Molecular Characterization of HCV Genotypes and Response rates of various Anti-viral Therapies among Patients with HCV Infection in Pakistan

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Molecular Characterization of HCV Genotypes and Response rates of various Anti-viral Therapies among Patients with HCV Infection in Pakistan

A thesis submitted in the partial fulfillment of the requirements for the degree of

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By

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Dedication

…To the Patients who are combating the menace of HCV courageously

Author's Declaration

I **Mr. Shahroz Khan** hereby state that my Ph.D. thesis titled **"Molecular Characterization of HCV Genotypes and Response rates of various anti-viral Therapies among Patients with HCV Infection in Pakistan"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

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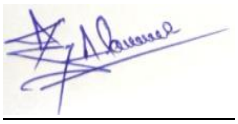

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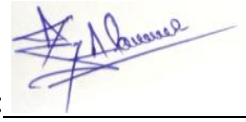

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Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Molecular Characterization of HCV Genotypes and Response rates of various anti-viral Therapies among Patients with HCV Infection in Pakistan"** was conducted by **Mr. Shahroz Khan** under the supervision of **Dr. Ijaz Ali** and Co. supervision of **Dr. Malik Badshah.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

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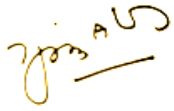
Supervisor Name: **Dr. Ijaz Ali** Signature: 

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LIST OF ABBREVIATONS

❖ µL	❖ Micro litre
❖ µm	❖ Micrometre
❖ ALP-Phos	❖ Alkaline phosphatase
❖ ALT	❖ Alanine Aminotransferase
❖ BLAST	❖ Basic Local Alignment Search Tool
❖ CBC	❖ Complete Blood Count
❖ CDC	❖ Center for Disease Control and Prevention
❖ cDNA	❖ Complementary DNA
❖ CHC	❖ Chronic Hepatitis C
❖ CLDN1	❖ Claudin 1
❖ DAADT	❖ Direct Acting Antiviral Double Therapy
❖ DAAs	❖ Direct Acting Antivirals
❖ DAATT	❖ Direct Acting Antiviral Triple Therapy
❖ DACLA	❖ Daclastavir
❖ DDBJ	❖ DNA Data Bank of Japan
❖ E 1	❖ Envelop Glycoprotein 1
❖ E 2	❖ Envelop Glycoprotein 2
❖ EIA	❖ Enzyme Immuno Assay
❖ ELISA	❖ Enzyme Linked Immuno Sorbant Assay
❖ EMBL	❖ European Molecular Biology Laboratory
❖ ETR	❖ End of Treatment Response
❖ EVR	❖ Early Virological Response
❖ FDA	❖ Food and Drug Administration
❖ g/l	❖ Gram per Litre
❖ H	❖ Hydrogen
❖ H2O	❖ Dihydrogen oxide
❖ H2O2	❖ Hydrogen peroxide
❖ H2S	❖ Hydrogen sulfide
❖ H2SO4	❖ Sulfuric acid
❖ HB	❖ Hemoglobin
❖ HCC	❖ Hepatocellular Carcinoma
❖ HCV	❖ Hepatitis C Virus
❖ HIV	❖ Human Immuno-deficiency Virus
❖ K2HPO4	❖ Dipotassium hydrogen phosphate
❖ KCl	❖ Potassium chloride
❖ L	❖ Litre
❖ MeS2	❖ Metal sulfide
❖ Mg	❖ Magnesium
❖ MgSO4.7H2O	❖ Magnesium sulfate heptahydrogen
❖ mL	❖ Millilitre
❖ mm	❖ Millimetre
❖ Na2S2O3.H2O	❖ Sodium thiosulfate
❖ NADH	❖ Nicotinamidedihydrogen
❖ Ni	❖ Nickel

❖ NR	❖ NR=Non Responders
❖ O2	❖ Molecular Oxygen
❖ PbS	❖ Lead sulfide
❖ PCR	❖ Polymerase Chain Reaction
❖ PEG-IFN	❖ Pegylated Interferon
❖ RBCs	❖ Red blood cells
❖ RBV	❖ Ribavirin
❖ RNA	❖ Ribonucleic Acid
❖ RNDRP	❖ RNA dependant RNA Polymerase
❖ rpm	❖ Rotation per minute
❖ S	❖ Sulphur
❖ S°	❖ Elemental sulphur
❖ SDO	❖ Sulfur dioxygenase
❖ SO42-	❖ Sulfate
❖ SOF	❖ Sofosbuvir
❖ SOR	❖ Sulfite acceptor oxidoreductase
❖ SVR	❖ Sustained Virologic Response
❖ Thio+	❖ Thiosulfate growing strain
❖ TLC	❖ Total Leukocyte count
❖ U	❖ Uranium
❖ Unt	❖ Untypable
❖ V	❖ Vanadium
❖ w/v	❖ Weight per volume
❖ ZnS	❖ Zinc sulfate

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Finally, all the errors that remains, are mine, alone.

Shahroz Khan Yousafzai

Abstract

The Hepatitis C virus (HCV) is a major cause of hepatitis C (hep C) which is a global problem with strong evidence of endemicity in several countries including Pakistan where more than 10% of the general population are suffering from the condition according to recent investigations. HCV infection reported to cause various phenotypes of the condition including acute infection; which progresses to chronicity among 80% of the infected individuals, fibrosis, cirrhosis as well as hepatocellular carcinoma (HCC). As an RNA virus, HCV is prone to mutate frequently giving rise to variants and new genotypes which may pose challenges to the existing diagnostic and therapeutic regimens. Currently, seven major genotypes and around 100 subtypes have been described prevailing in different parts of the world. The evolution of HCV genotypes depend upon a number of factors including ethnicity, infection rate and the use of different antivirals etc. The pattern of HCV genotypes distribution in a population is important with respect to understanding dynamics of the infection as the evolution of or introduction of variants or novel types have implications for diagnostic and therapeutic strategies. In Pakistan very few small scale studies have investigated the distribution pattern of HCV genotypes and response rates of various antiviral regimens while some newly approved therapeutics such as DAAs (Direct Acting Antivirals) have never been investigated against the prevalent HCV types. This study was therefore designed to investigate the existing pattern of HCV genotypes distribution and the pan-genotypic response rates of various antivirals in Pakistan.

The first objective of the study was to investigate the distribution pattern of hepatitis C virus genotypes in different geographical regions of Pakistan by using Type-specific PCR assay. The Type-specific results were further validated by sequencing of a representative number of HCV isolates at their 5' UTRs. In the current study, 5704 randomly selected subjects(from a larger pool of people seropositive for HCV infection) of both genders, different ethnicity, age groups and belonging to 24 geographical locations in different cities of Pakistan were included, out of which 3061 (53.6%) females were found with a relatively higher frequency of active HCV infection than male subjects 2198 (38.5%).

In male subjects the genotype distribution pattern demonstrated that HCV 3a was abundantly found (33.4%) followed by the Untypable genotypes (3.2%), HCV 3a3b (2.9%), HCV 3b, (1.4%), HCV 2a, (0.8%), HCV 2b, (0.1%) and HCV 1b (0.001%). In female subject, HCV 3a was slightly more frequent (46.5%) in comparison to male subjects followed by the Untypable genotypes (4.7%), HCV 3a3b (3.9%), HCV 3b (2.0%), HCV 2a (1%), HCV 2b (0.1%) and HCV 1b (0.001%). HCV genotype 4a (0.001%) was found in only one female subject. The subjects were divided into 6 age groups. Mean age of subjects enrolled was 44.23 ± 11.71 yrs. In age groups (26-40 and 41-55 yrs), the active infection was documented to be 36% and 43% respectively which was higher compared to rest of the age groups. HCV genotyping results indicated that out of the 5259 samples with active infection, Typable genotypes were identified for 92.12% subjects. Furthermore, HCV genotype 3a was found to be the most frequent one n=4203 (79.9%), secondly the Untypable HCV genotypes n=415 (7.9%) were found most abundant, similarly mixed infections of HCV 3a3b were n=355 (6.7%), HCV 3b were n=182 (3.5%), HCV 2a were n=92 (1.7%), HCV were 2b n=10 (0.2%) and HCV were1b n=2 (0.03%). Interestingly a single case of HCV 4 was also observed. The pattern of HCV genotypes distribution in 24 different cities of Pakistan reflected the same trend as detected in the entire study area with majority of infections due to HCV 3a, HCV Untypable genotype and HCV 2a while HCV 2b, 1a, 1b and HCV 4 were rare. Moreover, the overall HCV genotypes distribution pattern was the same in the case of both the genders and age groups indicating a uniform pattern of HCV genotypes distribution in different parts of the country.

The second objective of the study was to characterize the Untypable HCV isolates from various geographical areas of Pakistan by sequencing and phylogenetic analysis of their 5′UTRs. The emergence of large number of HCV Untypable isolates among chronically infected Pakistani subjects pose challenges to accurate diagnosis, optimal regimen, dose and duration of antiviral therapy as well as for estimating the response rate. During the course of this study, a total of 415 Untypable HCV strains were detected from different parts of the country in which 50 (12%) randomly selected serum samples were used for sequence analysis of 5′UTR of HCV.

The derived consensus sequences in case of all the 50 isolates were later used for genotype prediction using NCBI BLAST (ncbi.nlm.nih.gov) and online HCV genotype prediction tools. The results indicated that all the 50 samples (100%) were very close to HCV 3a. Self-alignment of all the 5' UTR sequences identified 10 diverse types circulating in Pakistan. Most common sequence variations were conserved (*), deletion (-) and transversion. Phylogenetic analysis of HCV Untypable isolates based on the 5' UTR sequences indicated that most of these isolates were genetically closer to Pakistani HCV 3a isolates with high bootstrap value as compared to some regional isolates. However, One Untypable isolate PK3 clustered with isolates from other regions and was distant from the Pakistani isolates which indicates that HCV 3a of different origins are distinctly evolving in Pakistan. The genetic diversity and phylogenetic analysis point towards the rise of variants of HCV 3a in this region in the form of the Untypable strains which need through characterization.

The third objective of the study was to evaluate the response rates of DAAs (Daclastavir/Sofosbuvir) or their combination with previous standard of care (SOC) which included Ribavirin and Pegylated Interferon) for the treatment of hepatitis C subjects. A total of 4411 subjects with chronic HCV infection were enrolled for this study out of which n=3779, (85.6%) completed the therapy in which n=2204 (58.3%) were females and n=1575 (41.7%) were males while rest of the patients with HCV were not treated because of either losing the follow up or discontinuation of therapy due to side effects. The subjects were divided into four treatment groups each receiving a different combination therapy and the response rates were assessed via monitoring the viral load, blood and liver enzyme profiles. Group-I included 1446 HCV infected subjects out of which 847 (58.5%) were females and 599 (41.5%) male subjects belonging to six age groups who were given SOFO-RBV combination therapy for 24 weeks. The End of treatment response rate (ETR) was found to be (91.7%) and SVR24 of (91.5%) among all subjects. The response rate in the age range of 26-70 years was comparatively higher than subjects older than 71 years. Response rate of the combination therapy among subjects infected with different HCV types were slightly variable, however there was no significant difference (p value >.05). Subjects infected with various HCV genotypes exhibited that the response rates of the double combination therapy against HCV 3a, HCV Untypable, HCV 3a3b mixed infections and HCV 3b were better than other HCV genotypes.

In Group-I, 120 (8.2%) non-responders and 3 (0.2%) relapsers were reported in the case of HCV 3a, 3a3b and HCV Untypable. In terms of gender wise response, high SVR24 rate (92.1%) was achieved in female subjects as compared to male subjects (88.8%) mostly among subjects in the age range of 26-70 years.

