IN THE NAME OF ALLAH, THE BENEFICENT
THE MERCIFUL

Read! And thy Lord is Most Honorable and Most Benevolent,
Who taught (to write) by pen, He taught man that which he knew not

(Surah Al-Alaq 30: 3-5)
Al-Quran

INTERFERON BASED THERAPY OF CHRONIC HEPATITIS C PATIENTS. A CASE STUDY OF KHYBER PAKHTUNKHWA.

SAJID ALI

APRIL 2012

INTERFERON BASED THERAPY OF CHRONIC HEPATITIS C PATIENTS. A CASE STUDY OF KHYBER PAKHTUNKHWA.

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By
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CENTRE FOR BIOTECHNOLOGY AND MICROBIOLOGY UNIVERSITY OF PESHAWAR

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INTERFERON BASED THERAPY OF CHRONIC HEPATITIS C PATIENTS. A CASE STUDY OF KHYBERPAKHTUNKHWA.

This dissertation is submitted by SAJID ALI as partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biotechnology and Microbiology

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Dedication

I dedicate this humble effort of mine to our Holy Prophet

Muhammad (S.A.W) and my Parents.

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ABSTRACT

INTERFERON BASED THERAPY OF CHRONIC HEPATITIS "C " PATIENTS. A CASE STUDY OF KHYBER PAKHTUN KHWA

Hepatitis C is the inflammation of the Liver caused by Hepatitis C virus (HCV), the leading cause of the liver cirrhosis and hepatocellular carcinoma. About 3% of the people have been affected by HCV world wide and in Pakistan being an underdeveloped country; an estimated 10 million people have Hepatitis C infection (WHO). As hepatitis C infection is asymptomatic and due to untimely diagnosis it leads to severe liver diseases and anuualy a lot of affected individuals lose their lives. In order to cure the infection Food and Drug Agency (FDA) has approved the use of Interferon (IFN) as the treatment remedy. In Pakistan National Institute of Health (NIH) has also given the recommendation for the use of IFN as the therapeutic agent

In KhyberPakhtunKhwa (KPK) mostly conventional IFN and Ribavirin combination therapy is considered due to the prevalence of responsive genotypes 2 and 3. Earlier no study has been conducted to sort out IFN response among chronic HCV patients in different districts of KPK province, therefore we attempted to find out response of conventional IFN combination therapy at districts level in KPK.

Samples were collected from chronic HCV patients referred by clinician/laboratories of different regions of KPK. The samples were analyzed for screening by ICT (Immunochromatographic Technique) and ELISA (Enzyme Linked Immunosorbant Asaay) followed by confirmation through polymerase chain reaction (PCR). We have also done genotyping for some of the chronic HCV patients. PCR confirmed positive patients were given IFN and Ribavirin combination therapy keeping in mind the therapy exclusion criteria. The dose of IFN and Ribavirin was 3 Million Units thrice a week and 800-1200mg daily depending on the age of the patients, respectively. This therapy was continued for six months with repeated testing of ALT (Alanin Transaminase) and CBC (Complete Blood Count), during and after therapy. At the end of six months of therapy, PCR test was done for all course completed patients.

Active HCV infection was present in 66.6% among 3075 anti-HCV positive patients while 33.3% anti-HCV positive patients were negative for HCV RNA. Rate of active

HCV infection was comparatively more in districts Bunir (72%), Dir (70 %) and in Mardan (69%). While lower in districts Swabi (66%), Peshawar (64%) and Kohat (59%).

HCV genotype analysis in chronic HCV patients of KPK revealed that the most abundant genotypes/subtypes among the patients analyzed were 2a followed by subtype 3a. Other common genotypes included the untypable type of the virus and genotype 3b.

Response of IFN and Ribavirn combination therapy in the 1st trial among 174 PCR positive patients was 74.71% and the resistance was 25.28%. Among different districts, high end of treatment response (ETR) was shown by district Mardan patients population (89.18%), followed by Bunir (69.23%). While low response was present in case of district Peshawar (60%) and Federally Administered Triable Area (FATA) (55.55%).

In the second trial of IFN therapy, out of total (341) selected patients for standard IFN-based therapy 81% showed ETR and 19% did not show response. Among the districts high ETR was shown by district Swabi (92%), followed by district Kohat (80%). Comparatively low response was present in case of district Bunir (71%).

In genotype specific response of IFN based therapy, out of total 51 selected patients. Responsive genotypes among these were 2a followed by 3a. Response rate among different HCV genotypes were as, 2a HCV genotypes had high (77.72%) ETR, followed by 3a genotypes (72.22%). Comparatively low response was present in case of 3b and 1b genotypes (66.66%) and (33.33%) respectively. While untypable genotypes showed no response.

Our results revealed that response of combination IFN therapy is good in some of the districts patients' population. High ETR rate in these districts may be attributed to prevalence of responsive HCV genotypes 2 and 3. In case of non responsive genotypes some new effective remedies should be discovered. While untypable genotypes should be sequenced so as to adopt some new therapeutic agents against them.

Introduction

Chronic Hepatitis C is the infection caused by hepatitis C virus (HCV). Hepatitis C infection leads to liver cirrhosis and hepatocellular carcinoma and is an indication for liver transplantation in many countries [1-3]. About 3% of the world population has been suffering from HCV infection [4]. Although symptoms of HCV infection may be mild for decades, but if it persist then 20% of patients having HCV infection ultimately lead to severe liver disease including cirrhosis and liver cancer [5]. According to World health organization (WHO), an estimated 308,000 and 785,000 persons die due to liver cancer and cirrhosis respectively [6]. In Pakistan, having total population of 170 million people, an estimated 10 million people have been infected with HCV infection [7].

HCV was discovered by molecular cloning method, from chimpanzee's plasma which was infected with non A-non-B hepatitis [8]. The HCV genome, which has marked similarities with the genus Pestivirus and Flavivirus of family Flaviviridae is single-stranded, enveloped, positive-sense RNA, approximately 9600 bases long and encodes a single polyprotein of 3,000 amino acids [9]. In the whole viral genome there is high sequence variability in different HCV isolates around the World. On the basis of sequence variability, HCV has been classified into multiple strains which include six known major genotypes and more than 50 subtypes of HCV [10, 11]. The cause of this great heterogeneity in HCV genome is the high rate of HCV RNA replication, the immune surveillance of the host and RNA-dependent RNA polymerase low fidelity. It is thought that this genetic heterogeneity is responsible for pathogenesis as well as for response to therapy in case of HCV-infected individuals [12 –14].

HCV is the leading cause of liver transplantation and organ shortage is the major associated problem [15]. The introduction of effective therapy for the prevention of such life threatening infection is the ultimate goal. It is especially more important in underdeveloped countries, where HCV infection rate is more and most of the patients have financial problems for its treatment.

Eradication of the virus is the primitive goal of hepatitis C treatment that is to achieve sustained virologic response (SVR) which may be defined as the undetection of HCV-RNA in serum after six months of treatment completion and is evidenced by sensitive molecular tests Polymerase Chain Reaction (PCR). It has been found over the past decades that there is great improvement in the SVR rates, when treatment strategies have been shifted from interferon (IFN) mono-therapy to combination IFN plus Ribavirin [16, 17].

Further improvements in SVR rates have been found very recently with the availability of pegylated IFN (Peg-IFN) [18, 19], such that by using peg-IFN in combination with Ribavirin more than half of the infected individuals have better hope of success [20–22]. Although improvements occurred in SVR rates, but in some patient's population less satisfactory results have been obtained due to some virus and patients associated factors. The Virus-related factors responsible for poor response include, the persons having infection with HCV genotypes 1 or 4 and having high initial viral load. While the factors related to patients are resistance to previous IFN therapy, the presence of cirrhosis, older age more than 60, adverse effects or contraindications to treatment and possibly obesity [23].

Different HCV genotypes have importance in different aspects like in epidemiology, in the development of vaccine and in the management of chronic HCV infection [24]. As HCV genotypes have been linked with sustained virologic response [25]. Some

studies have reported that individuals infected with type 2 and type 3 have better response to IFN therapy than those having infection with type 1 [26]. It has been found that patients infected with HCV-2/3 and HCV-1 genotypes, have SVR to combination therapy 65% and 30%, respectively [16, 27].

False positivity is a common problem associated with ICT devices [28-30]. Earlier studies have reported the prevalence of anti-HCV antibodies by using Immunochromatographic tests among the blood donors or general population from KPK province [31-33]. It has been found that in Pakistan, HCV prevalence in general adult population was 5% [34].

Hence there was need to screen out HCV infection among confirmed anti-HCV patients; early detection enables the patients to take possible early curable and preventive measures, so as to minimize the complications associated with end-stage disease. Before this study, many attempts have been done to determine the general prevalence of HCV infection in different regions of KPK. Different districts have different rates of HCV infection. But still no study has been performed to screen out viremia among confirmed anti-HCV patients. The objective of this study was to determine the frequency of active HCV infection among the confirmed anti-HCV subjects of KPK in order to help out the infected subjects decide about anti-viral treatment options.

In KPK, some studies have shown that abundant genotypes among HCV patients were 3 followed by 2 [35]. As HCV genotypes have earlier been implicated in response to therapy [25], therefore it was of prime importance to investigate the frequency distribution of HCV genotypes in KPK province of Pakistan, where genotype determination prior to therapy is not common in practice.

Response rates to standard IFN therapy in chronic HCV patients have been investigated in KPK province [36, 37]. But no study has been conducted on large scale which can present IFN response at districts level and also there was lack of genotype specific response to IFN therapy, therefore we attempted to found general as well as genotype specific response among chronic HCV patients of KPK. Genotype specific response study was very important to conduct as this reflects an epidemiology of an infection and some genotypes require more than six months of conventional IFN therapy. Due to the high prevalence of HCV and heavy economic burden of hepatitis C treatment in KPK, it was the need of the hour to conduct such a study so as to help standardize the treatment protocol and minimize the economic burden on the people of KPK.

1.1 Basic Virology

1.1.1 HCV Genome Organization and Function

Hepatitis C virus (HCV), the member of hepacivirus genus belongs to family Flaviviridae which also includes the genus Pestivirus and Flavivirus [38]. HCV is a positive sense, small virus the genome of which is single stranded RNA of 9600 nucleotide bases enclosed in a capsid shell which is surrounded by viral envelop (Fig. 1).

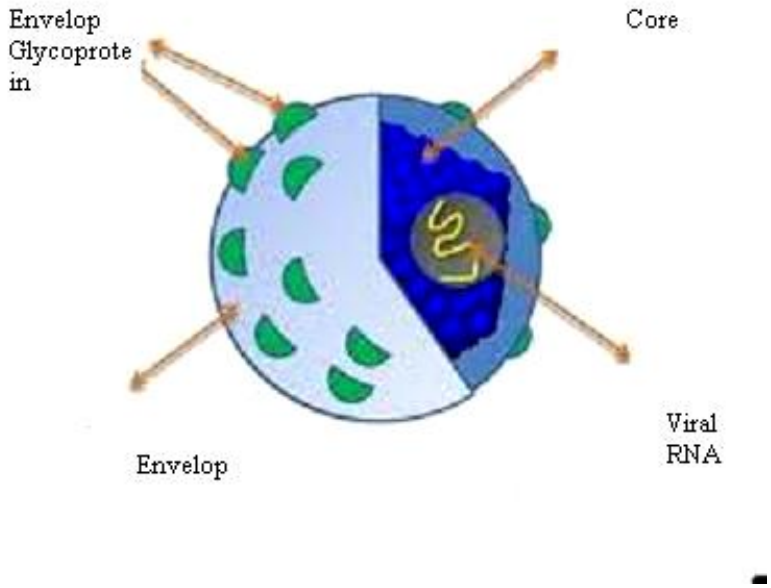

Fig 1: Structure of Hepatitis C Virus. [www.google/image.com]

ORF: HCV has single open reading frame (ORF), the sequence contains a 5'-untranslated region (5'- UTR) of 341 bases and a 3'-untranslated region (3'- UTR). The ORF encodes for a single polyprotein that is cleaved enzymatically into mature structural and non structural proteins (Fig 2). The structural proteins are the core (C) and two envelop (E1 and E2) proteins. While NS2, NS3, NS4A, NS4B, NS5A and NS5B are the non structural proteins [39]. The respective function(s) of these proteins are listed in Table (1). The most important and consistent functions of the most flavivirus proteins are; the three N-terminal HCV proteins (C, E1, and E2/NS2) are believed to be structural while the four C-terminal proteins (NS2, NS3, NS4, and NS5) are believed to play their role in HCV replication. The 3′-UTR comprises a tripartite structure consisting of a variable region immediately after the stop codon of the ORF, a poly (U/UC) tract varying in length between 30 and 150 residues and a highly conserved 'X-tail' or 3′X sequence. The 3′X is believed to be important in efficient HCV replication [40]. There is variation in the length of the ORF of each genotype. The ORF of the type 1 genotypes is approximately 9,400 ribonucleotides, type 2 have a length of 9,099 nucleotides and that of type 3 length is 9,063 nucleotides [10]. A brief overview of the viral gene products and their roles in the HCV lifecycle is given in table 1.

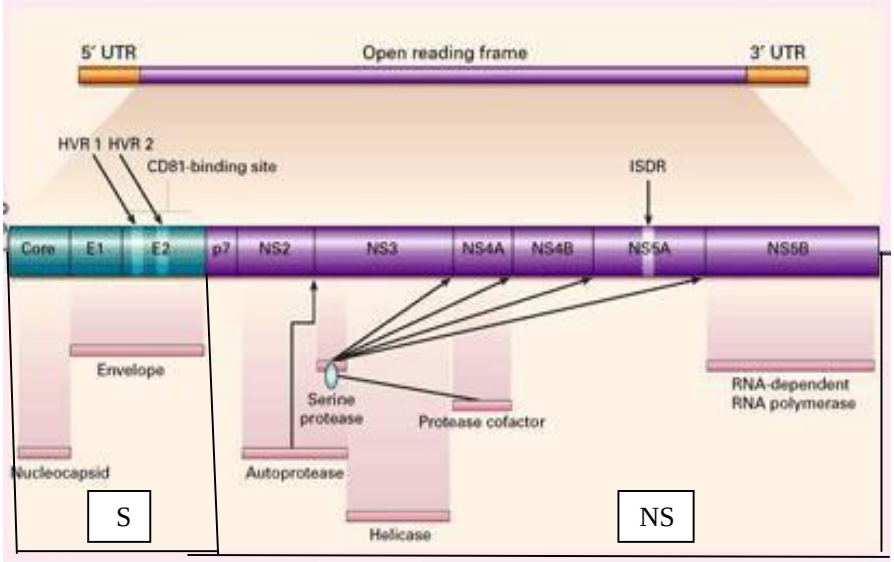

Fig 2: Schematic representation of the HCV genome and encoded viral proteins.

At the top is single open reading frame representing two ends, 5’ and 3’-untranslated regions (UTR). Below are the encoded proteins, structural (S) and nonstructural (NS) [41].

Table 1: HCV proteins and their functions in the viral life cycle [42].

HCV protein	**Function**	**Apparent molecular weight (kDa)**
Core	Nucleocapsid	23 (precursor), 21 (mature)
E1	Envelope, Fusion domain?	33–35
E2	Envelope, Receptor binding, Fusion domain?	70–72
p7	Calcium ion channel (viroporin)	7
NS2	NS2-3 autoprotease	21–23
NS3	Component of NS2-3 and NS3-4A proteinases NTPase/helicase	69
NS4A	NS3-4A proteinase cofactor	6
NS4B	Membranous web induction	27
NS5A	RNA replication by formation of replication complexes	56 (basal form), 58 (hyperphosphorylated form)
NS5B	RNA-dependant RNA polymerase	68

1.1.2 Hepatitis C Virus Replication

The primary goal of all viruses including HCV is to synthesize new copies by means of cell cycle replication. Replication in HCV and other viruses is only possible when they infect other cells and utilize the cell's translational apparatus including enzymes and other proteins. HCV, which is considered as hepatotropic, targets the human host hepatocytes [43]. After entry into the host cell, all the events of replication occur in the cytoplasm (Fig. 4). The progression in replication of HCV is done by both cellular and viral proteins throughout its replication cycle. HCV genome encodes for different viral structural and non structural proteins; the functions of many of these proteins are evident but the roles of these proteins in viral replication and host-viral interactions are still unclear [44]. HCV replication occurs in the following stages.

1. Attachment of the virus to host cells followed by entry and then uncoating.
2. Synthesis of mRNA and polyprotein and then processing of viral polyprotein in the cytoplasm.
3. Viral RNA replication.
4. Assembly and release of new virions from host cell.

1.1.2.1 Stage 1: Viral attachment, entry into the host cell and uncoating

Replication of virus starts with the entry into the host target cells. Generally the Flaviviridae members utilize three steps for entry into the host cells that is attachment, entry and uncoating (Fig. 3).

