain can further hinder to achieve a good ETR.

It is observed that most of the hepatitis C viruses belong to 3a and 2a genotype and highest Sustained Virological Response (SVR), after using PEG-Interferon alfa-2a (180µg) Plus Ribavirin, has been observed (87% and 85%, respectively) in these two groups. These results are in compliance with Jerzy et al. (2013) who demonstrated that HCV genotype 2 and 3 depicted 70-90% SVR. Similarly Saira et al. (2010) reported 80% SVR for HCV genotype 2 and 3. The most prevalent genotype in District Mardan is 3a but it responded well to anti-viral therapy. Other genotypes (1a and 1b) were also found but their presence and circulation was rare but it was evident that resistance to anti-viral therapy was seen more in 1a and 1b genotypes (33% and 25%, respectively) as compared to other genotypes. Similar observation were noted by Shepherd et al. (2004) that best sustained virological response (SVR) was seen for genotype 3 and 2 as compared to genotype 1. Micheal et al. (2001) reported 42% SVR for genotype 1 and 80% SVR for genotype 2 and 3 in response to PEG-interferon alfa-2a (180µg) plus ribavirin therapy

The dominant group of HCV patients in this study that didn't respond to interferon alfa-2a (3MIU) plus ribavirin was female (55%) as compared to male (45%) but resistance ratio after PEG-interferon alfa-2a plus

ribavirin was higher in male as compared to female patients. Resistance to anti-viral therapy was 25% and 6% observed in male and female, respectively. Female patients responded well as compared to male patients. Resistance to antiviral therapy (interferon plus ribavirin) was significantly noted in age group of 36-45 in both male and female patients that became 90% and 100% SVR, respectively after therapy with PEG-interferon alfa-2a (180µg) plus ribavirin. In age group 46-55 the SVR became 22% and 72% in male and female, respectively after treatment with PEG interferon alfa-2a (180µg) plus ribavirin. It clearly indicates a decrease in response to PEG-interferon alfa-2a(180µg) plus ribavirin therapy with increasing age especially in case of male.

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