Group-II consisted of 473 HCV infected subjects out of which 262 (55.3%) were females and 211 (44.7%) were males belonging to six age groups. In Group-II, the overall ETR and SVR of the DCV-SOFO combination therapy administered for 24 weeks duration were (93.2%) and (92.6%) in treated subjects. Subjects infected with various HCV genotypes exhibited that the response rates of DCV-SOFO combination against HCV 3a, HCV 3a3b and the Untypable HCV were better than the other HCV genotypes. In the age range of 26-70 years, the response rate was higher in treated subjects. SVR24 was achieved among (91.7%) female subjects and (92.3%) in male subjects. In Group-II, 32 (6.7%) non-responders and 3(0.6%) relapsers were reported who were infected with HCV 3a, 3a3b and the Untypable HCV.

In Group-III, a triple combination therapy of DCV-SOFO-RBV was administered for 24 weeks. The group included 747 subjects infected with different HCV genotypes, out of which 449 (60.1%) were females and 298 (39.9%) males belonging to five age groups. The overall ETR and SVR rates of the combination in Group-III turned out to be (93.9%) and (93.8%) in treated subjects. Interestingly, in the age range of 26-70 years, the response rate was higher than the rest of the age range as noted in the case of DCV-SOFO combination therapy as well. Response rate of the combination turned out to be more favorable against HCV 3a, HCV Untypable, HCV3a3b and HCV 3b than other HCV genotypes**.** Similarly SVR24 was achieved among (93.0%) female subjects and (93.9%) male subjects. In Group-III, 45(6.0%) non-responders and 1(0.1%) relapse patients were reported in the cases of HCV3a 3a3b, 3b and the Untypable HCV infections.

In Group-IV a triple combination therapy of SOFO-RBV-PEG INF-α-2a was administered for 24 weeks. The group-IV included 1113 subjects infected with different HCV genotypes, out of which 649 (58.3%) were female and 464 (41.7%) male subjects. The overall ETR and SVR rate of the combination in Group-IV turned out to be (93.3%) and (91.1%) among all the treated subjects. SVR rate of (90.3%) was achieved in female subjects and (90.0%) in male subjects.

In the age range of 26-71 years, the response rate was higher than the other age groups. Subjects infected with various HCV genotypes exhibited that the response rates of the triple combination against HCV 3a, HCV Untypable and HCV 3a3b mixed infections were higher than other HCV genotypes detected in the group**.** In Group-IV, 74 (6.6%) non-responders and 24 (2.3%) relapsers were reported against the SOFO-RBV-PEG INF-α-2a, combination therapies including HCV 3a and 3a3b and Untypable.

This study concludes that there is a uniform pattern of HCV genotypes distribution across the country with HCV 3a and its subtypes a/b as the most dominant types followed by 2a while other HCV genotypes are rarely prevalent in different areas. Although partial genetic information reveal that the Untypable HCV may be slight variants of HCV 3a, yet whole genome sequencing on a representative scale could help reveal their entire genetic divergence and their true identity as variants or novel types. Response rates of the combination therapies used against the Untypable HCV also indicated variations with respect to response rates achieved in the case of HCV 3a thus further re-inforcing the need for through full length characterization of such strains. Although combination of the newly approved DAAs or their combinations with RBV and PEG-IFN exhibited higher response rates (>90%) against various HCV subtypes, DCV-SOFO-RBV with SVR (93.8%) and DCV-SOFO with SVR (92.6%) combination therapy proved relatively more effective against the most abundant HCV 3a and 3b infection in Pakistani population.

Introduction

Hepatitis C virus (HCV) is the main reason of chronic liver infection called hepatitis C which is a grave health problem faced in routine clinical practices across the globe. In case of severe problems like Cirrhosis and Hepatocellular Carcinoma (HCC) liver transplantation is required (Hoshida et al., 2014). HCV has affected 350 million people all over the planet including 18 million of the total population in Pakistan (Idrees et al., 2009) which makes the country among one of the most affected states in the world (Waheed et al., 2009).

Hepatitis C virus is a linear, single stranded, (+) sense RNA virus classified as a separate *Hepacivirus* genus in the family of *Flaviviridae* (Ndjomou et al., 2003). The size of the HCV genome is about 9.6 kb with a 5' un-translated region (UTR) which comprises of almost 340 nucleotides and is an extremely preserved part of the genome of HCV used for genotypes detection (Husain et al., 2009, Alfaresi, 2011, Beales et al., 2001). Downstream to the 5'UTR, is a large solo (ORF) consisting of about 9,000 nucleotides that encodes a large poly-protein precursor of about 3,010 amino acids. The poly-protein is cleaved by cellular and viral proteases into 10 various kinds of proteins; the structural proteins such as the core (c), two Envelope proteins (E1) and E2, trailed by p7 protein which is taken from an ion channel. The core protein makes the viral capsid while E1 and E2 form envelope glycol-proteins. The non-structural (NS) proteins encoded by the HCV genome are NS2, NS3, NS4A, NS4B, NS5A as well as NS5B (Quinkert et al., 2005). NS2, together with the NS3 amino terminus, formulate the protease of virus (Hijikata et al., 1993). Protein NS3 serves as a helicase as well as NTPase while NS4A protein is a cofactor for protease of virus (Pawlotsky, 2003a) and NS4B makes the membranous net (Yan et al., 1998). NS5A is significant for regulation of cellular pathways and HCV replication (Penin et al., 2004). NS5B produces the RdRp (Waheed et al., 2012).

Genetic heterogeneity is a fundamental characteristic of HCV with vital impacts in diagnosis, infection progression, treatment management and vaccine production. The recognition of HCV genotypes, subtypes and quasi species has been very useful to understand the progression and the epidemiological advancement of hepatitis C. It is a significant characteristic in the prophylactic assessment of subjects as well (Akhund et al., 2008, Qazi et al., 2006, Umer and Iqbal, 2016). Due to RNA changeover speed of 1.44 × 10-3 and 1.92 × 10-3 per cell per year HCV RNA shows a great substantial genetic heterogeneity (Attaullah et al., 2011) exhibited in the form of emergence of variants and novel types.

HCV variants are categorized into a minimum of six major genotypes (genotypes 1–6), by 31–33% difference in nucleotide sequences (Simmonds et al., 2005) while some investigators have claimed up to 11 major genotypes with approximately 100 subtypes identified as A, B, C etc (S. Z. Safi et al., 2010). HCV genotype 7 through 11 should be known as variants of the same group and are considered as a solo genotype, type 6 (Ali et al., 2011b, Inamullah et al., 2011). HCV variants have four phases of genetic variation: major genotypes (66% - 69% sequence similarity), subtypes (77% - 80.0% sequence similarity), isolates as well as quasi species (91%-99% sequence similarity) (Afzal et al., 2014). The most frequent subtypes of various HCV genotypes are HCV 1a, HCV 1b and HCV 1c of genotype 1; HCV 2a, HCV 2b and HCV 2c of genotype 2; HCV 3a, HCV 3b and HCV 3k of genotype 3; HCV 4a of genotype 4; HCV 5a of genotype 5 and HCV 6a, 6b and 6d of genotype 6 (Simmonds et al., 2005). Besides this, prominent isolates of same subtype might vary by 5–15% heterogeneity (Abdo and Lee, 2004). HCV genotypes are mostly assigned on the basis of phylogenetic analysis of the 5'UTR, core/E1, NS5B, and/or whole genome sequences (Simmonds et al., 2005).

HCV frequently develops into chronic hepatitis, with occasional cases of spontaneous viral eradication (Ali et al., 2016a, Idrees et al., 2009). Hepatitis C is becoming a most important source of liver cancer. Chronic HCV infection leads to cirrhosis of liver as well as HCC (Simmonds, 2004) is considered to be one of the main indications for liver transplantation (Pawlotsky, 2003b). Among all the subjects with HCV infection, 55 to 85% in the later stage suffer from chronic HCV infection. Besides this, 10 to 20% of subjects with chronic HCV infection lead to cirrhosis and HCC, resulting in high mortality and morbidity around the globe (Ashfaq et al., 2011).

According to recent statistics on the prevalence of HCV infection around the world, it is particularly common in the developing world as compared to the developed world. The reason for higher prevalence of the infection in the developing world may be numerous risk factors including unsafe blood transfusion, poor healthcare facilities etc. Individuals with haemodialysis are highly vulnerable to develop HCV infection due to several risk factors, like numerous transfusions, medical history of surgeries and multiple injections (Khokhar et al., 2005, Gul and Iqbal, 2003, Asghar and Hahiz, 1998). Barbers in the developing world have been linked with high HCV transmission as well (Muhammad and Jan, 2005).

Molecular epidemiological studies have shown that different geographical parts of the world have prevalence of different HCV genotypes among the infected subjects, however the distribution of some genotypes is global. HCV genotype 1a is generally reported in the US and Northern areas of Europe. HCV 1b is considered to be globally distributed while HCV 2a and HCV 2b are mostly reported in Northern America, Europe, and Japan (Simmonds, 2004). HCV 3a has been reported from Pakistan, India, US and Europe. HCV 4a is mostly reported from Northern Africa and Middle East. HCV 6a is prevalent in Hong Kong and in most parts of South East Asia, whereas HCV 5a and 7 are reported from South Africa (Hoofnagle, 2002, Legrand-Abravanel et al., 2004, Sy and Jamal, 2006) and the Democratic Republic of Congo (Pineda et al., 2007).

The prevalence of HCV differs significantly in various areas of the same country even though among various individuals of the same population (Shakeri et al., 2013). Pakistan stands second in case of hepatitis C prevalence (Waheed et al., 2017) where HCV infection is alarmingly high and has been reported to have affected more than 18 million people having an average prevalence rate of 7% in many areas of the country (Afridi et al., 2014, Khan et al., 2013). The rates of anti HCV and active HCV infection varies from province to province and different investigators have reported considerably high prevalence rates from different areas. Umar & Bilal reported the highest prevalence in the most populous province of the Punjab (7%) followed by Sindh (5.3%) , Baluchistan (1.7%), and KPK (1.2%) (Umar and Bilal, 2012). Some studies have reported HCV prevalence from various cities of the Punjab province; with considerably high prevalence 12.85% in Lahore, 7.03% in Faisalabad, 12.96% in Gujranwala, 10.65% in Gujrat and 9.44% in Sialkot (Afridi et al., 2014). In KPK province, HCV prevalence in different areas has been documented to be 3-9% (Ahmad et al., 2012, Khan et al., 2011, Nawaz et al., 2015). A prevalence rate of 4.95% has been estimated from 1994 to 2009 in the general population while an extremely elevated predominance (57%) has been noticed among the IDUs (Waheed et al., 2009). The sero-prevalence rate of HCV in Pakistani population is considerably higher as compared to neighboring states such as India (0.66%), Nepal (1.0%), Myanmar (2.5%), Iran (0.87%) (Umar et al., 2010), China (1%) (Attaullah et al., 2011) and Afghanistan (1.1%) (Khan and Attaullah, 2011).

The HCV genotypes indicate unique pattern of distribution (Sy and Jamal, 2006, Alfaresi, 2011, Qazi et al., 2006). In Pakistan, the commonly prevalent HCV subtype that infects the

general populace, has been reported to be HCV 3a, with areas different in the frequency distribution of other subtypes (Arshad and Ashfaq, 2017), (Hamid et al., 2004, Waheed et al., 2009). HCV 3a has been reported with high frequency in different provinces of the country such as 69% in Punjab, 77% in Sindh, 58.5% in KP and 61% in Baluchistan (Khan et al., 2013). Besides HCV 3a, a considerable burden of other HCV genotypes have also been reported including HCV 3b, HCV 2a, HCV 2b, HCV 1a, HCV 1b, HCV 4 and the Untypable HCV. Although subtypes of HCV 1 have been rarely reported by several investigators from different parts of the country, yet some studies have reported a relatively higher number of subjects (12.0%) infected with HCV 1 in the Punjab province followed by the Untypable HCV genotype reported with high distribution rate in Sindh (8.33%), KPK (20.16%) and in Baluchistan (32.14%) (Attaullah et al., 2011, Idrees et al., 2008).