Attachment: The primary cell surface receptors which are thought to be capable of binding to HCV virions are "the low and very-low-density lipoproteins receptors (LDLR), low density lipoprotein receptor (LP), glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1). CLDN1 functions at a later stage of cell entry, possibly at tight junctions of

polarized hepatocytes [45, 46]. Besides infecting the hepatocytes, HCV has also been found outside of the liver in a variety of cells which may include white blood cells (WBCs) and components of the immune system [47].

The attachment of virions become possible through interactions in between HCV envelope attachment proteins and host cell surface receptors. E1 and E2 are the two envelop proteins of HCV which are produced by the virus itself. Heterodimers are formed when E1 and E2 bind together on the envelop [48]. For attachment to the host cell surface receptors, HCV uses one or both of these envelop proteins. Besides these, many other such HCV receptors have been proposed but still their roles in viral entry are unclear. HCV may require more than one type of receptors like HIV for their attachment [45].

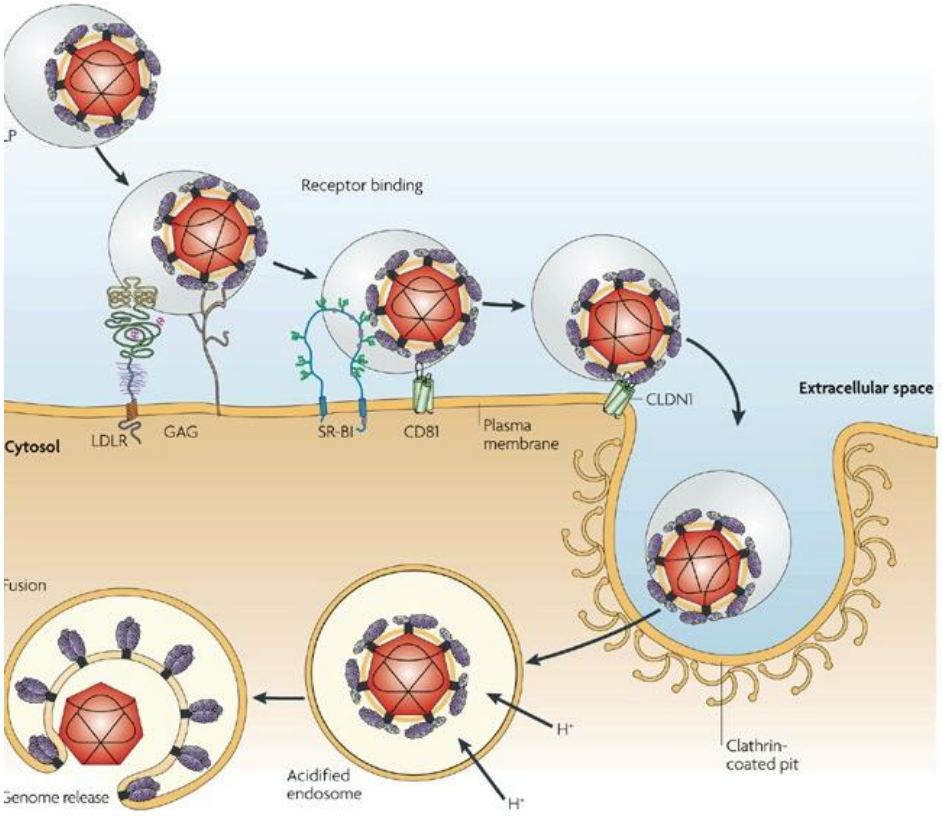

Fig 3: HCV attachment, entry and uncoating of its genome.

HCV host cell surface attachment receptors; low and very-low-density lipoproteins (LP), low density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1) [49].

Entry: Receptor-mediated endocytosis process causes the entry of HCV particles into the target cells by using class II fusion proteins [50]. HCV glycoprotein is believed to belong to class II fusion proteins [51]. After internalization, this complex is encapsulated in a pocket of fluid surrounded by a thin membrane (vesicle). It has been observed that HCV entry into the cell is pH dependent [52].

Uncoating: The process of release of viral genome into the host cell cytoplasm. Uncoating of HCV is still not characterized but it resembles other enveloped viruses that adopt receptor mediated endocytosis process for their entry. In this process HCV envelop fuses with vesicle membrane causes degradation of viral capsid and subsequently viral genome is released into cytoplasm (Fig. 3), which is then transported to the endoplasmic Reticulum (ER) [50].

1.1.2.2 Stage 2: Polyprotein translation and processing

Polyprotein synthesis: After decapsidation, HCV-RNA released into the cells cytoplasm acts as free positive strand viral genomic RNA, serving together with newly synthesized RNAs as messenger RNAs and synthesize polyprotein. The polyprotein synthesis is Internal Ribosomal Entry Site (IRES) dependent (Fig. 5). The region of IRES spanning domains II to IV of the 5' UTR and the first nucleotide of the core coding region plays an important role in initiating translation [53].

Post translation processing: After translation into polyprotein, the HCV polyprotein is broken down into different products, with the structural proteins located in the 5' terminal and the nonstructural replicative proteins in the 3' terminal [54, 55]. Cellular signal peptidases (denoted as open diamond), localized in the lumen of the ER [56, 57], catalyze the cleavage of the structural region (Fig. 4). NS2 and NS3 cleavage is done by protease comprised of the NS2 and NS3 proteins themselves through a process described as auto cleavage [58, 59]. The separate N3 serine protease causes

the cleavage of other down stream NS protein NS5B, RNA dependant RNA polymerase (RdRp) [60, 61]. When cleavage is done then further post-translational modifications of E1, E2 and some other proteins occur before these proteins starts their actual function (Fig. 4).

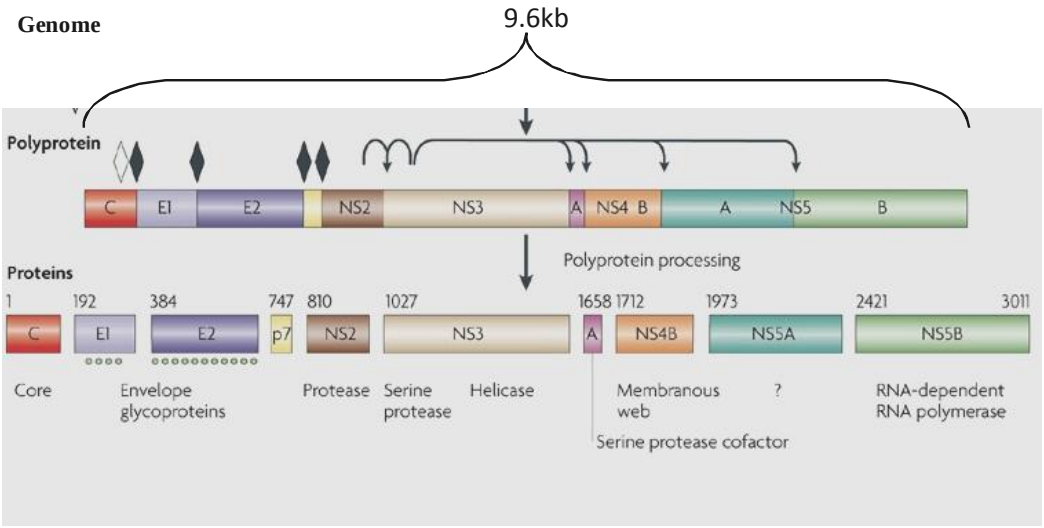

Fig 4: Poly protein synthesis and Post translational Cleavage of Polyprotein.

At the top is HCV 9.6-kb genome. Second line representing a polyprotein synthesis and the third line representing cleavage of polyprotein. Above each protein is their respective number of amino acid. HCV polyprotein precursor cleavage sites by the endoplasmic reticulum signal peptidase are shown with solid diamonds. Further C-terminal processing of the core protein by signal peptidase is denoted with the open diamond. Dots in E1 and E2 indicate the glycosylation of the envelope proteins [62].

1.1.2.3 Stage 3: Viral RNA replication

The pivotal event in the HCV life cycle is the replication of its Genome. Before replication starts, virions will bind to the cell membrane followed by entry and uncoating of its genome. After IRES mediated translation and polyprotein processing, RNA replication starts (Fig. 5). The characteristics of each viral particle is that each one require its own RNA genome which are the RNA strands that have been copied or transcribed from the original strands of HCV RNA. Viruses are not capable to copy directly its positive sense RNA from the original positive Sense RNA strand. The replication occurs in two steps:

1) Complementary negative sense RNA strand originates from the original positive-sense RNA strand, and

2) The negative-sense RNA strand originated in the 1st step then directs the synthesis of new, genomic, positive-sense viral-RNA [63].

This whole process is accomplished virtually by all nonstructural viral proteins as well as some cellular proteins. Viral replication complex is formed where RNA strand synthesis occurs. Among those proteins, the most important role in the RNA replication process is played by viral NS5B RdRp and NS3 helicase/NTPase. RdRp activity is to assemble nucleotides complimentary to original RNA strand and Helicase/NTPase, with NS4A acting as a cofactor, moves along the template RNA stand to prevent the formation of double helix of original and newly synthesized RNA strands (Fig. 5) [64]. Turnover rate of HCV is very high that is, between 10^{10} to 10^{12} virions per day and a predicted viral half-life of 2 to 3 hr. The reasons of high mutation rates in HCV are the rapid viral replication and lack of proof reading ability of the viral RNA-polymerase enzyme [65].

1.1.2.4 Stage 4: Virion assembly and release

After replication the new HCV virion is composed of three components: single-stranded HCV-RNA, a capsid shell and the viral envelope. These are the resultant products of the translation (core protein and envelope protein) and viral RNA replication (genomic HCV-RNA), respectively. The genomic RNA forms the nucleocapsid by packing within the capsid from core protein in the endoplasmic reticulum and then enveloped in a section of cellular membrane. The new virion assembly is then completed by incorporating viral envelop proteins into the nucleocapsid. After enveloping and maturation in the Golgi apparatus, the new virions are released in the pericellular space by exocytosis [66, 67] to infect new cells. It is thought that the viral protein, p7 plays its role in viral release by increasing cell permeability and by forming ion channels [68].

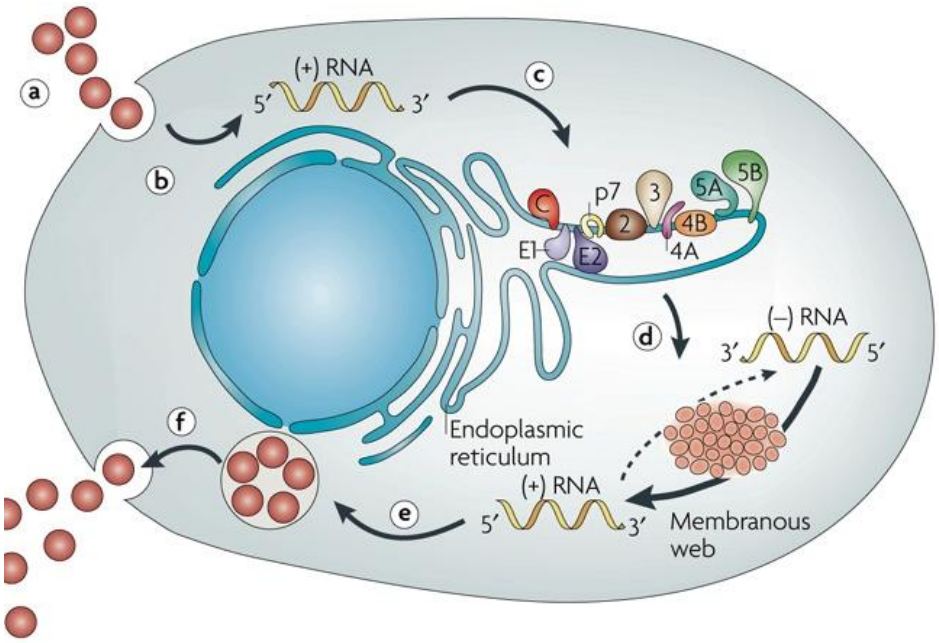

Fig 5: Viral Replication

(a) Viral entry (b); release of RNA in cytoplasm (c); IRES-mediated translation and polyprotein processing (d); RNA replication (e); packaging and assembly (f); virion maturation and release. Replicative and structural proteins have been shown in stage c [63].

1.2 Hepatitis C Virus Infection

1.2.1 Acute Hepatitis C

The hepatitis which last for the first six months is referred to as acute hepatitis. Among patients having acute HCV infection, about 60-70% of patients have no symptoms, 20-30% has jaundice and 10-20% have non-specific symptoms like; loss of appetite, lack of interest in general activities, fatigue, jaundice, abdominal pain and flue like symptoms which mostly lead to non specific diagnosis [69-71]. In acute phase of infection spontaneous viral clearance of 10-60%, means the body clears the virus itself and is indicated by normal level of Alanine Transaminase (ALT) and also undetectable HCV-RNA from the plasma [72]. Acute HCV incubation period ranges from 2-6 months with an average of 6-7 months [73, 74]. However, viral-RNA can be detected within the first week of exposure to HCV infection.

1.2.2 Chronic Hepatitis C

The hepatitis lasting for more than 6 months after infection with HCV is defined as chronic hepatitis C. It has been estimated that 85 % of patients or more having acute HCV infection develop into chronic HCV infection [75-77]. It has also been noted that chronic active hepatitis C develops in 26 or more than 50 % of the patients having HCV infection. Among these, cirrhosis develops in 8-42% of patients in an average time span of 3 years. More follow-up studies showed that in 10-20% of patients, cirrhosis develops during the first 20 years of HCV infection [78]. It is believed that HCV infection is the cause of primary hepatocellular carcinoma and its rate is high upto 4% per year after the development of cirrhosis [79]. The factors which influence the severity of liver disease are the age more than 40 years, male sex and take of alcohol 50g /day or more [78]. Every individual has considerable variation in the natural course as well as in the progression of liver fibrosis in chronic hepatitis C

infection. The circulating levels of liver enzymes, lack of definitive symptoms and histology of liver are the best predictors of liver progression [80, 81]. The progression is usually slow and limited when liver inflammation and fibrosis is mild [81]. Hence the individuals having high level of liver enzymes will progress rapidly as compared to those having low level of liver enzymes [80].

The associated factors affecting the rate of progression of chronic hepatitis C to cirrhosis and liver cancer are the duration of infection, viral load and alcohol consumption. Beside this some other factors have also been found to play their roles in the progression of the disease, like co-infection with other type of hepatitis virus and/or with human immunodeficiency virus (HIV), viral genotype and gender [80]. Associated factors leading to cirrhosis and carcinoma have been shown in Figure 6.

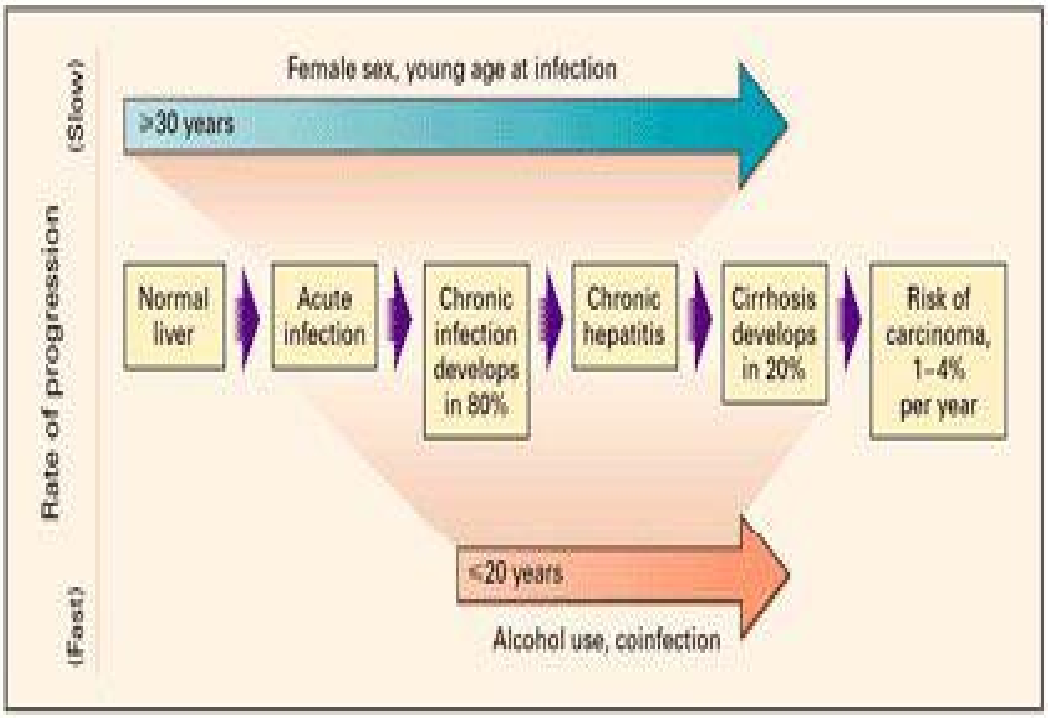

Fig 6: The Natural History and course of progression of HCV Infection [41]

1.3 Molecular Epidemiology of HCV

1.3.1 Epidemiology of HCV World Wide

HCV infection has reached an epidemics proportion and about 3% of the world population have been suffering from this infection world wide [4]. HCV prevalence has been noted to be 1% in developed countries of the world like Australia and the United States (US) [82, 83]. In Asia more prevalence has been recorded and is about 6% [84, 85]. Different developed and populous nations of the world have relatively low rates of HCV seroprevalence such as Germany (0.6%), Canada (0.8%), France (1.1%), Japan (1.5-2.3%), and Italy (2.2%) [83, 86-92]. China, which is highly populated nation of the world, has HCV prevalence of 3.2% [93]. In developing countries such as India, Indonesia and Egypt, HCV prevalence has been found to be 0.9-22% [94-96]. It has been observed that chronic HCV infection in African American, injection drug users' population was 95% [97].