Nevertheless, latest data shows that although genotype 3a may be the major HCV subtype in Pakistan, the distribution pattern of different genotypes have gone through considerable variation (Ali et al., 2011b, Khan et al., 2014). Some studies demonstrated a pattern change in the distribution of HCV genotypes and an increased prevalence of genotype 2a, mostly in the areas of Khyber Pakhtunkhwa (KP) (Ali et al., 2011b, Khan et al., 2014). In the KP province, the bulk of infections are due to HCV subtype 3a (Gul et al., 2016) followed by 2a and 2b genotypes although some studies have come up with different results (Inamullah et al., 2011). Similarly, in Punjab; which is the largest province of Pakistan in terms of population with a total population of 204.60 million in 2017, a number of studies have concluded that genotype 3a has been the most abundant type over the past decade and a half (Idrees et al., 2008, Umar and Bilal, 2012, Waheed et al., 2017, Aziz et al., 2013), however the percentage prevalence mentioned in different studies is variable in the range of 75 to 80 % and the distribution of other HCV subtypes has never met a consensus because of limitations of different studies in terms of geographical or numerical aspects.

Various studies from Sindh province; which is the second most populous province of Pakistan, indicate that again HCV 3a leads all other subtypes with temporo-spatial variation in the percentage prevalence of other genotypes (Umer and Iqbal, 2016, Arshad and Ashfaq, 2017) while only a couple of studies are available from Baluchistan (Umer and Iqbal, 2016, Afridi et al., 2009) indicating a similar trend with abundance of HCV 3a and variations in the distribution of other types.

Costly treatment and diagnosis of hep C have been major impediments in curtailing the burden of infections in Pakistan and several other developing countries where majority of the population lives below the poverty line. The first antiviral therapy approved for the treatment of hep C was Interferon monotherapy(IFN) which usually resulted in minimal rates of response (Ali et al.,2016). In 1998, the adding of the guanosine analog, RBV to the traditional interferon did achieve little progress in sustained virologic response (SVR) even though it was not up to the mark especially in the case of HCV genotypes 1 and 4. In 2001, pegylation of IFN led to modify the pharmacokinetic sketch of IFN-α-2. Both PEG-IFN-α-2a and PEG-IFN-α-2b have slower absorption, minimal distribution and least eradication rate as compared to the non pegylated IFN-α. The sustained concentrations of peg-IFNα restricts viral replication for longer time of dose once in a week (Kamal, 2011, Ali et al., 2016a, Edwards et al., 2015).

Prior to 2011 peg-IFN and RBV therapy led to an improvement in SVR after the completion of 24 weeks of treatment. With PEG-INF α-2 and RBV, the rates of response in genotypes 3 and 2 were in the range of 70 and 80%. Nonetheless, the rates of SVR against HCV genotypes 1 as well as 4 infections were suboptimal. The related serious side effects had resulted into a much needed initiative to produce latest treatments that are more safe and effective (Zuberi et al., 2008, Sharafi et al., 2015). In 2016, the invention of DAAs, in the form of double and triple therapies, resulted into a new age of HCV treatment which was previously thought as a fantasy. The SVR rate of these therapies was more than 90% (Foster et al., 2016).

In 2013, the first polymerase inhibitor Sofosbuvir (SOFO) was approved as a key to the combination therapy for HCV infection. SOFO is a once-daily direct-acting nucleotide NS5B polymerase inhibitor that is affirmed as an oral prescription for the cure of chronic HCV infection (Bourlière et al., 2011). Since 2013, HCV genotype 1, 4, 5 or 6 and their related conditions are checked by administering a combined dose of SOFO-RBV-IFN, which was found to be 90% effective in fighting the viral diseases (Imran et al., 2013). Since 2016 daclatasvir (DCV) was approved in Pakistan which is a pangenotypic nonstructural protein 5A (NS5A) inhibitor (Gao et al., 2010). For subjects without cirrhosis, RBV- free treatment with DCV-SOFO for 12 weeks is exceedingly viable for treatment of genotype 3 infection. The sustained virologic response (SVR) rate at post-treatment week 12 (SVR-12) was reported to be 96% in genotype 3-infected subjects without cirrhosis, irrespective of previous HCV treatment experience, with great tolerability (Nelson et al., 2015). The DCV and SOFO combination, with

RBV, brought about high (96% to 98%) rates of SVR in subjects having HCV genotype 3 and 1 infection who had taken earlier medication with IFN-based therapy, including subjects who had taken a protease-inhibitor therapy and those with compensated liver cirrhosis (Pol et al., 2016, Sulkowski et al., 2014).

As HCV genotype determination is essential prior to making decisions about the type and duration of various treatment options (Gupta et al., 2014) therefore molecular characterization of HCV genotypes in various geographical areas of the globe is always needed for figuring out the prevalence of various genotypes or subtypes in respective populations which ultimately would help with disease eradication by designing effective control strategies. As an RNA virus, HCV is prone to mutate (Gul et al., 2016) particularly in social settings with uncontrolled outbreaks due to getting more chances to replicate in particular populations and hence variants, novel subtypes and genotypes are expected to emerge over times (Iqbal et al., 2017). This aspect of accumulating variations in its genome and the rise of variants makes it utmost necessary to carry out characterization of HCV genotypes over certain periods of time particularly in endemic regions. Moreover, globalization has increased chances of changing the epidemiological pattern of pathogens due to extensive migrations of infected people (Umar and Bilal, 2012) which re-enforces the need to put the surveillance program for characterization of HCV genotypes in place to figure out the temporo-spatial distribution of HCV genotypes. As there was no consensus on the current distribution pattern of HCV genotypes (Umer and Iqbal, 2016, Waheed et al., 2009, Ramia and Eid-Fares, 2006) and some small scale studies had claimed a pattern shift of HCV genotypes over the past couple of years in different parts of the country (Gul et al., 2016; Idrees, 2008), It was of much interest to figure out the existing distribution pattern of HCV genotypes on a relatively larger scale so as to update the current knowledge on the distribution of HCV genotypes in different regions for designing better control strategies and for understanding the dynamics of HCV infection in Pakistan.

Various strategies are being employed in order to characterize the HCV genotypes including Type-specific PCR and sequencing (Gul et al., 2016, Zein et al., 1996). Although sequencing is thought to be the golden standard for HCV genotyping, yet, it is not applicable on a massive scale in endemic regions which are mostly resource-limited such as Pakistan, Egypt and other countries (Farag et al., 2015, Kumar et al., 2018). Majority of the studies from Pakistan (Idrees et al., 2009)and elsewhere (Messina et al., 2015, Gower et al., 2014) have employed Type-specific

PCR for characterization of HCV genotypes but due to limitations of the procedures to amplify variants that arise over times, several studies have reported Untypable HCV genotypes from various regions across the globe including several studies from Pakistan (Afzal et al., 2014, Lovo et al., 1996). As HCV genotype is an vital factor for calculating possible response to therapy (Waheed et al., 2017), therefore it was utmost necessary to characterize the Untypable genotypes prevalent in particular regions in order to guide treatment decisions and to understand viral evolution. It was due to the high percentage of the Untypable HCV genotypes reported from different parts of Pakistan that motivated us to characterize them on a representative scale.

Although new antiviral therapies with better response rates and fewer side-effects have been developed recently (Manns et al., 2006, Cornberg et al., 2006), however in Pakistan, IFN-based combination therapies are still being practiced in various parts of the country (Ali et al., 2011a, Ahmad et al., 2012) due to a variety of reasons including accessibility and affordability. Response rates of IFN-based regimes against HCV 3a and some other types have extensively been reported from various parts of the country with considerable variations (Ali et al., 2011a, Ali et al., 2016a) and some studies have also reported increasing resistance to these regimes in the case of HCV 3a as well (Ali et al., 2016b). Moreover, there are very few small scale studies that have reported response rates of SOFO-based combination therapies against HCV types (Waheed et al., 2017, Akhter et al., 2017) but as per the updated information, response rates of the newer IFN-free formulations or their combinations against HCV 3a or other prevalent types have not been reported from Pakistan so far. It was because of these reasons that we planned this study to investigate the comparative efficacy of various anti-viral regimen (with or without IFN) including the newly approved DAAs in Pakistani population on a representative scale as response rates of several of the combinations have not been assessed against HCV types prevalent in different ethnic groups of the country.

Hypothesis:

"As compared to conventional combination therapies (IFN-based) DCV-RBV-PEG-IFN, the newly approved anti HCV oral combination therapies (IFN-free) SOFO-RBV, DCV-SOFO and DCV-SOFO-RBV will show a better response rate against infections with different HCV genotypes among chronically infected patients with HCV in Pakistan".

Aims and Objectives

The aim and objectives of this research were

Aim:

- Molecular "Molecular characterization of HCV Genotypes via PCR as well as partial sequencing and assessment of response rates of various (IFN-based) DCV-RBV-PEG-IFN and (IFN-free) new direct-acting antiviral therapies combinations SOFO-RBV, DCV-SOFO and DCV-SOFO-RBV among patients of Pakistani origin who are chronically infected with different HCV subtypes".

Objectives:

- To investigate the distribution patterns of hepatitis C virus genotypes in different geographical regions of Pakistan.
- Molecular characterization of Hepatitis C Untypable genotypes through partial sequencing among chronically infected patients in Pakistan
- To figure out the Response rates of (IFN-based) combination therapies DCV-RBV-PEG-IFN and the newly approved anti HCV oral combination therapies (IFN-free) SOFO-RBV, DCV-SOFO and DCV-SOFO-RBV among chronically infected hep C patients in Pakistan.

2. Literature Review

2.1. Hepatitis

Hepatitis is inflammation of the liver cells (called hepatocytes) which happens due to a variety of reasons including viral or bacterial infections. Hepatitis of viral etiology usually leads to a number of complications including steatosis, fibrosis, cirrhosis and heaptocellular carcinoma (HCC). Liver is a major organ which occupies the whole upper right quadrant of the abdomen in the human body. It does more than 500 important biological functions including detoxification, production of immunoglobulins, storage of carbohydrates etc. The ultimate damage to the liver disturbs all its processes. The severity of hepatitis varies from a self-limited status with complete revival to chronic liver diseases. More over certain environmental toxins, chemicals, drugs, alcohol use and other medical complications also result in liver inflammation (Idrees et al., 2008, Idrees et al., 2009).

Hepatitis might be acute or chronic. Acute hepatitis advances into a chronic one. Generally acute hepatitis comes abruptly or gradually and lasts for four to eight weeks. In acute hepatitis, usually there is liver steatosis which indicates cell damage due to activity of the immune system, but some time it leads to various degrees of severity. Relatively chronic hepatitis comes slowly and lasts for long period of time. On the basis of severity, sign and symptoms of the condition, chronic hepatitis is categorized and characterized as (a) Chronic non active hepatitis which is typically mild or slowly progressive and results in limited liver damage and (b) chronic active hepatitis which causes widespread liver harm and cell damage away from the portal tract (Idrees and Riazuddin, 2008, Idrees et al., 2009).

2.2. Hepatitis Viruses

The infection of liver cells due to viruses leads to hepatitis. For centuries these viruses are co-evolving with humans, since then these viruses are existing and transmitting the disease inside humans. (Szabó et al., 2003). The emergence of viral hepatitis is an alarming medical health issue across the globe affecting 200 million individuals (Umar et al., 2016). Substantial morbidity and mortality in the individual's population are due to viral hepatitis caused by a number of DNA and RNA viruses known as HAV, HBV, HCV, HDV, HEV etc.

2.2.1 Hepatitis A Virus (HAV)

HAV was characterized in 1973 (Feinstone et al., 1975), categorized under the umbrella of hepatovirus. HAV is a non-enveloped symmetrical RNA virus, with a 7.5 kb single-stranded positive-sense RNA genome. It has fecal-oral transmission route which causes infectious or epidemic hepatitis (WHO., 2018).

2.2.2 Hepatitis B Virus (HBV)

HBV was discovered in 1965, which comes under the umbrella of hepadna virus cluster with double stranded DNA. A remarkable character of HBV is its genome. The HBV DNA is a relaxed, circular, partial double strand and about 3.2 kb long. Globally HBV infection is a major health issue, affecting about 2 billion individuals, out of which 350 million individuals have chronic hepatitis B infection (CHB). In general hepadna virus can also infect mammals and birds (Datta et al., 2012).

2.2.3 Hepatitis D Virus (HDV)

HDV virus was discovered in 1977 (Rizzetto et al., 1977). Hepatitis D virus (HDV) replicates in unusual fashion and is characterized as, single stranded, spherical RNA virus with resemblance to definite plant viruses as well as viroids. There is lengthwise variation in the genome of DNA virus which ranges from few kilo-bases (kb) to several hundred kb. The genome size of HDV is approximately 1.7 kb, making it the smallest genome of any virus (Huang et al 2010). This virus requires hepadna virus helper functions for propagation in hepatocytes, and is prime reason of both acute and liver damage across the globe (WHO., 2018).

2.2.4 Hepatitis E Virus (HEV)

In 1983 Hepatitis E virus (HEV) was extracted from the feces of a human with HEV infection (Balayan et al., 1983). Hepatitis E virus comes under the umbrella of caliciviruses, the distinctive features of this virus are that it is with single stranded (+) sense RNA genome which varies from 6.6 to 7.3 kB in length. It is non enveloped shape virus and can be transmitted enterically. Usually acute hepatitis occurs due to HEV in Indian subcontinent, Southeast Asia and Central Asia, some countries of Middle East and African countries (WHO.,2018).

2.2.5 GB Virus C Hepatitis G Virus (GBV-C)

GBV-C as well as hepatitis G virus (called HGV) were consecutively discovered in 1995 year and 1996 members of the family *Flaviviridae* (Simons et al., 1995, Linnen et al., 1996). Recently the GB hepatitis viruses variants were generated and basic genomic description demonstrated their association with other viruses of (+) RNA strand, with limited parts of bases similarity with different flavi-viruses. Phylogenetic analysis of genomic sequences strongly demonstrates that these viruses were not originated from HCV genotypes.

2.3 The Hepatitis C Virus Discovery

For the first time in 1970 the word Non-A, Non-B (NANBH) was introduced to illustrate the liver inflammatory diseases which were not caused due to the infection of hepatitis A or B viruses (Alter et al., 1975, Feinstone et al., 1975, Prince et al., 1974). The Serology examination affirmed that HAV and HBV were not involved in about 90% of the hepatitis infection cases (Knodell et al., 1975). Moreover in 1978 it was observed that NANBH can also infect chimpanzees (Alter et al., 1978, Hollinger et al., 1978, Tabor et al., 1978). Filtration investigation demonstrated that the size of the agent (NANBH) is smaller than 80nm, and therefore it was most probable to be a virus (He et al., 1987). Sensitivity to chloroform showed that NANBH virus was enveloped one (Bradley et al., 1983, Farag et al., 2015). In 1989 the molecular cloning of (NANBH) agents was documented and it was confirmed that it was (+) stranded RNA virus and was termed as HCV (Choo et al., 1989).

2.4 HCV Genome

HCV is a small, single stranded, (+) sense RNA virus. Size of genome is about 9600 bases, encircled by viral envelop and a capsid shell originated from the lipid bilayer of the host (Inoue and Tsai, 2013). Genome of the HCV is comprised of an open reading frame (ORF), which encodes a poly-protein precursor of about 3,011 continuous amino acids chain which is edged by 5′-NTR of 341bases as well as 3′-NTR of about 200-300 bases (Oh et al., 1999). ORF induces HCV translation through 5'NTR, operating as an internal ribosome entry site (IRES). It helps out the ribosome's attachment to the ORF start codon (Khawaja et al., 2015). The 3′-NTR

consists a tri-partite structure that covers a variable region directly after the stop codon of the ORF, a poly (U/UC) zone that varies in measurement lengthwise in the range of 30 to 150 nucleotides as well as a fine conserved "X-foot end" of about 98 bases in length (Ivanyi-Nagy et al., 2006), necessary for viral replication. HCV polyprotein is converted into ten structural and non structural proteins by the action of protease enzymes (Arumugaswami et al., 2008, Isken et al., 2015). Schematic diagram of the genome of HCV which encodes different proteins is revealed in (Fig 2.1).

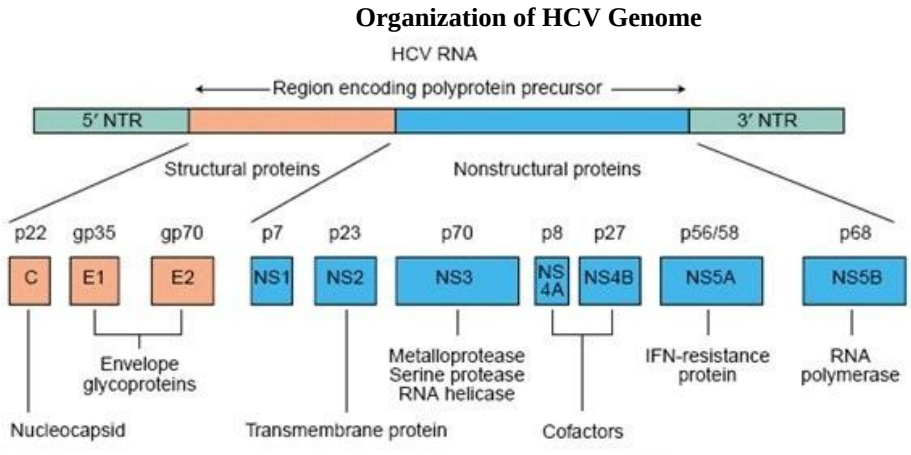


Figure 2.1: HCV genome diagrammatic representation

Like other *Flaviviruses* family members, Core, E1 and E2 are the major structural elements of the virus poly protein and (p7, NS2 through NS5B) NS proteins are considered to be of prime importance in the replication of viral RNA (Kamili et al., 2012). Fundamental physiology of hepatitis C virus (S and NS) proteins are given in (Table2.1).

Table 2.1: Description of HCV proteins

Region	Description	Action
Core	Capsid protein	Nucleocapsid formation, contains IRES, interacts with viral RNA (73)
Envelop E1/E2	Type 1 transmembrane-glycoproteins	Surround the viral particles; involved in viral entry thru receptor binding and fusion (74)
P7	Viroporin	Essential for viral assembly and release (75)
NS2/NS3	Viral auto-protease	Main target for HCV antiviral drug development (76)
NS4A	NS3 protease co-factor	Forms a stable complex with NS3 (77)
NS4B		Membranous web formation (78, 79)
NS5A	Zinc-binding metallo-protein	Regulator of replication and viral assembly (80)
NS5B	RNA dependent RNA polymerase	Production of new viral RNA genomes (81)

2.4.1 HCV 5' UTR

In most of the viral isolates, the length of 5'UTR is 341-nt of the HCV genome. Above 90% of homogeneity exists among various HCV genotypes, while some of the region are closely identical among the various strains (Bukh et al., 1992). The secondary structure as well as tertiary structure of this segment are almost preserved (Brown et al., 1992, Honda et al., 1999). Majorly four structural domains (domains I-IV) of the HCV 5'UTR, have been acknowledged by the combination of phylogenetic, computational and mutational analyses (Fig 2.2), most of which are preserved among the HCV genotypes (Brown et al., 1992, Honda et al., 1999, Honda et al., 1996, Smith et al., 1995). General structures contain a pseudoknot (psk) and a large stem-loop III. 5'UTR sequences of HCV consists of two smaller stem-loops, stem-loop-Ia close to the 5' extreme end and stem-loop-IV having the starting codon for translation (Honda et al., 1996).

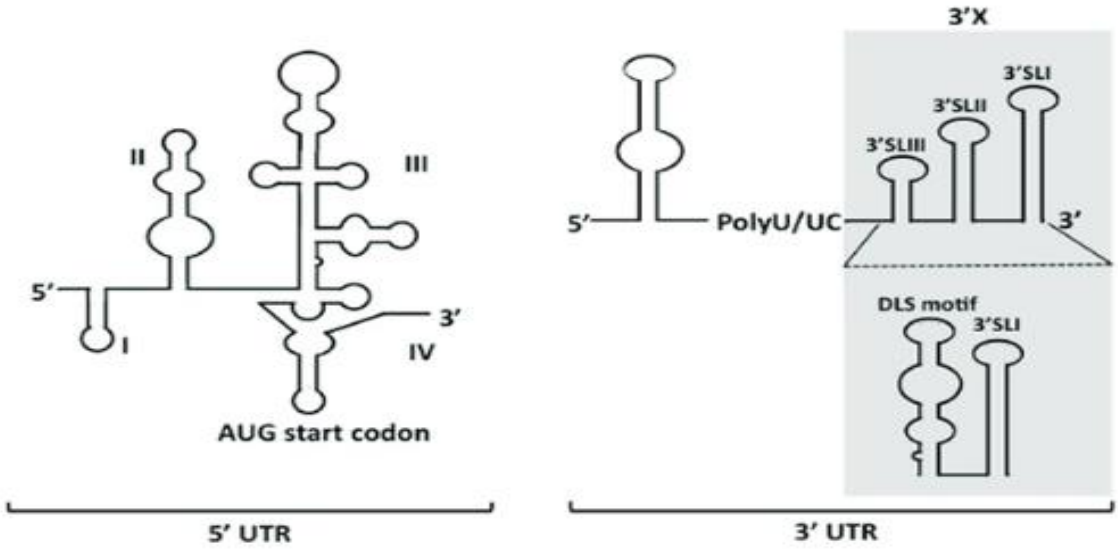


Figure: 2.2 Depicted secondary structures of 5-and 3 UTRs of HCV genome. The 5 UTR contains four structured domains (I-IV). The translational start codon is indicated. Two types of conformations for the 3 X region (shadowed region) within the 3 UTR have been proposed.

Domain-I is formed by the initial 40 nt of 5'UTR, which are involved in RNA replication but do not play a necessary role in translation; because of this the physiology of this segment is distinguished from the remaining of the 5'UTR, which helps in translation (Friebe et al., 2001, Luo et al., 2003). Rest of the domains II-IV form an IRES (Fig 2.2) (Brown et al., 1992), which play role in cap independent translation of the ORF of HCV (Tsukiyama-Kohara et al., 1992). Domain-IV is not so much complicated in comparison to domains II and III and include several loops and stems (Honda et al., 1999, Lemon and Honda, 1997). NMR and numerous electron microscopy studies have provided comprehensive structural data (BEALES et al., 2001, Spahn et al., 2001) on the IRES main domains (Lukavsky et al., 2000). From a minute central domain that contain junctions IIIe–IIIf and stem loops, domains IIIa–IIIc and II extends in opposed directions (Spahn et al., 2001). The hairpin loop of the minute subdomain IIIe constitute a new tetra-loop fold with three uncovered Watson–Crick appearance that may play important role in ribosome 40S attachment (Lukavsky et al., 2000). The IIId sub domain stem constitute an E loop motif similar to those noted in ribosomal RNA of eukaryotic and prokaryotic cells, and hairpin loop with six nucleotide comprising an S turn motif (KLINCK et al., 2000, Lukavsky et al., 2000). Hairpin loops sequences of the IIIe and IIId sub domains are preserved in total HCV isolates and involved in initiation of translation.