1.3.2 Epidemiology of HCV in Pakistan

HCV prevalence may be different in different regions and various groups of the same community [98]. According to hospital-based studies prevalence of HCV in different regions of Pakistan noted was as, in capital Islamabad region, HCV prevalence was 5.31% [99]. In various parts of the Punjab province, HCV prevalence was 2.45-20.89% [100-102]. In the province of sindh, especially in Karachi was 4-6% [103]. In KPK and Northern Areas of Pakistan, HCV prevalence has been found to be 5-9% and 25.7% respectively [31,104,105]. While in 2005 in the earth quake affected areas of Pakistan, prevalence of HCV was recorded slightly higher [106]. In 2011, it has been found that about 1.68% of the blood donors had active HCV infection in KPK [107].

1.3.3 Epidemiology of HCV Genotypes

1.3.3.1 Epidemiology of HCV Genotypes World Wide

Genetic differences make the basis of classification of different HCV isolates into genotypes and subtypes. HCV has been classified into six major genotypes (1-6) and more than 50 subtypes (a,b, c, etc), termed as isotypes or quasispecies [10, 11]. The heterogeneity of HCV major genotypes, subtypes and quasispecies in respect to sequence variability, have been shown in table (2). Sequence variability was mostly found in the 'hypervariable' regions (HVRs) of the E1 and E2 glycoproteins. The conserved sequences were found in the core gene and some of the non-structural protein genes, such as NS3. The 5´ NTR has the lowest sequence variability in between the six genotypes [53].

Different countries of the world have different genotypes distributions, like genotypes 1, 2 and 3 appear to have a worldwide distribution, while the most common genotypes in the US [25] and Europe [27, 108, 109] are 1a and 1b subtypes. In Japan, the most prevalent genotype is subtype 1b [110]. The common genotypes in the North America, Europe, and Japan are subtypes 2a and 2b, while in Italy subtype 2c is highly prevalent. The prevalent genotype in North Africa and the Middle East is 4 [111, 112]. In South Africa and Hong Kong mostly genotypes 5 and 6 are prevalent genotypes [113, 114]. Genotypes 7, 8 and 9 are prevalent genotypes among Vietnamese patient's population [115], while in Indonesian patient's population; genotypes 10 and 11 are found [116]. World wide distribution of HCV genotypes has been shown in Figure 7.

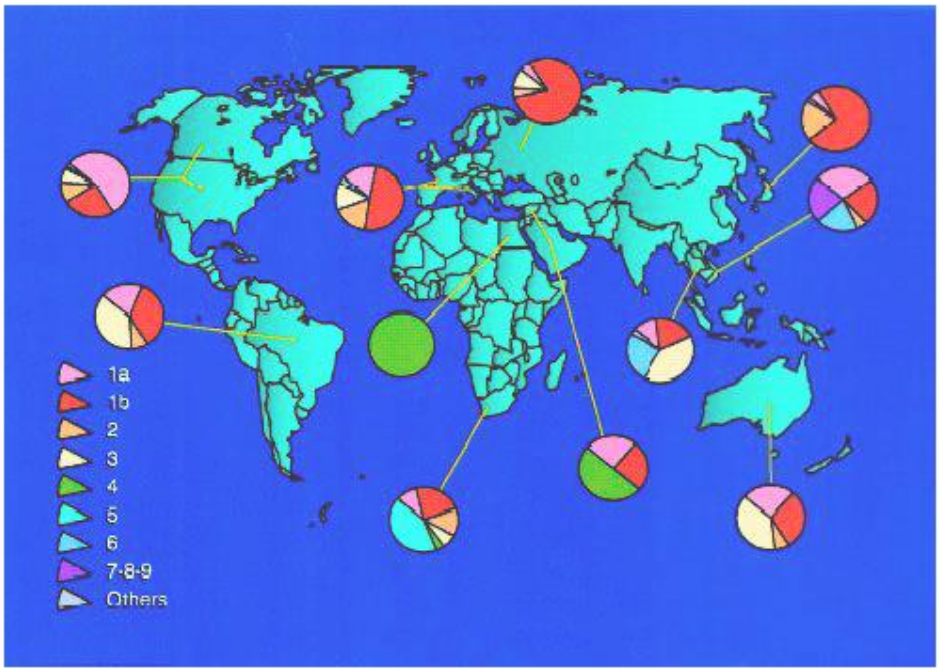

Fig 7: World wide HCV genotypes and subtypes distribution. Others indicate unknown sequences [117].

Table 2: Common terminologies related to HCV genomic heterogeneity

Terminology	Definition	% Nucleotide similarity a°
Genotype	Genetic heterogeneity among different HCV isolates	65.7–68.9
Subtype	Closely related isolates within each of the major genotypes	76.9–80.1
Quasispecies	Complex of genetic variants within individual isolates	90.8–99

a° % Nucleotide similarity refers to the nucleotide sequence identities of the full length sequences of the HCV genome.

1.3.3.2 Epidemiology of HCV Genotypes in Pakistan

A study in 1997, reported that 87% of patients had the prevalence of genotype 3 in Pakistani population [118]. A panel of 30 gastroenterologists reported in 2004, that affected individuals in Pakistan had 75-90% of HCV genotype 3a [7]. In 2006, Qazi et al. reported that HCV genotype 3 is present in 71% while genotype 1 is present in 10% of patients having chronic HCV infection [119]. Another study in 2007 revealed that 81% of patients had genotype 3 while only 9.5% of patients had genotype 1 [120]. Hakim et al. in 2008 reported that 3a is present in 51% of HCV patients, 3a/3b co-infection in 24% and 3b in 16% of patients [121]. Similar results were also reported by Afridi et al, [122] that 50% of HCV infected individuals had genotype 3a followed by 3b and 1a. In 2008, Idrees and Riazuddin conducted a detailed study and reported that the most prevalent genotype in Pakistan is 3a [98]. In 2011, in KPK province, the most abundant genotypes found were 2a followed by 3a [123].

1.4 Risk Factors and transmission of HCV

In Pakistan there is great lack of national data which evaluate risk factors associated with hepatitis C infection. Most of the risk factors have been evaluated in selected areas of Pakistan [124]. Some of the common risk factors associated with HCV transmission in Pakistan are as follows,

Major risk factors

Unsafe injections

According to WHO, Billions of injections (not required or not sterilized) are administered every year in developing countries [125]. Overuse of injections are common in Pakistan as it has been proved by Janjua et al. that 71% of people from different urban and rural areas of Pakistan believe that use of injections are an easy approach to treat the disease [126]. In a study conducted at semi urban area of Karachi

reported that people (94%) using unsafe injections are having high HCV prevalence (44%) [127]. Therefore, it is clear that unsterilized and unnecessary use of injections is the major risk factors associated with HCV transmission.

Blood transfusions

Blood transfusion in Pakistan is also a major risk factor responsible for transmission of HCV infection. Although screening of the blood has reduced the role of this way of transmission but still it is considered as the major risk, due to lack of proper screening procedures and the use of paid blood donors [128]. In patients with thalassaemia major, who require frequent blood transfusions, HCV prevalence of 34.8% to 60% has been recorded which reflects the role of blood transfusion in the spread of HCV infection [129-131].

Surgery and the use of contaminated instruments

People who are exposing to contaminated equipment during major or minor surgery and daycare procedures like hemodialysis, endoscopy etc are supposed to have greater chances of acquiring HCV infection. The risk of acquiring infection is 1.7-2.4 times [125, 132, 133] higher among the people who are receiving dental treatment and the risk even more increases with the unqualified persons performing dental surgery (3.5-29.3 times) [134, 135].

Minor risk factors

Besides above, the following are the some minor risk factors associated with HCV transmission:

Sharing of objects

As barbers have low level of awareness about the spread of HCV, therefore they are reported as significant predictors of spreading of HCV infection [124, 133].

Healthcare workers

HCW are also considered at major risk of acquiring and transmitting HCV, due to high chances of exposure with contaminated equipments and body fluids [136].

Others

The women are at major risk due to piercing particularly of ear and nose, tattooing and body piercing with contaminated needles are also the possible sources in case of HCV transmission in Pakistan. Contributions of various risk factors are shown in Figure 8.

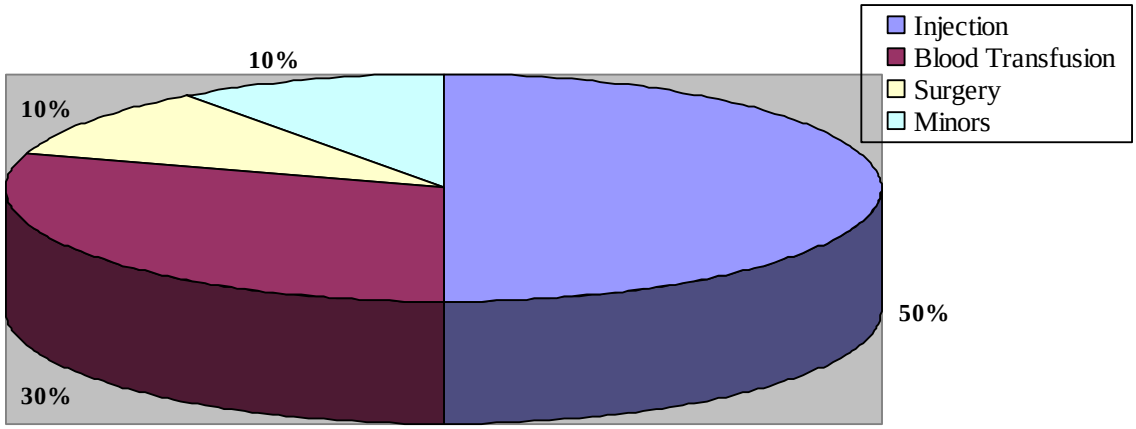

Fig 8: Risk factors associated with HCV transmission.

1.5 Diagnosis of HCV

Diagnosis of HCV is based on the

Clinical picture,

Ultra sound and elevated LFTs level.

HCV infection in suspected persons are further confirmed by using the following tests:

1. Anti-HCV (serological Assays)
2. HCV RNA testing (Molecular assays) and
3. Genotyping

1.5.1 Serological Assays

Serologically suspected persons are diagnosed through screening by ICT and ELISA techniques [137]. Both of these are based on the antibodies detection in the sera of the infected persons. WHO has recommended the use of 1st, 2nd and 3rd generation ELISA technique for diagnosis of HCV infection in the bodies of infected individuals.

1.5.2 Molecular Assays

1.5.2.1 Nucleic Acid Detection

Further HCV is diagnosed by detecting HCV-RNA through polymerase chain reaction (PCR) test. The RNA can be detected within one to two weeks of infection with HCV, some weeks before the elevation of ALT level. Some patients have only evidence of HCV-RNA [138].

1.5.2.2 Genotyping Methodologies

Genotypes are important for epidemiology, duration of treatment and vaccine development so its need is becoming more and more [24]. Until now many methods have been discovered for HCV genotyping including direct sequencing of DNA [139, 140], type specific PCR [141], Rrestriction fragment length polymorphism (RFLP),

line probe assays [142], primer-specific and mispair extension analysis [143], heteroduplex mobility analysis by temperature gradient capillary electrophoresis [144] and denaturing high preference liquid chromatography [145]. As sequencing of large fragment is difficult and laborious therefore many rapid methodologies have been adopted by many laboratories. Regarding genotyping assay crucial point is the choice of genomic region. The selected region should have subtypes and type specific motifs which in real sense represent entire genome diversity. The genomic regions analyzed for genotyping classification are the 5' NCR, CORE, E1 and NS5B regions. These have been frequently amplified and studied for the purpose of genotypic classification [146-148]. Comparatively NS5B is more often used for differentiation of subtypes and confirmation of genotyping results in research settings.

Practically the 5' NCR is the preferred target for HCV genotyping despite limited sequence variability in the HCV 5' non coding region (NCR), in most diagnostic laboratories [149]. Recently many HCV genotyping assays are currently commercially available which include the TRUGENE HCV 5'NC genotyping kit (TRUGENE 5'NC; Bayer HealthCare LLC, Berkeley, Calif.) and the VERSANT HCV genotype assay (LiPA; Bayer HealthCare LLC). As more sequence data is available now therefore these methods are often found not to be definitive, suggesting 5' NCR might not contain enough sequence characteristics that can be used to differentiate all genotypes and subtypes.

Apart from the above mentioned genotyping methodologies, HCV genotyping can also be done through Real Time PCR based technique. This is the latest technique developed for the first time by Abbot Company. The sensitivity of the assay developed by the abbot is eleven genotypes. There are some companies which have designed Real Time PCR based genotyping but that can only detect the common 1, 2,

3 major genotypes (Sacacea, USA). The real Time PCR based genotyping is more accurate than the above mentioned conventional methodologies.

1.6 Treatment of Chronic Hepatitis C

Food and drug agency (FDA) has approved IFN based therapy for the treatment of chronic hepatitis C. Two types of IFN have been approved, conventional and Peg-IFN with Ribavirin combination. Hepatitis C therapy started with a small trial of recombinant human IFN Alfa almost 25 years ago [150]. IFN was approved for the hepatitis C treatment in the US in 1992.

IFN was selected because of its broad activities against the viruses and it was thought that it might also be active against still-undiscovered agent of non-A, non-B hepatitis. No doubt, IFN was found very active against HCV and resultant effects were decrease in the level of serum Alanin aminotransferase (ALT). So far HCV was not discovered, the effects of IFN were not understood but the result of using IFN was the reduction in HCV-RNA level, which led to a sustained absence of virus in a proportion of patients [151]. Nevertheless,

SVR is the primitive goal of HCV treatment, which is the absence of HCV-RNA after being tested by molecular sensitive test 24 weeks after completion of therapy [151]. There are 95% chances of having no virus for the 5 years in the body of affected individuals who get SVR [21]. These individuals will have fibrosis regression, low incidence of hepatocellular carcinoma and hence low morbidity and mortality [152].

1.6.1 Mechanisms of action of Interferon in chronic hepatitis C

IFNs type 1 [IFN alpha and beta (IFN-α/β)] comprise a family of distinct proteins [153], produced by different types of the body cells like epithelial cells, fibroblasts, and hepatocytes [154], although the major source of production is the plasmacytoid dendritic cells (DCs) during viral infection. While type II IFN [IFN gamma (IFN-γ)] having no structural similarities with IFN-*α*/*β*, are mostly produced by natural killer (NK) cells, macrophages and T lymphocytes. Interaction of Both types of IFNs with cells occurs via distinct cellular receptors. The details that how IFN-*α*/*β* and IFN-*γ* induces the transcription of IFN-stimulated genes (ISGs) and depress the transcription of others are still not being defined [155].

The outcome of the infection in most of the viral attacks depends on the innate and acquired immune responses. The triggering of immunological reactions occur firstly by the engagement of pathogen-associated molecular pattern (PAMP) receptors that after sensing the viral attack inducing the antiviral state through a cascades of reactions including cellular translation limitation through protein Kinase R (PRK R), viral RNA modification and degradation via RNA specific adenosine deaminase, 2′,5′-oligoadenylate synthetase (OAS) and ribonuclease L, alteration in cellular vesicles trafficking through guanosine triphosphatases (GTP) and many more undiscovered anti viral mechanisms [153]. After engament of the PAMP receptors like Toll Like receptors (TLRs) and the retinoic acid–inducible gene I (RIG-I)–like helicases, causes the activation of signaling pathways leading to the synthesis of IFN-α/β, tumor necrosis factor (TNF) and other cytokines like interlukein12 (IL-12) and interlukein 15 (IL-15) [153] (Fig. 11). Majority of these are produced by Dendtritic cells (DCs) that express TLRs in abundance. Produced IFN-αβ acts to induce Natural Killer cells (NK), enhancing their cytotoxic activities by stimulating the production of

IFN- γ. the IL-15 in turn stimulates the proliferation and accumulation of NK cells [156, 157]. The additional IFN- γ is produced by the activation of CD-8+ T cells, playing a central role in adaptive immunity [158].