A pseudo-knot an extremely preserved region constituted in the bottom of III domain, plays important role in IRES action (Wang et al., 1995). For the ribosome 40S subunit pseudo-knot is the region of the attachment site (Kolupaeva et al., 2000). In II domain other tertiary structural element acknowledged by RNA-RNA cross linking, may play role in ribosome attachment (Lyons et al., 2001). Domain IV is made up of a minute stem-loop in which AUG is the starting codon which is positioned inside the single stranded loop part (Honda et al., 1996). Stem loop IV is not mandatory for ribosomes internal entry. In reality, the constancy of this stem loop structure is negatively associated with the viral RNA translation (Honda et al., 1996).

2.4.2 3'UTR region of HCV genome

The 3'UTR of HCV lengthen about 200-235 nt, which comprised of 3 different segments, in the direction of 5' to 3', a variable region poly(U/UC) sequence and an extremely preserved 98

nt X part (Blight and Rice, 1997, Kolykhalov et al., 1996, Tanaka et al., 1996, Yamada et al., 1996). Without delay the termination codon of the HCV poly protein is followed by the variable region, and is variable lengthwise (27 to 70 nt in range) and in structure among various genotypes. Still, among viral strains of the same genotype it is extremely preserved (Kolykhalov et al., 1996, Yanagi et al., 1997, Yanagi et al., 1998).

Two possible stem loop structures have been identified through computer analysis in the variable segments, of which the 1st stem loop lengthen into the 3' end of the NS5B coding sequence (Han and Houghton, 1992, Kolykhalov et al., 1996). The poly(U/UC) tract comprises of a poly(U) chain and a C(U)n-repeat region (referred to as the transitional segment) and varies to a great extent in length and somewhat in sequence between various viral isolates (Tanaka et al., 1996). Numerous preserved A residues are present in the transitional regions of genotypes 2a, 3a, and 3b, which are absent in genotypes 2b and 1b (Tanaka et al., 1996, Yamada et al., 1996, Yanagi et al., 1999). Among flaviviruses, the polypyrimidine tract availability inside the 3'UTR is distinctive to HCV (Simons et al., 1995). Replication capacity of HCV RNA has been associated to the length of this region (Friebe and Bartenschlager, 2002, Kolykhalov et al., 1997, Yanagi et al., 1999, Yi and Lemon, 2003). The region-X constitute 3 constant stem loop structures that are extremely preserved throughout all genotypes (Blight and Rice, 1997, Ito and Lai, 1997, Kolykhalov et al., 1996) (Fig 2.2).

Enzyme probing and certain chemicals have confirmed the availability of SL1 and SL3, which is the structure of the X-region reported by recent studies, but suggested that inspite of one and may further form a imaginary pseudoknot, the segment between the two stem-loops folds into two hairpins (Dutkiewicz and Ciesiołka, 2005). In comparison to this, the complementary sequence of the X region form a 3-stem loop structure in this region (Dutkiewicz and Ciesiołka, 2005). In the 3'UTR poly(A) sequence is absent. Instead, the sequence of 3'UTR, specifically the region-X, play important role in the translation regulation, greatly in the similar fashion as the sequence of poly(A) in the in mRNAs of other RNA viruses. Conceivably, these sequences are involved in the replication, stabilization and also packaging of viral RNA.

Consequently the stem loop creation in the X-region, the HCV genome is estimated to finish with a double-stranded stem. The assessment of the 3'-terminal sequences of HCV genome in sera of infected subjects demonstrated that the largest part of HCV RNAs contain identical 3'

ends with no additional sequence downstream of the X foot end (Tanaka et al., 1996). On the other hand, one specific cDNA genetic copy extracted from a subject serum contain 2 extra nt (UU), hence creating a single-stranded tail (Yamada et al., 1996). For the commencement of RNA replication the structure of 3'-end will have certain implications.

2.5 HCV Replication

The replication process of the RNA of hepatitis C virus has been demonstrated in the hepatocytes of human or in chimpanzees (Frentzen et al., 2015). The virus also replicates in peripheral blood mononuclear cells (PBMCs) and practically infects B and T-cell lines (Tariq et al., 2012). HCV replication rate can be comparatively elevated in the range of 10^{10}-10^{12} virions each day, and projected half life is about 2-3 hours (Chen and Morgan, 2006). Replication cycle sketch of HCV in liver cells is demonstrated in (Fig 2.3).

2.5.1 Virus attachment to the host receptor cells

For cell attachment, HCV use encoded viral envelope proteins, considered as very initial step of viral infection (Jiang et al., 2012). The Hypervariable Region (HVR1) positioned inside the envelope of E2 glycoprotein in connection with different lipo-proteins play important role in attachment of the virus (Urbaczek et al., 2015). The primary proteins of cell surface which are involved in attachment to the viral envelope glycoproteins E1 and E2 and start binding and cell entry are Heparan Sulfate Proteoglycans (HSPGs), Low Density Lipoprotein receptor (LDLr), CD81, Claudin-1 (CLDN1), Scavenger Receptor Class B type 1 (SRB1) and Occludin (OCLN (Ujino et al., 2016).

2.5.2 Entry of the Virus

It is presumed that receptor mediated endocytosis is involved in the entry of HCV into hepatocytes cells and ensuing fusion among the virus and membranes of the cell (Jiang et al., 2012). CD_{81} receptor cells positioned on the surface of hepatocytes cells play important role in attachment to E2 envelope protein, originate a conformed modification in the E1 or E2 envelope proteins, thus assist the pH reliant union of the viral elements with cells of the host and successive clathrin mediated endocytosis (Ujino et al., 2016).

2.5.3 Translation of Viral RNA

After internalization of virus by the host hepatocytes, the positive strand HCV genome undergoes a process called cap-dependent IRES mediated translation (Lupberger et al., 2015). The positioned IRES inside the 5′UTR segment directs the attachment of ribosomes to RNA of HCV and also starts translation through attachment in near end to the (AUG) start codon of large poly-protein with minute or no scanning. Rough Endoplasmic Reticulum (RER) has a vital role in HCV polypeptide processing. At the RER membrane, the replicase compound is accumulated, which leads to the formation of a complementary negative-sense RNA intermediate, which is later on, used as a amplified segment of RNA for the manufacturing of fresh (+) sense genome of RNA (Kallio et al., 2013, Moradpour and Penin, 2013).

2.5.4 Viral poly-protein processing

Proteases of both the host cell and virus cleave the poly protein precursor into total of ten structural and non structural proteins which form full grown hepatitis C virus (Hundt et al., 2013). At the junctions, C-E1, E1-E2, E2-p7, p7-NS2, ER signal peptidases chop up the Core-NS2 segment (Vieyres et al., 2014), while HCV non structural proteins manufacturing is completed through NS2/NS3 as well as NS3/NS4 viral proteases which cleave at junctions of NS2-NS3, NS3-4A, NS4A-B, NS5A-B as well as NS4B-5A. Apart from virus proteins, a lot of cellular aspects play role in the replication of viral RNA such as Cyclophilin, which changes RNA attachment ability of NS5B polymerase as well as interact with NS5A. With ongoing clinical development Cyclophilin inhibitors have anti-HCV effect (Germain et al., 2014). A supplementary vital protein of the host cell needed for the replication of virus is Phospha-tidyl-inositol 4-Kinase III α (PI4KIIIα), which is a lipid kinase and is employed through NS5A to manufacture and integrate the membranous complex of viral replication (Reghellin et al., 2014).

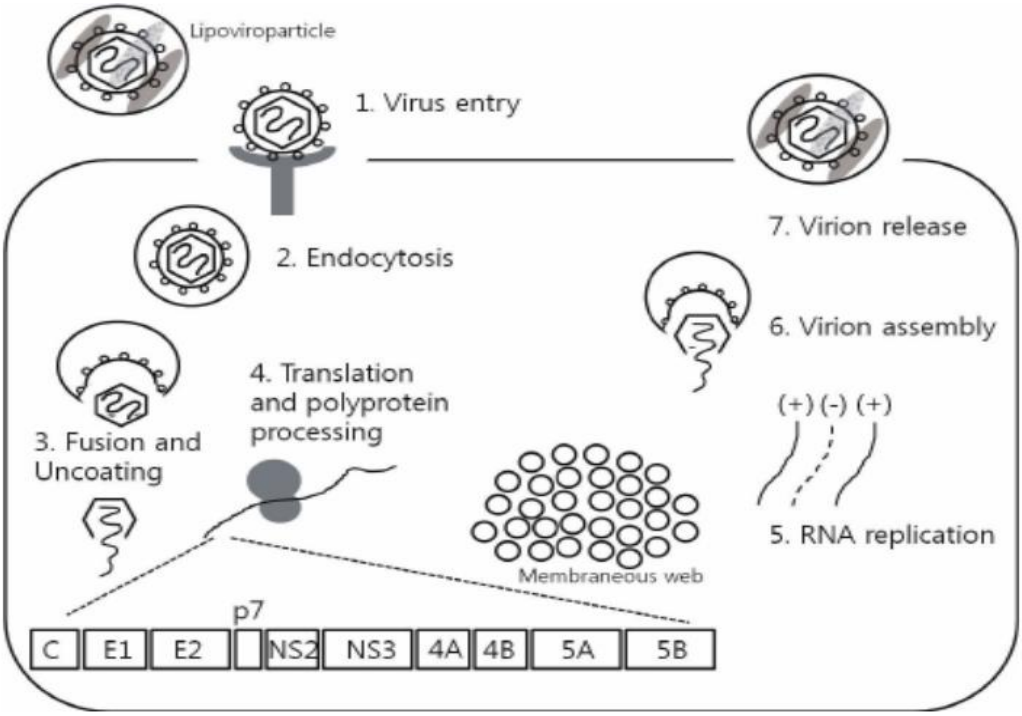


Figure 2.3: HCV replication cycle

2.5.5 Assembly and release of the Virus

At the lipid droplets surface, fresh transcribed viral RNA is accumulated at a site where ER membrane is linked, most likely through contact among NS5A and Core protein (Zayas et al., 2016). Nucleocapsid structure and encapsidation consequently results when the RNA of virus is permitted to relate with (Core) protein (Suzuki, 2012). Assembly of the new viruses carries on by unknown processes in which E1/E2 envelope glycoproteins are added to the nucleocapsid. Interaction between p7-NS2 and NS3-4A is necessary to recruit C protein from lipid droplets into sites of virus assembly (Guo et al., 2015). In the manufacturing process of lipoprotein of HCV elements and their release out of the cells, the secretion pathway of very-low-density lipoprotein are involved (Jammart et al., 2013). During egress, viral particles depend on p7 to neutralize acidic compartments within the secretory pathway (Dubuisson and Cosset, 2014).

2.6 Pathogenesis of HCV

HCV is a major reason of acute as well as chronic hepatitis (El-Shamy and Hotta, 2014, Ozaras and Tahan, 2009). Mostly hepatitis C is considered an unvoiced killer disease where the acute stage is considered to be related with jaundice but most of the time the infection is asymptomatic (Kamal, 2008). The acute phase converts to chronic phase resulting in rigorous appearance like cirrhosis of liver and HCC (Chung and Baumert, 2014, McCombs et al., 2014). Summary of innate evolutionary history of the infection of hepatitis C virus is given below (Fig 2.4).

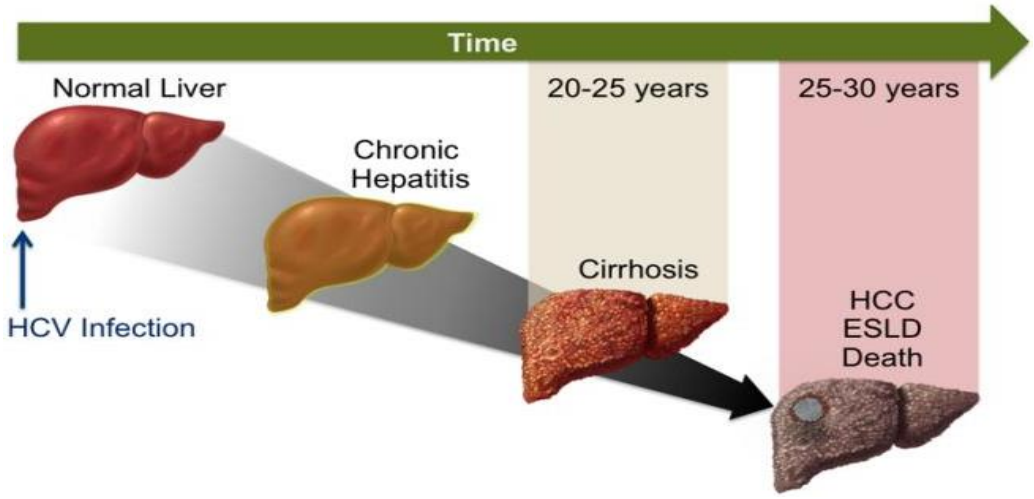


Figure 2.4: HCV infection innate history

2.6.1 Acute HCV infection

In general HCV acute infection is less severe as compared to chronic HCV infection and 15-25% chance is there of curing infection which is instinctively indicated by HCV aviremia (Hajarizadeh et al., 2013). In acute phase of infection (up to 24 weeks), the subjects generate general symptoms like loss of appetite, malaise, jaundice, anorexia, fatigue and fever. Yet some times HCV acute infection typically progresses to chronic infection and about 60 to 85% of the subjects extend to chronic hepatitis (Tsertsvadze et al., 2016).

2.6.2 Chronic HCV infection

Infection lasting for more than 24 weeks is designated as chronic HCV infection. The infection relies on several viral as well as host factors including age of the subject, gender, ethnicity, primary sickness, genotype of virus and significantly the effectiveness of the immune

response of infected subject. HCV chronic infection is commonly without symptoms, slowly and gradually developing infection distinguished by constant liver inflammation with fibrosis which finally leads to the advancement of cirrhosis in nearly 10–20% subjects in duration of 2 to 3 decades. In general, subjects with HCV related cirrhosis have a 3–6% yearly risks of hepatic decompensation and 1–7% yearly risk of hepatocellular carcinoma (Hoshida et al., 2014). The death risk in hepatic decompensation is about 15-20% in the following years (Westbrook and Dusheiko, 2014). Different complications are related with Liver cirrhosis which includes hepatocellular carcinoma, varices bleeding and ascites. Other cofactors like alcohol use, fatness and HIV/AIDS are significant features in the development of liver fibrosis.

2.7 Global prevalence of HCV

HCV is a blood born pathogen and its prevalence varies across the globe (Pybus et al., 2009). The current situation of disease burden clearly indicates the rise in its prevalence which has been documented above 3% during the last 15 years, correlating to it the infections of about 200 million around the globe. However in developed countries the incidence rate has fallen down (Messina et al., 2015). A study carried out in 2014 demonstrated that generally, 31 nations are accountable for above 80% of the total HCV infections which includes Egypt, Pakistan, China, , Russia , India, and Nigeria (Gower et al., 2014). In a few developed countries of western Europe and in Australia, the load of the disease is comparable to that in the US, at less than 2% (Mohd Hanafiah et al., 2013, Kretzer et al., 2014). Comparatively elevated infection rates (≥3%) have been documented from most of the African countries, East regions of Europe, Latin America, South Asia and Middle East (114). So far Egypt stands first in hepatitis C infections all around world (≥10%) (Averhoff et al., 2012, Fadlalla et al., 2015).

2.7.1 HCV Prevalence in Pakistan

HCV prevalence significantly fluctuates in various locations of the same state and even inside different individuals of the same population (Shakeri et al., 2013). Hepatitis C infection affects about 18 million population of Pakistan and has an average occurrence rate of 4.7-6% in different regions of the country (Afridi et al., 2014, Khan et al., 2013). The infection rate has been reported to vary in different provinces with documented frequency of about 7% in Punjab

province, 5% in Sindh province, 1.7% in Baluchistan province, and almost 1.1% in KP province (Umar and Bilal, 2012).). However the prevalence rates of hep C virus might be more than the documented frequencies in different provinces. For instance in various cities of Punjab which is the biggest province of Pakistan in terms of population, the prevalence rate of HCV infection was in the range of 317/995(31.85%) in Lahore, Faisalabad 70/995(7.03%), Gujranwala 129/995(12.96%), Gujrat 106/995(10.65%), Sialkot 94/995(9.44%), Sargodha 60/995(6.03%), Mandibaha-ud-din 135/995(13.56%), Jhang 86/995(8.64%) has been documented (Afridi et al., 2014). Similarly studies from the Sindh, KPK and Baluchistan provinces have reported HCV prevalence in the range of 3-5%, 3-9% and 1.5-2.0% respectively (Ahmad et al., 2012, Khan et al., 2011a, Nawaz et al., 2015).

2.8 Classification of HCV genotypes

The heterogeneity between HCV genotypes is 31-33% (Timm and Roggendorf, 2007). Further the genotypes of HCV are classified into various distinctive subtypes with 20-25% nucleotide variation from each other (Table 2.2). Taxonomic classification and identification of novel genotypes as well as subtypes of hepatitis C virus is completed either by sequences analysis of phylogenetic information or through pairwise sequence match (Simmonds et al., 1996). Long sequences are relatively more useful for classification; generally there is possibility to classify the genotypes of HCV through sequence matching of relatively small sub-genomic segments (Marascio et al., 2014). Phylogenetic analysis of amplified RNA sequences in genome segments encoding the 5′UTR, Core as well as NS5B which are regularly used to classify the genotypes as well subtypes of hepatitis C (Saludes et al., 2014, Li and Lo, 2015). The Core along with NS5B of HCV have enough genetic heterogeneity and reflect the whole genome-based phylogeny (Simmonds et al., 1993).

Table 2.2: Terms used in studies associated to HCV genomic heterogeneity on the basis of whole HCV genome sequences.

Terminology	Definition	%Nucleotide Dissimilarity
Genotype	Genetic heterogeneity among different HCV isolates	31-33%
Subtype	Closely related isolates within each of the major genotypes	20-25%
Quasispecies	Complex of genetic variants within individual isolates	1-5%

2.9 HCV Genotypes Distribution

2.9.1 Global HCV genotypes distribution

HCV shows a great deal of genetic diversity in unusual fashion (Echeverría et al., 2015) its genotypes prevalence varies in different geographical locations across the globe (Altuglu et al., 2008). It has the capability to challenge the innovation of both the pan genotypic therapies as well as HCV vaccines a diagnostic strategies (Messina et al., 2015). In recent times, the HCV genotypes and subtypes are useful to direct the type and duration of therapy prescribed (Myers et al., 2015). On the basis of whole genome sequence, 7 major HCV genotypes have been identified through phylogenetic and sequence analysis (Smith et al., 2014). All HCV genotypes are further classified into 67 affirmed and 20 temporary subtypes, which vary in their genetic composition (Messina et al., 2015). Genotypes 1, 2 and 3 have global distribution (Rasheed et al., 2014) with genotype 1 found abundantly, followed by genotypes 3 and then 2 respectively (Li and Lo, 2015).

HCV genotype 1a the model type used in the improvement of primary diagnostic tools common in Europe as well as US (Kamili et al., 2012). Similarly HCV 1b is found in the US, Japan and European countries (Njouom et al., 2012). HCV 2b and 2a are commonly reported in Japan and Northern America, while HCV 2c genotype is generally prevalent in Northern parts of Italy (Ali et al., 2011c). HCV genotype 3a, is frequently found in South-east Asia, the India subcontinent and Indonesia (Wasitthankasem et al., 2015). Genotype 4 is reported to be prevalent

in the Middle East and Northern parts of Africa (Thong et al., 2014), whereas genotypes 5 and 6 are commonly found in South Africa as well as Hong Kong respectively (Al Naamani et al., 2013). So far, genotype 7 has been reported in Canada only (Murphy et al., 2015).

2.9.2 HCV genotypes distribution in Pakistan

A systemic review on geographical distribution of HCV genotypes and subtypes in four provinces of Pakistan revealed that majority of HCV infections in patients were attributed largely to HCV genotype 3, with an overall prevalence of 68.94% in Punjab, 76.88% in Sindh, 60.71% in Balochistan and 58% in KP. In a small scale study conducted in 1997 genotype 3 was identified as the most common strain (87%) in HCV associated chronic liver disease in Pakistan followed by genotype 1 (9%) and genotype 2 infections (Ali et al., 2011c). In 2004 similar results were reported in which genotype 3a alone contributed to at least a 75-90% of all isolates (Hamid et al., 2004). Between 2006-2007 two studies identified HCV genotype 3a as the most abundant genotype affecting 71% and 81% of Bahawalpur and Faisalabad poulation respectively, followed by type 1 infections ranging 9.5-10% as reported earlier (Waheed et al., 2009). In 2008 a study conducted in Karachi reported genotype 3a in 51% of young healthy females followed by high proportion (24%) of mixed 3a/3b infection while 3b and type 1 infections were present in only 4% and 3% of studied subjects (Ali et al., 2010). Almost similar results were reported from Balochistan province in 2009 by Afridi *et al.,* with HCV genotype 3a present in 50% of HCV isolates followed by type 3b and type 1 infections (Afridi et al., 2014).

A comprehensive research study conducted in 2008 indicated that in Pakistan, the prevalence of various HCV genotypes is as follows; genotype 3a (49.7%) followed by 3b (17.6%), 1(8.2%), 2a (7.5%) and 1b (3.0%) (Idrees et al., 2008). A study was carried out in 2013 to estimate the burden of HCV genotypes in Punjab, which showed that HCV genotype 3a (88%) was found in abundance followed by 1a (3.5%), 3b (3.0%), 1b (1.0%), 2a (1.0%), 1a1b (0.3%), 3a3b (3.1%), and 3a1b (0.2%). There is slight variation among HCV genotypes distribution pattern in various cities of the province (Aziz et al., 2013). A study conducted by Aziz et al., reported that HCV genotype 3 (94%) was the most prevalent in Rawalpindi division, with frequency rate of subtype 3a (89 %) followed by 3a3b (3.5%), and 3b (0.6%). HCV genotype 1 (5.6%) includes the subtypes 1b (1.5%), 1a (3.5%) and 1a1b (0.6%). 3a1b was found as (0.3%) (Aziz et al., 2013). In the same study, the distribution pattern of HCV genotypes in Sargodha division was reported as 93.0% HCV genotype 3a, while (n=6, 4.0%) had

genotype 1a and 3a3b was noted in four (2.7%) subjects (Waqar et al., 2014). Only HCV genotype 3a was found in Gujranwala Division; no other genotype was detected in the mentioned division (Aziz et al., 2013). Two diverse research studies from Punjab reported that HCV 3a was affecting (88.1%) and (71%) of chronically infected subjects followed by 3b, 1a and 1b infections (Aziz et al., 2013, Marwaha and Sachdev, 2014). So far no data is available for review in Azad Kashmir and Islamabad regarding HCV genotypes distribution.

In 2010 among the typeable samples from Peshawar and other districts of KP, HCV 3a was the prevalent type (72.22%) followed by 3b and a relatively higher (19.44%) percentage of untypeable samples among Peshawar population (Ali et al., 2011a). Among HCV infected population of Hazara division of Pakistan Ali *et al.*, in 2011 showed 54.42% of HCV genotype 3a followed by an equal proportion 8.19% of genotype 3b and mixed genotypic infections, while genotype 1a was present in 6.8%, 1b in 4.6% and 2a in 1.31% of patients (Ali et al., 2011a). Another study in 2011 in hemodialysis patients of Peshawar reported a high rate of HCV 3a (42.85%) and 3b (23.21%) genotypes as compared to other types (Safi et al., 2012). In two separate studies from district Swat of KP province genotype 3a was the predominent genotype (45.5%) followed by a higher percentage (16%) of mixed genotypic infection (Mahmood et al., 2011).