Type 1 IFN receptors convey signals to the nucleus through Janus kinase-1 (Jak1) and tyrosine kinase 2 (Tyk2) phosphorylation of the signal transducers and activators of transcription (STATs) [159]. There are total seven different STATs, 1 through 6 with 5a and 5b, when these are activated, assembled as homodimeric and heterodimeric signaling complexes. STAT1/STAT2 heterodimers are stimulated by the classic IFN-α/β signaling pathways; this leads to the expression of subset of genes, controlled by promoters containing IFN stimulated response elements (ISRE) (Fig. 9).

These pathways and signal transduction leads to the different antiviral activities like disrupting viral RNA, instability of viral RNA, transcription and translation. The exogenous IFN has the same action like endogenous produced IFN.

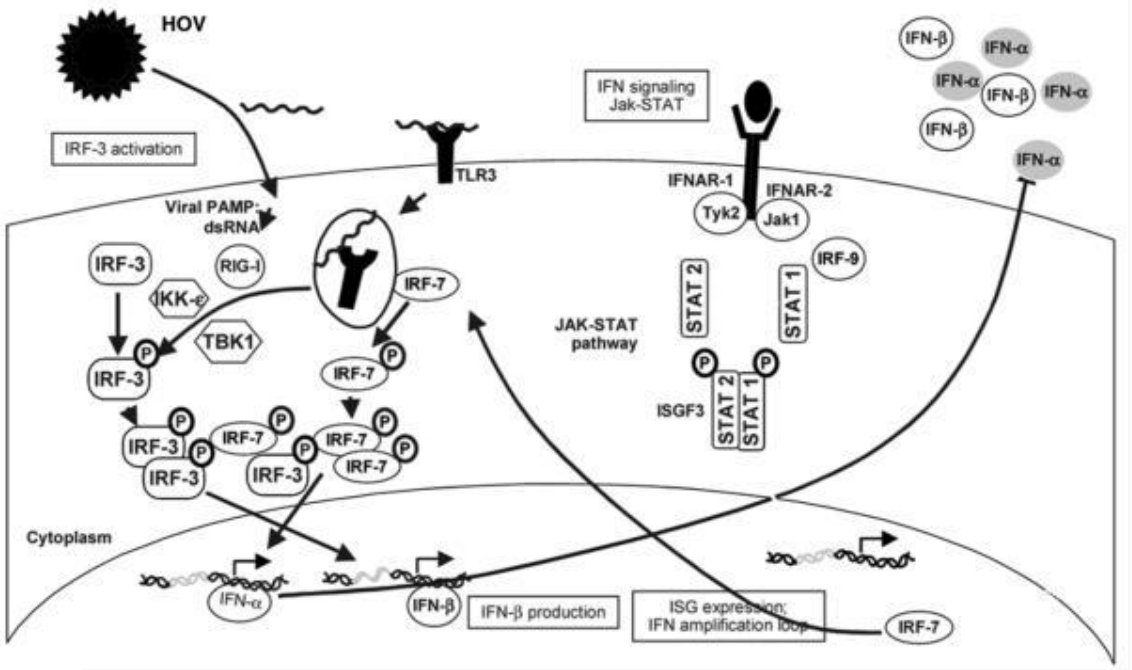

Fig 9: The host innate response to HCV infection.

HCV, hepatitis C virus; dsRNA , double-stranded RNA; IRF, interferon regulatory factor; IFN, interferon; ISG, interferon-stimulated gene; IKK-ε, I kappa B kinase ε; Jak, Janus kinase; RIG-I, retinoic acid-inducible gene I; PAMP, pathogen-associated molecular pattern; STAT, TLR3, toll-like receptor 3; signal transducer and activator of transcription; and Tyk2, tyrosine kinase 2; TBK1, TANK-binding kinase 1 [160].

In 1998, Ribavirin as an adjuvant to IFN was approved for the treatment of hepatitis C. The discovery of Ribavirin, a nucleoside analogue was the second improvement in hepatitis C treatment. Ribavirin was known to have activity against several Flaviviruses. When it came to know that HCV belongs to Flaviviruses then no hesitations were there in treating HCV with Ribavirin. The effects of using Ribavirin were the reduction of ALT level and improving the histological features of the liver but its effects were found low on serum HCV-RNA levels [161].

Moreover, when ribavirin was combined with IFN then it increased SVR [162]. The combined effect of using IFN and Ribavirin when given for 48 weeks was three times more than that of IFN alone and the SVR was 40-50%. [163].

1.6.2 Ribavirin: Mechanism of action in chronic hepatitis C

There are several hypotheses that can explain mechanism of action against HCV as is depicted in Figure 10:

(1) Ribavirin has direct antiviral action against RNA-dependent RNA polymerase;

(2) Intracellular GTP pools depletion because of its action as an inhibitor of inosine monophosphate dehydrogenase (IMPDH);

(3) Induction of mis incorporation of nucleotides mis incorporation by the viral RNA polymerase induction that causes mutation and non lethal virus production and

(4) Host T cell-mediated immunity induction against viral infection by switching the T cell phenotype from type 2 to type1 [161].

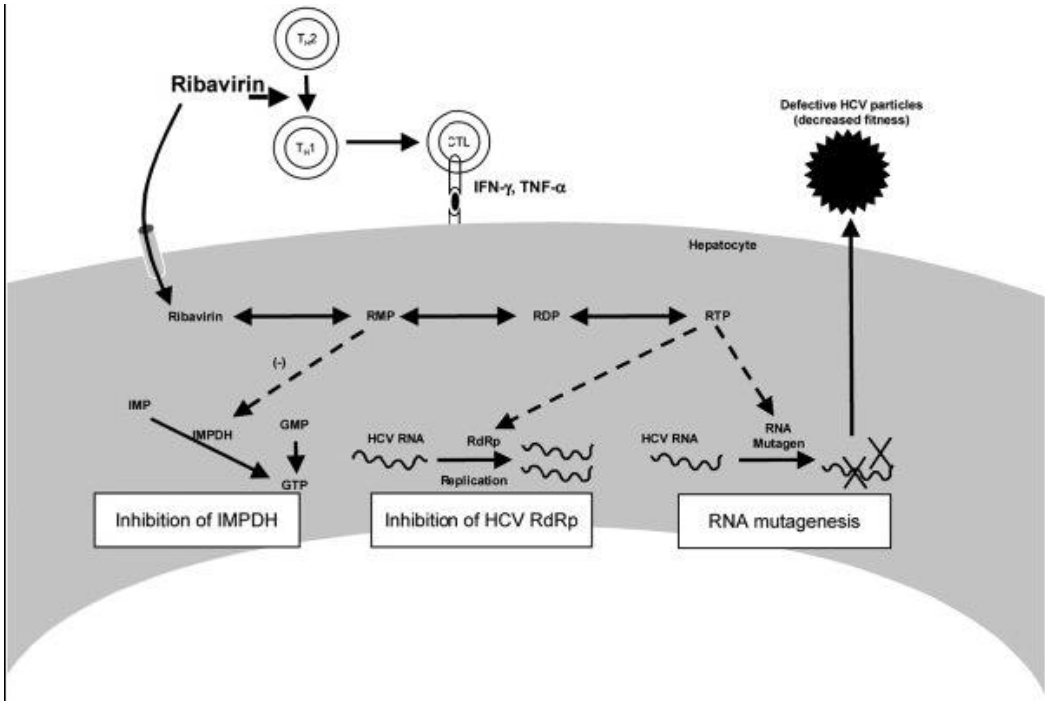

Fig 10: Mechanism of action of Ribavirin.

IFN,interferon; GMP, guanosine monophosphate; IMPDH, inosine monophosphate dehydrogenase; , RDP, ribavirin diphosphate; RdRp, RNA-dependent RNA polymerase; GTP, guanosine triphosphate; RTP, ribavirin triphosphate; RMP, ribavirin monophosphate; Th2, T helper 2 and Th1, T helper 1 [163]

In 2001, Peg-IFN was approved for the treatment of hepatitis C in the US. Introduction of Peg IFN was the third improvement in therapy of hepatitis C with reduced dose that is once in a week rather than thrice a week injections. The response rate was 45-55% when using peg-interferon and ribavirin for 48 weeks which is comparatively higher than that of standard IFN therapy [21]. Response rate varies according to HCV genotypes. The sustained response rate in HCV genotypes 2 and 3, when treated with 24 week course of peg-interferon and reduced dose of ribavirin was 70-80% [21]. Comparatively response rate was low ranging from 40-50 % with 48 weeks of full dose of ribavirin. No doubt the increase in the sustained response rate was found with higher doses and prolongs treatment duration but associated side effects were also increased with such treatment strategies [161].

Other advanced research drugs designed include:

Telaprevir was designed according to HCV molecular structure and is a specific HCV protease inhibitor [164]. This molecule has great similarities with the polypeptide of HCV which is cleaved by the viral proteases, a necessary step in HCV replication. The effects of using telaprevir molecule found profound in cell culture and in animal models on HCV replication [164]. It has been found that with the new combination of telaprevir and peg-interferon, the effect against viruses was more profound and less chances of viral resistance were there [165, 166]. Telaprevir a crucial improvements in the treatment of hepatitis C, started an era of specifically acting antiviral agents, developed against hepatitis C. Some others agents which are specific to HCV like other protease inhibitors, helicase and polymerase inhibitors and viral replication interfering molecular agents, [167, 168] are likely to follow. The desired goal of hepatitis C treatment can be achieved by combination of these new agents with currently available drugs so as to provide effective therapy for the hepatitis C patients.

1.6.3 Predictive Factors of Interferon Treatment Response

The efficacy of IFN based therapy depends on the dynamic interactions between the virus and host factors in chronic HCV infection treatment. The virus related factors influential in the treatment of HCV are virus titer, viral genotype and viral kinetics on treatment. The host factors associated with response to treatment are age, race and gender. Beside this host genetic factors are also playing role in response to therapy. It has been noted that HCV viral load is the determining factor in response to therapy either monotherapy or combination of IFN and Ribavirin and also combination of Ribavirin with pegylated IFN [169]. Patients having Low viral load (less than 2*105 copies/ml) are more likely to have SVR. In such case the response rate could reach 60-70%. However the rate even dropped to about 11% in patients having higher viral load. Different HCV genotypes have different response rate in HCV infected patients. Patients having genotype 1 and especially genotype 1b have the lowest response rate to therapy. The response rate in HCV genotype 2 and 3 infection predicts significantly higher than genotype 1 infection [170, 171]. Some host factors also predicts the response efficacy as well. Studies have shown that some host factors predict HCV treatment response as well. African Americans showing lower response to antiviral therapy receiving combination IFN plus Ribavirin or combination peg-interferon plus ribavirin [172]. In patients having age less than 40 years were identified as better responders and for long duration of time to IFN therapies [173]. The association of age and IFN therapy response was more evident in women than in men. It has been shown by a study in Japan that age and gender interaction was associated with complete response to IFN therapy [174]. Still it is not clear that patients having same genotype, same viral load, same age and genders but having different response rates.

It is likely that host genetic constituents may play an important role in viral clearance, disease outcome and treatment response. .

1.6.4 Viral kinetics of interferon-based therapy

IFN based therapy usually produces a biphasic decrease in serum HCV-RNA level: first phase is characterized by a decrease in HCV-RNA level by1–2 $\log_{10}$ IU/ml and lasting for approximately 1-2 days in genotype 1 infected patients. It is followed by second phase of decline which is characterized by further decrease in HCV-RNA level as much as 3–4 $\log_{10}$ IU/ml in genotype 2 of HCV infected patients [175], but variation exist because some time there is an initial decline followed by an intermediate increase in HCV-RNA level. In some patients, reduction in HCV RNA occurs rapidly but after some time, rebound occurs in HCV-RNA level. Some time there is no first phase or second phase decline and hence no response to therapy (null response) or alternatively having first phase decline followed by little or no second phase decline (flat response) [176].

It is stated that initially IFN causes the blocking of viral replication in the rapid phase of viral decline and the efficacy of IFN depends not only on the treatment regimes but also on HCV genotype. The patients showing response to IFN, the second phase of decline reflects the clearance and net loss of the infected cells. This at end leads to further reduction in overall viral production and RNA levels [175].

The overall pattern of viral response could be used as an indication for the success of treatment or it can help in making decision about the treatment duration in case of IFN therapy. This is helpful in determining an SVR, which is the primitive goal of HCV treatment. It has been shown that patients who fail to achieve an early virologic response (EVR), that is as either absence of HCV-RNA or a decline in HCV-RNA levels of at least 2 $\log_{10}$ IU/ml after 3 months treatment, are difficult to have an SVR,

the negative predictive value in such case is around 97% [20] , this type of study help in establishing 12 weeks stopping rule in case of HCV genotype 1 patients [20]. In comparison to rapid virologic response (RVR), which is the absence of HCV-RNA in serum (<50 IU/ml) at 4 weeks of treatment, has positive predictive value and are considered to have an SVR according to treatment-related viral kinetics [177].

Table 3: Selected IFN-based therapies for the treatment of HCV infection

Drug name	Clinical phase
Monotherapy	
Intron A (IFN-α2b, recombinant)	FDA approval, 1995
PEG-INTRON (PEGylated IFN- α 2b)	FDA approval, 2001
Roferon A (IFN- α 2a, recombinant)	FDA approval, 1996
Pegasys (PEGylated IFN- α 2a)	FDA approval, 2001
Infergen A (IFN alfacon-1)	FDA approval, 1997
Wellferon (lymphoblastoid IFN- α n1)	FDA approval, 1999
Omniferon (natural IFN- α)	Phase II
Omega IFN (IFN- α)	Phase II
Combination therapies	
Rebetron (Intron A and ribavirin)	FDA approval, 1998
PEG-INTRON and ribavirin	FDA approval, 2001
Pegasys and ribavirin	FDA application submitted
Intron A and Zadaxin (α 1-thymosin)	Phase III
Pegasys and Ceplene	Phase III
IFN- α and EMZ701	Preclinical

FDA approval for the treatment of relapsing forms of multiple sclerosis. HCV, hepatitis C virus; IFN, interferon; PEG, polyethylene glycol [178].

Table 4: Currently pipeline drugs for hepatitis C and related treatments

Target	Drug name	Mechanism	Clinical phase
IRES	ISIS 14803 Heptazyme	Antisense, Ribozyme	Phase II Phase II*
NS3	BILN-2061 VX-950/LY-570310	Serine-protease inhibitor Serine-protease inhibitor	Phase II Preclinical
NS5B	JTK-003	RdRp inhibitor	Phase I/II
E1	Not known; a recombinant E1	Therapeutic vaccine	Phase IIa
E2	XTL-002	Monoclonal antibody	Phase Ib
IMPDH	VX-497 Levovirin Viramidine	IMPDH inhibitor IMPDH inhibitor IMPDH inhibitor	Phase II Phase I Phase I
Liver fibrosis	Actimmune (IFN-γ) IP-501	Antifibrotic Antifibrotic	Phase II Phase III
Liver apoptosis	IDN-6556	Caspase inhibitor	Phase II
HCC	T67	tubulin inhibitor	Phase III
HCV reinfection	Civacir CellCept	HCV IgG Immunosuppressant, (mycophenolatemofetil)	Phase I/II Preclinical
Target unknown	Ceplene (histamine dihydrochloride) Zadazin (thymosin α-1) Symmetrel (amantadine Hydrochloride)	Immune modulator Immune modulator Broad antiviral	Phase II Phase III Phase IV

* Suspended pending toxicology investigation. HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IgG, immunoglobulin G; IMPDH, inosine monophosphate dehydrogenase; IRES, internal ribosome-entry site; NS, non-structural protein; RdRp, RNA-dependent RNA polymerase [178]

Aims and objectives

Aims and objectives of this study were to find out

1: Rate of active HCV infection among confirmed anti-HCV patients of KPK.

2: Frequency distribution of HCV genotypes among chronic HCV patients.

3: Response rate of conventional IFN and Ribavirin combination therapy in chronic HCV patients, (General response) and

4: Genotype specific response of conventional combination therapy in active HCV infected patients of KPK.

CHAPTER 2

MATERIALS AND METHODS

List of Abbreviations

PGMIIRE:	Post Graduate Medical Institute Institutional Research and Ethics Board
ICT:	ImmunoChromatographic Techniques
ELISA:	Enzyme Linked Immunosorbant Assay
PCR:	Polymerase Chain Reaction
KPK:	KhyberPakhtunKhwa
GDP:	Gross Domestic Product
RPM:	Revolution Per Minute
HRP:	Horseradish peroxidase
IC:	Internal control
Ct:	Threshold cycle
NTC:	Non-template controls
5’ UTR:	5’ Untranslated Region
RT-PCR:	Reverse Transcription PCR
IFN:	Interferon
MIU:	Million International Unit
ALT:	Alanin Transaminase
CBC:	Complete Blood Count

This study has been approved by the Post Graduate Medical Institute Institutional Research and Ethics Board (PGMIIRE), Annex 1.

2.1 Area Selection

Area selection was mainly based upon the status of the health care infrastructure, level of awareness among the masses and previous history of epidemiological surveys. As KPK is home to relatively economically poor population having access to very few health care facilities and several districts of the province have never been investigated for HCV infection at Molecular level, therefore the province was selected for this study.