A small level study conducted in 2012 (Khan et al., 2014b) demonstrated that HCV genotype 3a affecting about (43%) of chronically infected subjects in KPK in which genotype 3b (22.32%), 1a (12.50%), 1b (8.92%), 2a (9.82%) and 2b (1.78%) were found respectively (Afridi et al., 2013). In 2013 a study carried out in district Mardan documented that HCV genotype was found with the highest frequency of 3a was (90%) followed by 1a,3b and genotype 4 which was rarely found in Pakistan (Afridi et al., 2013). In 2014 a study was carried out by (Khan et al., 2014a) in which 537 isolates were included from DI Khan and district Peshawar. This study reported that the prevalence rate of HCV 3a was (66.11%) followed by 2a (7.45%), 3b (2.61%) and genotype 2b (0.93%), 1b (0.92%) and 1a (0.90%) and Untypable HCV genotypes (18.81%) (Ali et al., 2014).

2.10 HCV diagnosis

There are two general categories for HCV diagnostic tests. 1) Serology assay which detects antibody to HCV (anti-HCV); and 2) Molecular assay which completely characterize the RNA genome of HCV infected subjects (Saludes et al., 2014). Serological assays have further

subdivided into screening tests for anti-HCV like enzyme immunoassay (EIA) (Li and Lo, 2015), and supplemental tests like recombinant immunoblot assay (RIBA). So far 3 generations of anti-HCV have been introduced and each generation has improved the sensitivity of anti-HCV detection. Supplemental anti-HCV tests are developed to fix the problem of false-positive testing through EIA, and are properly used in low-prevalence settings in which false-positive anti-HCV tests still exists as a problem. EIA-3 and RIBA-3 the third-generation anti-HCV tests comprises of antigens from the HCV core, NS-3,NS-4 and NS-5 genes (Batool et al., 2009). PCR is used for the detection of HCV RNA in chronically infected HCV subjects and is useful for the confirmation of diagnosis and monitoring the response of anti viral therapy. Currently optimal HCV PCR assays have sensitivity of not more than 100 copies of HCV RNA/ml of serum or plasma. Standard and proficient testing of diagnostic laboratories performing HCV PCR, still have significant issues in coming future. There are two major techniques for the measurement of HCV RNA and viral copies (Liver, 2014). The most sensitive technique for the estimation of HCV RNA level is Quantitative PCR (Ghany et al., 2011, Chevaliez et al., 2014), on other hand branched-chain DNA test is considered to be the most accurate technique (Saludes et al., 2014). However these techniques have some limitations which are insufficient dynamic range and high variability of PCR-based assays, and low sensitivity of the branched-chain DNA test. The classification of HCV into prominent genotypes relied upon the development of Molecular tests (Li and Lo, 2015, Pawlotsky et al., 1997); For future research the clinical importance of HCV genotypes determination remains an important subject to discuss (Gretch et al.,1997).

Currently commercially available (Murex serotyping 1-6 HC 02) is generally used for detection of HCV serotyping (Gault et al., 2003). 5′UTR, Core, E1 or the NS5B parts of the genome sequencing and subsequent phylogenetic analysis are the reference standard techniques for the detection of HCV genotypes (Fattovich et al., 2001). So far in reality it is not easy to undertake large level direct sequencing as far as the complication of the method is thought. Few limited research laboratories have updated equipments to undertake these procedure in daily routine, inspite of the introduction of automated sequencing methods without the involvement of radioactive elements (Qu et al., 1994). Substituted techniques that recommend rapid and reasonable genotyping primarily depends on extraction of viral RNA from clinical isolates, followed by hybridization with type specific probes (Okamoto et al., 1992) or amplification of specific primers with related genotype (Christdas et al., 2013).

Or through PCR products amplification with specific restriction enzymes able to identify the type specific cleaved up sites (Widell et al., 1994).

2.11 Evolution of antiviral therapy against Hepatitis C

The main objective for the treatment of hepatitis C is to diminish the incidence of end-stage of liver disease along with its complexities, which are decompensated cirrhosis, transplantation of liver as well as hepatocellular carcinoma. Therapy achievement is estimated through SVR (Singal et al., 2010). For hepatitis C infection standard of care (SOC) was IFN based therapy for last 20 years. IFN-α has effective anti-viral action due to its ability to stimulate ISGs or IFN stimulated genes that encode proteins which restrain different phases of the virus replication (Takaoka and Yanai, 2006). Primarily, conventional IFN single therapy was used for the treatment of chronic hep C which responded very poorly. Adding together guanosine analog (RBV) to IFN results a bit progression in SVR, although the progression was relatively reasonable specifically in 1 and 4 HCV genotypes. IFN molecule pegylation leads to amendment in IFN-α-2 pharmacokinetic profile. In comparison to non pegylated IFN-α both peg-IFN-α-2a as well as peg-IFN-α-2b have less distribution, slower absorption and lower removal rate.

The sustained absorption of PEG-IFN-α permitted longer time of viral hang-up with one time dose in a week. Pegylated IFN and RBV therapy relatively achieved better SVR-24. With pegylated IFN-α-2 and RBV combination therapy, a response rate is between 70-80% against genotypes 3 and 2. IFN-based general adverse side effects are fatigue, anxiety, flu-like symptoms, skin rash with gastrointestinal indications like nausea as well as diarrhea.

Frequent RBV use results in hemolytic anemia. Some subjects used PEG-IFN and RBV may develop cardiac arrhythmias or unpleasant effects of rigorous neuro-psychiatric such as depression as well as suicidal tendencies. The different adverse events are the lengthy duration of therapy as well as the need to insert IFN less compliance and treatment adherence. These aspects have motivated the urgent need to innovate novel treatments that are more useful and relatively safer. The DAAs innovation acknowledged the emergence of a novel era of HCV treatment which became fact.

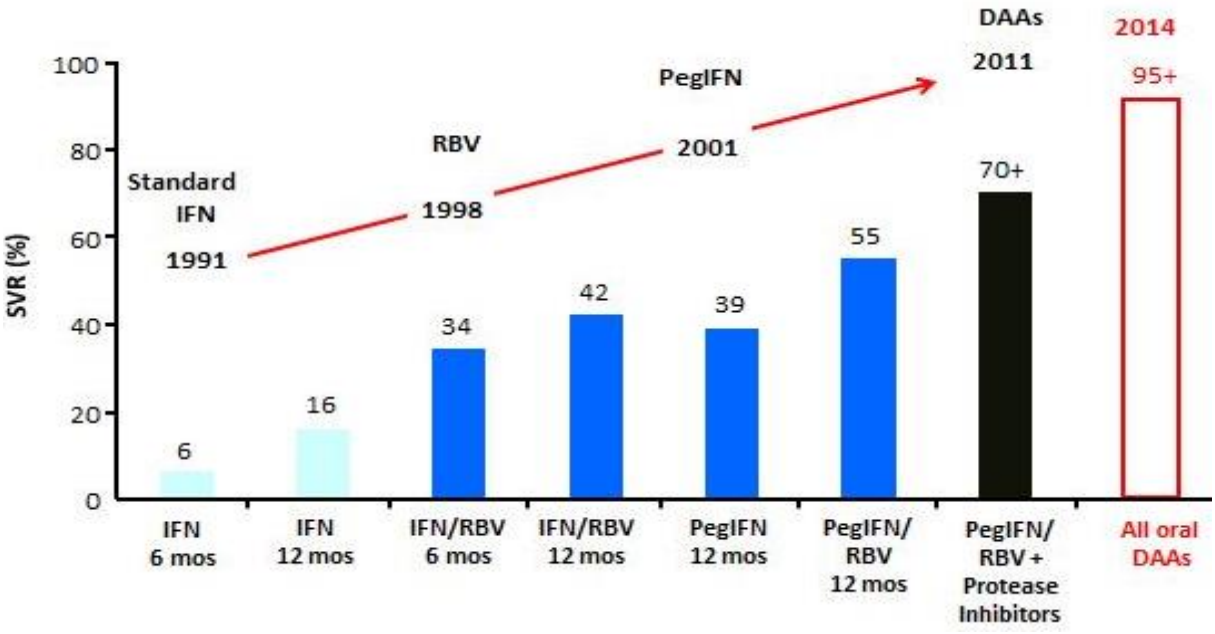


Figure: 2.5 Evolution of HCV Therapies

2.12 Direct-acting antiviral agents (DAAs)

The innovation of DAAs has brought a revolution in HCV drug discovery. DAAs were designed to achieve the better rates of SVR, minimize severe effects and advance adherence to combination therapy in HCV infected subjects. Prior standard of care (SOC) DAAs were primarily launched as add-ons comprising of peg-IFN-α plus RBV. Lately, a step forward in the therapy of HCV has been achieved with the introduction of IFN-free all-oral DAAs, with achieving rates of SVR more than 90% after 3 months of therapy against genotype 1. DAAs targets particular phases inside the life cycle of HCV and upset the replication of virus, an effort to terminate the cycle before its finish (Pockros, 2010). Cell attachment and the insertion of HCV RNA via liver cells surface receptors is the primary step in the virus life cycle.

Later on a single poly-protein of 3010 amino acids was formed due to the translation of HCV RNA which is later on consequently cleaved up by the enzyme protease. It is afterward categorized into 4 “S” proteins (Core, E1, E2, and P7) and NS proteins (NS2-3 along with NS3-4A proteases, NS3 helicase as well as NS_5B RdRp). All of these mentioned enzymes are necessary for viral replication and are probable drug discovery targets (Kim and Chang, 2013, Bartenschlager et al., 2011, Moradpour et al., 2007).

2.12.1 HCV Goals and DAAs treatment endpoints

The main purpose of combination therapy is to root out HCV infection and to prevent hepatic cirrhosis, HCC, and other adverse complications. The end result of therapy is eradicate HCV RNA from the blood of infected subjects through a sensitive assay.

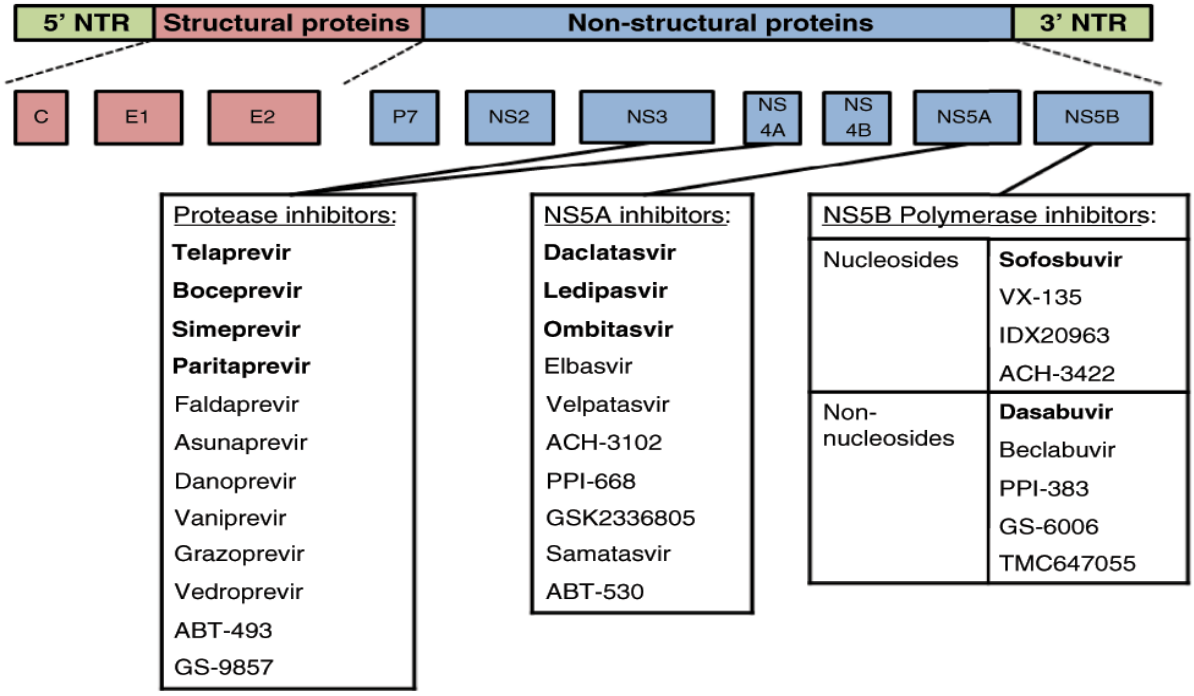


Figure 2.6: Targets of Direct Acting Antiviral therapies

2.13 DAAs Classes

There are four classes of DAAs, which are acknowledged though therapeutic target and their action mechanism. Following inhibitors included in DAAs are.