The total area of KPK province is about 28,773 mi^2 or (74,521 km^2) and has been divided into 25 districts. The population of this province was approximately 17 million, according to the 1998 census, of whom 52% are males and 48% are females. Among these, the largest ethnic group is Pashtun (ethnic afghan). Besides this, about 1.5M Afghan refugees are also living in this province. The total shares of the KPK in Pakistan's GDP is about 10.5%, although the population of KPK accounts for 11.9% of Pakistan's total population, rendering it the second-poorest province after neighboring Balochistan. The literacy rate is increasing day by day, in 1972 the literacy rate was about 15.5% and in 2008, the rate has been jumped upto 49.9% .The location of various districts and the borders of the KPK province are shown in Figure 2.1.

Fig 2.1: The map and borders of the KPK Province

2.2 History and Data Collection

The demographics and other relevant history of the patients were recorded in a proforma. A standard proforma annix-II was developed with the help of researchers, clinicians and epidemiologists. The proforma contained informations about the name of patient, age, gender, address, treatment and risk factors etc.

2.3 Sample collection and serum isolation

In case of each subject, 5ml of blood sample was collected in a sterile Gel tube. Waited for ten minutes and then serum was isolated from each blood sample by centrifugation at 8000 rpm for 5 minutes. Sera were stored at -20 $C^{o.}$

2.4 Screening of the samples

2.4.1 Serological Methods

2.4.1.1 Immuno Chromatographic Techniques

Screening for anti-HCV positive samples was carried out with the help of Immuno-Chromatographic Assay (Accurate, USA). ICT was used to detect antibodies against HCV in human serum. 10 µl serum was applied on to the sample well and two drops of sample diluent was added immediately. Appearance of colored test band was considered as positive for anti-HCV in a particular sample.

Principle of ICT

This is based on antigen-antibody reaction; Recombinant antigen is coated in the sample pad of the strip (ICT Device), having high reactive region of HCV. Serum antibody when allowed to migrate along the strip by putting serum sample into the well of the strip. The antigen-antibody complex is formed showing a color test band in case of positive sample while negative sample does not produces the band.

2.4.1.2 ELISA

Third generation ELISA is more sensitive for the detection of anti-HCV in serum as compared to ICT procedures. In this study, we used 3rd generation ELISA (Monobind, USA) to detect anti-HCV among the ICT positive samples, according to the manufactures instructions. Briefly, the procedure adopted was as follows:

1: Serum was added to the wells of the plate containing coated antigen and incubated for 10 minutes.

2: The wells were washed during the second step in order to remove unbound serum proteins.

3: Added anti-anti bodies conjugated to horseradish peroxidase (HRP) to each well of the plate and again incubated for 5 minutes.

4: Washed the plate with washing solution in order to remove unbound HRP-conjugate.

5: Added coloring agent into the wells of the plate and measured the intensity of color. The quantity of antigen in a sample was measured by the intensity of color which appeared in anti-HCV positive samples.

2.4.2 Confirmation of active HCV infection by Molecular Methods

Simply 5-ml blood was taken from anti-HCV confirmed patients. About 3075 patients were included in the study. Sera were isolated by centrifugation at 8000 rpm for 5 minutes and was stored at -20C^{o}. RNA was extracted from the sera by following instructions of column based extraction kit (Roboscreen, Germany). PCR was performed for each sample by using Real time PCR (Miniopticon, Bio-Rad, USA) and Roboscreen amplification kit having internal control for each sample in order to rule out false negative results. We analyzed the results by calculating the Ct values for each sample. The Ct value less than 40 were considered as positive and more than 40 were considered as negative for HCV

The protocols adopted for RNA extraction and Real-time amplification were as follows:

2.4.2.1 Procedure for RNA extraction

RNA extraction from serum samples was carried out using the following procedure as recommended by manufacturer (Cat.No. 0209200503). The contents of the kit are given in annex-III.

- Opened the Extraction Tube and added 450 µl lysis solution and 150 µl of the serum sample, mixed vigorously for 10 sec. Incubated at room temperature for 15 minutes with continuous vortexing for 3-4 times. After incubation, extraction Tube was centrifuged shortly to remove condensate from the lid of the tube.
- Added 600 µl Binding Solution to the lysed sample and mixed by vortexing to get a homogeneous solution.
- Applied 650 µl of the sample to the Filter located in a 2.0 ml Receiver Tube. Closed the cap and centrifuged for 1 minute at 10,000 rpm.

- Discarded the tube along with the filtrate. Placed the filter in to a new 2.0 ml tube and loaded the residual sample onto the filter. Closed the cap and centrifuged for one minute at 10,000 rpm.
- Again took a new tube and placed the filter into that.
- Added 500 ul washing solution 1 onto the filter having bonded RNA and centrifuged for 1 minute at 10,000rpm.
- After the 1st washing, added second washing solution 2 onto the filter and again centrifuged for 1 minute at 10,000 rpm.
- Finally centrifuged the tube having filter at maximum speed for 5 minutes in order to remove all traces of ethanol.
- Took elution tube and placed the filter into that and added 50 ul RNase-free water. Allowed to incubate for 3 minutes and then centrifuged for 30 seconds at 8,000 rpm.
- The final filtrate contains RNA which may be stored and used for down stream applications.

2.4.2.2 Amplification and detection

After RNA extraction, the next step was amplification. In Real time PCR amplification and detection is done simultaneously in a single step. Real time PCR technology is more superior than that of conventional PCR as this does not require post PCR step that is Gel Electrophoresis. Real Time PCR is basically designed to calculate the initial viral load that is shown as Ct value while conventional PCR does the end point analysis. In performing Real Time PCR, there are two steps that are plate setup and thermal protocols but before performing PCR, there is need of master mix preparation which was prepared by using Roboscreen detection module (Cat.No. 0203000171) and the contents of it are given in annex-III.

Master Mix preparation and Plate setup

Before master mix preparation, prepared stock solution of the primer probe by adding 40 ul PCR grade water to the amber vial containing lyophilized primer and probes. Incubated for 20 min at 37 C^{o} before using. Took sample and standard strips and prepared master mix as is given in Table 2.1.

Added 20 µl aliquots of master mix to each well of 0.2ul PCR reaction tubes, of the control and sample strips and 5 µl aliquots of PCR grade water to the coated standard and negative control tubes and 5ul RNA samples to each well of the sample strip. Each strip has been numbered according to each sample. Closed the cap of the strips by means of flat caps and placed the sample and control strips into the block of Real Time PCR machine. Selected the wells that contained the controls ("standards"), non-template controls ("NTC") and samples ("unknown"). Entered a number or text that identifies the sample or control and entered a number that represents the quantity of the controls. Selected the detector dyes "FAM", "Yakima Yellow" (VIC/JOE channel), both quenched by dark quencher, and the passive fluorescence dye ROX.

Set up the thermal conditions as shown in Table 2.2 and started the run.

Table 2.1: Pipetting scheme for the preparation of HCV/IC Master Mix

Reagents	**Color coding of vials**	**HCV/IC master mix [µl]**	
		1 reaction	**Final concentration**
PCR grade water	Clear vial/clear cap	2.0	-
10x Passive reference dye Solution	Amber vial/ green cap	2.5	1x
2x Reaction mix (buffer containing RT-PCR buffer, 6 mM-sulfate and dNTP)		12.5	1x 3.0 mM Mg-sulfate
Mg-sulfate (50 mM)		1.0	2.0 mM Resulting final Mg-sulfate Concentration 5.0 mM
25x reagent mix (containing primers and probe)	Amber vial/amber cap	1.0	1x
RT-PCR Enzyme Mix		1.0	

Table 2.2: Thermal profile for the Real Time PCR

iCycler, DNA Engine ,Opticon 2 Real time PCR	RT	HOLD	CYCLE
Temperature (c^{o})	59	95	95 57
Time (min:s)	60:00	2:00	00:20 01:00
Cycles			45

Detection

Detection of sample was done at the exponential phase of amplification curve using threshold cycle value (Ct value). The sample crossing Ct line was considered positive while sample failing to cross Ct line was considered as negative for HCV RNA as shown in Figure 2.2. In order to rule out false positive and negative results, an internal control was run with each sample. Sample and internal control was detected by FAM and VIC channel respectively.FAM and VIC are the dyes, attached to Probe (Taq Man Probe), that are specifically designed for HCV targeted sequences. These have their specific wavelengths at which these are detected by the Real Time PCR camera (detector).

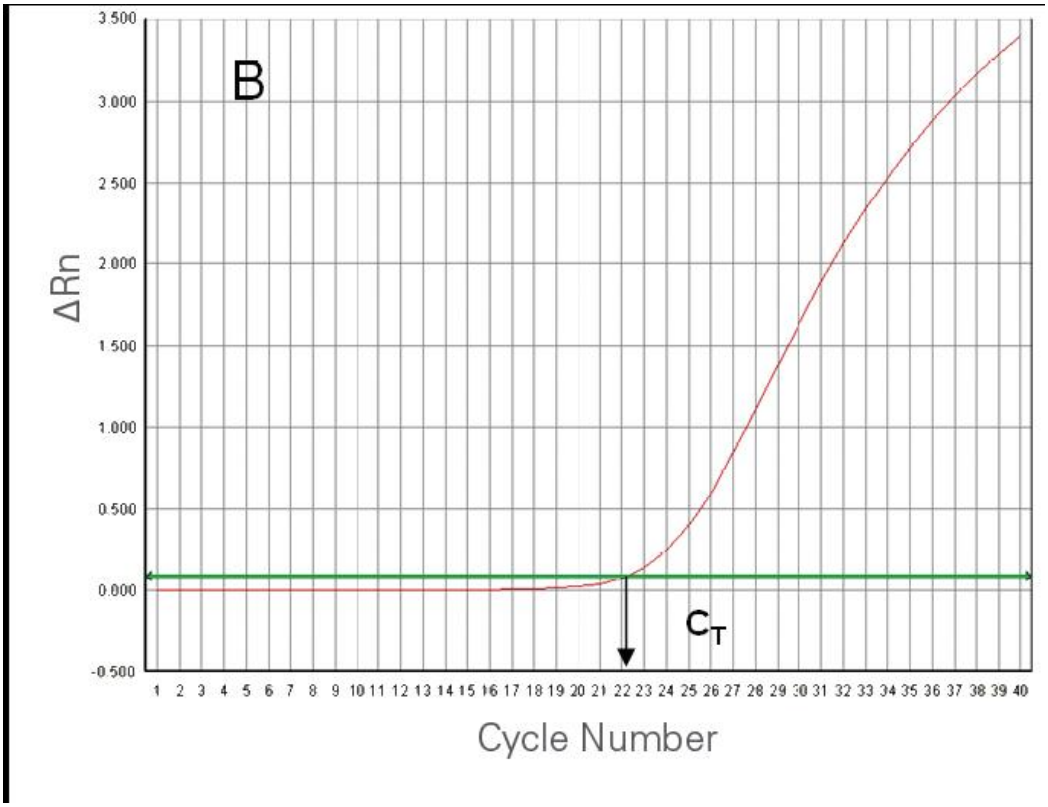

Fig 2.2: PCR Amplification curve representing Ct (Threshold cycle) value.

2.4.3 Determination of HCV genotypes.

HCV genotyping was carried out for the determination of different genotypes in different geographical regions of KPK as well as for the evaluation of HCV genotype specific response to Interferon based therapy. Samples were collected from patients chronically infected with HCV. None of the patients selected for the study had started with anti-viral therapy. All the patients duely signed a proforma containing their demographics, previous history of viral infection etc. Serum was isolated and extraction of RNA was carried out with spin column based extraction kit (Roboscreen, Germany) according to the manufacturer's instructions. HCV RNA was amplified using reagents and equipment from the Cepheid (Cepheid, USA).

HCV genotyping was carried out according to Ohno et al. [179]. The procedure adopted was as follows.

For determination of different HCV genotypes in the serum samples, reverse transcription-PCR (RT-PCR) was used for the amplification of HCV RNA. RT-PCR was based on the highly conserved HCV 5' UTR genomic region. Primers and PCR conditions used for HCV genotyping of HCV isolates in this study have earlier been described by Ohno et al. (1997). Briefly, HCV RNA was extracted by following manufactures instructions as discussed above [2.5.1.1] (Roboscreen Germany). The RNA was Reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (MMLV) (Fermentase USA), with random hexamer (GIBCO BRL, Gaithersburg, Md.) The Master Mix and thermal protocols used were as follows:

Master Mix

5x first strand buffer	4ul
dNTPs (100mM)	2ul
primers (Sense and Antisense)	1ul
M-MLV (20u/ul)	1ul
RNase inhibitors	0.5ul
Nuclease free water	1.5ul
RNA	10
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Total	20ul

Thermal protocols for RT-PCR was as follows

42C^{o}	60 min
95 C^{o}	2 min
4 C^{o}	10 min

The resultant cDNA was stored at -20 C^{o} before being proceeded for the first round PCR.

First round of PCR

First round of PCR was performed by using Sc2 and Ac2 primers (Table 2.3) and the resultant cDNA obtained during reverse transcription. The master mix and thermal Protocols used were as follows

Master Mix

10 x PCR buffer	5 ul
MgCl2 (50mM)	1.5 ul
Taq DNA polymerase 4u/ul	0.4 ul

Primers sense and antisense	1 ul each
Nuclease free water	9 ul
cDNA	2 ul
……	
Total	20 ul

Thermal protocols

Preliminary 20 cycles of amplification were carried out at 94C for 1 min, 45C for 1 min and 72C for 1 min, followed by 20 additional cycles of

94C^{o}	1 min
60C^{o}	1 min
72C^{o}	1 min

Type specific second round PCR

The first round PCR product was amplified using two primer mixtures, Mix A and Mix B. The contents of mixtures, Master Mix and thermal protocols are given as

Mix A or Mix B:

Mix A: S7, S2a, G1b, G2a, G2b, and G3b primers (Table 2.3)

Mix B: S7, G1a, G3a, G4, G5a, and G6a primers (Table 2.3)

Master Mix

10x PCR buffer	5ul
MgSo4	1.5ul
Taq DNA polymerase (4u/ul)	0.4ul
Primers Mix A or Mix B	1ul each
Nucleas free water	9ul
First round PCR product	0.5ul
……..	
Total	20 ul

Thermal protocols

30 cycles amplification

94C^{o}	1 min
62C^{o}	45 s
72C^{o}	1 min

Detection

About 8ul of second round PCR products were run on agarose gel prestained with Ethidium bromide, along with 50 bp DNA ladder (Gibco BRL). Amplified PCR products in the case of various HCV genotypes were recognized with the help of the genotype-specific PCR product key (Table 2.4) [179].

Table 2.3: Oligonucleotide primers used for PCR and genotyping [179]

Primer[a]	PCR Round[b]	Sequence (5'-3')[c]	Nt position
Sc2	1	GGGAGGTCTCGTAGACCGTGCACCATG	224–3
Ac2	1	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	417–391
		Mix A	
S7	2 g	AGACCGTGCACCATGAGCAC	212–8
S2a	2 g	AACACTAACCGTCGCCCACAA	40–60
G1b	2 g	CCTGCCCTCGGGTTGGCTA(AG)	222–203
G2a	2 g	CACGTGGCTGGGATCGCTCC	178–159
G2b	2 g	GGCCCCAATTAGGACGAGAC	325–306
G3b	2 g	CGCTCGGAAGTCTTACGTAC	164–145
		Mix B	
S7	2 g	AGACCGTGCACCATGAGCAC	212–8
G1a	2 g	GGATAGGCTGACGTCTACCT	196–177
G3a	2 g	GCCCAGGACCGGCCTTCGCT	220–211
G4	2 g	CCCGGGAACTTAACGTCCAT	87–58
G5a	2 g	GAACCTCGGGGGGAGAGCAA	308–289
G6a	2 g	GGTCATTGGGGCCCCAATGT	334–315

a: For the naming of primers, S sense, A or G antisense, and C core region;

The notations 1a to 6a are in accordance with the HCV genotype nomenclature proposed by Simmonds et al. [11]. Numbering is from the authentic start codon of the open reading frame.

b: 2g, second-round PCR for genotyping.

c: Pairs of nucleotides inside parentheses are degenerate nucleotides.

Table 2.4: Genotypes and their respective product size [179]

S. No	Genotypes/subtypes	Product size
1	1a	208bp
2	1b	234bp
3	2a	139 or 190bp
4	2b	337bp
5	3a	232bp
6	3b	176bp
7	4	99bp
8	5a	320bp
9	6a	336bp

2.5 Response rate of combination therapy among chronic HCV patients in KPK (1st Trial).

To evaluate the response of standard IFN-combination therapy against chronic HCV infection, we selected four different regions of KPK province. The regions were district Bunir, district Peshawar, district Mardan and FATA region. Samples were collected from the suspected patients. After initial screening with ICT and ELISA, PCR test was performed for each patient sample according to instructions of manufacturers (Roboscreen Germany). Among the confirmed anti-HCV patients only 174 patients whose PCR was positive, were selected for IFN therapy keeping in mind the exclusive therapy criteria that is, the age of the selected personals should not exceeds 60 years, having no co-infection with either HIV or HBV, having high ALT level, Complete Blood count (CBCs) should be in normal values and having no cirrhosis. We selected 52 patients from Bunir, 22 from Peshawar, 74 patients from Mardan and 18 from FATA regions.

PCR positive patients were given standard IFN combination therapy i.e. IFN alpha 2a thrice a week with a dose of 3 MIU and Ribavirn with a dose of 800-1200mg/day of the body weight. This combination therapy was continued for six months with repeated testing of ALT level during IFN therapy. After completion of 72 injections, PCR test was repeated for all of the patients' samples.

2.6 Response rate of combination therapy among chronic HCV Patients in KPK (2nd trial).

In the second trial of IFN based therapy, we selected district Swabi, district Bunir and district Kohat. In these districts, we randomly selected patients of different age and sex from different villages. A total of 198 patients were selected from district Swabi, 54 from Kohat and 89 from Bunir for standard IFN and Ribavirin combination therapy.

All the patients were subjected to screening (ICT and ELISA) process followed by confirmation of active HCV infection by PCR. Before starting therapy, all the essential tests including CBCs, LFTs, ultrasound and the exclusion criteria that is previous history of non response, cirrhosis, age etc was duly considered. All the patients were given 3MIU dose of standard IFN thrice a week and Ribavirin with a dose of 300-400mg thrice a day depending on the weight of the person. This therapy combination was continued for six months with repeated investigations of CBCs and LFTs during and at the end of course. PCR test was also repeated during (3months) and at the end of treatment (six months).

After completion of six months therapy and confirmation of IFN response by PCR, the responders and non responders were separated. All the non responders from Swabi district were again treated with the same IFN but with the different dose and duration. This time the dose of IFN was 6MIU and for extra two months. Again at the end of two months PCR test was repeated followed by analysis of all the enrolled patients.

2.7 Genotype specific response of combination therapy among chronic HCV patients in KPK.

To evaluate the response of standard IFN against specific HCV genotypes, we selected 51 anti-HCV and PCR confirmed patients from different regions of KPK. Blood samples were taken from all patients followed by serum isolation. We determined genotype of each of the patient sample by using type specific PCR method as discussed above (2.4.2.2).

All the patients were given standard IFN and Ribavirin combination therapy. Therapy was continued for six months with a dose of 3MIU standard IFN thrice a week and Ribavirn with a dose of 800-1200mg/day. At the end of six months of treatment, PCR test was done for each of the patient's sample followed by analysis of results in the case of all enrolled subjects.

Results and Discussions

Molecular epidemiological studies have earlier revealed that there was considerable genetic heterogeneity among the Pakistani HCV isolates [98]. As the response rate of IFN based therapies depend upon the type of virus, ethnic background and initial viral load [23], therefore it was very important to investigate the response rate of combination therapy in Pakistani population. Prevalence studies described earlier are limited in scope either because of regional limitations or because of the limited value of anti-HCV determination as compared to active HCV infection. Majority of the studies from various geographical regions have reported anti-HCV among the general population or specific groups [99-107]. In this study, we have targeted comparatively larger population groups in various districts of the KPK province in order to figure out active HCV infection among the population.

3.1 Rate of active HCV Infection among chronic HCV patients in KPK.

As screening tests are associated with false positivity and could not confirm the viremia, therefore we carried out our study by using Real time PCR technique for confirmation and screening out of viremia. Out of 3075 anti-HCV positive samples, HCV RNA positive and negative samples were 2055 (66.6%) and 1020 (33.30%) respectively, while male and female positive samples were 1200 (57.67%) and 855 (42.43%) respectively (Table 3.1).

Our study revealed that the rate of active HCV infection was found higher in district Bunir (72%) followed by districts Dir (70.06%) and Mardan (69.06%), while low active HCV infection was found in district Swabi (66%) followed by districts Peshawar (64.06%) and Kohat (59%) (Table 3.1).

Determination of viremia is important for early decision about whether a patient should take the IFN-based therapy or not. Untimely diagnoses and treatment in the case of chronic HCV

infection have serious complications as the infection leads to cirrhosis, hepatocellular carcinoma and finally death [3]. Therefore it is necessary to investigate viremia among confirmed anti HCV patients by using PCR techniques as anti-HCV test is not informative about active infection.

Hepatitis C infection is spreading rapidly due to poor and unsatisfactory health care conditions and its prevalence is very high in the general population of Pakistan [34]. In KPK, where the health care facilities are poorly equipped with essentials for screening and Sterilization, HCV has become an economic burden over a population with considerable number of people living below the poverty line.

In KPK, according to our observations, internationally approved treatment and diagnostic procedures are not properly followed. As anti-HCV positivity and elevated Liver Function Tests (LFTs) are least informative about whether a person is actively infected, so according to the accepted norms, the patients should not be subjected to antiviral treatment before confirmation of the active infection. Lack of facilities and knowledge about the proper diagnosis and treatment of HCV infection, both on the part of the general population and physicians is contributing towards the gravity of the problems in KPK.

In the current study active HCV infection was found in different districts of KPK among confirmed anti-HCV patients. The average viremia rate (66.6%) was found in these selected districts. The viremia rate was high in district Bunir (72%) followed by districts Dir (70.06%) and Mardan (69.06%). The low viremia rates were present in the people of districts Swabi (66%), Peshawar (64.06%) and Kohat (59%). Among the infected individuals active HCV infection was more common in male as compared to female [Table 3.1].

High viremia rate (66.06%) determined in this study was an indication of high prevalence of active HCV infection among anti-HCV positive subjects .The percent positive frequency noted was higher in district Bunir followed by district Dir and district Mardan [Table 3.1]. This is almost in agreement with the previous hospital based study about the general prevalence of anti-HCV positivity in districts Bunir [104] and Mardan [31], showing high HCV infection in these two districts. High infection rate in district Bunir may be attributed to high rate of dental surgery [98]. In district Dir, previously no study has been conducted to determine HCV prevalence and we, for the first time, determined rate of chronic HCV infection among confirmed anti-HCV patients. High viremia rate in these districts may be attributed to low literacy rate and hence less awareness about the spread of HCV infection and its pathogenesis. This was revealed by a census conducted in 1998, that literacy rate was lowest in district Bunir (22.60%) and Dir (29.90%). While literacy rate found in district Mardan (36.45%) was comparatively higher than both of districts Bunir and Dir. Comparatively less positive percent frequency of active HCV infection was found in districts Swabi, Peshawar and Kohat respectively [Table 3.1]. Previously no study was conducted on the general prevalence rates in the above-mentioned districts. Among these districts the highest literacy rate is that of Kohat (44.06%) followed by district Peshawar (41.79%) and Swabi (36.00%). Accordingly the lowest active HCV infection was found in district Kohat followed by Peshawar and Swabi. This reflects the awareness about the transmission and prevention of HCV infection in these mentioned districts. It was also noted that Chronic HCV infection was high in male as compared to female [Table 3.1], which has also been shown by different studies.

It is evident from ours and some previous studies that active HCV infection frequency is high in our country, to identify people who have active HCV infection there is need of efforts

nationwide. Awareness and Mass education of the people about the spread of HCV infection through blood transfusion, repeated usage of the same syringe and reuse of the medical and dental equipments, gloves and other instruments, tattooing, piercing of ear and nose from the general market and shaving from the barbers etc is very crucial. In rural areas of our country prevalence of HCV infection is alarmingly high due to lack of healthy resources and less trained medical personals. Hence there is need to prevent this infection by creating awareness and health education among the general public. This job should be performed by the involvement of electronic as well as print media, locally adopted health educator's programmes, Non Government Organizations (NGOs) and religious scholars. To save our generations' awareness programs regarding Hepatitis C at school level should also be started.

As there is lack of national database about HCV prevalence, further studies will be helpful in determining the areas of high HCV prevalence, at individuals and at districts levels. In return this will be helpful to determine risk factors responsible for transmission of infection.

Table 3.1: District wise distribution of Chronic HCV RNA positive and negative samples.

Districts	**Total Samples**	**Positive Samples**	**Negative Samples**	**Male Positive**	**Female Positive**
Peshawar	1587	1016 (64.04%)	571 (35.91%)	598 (58.8%)	445 (41.14%)
Mardan	514	366 (69.06%)	148 (31.04%)	221 (60.3%)	145 (39.61%)
Bunir	378	275 (72%)	103 (28%)	165 (60%)	110 (40%)
Kohat	157	93 (59%)	64 (41%)	51 (54.8%)	42 (45.16%)
Swabi	126	84 (66%)	42 (34%)	49 (58.3%)	35 (41.16%)
Dir	313	221 (70.06%)	92 (29.04%)	116 (52.4%)	105 (47.51%)
Total	3075	2055 (66.6%)	1020 (33.30%)	1200 (57.67%)	855 (42.43%)

3.2 Frequency distribution of HCV genotypes among chronic HCV patients in KPK.

For determination of different HCV genotypes we used type specific method of detection. This method is based on sequence specific primers and can detect 11 types and subtypes of HCV. We determined genotype frequency in different geographical regions of KPK.

HCV genotype analysis in chronic HCV patients (age 15-60 years) of Khyber Pakhtunkhwa revealed that the most abundant genotypes/subtypes among the patients analyzed were 2a followed by subtype 3a (Table 3.3). Other common genotypes included the untypable type of the virus and genotype 3b. Comparatively less common genotypes were 1b, 1a and 2b (Table 3.3). Major risk factors associated with HCV infection were blood transfusion and Dental surgery followed by major surgery (Table 3.2). The patient's history indicated that comparatively more patients had a history of blood transfusion in Peshawar and Mardan districts while a history of dental surgery practices was common in less developed District Bunir. Moreover, district Bunir and Peshawar had more cases of a positive family history of HCV infection as well. In the studied individuals, there were no co-infections. It was also noted that the overall prevalence of chronic HCV was more in male as compared to female (Table 3.3).

HCV prevalence is alarmingly high in Pakistan and due to unsatisfactory health care conditions; the infection is spreading at a rapid pace [34]. HCV has great heterogeneity in its genome and on the basis of this heterogeneity; HCV has been classified into eleven genotypes and more than 50 subtypes [10].

Different geographical regions have different HCV genotypes distributions. Like distribution of genotypes 1, 2, and 3 appear to be worldwide, while the most common genotypes in the US [25], Europe and Japan [108, 109] are 1a and 1b subtypes. The common genotypes in the North America, Europe, and Japan are subtypes 2a and 2b and in Italy is subtype 2c. North Africa and

the Middle East appear to have prevalence of genotype 4 [111, 112]. South Africa and Hong Kong have genotypes 5 and 6 distribution respectively [113, 114]. In Vietnamese patient's population, genotypes 7, 8 and 9 are identified [115], and in Indonesian patients' population genotype 10 and 11 are found [116].

A study in 1997, reported that 87% of patients in Pakistan had the prevalence of genotypes 3 [118]. A panel of 30 gastroenterologists reported in 2004, that HCV infected individuals in Pakistan had 75%-90% of HCV genotype 3a [7]. In 2006, it was reported that genotype 3 is present in 71 % while genotype 1 is present in 10 % of chronic HCV patients [119]. Another study in 2007 revealed that 81% of patients had genotype 3 while only 9.5% of patients had genotype 1 [120]. Hakim et al. [121] in 2008, reported that 3a is present in 51% of HCV patients, 3a/3b co-infection in 24% and 3b in 16 % of patients. Similar results were also reported by Afridi et al, [122] that 50% of HCV infected individuals had genotype 3a followed by 3b and 1a. In 2008 Idrees and Riazuddin conducted a detailed study and reported that the most prevalent genotype in Pakistan is 3a [98]. In 2011, in KPK province, the most abundant genotypes found were 2a followed by 3a [35].

Genotypes are important in determining epidemiology, duration of treatment and vaccine development so its determination prior to therapy is ufmost important [24]. Until now many methods have been discovered for HCV genotyping, including direct sequencing of DNA [139, 140], type specific PCR [141], RFLP, line probe assays [142], primer-specific and mispair extension analysis [143], heteroduplex mobility analysis by temperature gradient capillary electrophoresis [144] and denaturing high preference liquid chromatography [145].

Genotype determination was not carried out prior to treatment in KPK and the treatment strategies were solely based on qualitative or quantitative viral detection. Reasons for variable

response rates in the case of different patients for both conventional and Peg- IFN therapy could therefore not properly be sorted out. We attempted to figure out HCV genotypes prevalent among the population of KPK, so as to create awareness among the practitioners about the importance of the HCV genotype and find a preliminary answer to the resistance phenomenon experienced here.

Our study indicated that HCV genotype 2a was the most abundant followed by 3a and 2b [Table 3.3]. The distribution of type 2 and 3 is thought to be worldwide [25] including Pakistan [98]. It has earlier been reported that HCV 2a and 3a are most susceptible to combination therapy [25] and good response rates in KPK may partly be attributed to the most abundant types of HCV prevalent here. Other types of the virus including type 1a, 1b and 2b are less prevalent among the population [Table 3.3]. Genotype 1a and 1b are prevalent in the USA [25], Europe and Japan [108, 109]. These genotypes are comparatively less responsive to combination therapy [25] and the response rates in the case of Peg- IFN have been reported to vary from 50-70% [21].

HCV genotypes among a considerable number of chronic HCV patients (17%) could not be determined by the assay used in this study. Untypeable genotypes have earlier been reported by other studies conducted in Pakistan [98, 123]. Sequencing of the untypeable genotypes is needed not only for academic reasons but also for future treatment directions.

This study concluded that the most abundant types of HCV among chronic HCV patients of KPK province of Pakistan were HCV type 2a, 3a and 3b. Although these abundant types are responsive to combination therapy and Pegasys, yet proper diagnostic and treatment strategies should be adopted in order to minimize the economic burden on the population and to prevent people from an extra stress of undergoing successive therapies.

Table 3.2: Demographics of Chronic HCV infected patients.

District	Age	M/F Ratio	Family History of HCV infection	Co-infection	Major surgery	Blood transfusion	Dental surgery
Bunir	22-56	11/5	5%	Nil	2%	5%	9%
Swabi	18-53	13/8	3%	=	4%	6%	7%
Dir	16-58	18/12	2%	=	3%	5%	8%
Kohat	20-45	16/9	2%	=	3%	4%	6%
Mardan	19-56	29/18	3%	=	4%	8%	7%
Peshawar	15-60	41/23	4%	=	4%	9%	5%

M (Male), F (Female).

Table 3.3: Prevalent HCV genotypes among Chronic HCV infected patients of KPK.

S. No	Genotypes	Subtypes	Number of isolates	% Frequency	Total Number of isolates
1	2	2a	78	39%	80
		2b	2	1%	
2	3	3a	62	31%	78
		3b	16	8%	
3	1	1a	3	1.5%	8
		1b	5	2.5%	
4	Untypable	Untypable	34	17%	34
Total				100	200

3.3 Response rate of combination therapy among chronic HCV patients in KPK (1st trial).

In order to determine response rate of combination therapy (IFN alpha and Ribavirin), we selected anti-HCV and PCR positive patients from different regions of KPK. The patients were subjected to standard combination therapy for a period of six months keeping in mind the exclusion criteria (Table 3.4). We had taken all the required background history from each patient on the consent Proforma.

After completion of the six months long therapy, the results obtained were as follows. Out of total 174 patients, 130 (74.71%) were negative for HCV RNA showing end of treatment response (ETR) while 44 (25.28%) were positive for HCV RNA and did not show the ETR. In district Bunir, out of 52 patients who had completed therapy, 36 patients (69.23%) showed ETR and 16 (30.79%) did not show the ETR. In district Mardan, we found that out of total 74 patients who had taken six months therapy, 66 (89.18%) were negative for HCV RNA and 8 (10.81%) were resistant to therapy. In Peshawar district, out of 22, 16 (60%) were negative and 6 (40%) were positive while in Federally Administered Triable Area (FATA), out of 18 only 10 (55.55%) were negative and 8 (44.45%) were positive for HCV RNA (Table 3.5).

Our study revealed that response rate of combination therapy was comparatively higher in districts Mardan (89.18%) and Bunir (69.23%) than in districts Peshawar (60%) and FATA region (55.55%) (Fig 3.1).

The study further revealed that response rate among the males was lower as compared to females (Table 3.5). Among different age groups, IFN responsiveness was observed to be higher in the case of age groups lower than 40 years, especially in districts Mardan and Bunir and found lower in age groups more than 40 years in districts Peshawar and FATA regions (Table 3.6).

Hepatitis C infection is spreading rapidly and world wide its prevalence is nearly 200 million people and every year it infects 3-4 million more [180]. The seroprevalence of HCV in different parts of Pakistan, reported in the last five years, is from 2.2% to 13.5%. The highest seroprevalence of hepatitis C has been reported from Lahore (13.5%) [181] Jasmshoro (9%) and Mardan (5%) [182, 31].

Pakistan is a developing country and the literacy rate is very low. Hence lack of informations among the general masses regarding the pathogenecity, routes of transmission and proper procedures of diagnosis and treatment has turned HCV infection an economic burden on the people of Pakistan and especially in KPK.

It has been studied that hepatitis C infection persists in 55% to 85% of individuals who have acute hepatitis C [183-185]. The range of cirrhosis development is from 5% to 25% in 25 to 30 years of period [186, 187]. In persons who are older in age, have obesity, co-infection or who are immunosuppressed and consume more than 50gm of alcohols/day, cirrhosis progression may be accelerated [188-191].

The main theme and goal of hepatitis C treatment is the prevention of complications and death in HCV infected individuals. Different parameters exist to monitor the response of therapy. Like short term response can be demonstrated biochemically; that is normalization of serum ALT levels, Virologically: as the absence of HCV RNA, determined by PCR based assay in serum, and with no worsening in fibrosis score and 2 point improvement in necroinflammatory score histologically [22, 20]. Virologically, several responses relative to treatment duration may occur. SVR, generally regarded as “virological cure” is the most important which can be defined as undetectable HCV RNA by PCR test after six months of completion of therapy [192]. ETR is referred to as the absence of HCV RNA at the end of treatment either 24 or 48 weeks of therapy.

An ETR prediction for SVR is not accurate but most of the time both have strong correlation. The absence of HCV RNA at the 4th week of therapy by sensitive PCR test is referred to as Rapid virological response (RVR). An RVR has strong predictive value for SVR to occur [193,194]. When there is reduction in HCV RNA by 2log IU/ml or when HCV RNA is found completely absent in serum by PCR test at 12 weeks of therapy then it is called as early virological response (EVR). An EVR is the most accurate predictor for an SVR; failing to achieve an EVR, it will be difficult for the individual to achieve an SVR [51, 195-197]. Viral kinetics monitoring is thus helpful to predict that whether an SVR is likely to develop or not.

The reappearance of HCV RNA during and after therapy is referred to as Virological breakthrough and virological relapse respectively. Null responders are those who are unable to suppress HCV RNA by 2log IU/mL after six months of therapy. The persons who are able to suppress HCV RNA by 2log IU/mL but with detectable HCV RNA are referred to as partial non responders.

National institute of health (NIH) has recommended the use of IFN alpha and Ribavirin as the standard treatment regimes in chronic HCV patients [4]. In poor countries, standard IFN therapy was recommended to treat Chronic HCV patients and also Pakistan Society of Gastroenterology and Endoscopy suggested the use of standard IFN therapy in case of genotype 3 patients [7, 198], as the use of Peg- IFN is out of the reach for the poor people in under developed and developing countries including Pakistan [199, 198].

IFNs are the natural cellular proteins products having different functions like antiviral state induction in the viral targeted cells along with cytokine secretion, immune cells recruitment and cells differentiation induction [200-202]. IFN-α when administered subcutaneously attaches to high affinity receptors present at the surface of targeted cells. This IFN-receptor fixation is

responsible to trigger series of intracellular reactions which in turn activate IFN-inducible genes [203-207]. The genes encoding different products are the mediators of IFN-α various cellular actions [208]. These genes products are creating antiviral sate in two distinct but complementary pathways. First the antiviral state induction, although not specific to the virus in the targeted cells, but results in a direct HCV replication inhibition. Secondly, immunomodulatory effects induction that is responsible for enhancing specific anti-HCV immune responses of the host. In infected cells, numerous IFN-induced proteins and enzymatic pathways are involved in the establishment of an antiviral state, but so far only a few have been identified [208]. In 1992, IFN was commended for the treatment of hepatitis C in the US.

The effects of using Ribavirin were the reduction of ALT level and improving the histological features of the liver but its effects were found very low on serum HCV RNA levels [161]. Moreover, when Ribavirin was combined with IFN then it increased SVR rate [162]. The combined effect of using IFN and Ribavirin when given for 48 weeks was three times more than that of IFN alone and the SVR was 40-50% [163]. In 1998, Ribavirin as an adjunct to IFN was approved for the treatment of hepatitis C.

In this study we determined the response rate of IFN combination (IFN and Ribavirin) therapy against chronic HCV infection. The average calculated ETR was 74.71% and resistance calculated was 25.28%. ETR rates among chronic HCV infected patients who had taken six months long combination therapy was variable such as, ETR was very high in district Mardan followed by district Bunir and lower in district Peshawar and FATA regions [Fig 3.1].

The average response rate of IFN combination therapy with Ribavirin in chronic HCV patients of KPK population was 74.71% [Table 3.5]. The results of the present study are almost in agreement with other studies, conducted internationally [209– 211] as well as locally [212, 36,

37], when treated with IFN and Ribavirin combination therapy, inspite the fact that KPK have different ethnic groups of population.

Different people have different response to IFN- based therapy. The factors associated with the response to IFN are the initial viral load, genotype, sex and age and specifically, genotype 2 and 3, female sex, age lower than 40 years and lower body weight [23].

In districts Mardan and Bunir, prevalence of HCV infection has been recorded to be 9% and 5% respectively [31, 104]. In the current study, high ETR was found in district Mardan (89.18%) followed by district Bunir (69.23%) and lower in Peshawar (60%) and FATA regions (55.55%) [Fig 3.1]. As it has been shown that people having age lower than 40 years will have lower relapse rate and showing better response to IFN based therapy [213]. The variable response rate in different regions of KPK could be attributed to different age groups patients. High ETR in districts Mardan and Bunir was observed in patients with age lower than 40 years while low response in other districts was recorded in patients' who were more than 40 years [Table 3.6].

There were more females than male patients in both districts Mardan and Bunir which may be a reason for high ETR in these regions as compared to other regions [Table 3.6]. High response rates associated with female sex has already been found in case of IFN based therapy [213].

Data from various clinical trials suggested that patients of different ethnicities may vary in their responses to IFN-based therapy. Asians and Hispanics may have a more favorable response as compared to African Americans [214-221]. The treatment response has been linked with ethnicity [222]. It has also been noted that response rate in African Americans was lower as compared to whites in case of IFN-based therapy [219]. The dominant cast in districts Mardan and Bunir is yousafzais, which is considered to be the largest Pathan tribes. Our study revealed

that ETR rate was higher among the yousafzai. The dominant yousafzai cast in districts Mardan and Bunir could be a reason for high ETR rate in case of IFN based therapy.

The ETR calculated in district Peshawar was 60%, lower than Mardan and Bunir while higher than that of FATA region [Fig 3.1]. In district Peshawar, the capital of KPK, having people of different casts and ethnicity, migrated from Afghanistan and different regions of KPK. Low response rate in district Peshawar might be attributed to different cast people and migration of people harboring resistant HCV types from Afghanistan, FATA and other associated regions due to the Floods and regional conflicts.

FATA stands for Federally Administered Tribal Areas (FATA). It is situated between the province of KPK, Baluchistan, and the neighboring country of Afghanistan. The total population of the FATA is 2% of the total Pakistani population. It is the most rural unit of Pakistan as only 3.1% of the population resides in the well established townships. The people of the FATA are mostly pashtun tribes. In this region, the response rate found was 55.55% and was the lowest response than all of the three districts [Fig 3.1]. The lowest response in this region might be due to low number of female sex, patient's age more than 40 years [Table 3.6], the prevalence of resistant HCV genotypes that may have resulted due to the influx of people from the Gulf and Central Asian Countries.

Our results showed that combination IFN therapy in the 1st trial was more effective in case of districts Mardan and Bunir; this reflects the availability of the associated responsive factors like the genotype 2, 3, age less than 40 years and favorable ethnicity. Moreover this also shows that patients were given right protocols for using antiviral drugs. The low response in district Peshawar and FATA regions reflects the partial availability of the above factors. The non

responders in all of the above regions should be studied further in order to explore the factors showing hindrance to antiviral therapy.

Table 3.4: Absolute contraindications to IFN- based therapy in Chronic HCV patients.

Active psychiatric disease like major depression
Organ transplantation like heart, kidney or lung
Autoimmune hepatitis
Severe hypertension, heart failure, poorly controlled diabetes
Hypersensitivity to drugs used for hepatitis
Pregnancy
Breast feeding

Table 3.5: Region wise distribution of positive HCV RNA samples and their respective ETR

Districts	**T. Samples**	**Age group**	**Sex M/F**	**ETR+**	**NR**	**%ETR+**	**%NR**
Bunir	52	18-55	30/22	36	16	69.23	30.79
Mardan	74	20-54	42/32	66	8	89.18	10.81
Peshawar	30	22-55	18/12	18	12	60	40
FATA	18	22-53	14/4	10	8	55.55	44.45
Total	174		104/70	130	44	74.71	25.28

ETR (End of Treatment Response), NR (Non Responder), M/F (Male, Female), T (Total).

Table 3.6: Age wise distribution of HCVRNA male and female samples along with ETR

Regions	**Total Samples**	**Age groups of M/F**	**%ETR+**
Bunir	52	M>40=12, F>40=8 M<40=18, F<40=14	69.23
Mardan	74	M>40=16, F>40=7 M<40=26, F<40=25	89.18
Peshawar	30	M>40=7, F>40=7 M<40=11,F<40=5	60
FATA	18	M>40=8, F>40=2 M<40=6, F<40=2	55.55
TOTAL	174	M>40=43, F>40=24 M<40=61,F<40=46	74.71

M/F (Male/Female), ETR (End of Treatment).

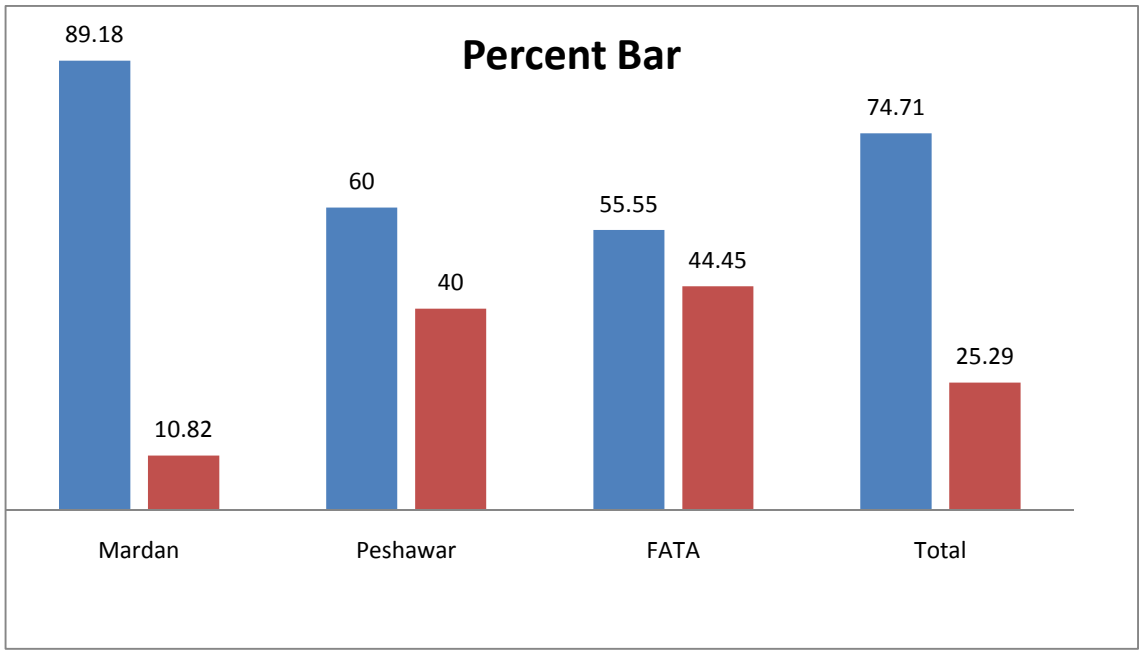

Fig 3.1: Region wise Percent ETR Bar.

3.4 Response rate of combination therapy among chronic HCV patients in KPK (2nd trial).

Anti-HCV and PCR positive patients were selected for IFN and Ribavirin combination therapy keeping in mind IFN therapy related contraindications. Before starting therapy, thirty three patients were dropped because of the contraindications [Table 3.4]. The remaining (341) patients completed six months combination therapy. The adverse effects noted during and after the therapy are given in Table 3.7.

Out of total (341) selected patients for standard IFN- based therapy, (81%) showed ETR and were negative for HCV RNA while (19%) did not show response to IFN therapy and were positive for HCV RNA. In district Swabi, (92%) patients were negative and (8%) were positive for HCV RNA at the end of six months treatment. In Bunir, (71%) showed ETR and (29%) did not show response to IFN therapy. In district Kohat, out of 54 patients, (80%) were negative for HCV RNA and only (20%) were positive for HCV RNA at the end of treatment [Table 3.8]. Among the non responders (16) from district Swabi who were given an extra dose of IFN and Ribavirin, (88%) were negative and only (12%) were positive for HCV RNA after 2 months additional treatment.

We did two trials of IFN response in different regions of KPK. At the same time we could not reach to all of the targeted regions therefore we did two trials of IF therapy. In the 1st trial we kept two remote regions, Bunir and FATA and two local districts, districts Peshawar and Mardan. Similarly we focused our study in the second trial on three districts, Swabi, Kohat and Bunir. In this we kept Bunir again because we have included some more number of patients having high different range of ages and to validate the 1st trial of IFN response.

Due to unhygienic conditions, poverty and low awareness regarding the spread and consequences of Hepatitis C, this infection is increasing alarmingly throughout the province. At

a district level as no study had been conducted to figure out response of IFN based therapy in chronic HCV patients therefore, we now has focused three more districts (districts Swabi, Bunir and Kohat) in the second trial of therapy response.

To eradicate the HCV from the infected individuals, IFN- based therapy is considered as the choice of treatment throughout the world [4]. IFN alone was used to treat the patients in early days but due to more chances of relapse and failure to decrease HCV RNA, it was not considered as the successful choice of therapy. In order to overcome the problem, Ribavirin was used as an adjunct with IFN to treat hepatitis C patients. The use of the IFN and Ribavirin combination therapy led to the increase in SVR rate by 40-45% than that of IFN alone [163]. As with increase in relapse rate and comparatively low SVR rate in case of standard IFN therapy the need for peg-IFN became more. This peg- IFN, was used to cure the disease because of its prolonged effects, is still considered as the gold standard of therapy in chronic HCV patients.

The enrolled anti-HCV and HCV RNA positive patients (341) were given IFN alpha three times a week with a dose of 3 Million International Unit (MIU) and Ribavirin 800-1200mg/day, respectively. The therapy was continuously given for six months and after six months of therapy, ETR was calculated for all the enrolled patients.

ETR of standard IFN and Ribavirin combination therapy, determined among chronic HCV patients in different districts of KPK was 81% and the resistance was 19%. High ETR was found in the district Swabi (92%) followed by district Kohat (80%) and district Bunir (71%). The adverse effects during and after the therapy were also noted [Table 3.8]. The most adverse conditions observed were fatigue, headache, fever and alopecia [Table 3.7].

In the selected three districts of KPK, the response of combination IFN therapy was almost similar to the response studies already done in Pakistan [36, 37] as well as internationally [209-

211]. As the response to IFN-based therapy has been linked with age, sex and ethnicity of the affected individuals [213]. Therefore similar response of the current study indicates the presence of these favorable factors.

In district Swabi, earlier IFN response study was not conducted and we for the first time determined response of IFN combination therapy in this district. ETR rate in district Swabi (92%) was comparatively higher than the other districts [Table 3.8]. High ETR rate in this district might be due to favorable factors like age groups, ethnicity and female sex. The most enrolled patient's age groups in district Swabi were less than 40 years [Table 3.9]. It has been studied that patient's age lower than 40 years are more responsive to IFN therapy than age more than 40 years [213].

Beside this, the second favorable characteristic of the enrolled patients to IFN therapy was the female sex. In the studied population, the proportion of the females was more than that of male in district Swabi as compared to other districts [Table 3.9]. It has been found that female sex is more responsive to IFN therapy than the male [213]. Therefore the response rate was found high in this district.

The ETR rate in case of district Kohat (80%) was comparatively lower than that of district Swabi (92%) and higher than that of Bunir (71%) [Table 3.8]. Comparatively lower ETR rate might be attributed to more proportion of male sex, patient's age more than 40 years compared to that of district Swabi [Table 3.9].

In district Bunir, the ETR rate was very low from both of the districts Swabi and Kohat [Table 3.8]. The lower ETR rate might be due to the more number of male populations, age more than that of 40 years [Table 3.9] and might be due to the difference in ethnicity.

Among the non responders from Swabi district who did not respond to standard six months therapy, when treated with an additional two months therapy with same IFN but with a high dose of 6 MIU thrice a week and 1200mg/day of Ribavirin daily respectively, the response rate in this additional two months therapy was 88% and the resistance was 12%. It has been shown that by keeping the dose of IFN high, the patients were able to show more response. It has also been shown that IFN dose [223] and duration of treatment [224] have greater importance in long term response to antiviral therapy. Therefore response of IFN- based therapy can be enhanced by some modifications. Like SVR rates of 44% and 28%, reported by Sánchez-Tapias et al. with 72 weeks and 48 weeks of treatment, respectively [225]. From this it is clear that by increasing dose and duration of IFN, SVR can be enhanced in slow virological responders especially in HCV 1 infected individuals. This has also been proved by viral kinetics that some time viral reduction occurs rapidly with an initial dose of IFN followed by accelerated increase in viral RNA [176]. At this time it might require continuous and high dose of IFN to suppress the viral RNA as is done by Peg-IFN, which due to its prolonged action keep the viral RNA suppressed. Guidelines regarding retreatment of non responders suggest that some considerations should be considered like severity of disease, adherence/compliance, previous treatment history, tolerance issues, viral genotypes and predictive value in response [4]. Hence it is proved that slow responders get relief with some additional course of IFN combination therapy.

Table 3.7: Commonly observed side effects with IFN and Ribavirin treatment

Adverse effects	**Percentage of adverse effects**
Fatigue	55-60
Fever	30-54
Headache	51-57
Nausea	32
Dyspnea	22
Alopecia	31-33

Table 3.8: End of treatment virologic response (ETR) in different regions of KPK

Regions	**T. No of samples**	**Age groups**	**M/F**	**ETR+**	**%ETR+**	**NR**	**%NR**
Swabi	198	16-45	97/101	182	92	16	8
Bunir	89	18-62	51/38	63	71	26	29
Kohat	54	17-52	28/26	43	80	11	20
Total	341		176/65	288	81	53	19

ETR (End of Treatment Response), NR (Non Responder), M/F (Male/Female)

Table 3.9: Age wise ETR rates in different districts of KPK

Districts	**T. Sample**	**M/F age groups**	**% ETR**
Swabi	198	M>40=18, F>40=14 M<40=79, F<40=87	92
Bunir	89	M>40=26, F>40=21 M<40=25, F<40=17	71
Kohat	54	M>40=12, F>40=11 M<40=16, F<40=15	80

3.5 Genotype specific response of combination therapy among chronic HCV patients in KPK. The heterogeneity of HCV has great impact on the sensitivity and specificity of serological and virological assays for the detection of HCV infection. Little is known about the role of HCV in the progression of the disease and response to IFN therapy. HCV heterogeneity is helpful in tracing sources of HCV epidemics. As different genotypes have difference in clinical management, therefore we conducted this study to find out response of different genotypes to six months of standard IFN combination therapy. In this regards a total of 51 patients who had active HCV infection, were selected randomly for genotype specific response.

Before starting therapy, genotype was determined for each patient by type specific PCR method as discussed above. Combination therapy was given to all patients for equal duration and equal dose of IFN and Ribavirin. After completion of therapy and performing PCR for all course completed subjects, the results obtained were as. Out of total 51 selected patients the abundant genotypes were 2a (22) and 3a (18) followed by 3b (6), 1b (3) and untypable (2). Percent frequencies of these genotypes were 43.13%, 35.29%, 11.76%, 5.88% and 3.94% respectively (Table 3.10). Responsive genotypes among these were 2a followed by 3a. Out of total (22), 2a HCV genotypes 17 (77.72%) showed response to standard IFN therapy and were negative for HCV RNA at the end of treatment and only 5 (22.28%) genotypes were resistant to combination IFN therapy. Among total (18) of 3a genotype, 13 (72.22%) showed response to IFN while 5 (27.77%) did not show the response. Total of 6 individuals having 3b genotypes, 4 (66.66%) showed response and only 2 (34.44%) were resistant to therapy. While the percent response in case of 1b and untypable genotypes was (33.33%) and 0% respectively (Fig .3.2).

Infection with any HCV genotype irrespective of the level of viremia can lead to cirrhosis, end-stage liver disease, and hepatocellular carcinoma. Such complications frequency is almost

similar with each genotype. The role of HCV genotypes in the progression of liver disease is one of the most controversial areas of HCV research. It has been investigated by Amoroso et al. [226] that in patients having HCV genotype 1b, 92% of patients were converted into chronic infection compared with 33% to 50% in patients having other genotypes. This suggests that genotypes play an important role in chronicity of infection.

This was proved by several studies that infections with 1b genotype proceed much faster into sever forms of hepatitis like cirrhosis [227] and hepatocellular carcinoma [228]. In 60-70% of patients having HCV associated hepatocellular carcinoma, HCV genotype 1b was present [227, 229]. It is unclear that whether HCV genotype 1b is an indicator or markers associated with sever liver disease, as it may reflect a longer time of infection rather than an aggressive form of infection.

Different genotypes have different virologic response to IFN-based therapy. Like in genotype 1, SVR rates were almost half of that found in HCV genotype 2 or 3 infected patients [16–22]. In genotypes 4, 5, and 6, the relative response rates to IFN therapy are less well defined, but their response is most probably similar to response found in genotype 1 rather than HCV genotypes 2 and 3. While in HCV subtypes (for example, HCV-1a and HCV-1b) the response rates are similar [230].

Response of antiviral therapy has been linked to genotype and different genotypes have different clinical management [25]. As data on general response of standard IFN against HCV infection exists but the data regarding HCV genotype specific response in KPK province of Pakistan is very rare. Therefore, we attempted to sort out genotype specific response to standard IFN and Ribavirin combination therapy administered for a period of six months.

The aim of this study was to determine the efficacy of IFN alpha and Ribavirin combination therapy in chronic HCV patients of KPK. Nowadays the standard therapy is the Peg- IFN, but as the response rate of conventional therapy is almost the same as Peg-IFN due to the prevalence of genotypes 2 and 3 in Pakistani population, therefore conventional therapy is considered as the choice of treatment in Pakistan [231].

To find out genotype specific response, we selected 51 patients, randomly from KPK population having chronic HCV infection. We determined genotype for each patient by type specific PCR method and put all the enrolled patients for six months combination therapy.

The present study indicated that frequency of HCV genotype 2a was higher followed by 3a and 3b. The lowest frequencies were that of genotypes 1b and untypable among chronic HCV infected patients [Table 3.10]. The highest ETR (77%) to standard combination therapy was observed in case of HCV genotype 2a patients, followed by 3a and 3b. The lowest response was observed in case of HCV genotype 1b, while null response was shown by patients having untypable HCV genotypes [Fig 3.2].

Different geographical regions have different HCV isolates distribution, like HCV genotypes 2 and 3 have world wide distribution [25]. But in KPK, according to our study the most abundant genotypes were 2a followed by 3a [Table 3.10]. This is almost an agreement with the previous study conducted in KPK regarding frequency distribution of HCV genotypes [123]. While in Pakistan the most abundant types are 3a followed by 2a [98]. The lowest frequency among chronic HCV patients was that of 1b and untypable [Table 3.10]. The distribution of genotype 1b is mostly in European states [92] but lower in Pakistan and also in KPK population [123]. While untypable distribution was the lowest one and it has also been shown by a study conducted in KPK that untypable genotypes are present in chronic HCV patients [123].

According to this study, combination therapy was more effective in case of chronic HCV patients with genotype 2 as compared to genotype 3 [Fig 3.2]. Other studies conducted in Pakistan have earlier revealed higher response rates (80-85%) in case of HCV 2a and HCV 3a, as these are predominant in Pakistan [36]. In Asians, approximately 90% response rate among HCV 2/3 patients has been found [232]. Comparatively higher response of HCV genotype 2 might be attributed to the responsive nature of this genotype. It was assumed that HCV genotype 2 and 3 require similar treatment duration. But evidence now indicates that this might not be the case. As patients having HCV genotype 2 seems to show better response to IFN–based therapy and consistently having higher SVR rate as compared to type 3, 80-93% compared with 66-80% respectively, when treated for 24 weeks [233-235]. Another predicting factor for the non response and relapse (23%) in case of genotype 3, might be High baseline HCV RNA levels (>600,000 IU/ml) in chronic HCV patients [23].

The response of other genotypes was comparatively low or null that is genotype 1b, has 50 % response while untypable genotypes had no response to IFN therapy [Fig 3.2]. The response rate in Asian patients having genotype 1 has been found to be 70% [17]. As genotype 1 is not prevalent in Pakistani population, mostly found in European states and it has been reported that response of genotype 1 and 4 to IFN therapy is 60-70% and may require 48 weeks of IFN-therapy [236]. Moreover the presence of HCV genotype 1 or 4 together has been considered as the strongest baseline predictor of non response to treatment [237-239]. Beside this it has been suggested that mutations in the HCV nonstructural (NS) 5A protein, considered as the IFN sensitivity region might be the cause of resistance to anti-viral therapy in case of HCV-1–infected individuals. Moreover it has been suggested by Viral kinetic studies that turnover of hepatocytes in case of HCV genotype 1 infected patients is slower than other genotypes after

initiation of anti-viral therapy [240]. Hence there is need of more aggressive and longer duration of treatment regimes [241, 242], having capability of continuous stress and pressure over the virus. While the null response in case of untypable might reflect some new isolates/quasispecies evolution. The evolution of viral quasi-species is another element regarding resistance to anti-viral therapy [243]. As most of the time quasi-species complexity has been observed in non responders than that of patients having undetectable HCV RNA after six months of treatment completion [244]. Due to migration of different cast, community and region people to this province the ratio of finding untypable genotype is becoming more .These genotypes should be explored and some serious attention must be made to investigate these nonresponsive genotypes. Hence it is proved that in KPK the abundant and responsive genotypes are 2 and 3.

Despite the improvements in virological response rates and in curing of hepatitis C by the introduction of IFN- based therapy, some challenges are still there in treating those who are difficult to treat and who are non-responders. There is continuously development of novel IFN – based products and most of the interest is focusing on anti-HCV drugs. In this regards several HCV specific inhibitors have been investigated and some are in preclinical and clinical trials. It is hoped that these may improve treatment options in case of chronic HCV infected patients. To date, some promising anti-HCV drugs are the HCV protease NS3-4A, responsible for maturation of viral protein during viral replication and the RNA dependent HCV RNA polymerase NS5B. No doubt development of new anti-HCV drugs is a challenge and in this regards many polymerase inhibitors have been discontinued due to unacceptable toxicity or low level of efficacy.

Table 3.10: Percent frequency of different HCV genotypes among chronic HCV patients.

Genotypes	**Isolates**	**Total numbers**	**%Frequency**
2	2a	22	43.13
3	3a	18	35.29
	3b	6	11.76
1	1b	3	5.88
Untypable		2	3.94

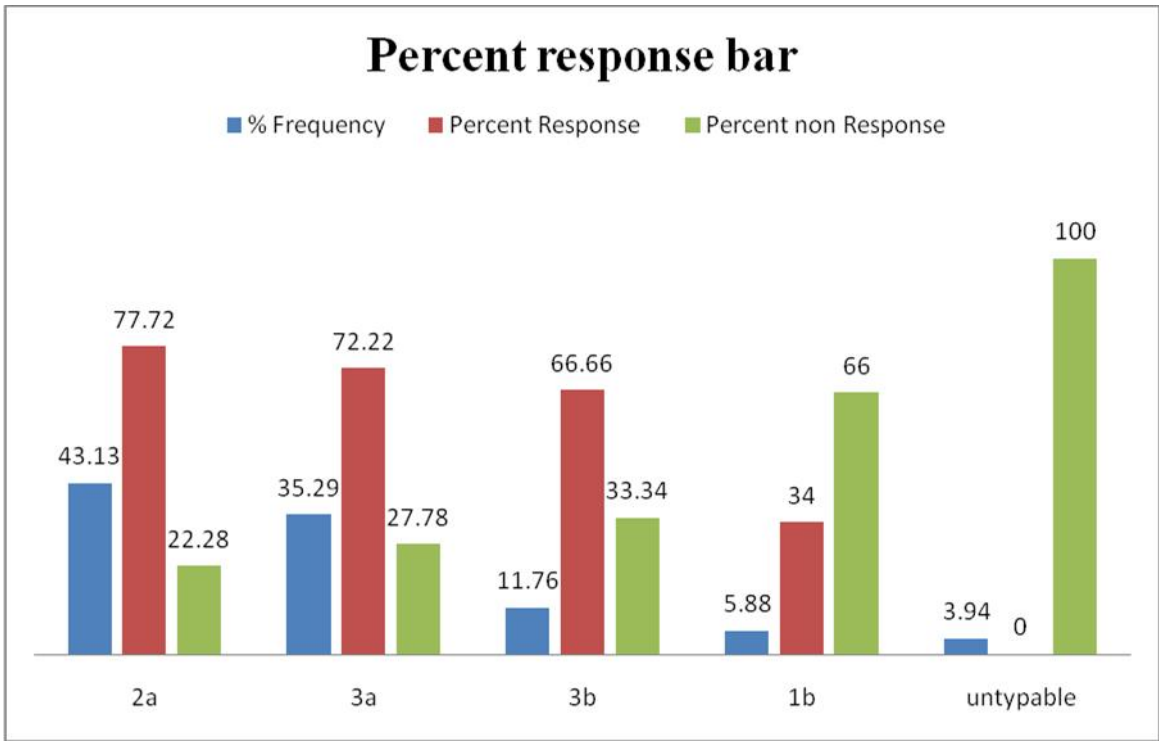

Fig 3.2: Percent response bar of different HCV genotypes

Conclusion

Our study concludes that active HCV infection is highly prevalent (66.6%) among the anti-HCV positive patients of KhyberPakhtunkhwa.

Further more, most abundant HCV genotypes found were HCV 2a (39%) followed by 3a (31%), while genotypes in a number of chronic HCV patients go undetected by the present genotyping system which indicate that novel types of the virus may have evolved during the course of time.

The trials of combination interferon therapy indicated that although the response rate was variable in different districts of KPK, however, the average response rate of the therapy was good enough against the prevalent genotypes.

In order to confirm that comparatively better response rate of the combination therapy is due to high prevalence of those HCV genotypes (3a and 2a) which have been locally and internationally investigated to be highly responsive to IFN-based therapy, genotypes of the chronic HCV patients included in the study were determined before initiation of the therapy. The genotype-specific response was comparably similar to the trials conducted earlier indicating that better response rates are due to high prevalence of the responsive types of the virus among the chronic HCV patients of KPK. In case of the Untypeable and genotype 1 infections, null or least response to combination therapy was observed which reveals that novel or variant types that have evolved over times in KPK are resistant to combination therapy and require characterization.

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Annexure II

Patient's enrollment proforma

Patient Name:

Age/sex	Location	Ethnicity	LFTs	Ultrasound	ELISA	PCR	Treatment

Consent: I agree to give blood for investigation and research purpose.

Sign: _____

Annexure III

RNA Extraction Kit components.

	10 extractions	**50 extractions**	**250extractions**
Lysis Solution RL	6 ml	25 ml	120 ml
Binding Solution RBS	8 ml	30 ml	160 ml
Washing Solution HS (1)	3 ml (final volume 6 ml)	15 ml (final volume 30 ml)	70 ml (final volume 140 ml)
Washing Solution LS (2)	2 ml (final volume 10 ml)	8 ml (final volume 40 ml)	36 ml (final volume 180 ml)
RNase-free water	1 x 2 ml	6 ml	30 ml
Spin Filter	10	50	5 x 50
Recevier Tubes (2.0 ml)	50	5 x 50	25 x 50
Elution Tubes (1.5ml)	10	50	5 x 50

HCV RNA amplification kit.

Label	Package size 140 Reactions	Package size 70 Reactions	Package formate	Description
HCV_D1	100 tubes	50 tubes	Plastic bag	Extaction tubes (coated with Internal controls and carrier nucleic acid)
HCV_D2	14 Strips (14 x 8 tubes)	7 strips (7 x 8 tubes)	Plastic bag	Sample RNA tubes (coated with amplification enhancer)
HCV-D3	4 strips (4 x 8 tubes)	2 strips (2 x 8 tubes)	Plastic box	Quantification standard tubes (coated with 8 different HCV RNA conc. IC RNA, and amplification enhancer)
HCV_D4	4 vials	2 vials	Amber vial / Amber cap	25x Reagent mix (lyophilized with HCV/IC-specific primers and probes)
Mg-sulfate solution (50 Mm)	2 x 0.18 mL	1 x 0.18 mL	Clear vial/ yellow cap	Mg-sulfate (50 mM)
RT-PCR enzyme mix	2 x 0.07 mL	1 x 0.07 Ml	Clear vial/ Clear cap	RT-PCR enzyme mix
2x Reaction mix	4 x 0.05 mL	2 x 0.05 mL	Clear vial/ Red cap	2x RT-PCR buffer (containing 6 mM Mg-sulfate and dNTPs)
PCR grade water	4 x 1.50 mL	4 x 1.50 mL	Clear vial/ Clear cap	PCR grade water
Caps	17	9	Plastic bag	