(1) NS_5B nucleotide inhibitors (NI)

(2) NS_5B no nucleoside inhibitors (NNI)

(3) NS_5A replication complex inhibitors and

(4) $NS_{3/4}A$ protease inhibitors (PI) (Fig 2.7).

Some of the review relevant classes are discussed as follows.

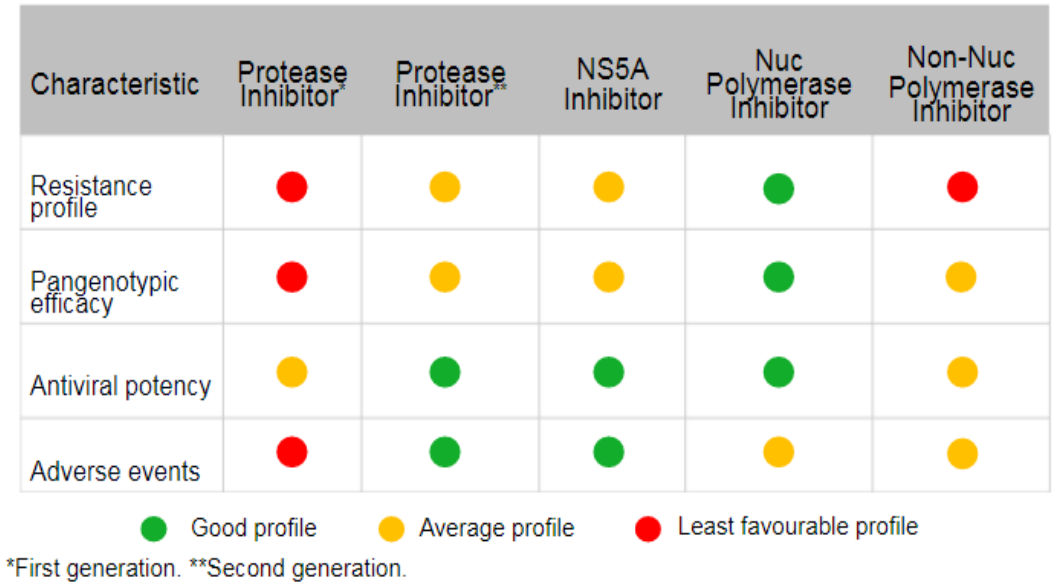


Figure 2.7: Pattern of Resistance in Various DAAs

2.13.1 NS5B (NPIs)/ Nucleoside polymerase inhibitors

NS5B is an RdRp which play important role in post-translational mechanism of HCV replication. Analogs of the natural substrates NPIs attaches to the active site of NS5B and terminate viral replication. As the NS5B part is extremely preserved throughout all the HCV genotypes, NPIs are considered as pan genotypic. NPIs reveal high anti-viral activities in all genotype and produce an elevated genetic barrier against resistance. Hence, NPIs are involved in a number of efficient oral combination therapies. As per their specificity and mode of action polymerase inhibitors are classified into NPIs and NNPIs. On the basis of specificity both of these classes usually vary from each other. The attachment of nucleoside inhibitors (NIs) to the catalytic site of RNA polymerase results in chain termination. Non nucleoside inhibitors attach to a bit preserved site resulting a conformational modification that disturb the position of residues attaching RNA, resulting in inhibition of polymerization (Soriano et al., 2013, Marascio et al., 2014).

a. Sofosbuvir (sovaldi)

Sofosbuvir (SOFO) is a nucleoside analog inhibitor of HCV NS5B polymerase. The sofosbuvir triphosphate structure mimics the natural cellular uridine nucleotide and is inserted by

the HCV RNA polymerase into the long RNA primer strand, results in the termination of viral chain. As a pro drug sofosbuvir is quickly changed after oral ingestion to GS-331007 which is utilized by liver cells. The GS-331007 is changed to its pharmacologically active uridine analog 5′-triphosphate form (GS-461203) through cellular kinases that is inserted by the HCV RNA polymerase into the long RNA primer strand, results in the termination of viral chain. SOFO is a strong pan genotypic NS_5B (PIs) with a high barrier to genetic resistance. It can be used as (400 mg) tablet taken single daily without or with meal. The innovation of SOFO has been a step forward in the treatment of HCV. At present, SOFO acts as backbone of numerous INF-free regimes for chronic hepatitis treatment due to different HCV genotypes (Rockstroh et al., 2015, Soriano et al., 2013, Bhatia et al., 2014).

b. Efficacy of SOFO plus Peg-IFN and RBV

The ELECTRON as well as ATOMIC trials (Arms studies 1-8) (Kowdley et al., 2013, Gane et al., 2013) developed the efficacy of a 12 weeks duration of SOFO and peg-IFN plus RBV for the treatment of naive subjects with HCV genotype-1 with SVR rates ranging between 87 and 100%. In genotypes 3, the SVR 12 rates were 71% with the 16-week SOF plus RBV regimen, 84% with 24 weeks of SOFO plus RBV, and 93% with 12 weeks of SOF plus RBV with PEG-IFN. The subjects infected with genotype 2 infections, the SVR 12 rates were 87% with the 16-week SOF plus RBV regimen, 100% with 24 weeks of SOF plus RBV, and 94% with 12 weeks of SOF plus RBV plus PEG-IFN (Foster et al., 2015).

2.13.2 NS5A inhibitors

The protein NS5A is very important for HCV viral replication and assembly. At the initial phase of assembly, NS5A inhibitors block viral production, so that no nucleocapsid protein or viral RNA is released (Gao et al., 2010). Therefore, agents that stop NS5B activity (PI) inhibit virus's RdRp (Gao et al., 2010). To induce the conformational changes that down regulates the activity of RdRp's, (NIs) attach to the active site of RdRp's, whereas the (NNIs) attach to the outer surface of the enzyme active site. Because of the potency differences and proper mechanistic, the NIs have wide effectiveness for multiple genotypes of HCV in comparison to the NNIs that have less chances of selection for resistant strains (Schinazi et al.,

2014). With NS5B, a host protein cyclo-philin interacts and seems to advance the attachment ability of HCV proteins. The interaction of NS5B with host protein (Cyclophilin) appears to advance the HCV protein's ability of binding.

a. Daclatasvir (Daklinza)

Daclatasvir (DCV) is first-in-class inhibitor of HCV NS viral protein (NS5A) or 5A a phospho-protein that play important role in the replication of HCV. To slow down the replication complex (NS5A) by DCV, the precise mechanism is not so clear, but it is confirmed that DCV stops viral RNA replication and virion assembly. The phosphorylation of NS4A may also be stop through this, and consequently the generation and commencing of the HCV replication complex. On the basis of in vitro information, DCV has revealed its activity against 1 to 6 HCV genotypes, with EC_{50} values from pico-molar to low nano-molar against HCV wild type genotype (Gao et al., 2016). DCV with SOFO and with RBV demonstrates high efficacy in all oral pan genotypic regimes. As per the findings of the ALLY-3 and AI444040 trials (Sulkowski et al., 2014, Nelson et al., 2015), DCV with SOFO for 12-week regimen against genotype 1 or 3 infection with chronic HCV non cirrhotic subjects resulted in elevated rates of SVR-12, irrespective of former treatment experience.

The elevated rates of SVR-12 was confirmed in the trial ALLY-3 (Nelson et al., 2015) with a 12 or 16 week regimen of DCV with SOFO and RBV for genotype 3 infections. In the ALLY-1 trial-A DCV with SOFO-based regimen confirmed efficacy against genotype 1, 3, or 4 infected subjects with developed cirrhosis or post transplant reappearance (Poordad et al., 2016), and in co-infected subjects with 1, 3, or 4 HCV genotype in ALLY-2 and HIV-1 trials (Wyles et al., 2015). DCV with SOFO or with RBV was commonly well tolerated, however nausea, headache fatigue and diarrhea were the severe side effects found in a few subjects treated with DCV (Nelson et al., 2015, Wyles et al., 2015). DCV and SOFO combination therapy can strongly originate serious bradycardia when co-administered with amiodarone. As DCV is a substrate of CYP3A, and it have contraindication effects when utilized with drugs that are well built inducers of CYP3A, containig rifampin, phenytoin and carbamazepine (Liver, 2014). Clinical trials information demonstrated resistance related change in gene of NS5A (Lontok et al., 2015). Hence, the IDSA/AASLD HCV guidelines suggested testing for these replacement

when NS5A inhibitors were un successful (Panel et al., 2015). The resistance appearance of NS5A may also be effected through basic NS5A polymorphisms (Panel et al., 2015).

DCV plus SOFO with RBV is a significant choice to be used for treatment-experienced or treatment-naïve subjects with 1, 3, or 4 chronic HCV genotype infections, including subjects with highly developed diseases of liver, post transplant reappearance and HIV-1 co-infection. DCV and SOFO without RBV is the most useful oral choice for HCV infected subject with genotype 3. Testing for the presence of NS5A polymorphisms is suggested at basic level for subjects with HCV genotype 1a earlier to the commencement of treatment with in subjects with genotype 1a and also cirrhosis earlier to SOFO with DCV treatment.

2.13.3 Protease inhibitors (PIs) NS3/4A

PI stop the action of NS3/4A serine protease, which is an enzyme that prevents TRIF-mediated Toll like receptor signaling and Cardif-mediated retinoic acid–inducible gene 1 (RIG-1) signaling resulting in impaired stimulation of IFN as well as jamming virus eradication (Rupp and Bartenschlager, 2014, Chang et al., 2012).

a. PIs 1st generation

Telaprevir and Boceprevir were the first DAAs for HCV treatment which are acknowledged as 1st generation PIs. For the treatment of HCV genotype 1, Boceprevir or Telaprevir was utilized in combination with peg-IFN and RBV (Chang et al., 2012, Matthews and Lancaster, 2012). Even though boceprevir or telaprevir regimenimproved the rates of SVR; the clinical effectiveness of the triple regimen was restricted by narrow specificity of genotype, small barrier to resistance as well as drug to drug interactions. This regimen develop severe side effects like rashes and mild to rigorous anemia to a limit that might necessitate to decrease the dose of RBV. The tolerability and subject's observance to the triple combination regimenwith TPV/BOC is a difficult task to achieve as the two DAAs should be administered with food thrice daily. Triple combination therapy has less efficiency with earlier peg-IFN/ RBV double therapy, no responders.

On the basis of economic point of view, the triple combination therapy spectacularly raised the expenses of HCV treatment which were formerly affordable. Therefore, the clinical

significance of these therapies reduced considerably with the innovation of subsequently generated protease inhibitors drugs (PIs). HCV protease inhibitor drugs (PIs) include asunaprevir, simeprevir, faldaprevir, aniprevir and danoprevir (Asselah and Marcellin, 2014, Clark et al., 2013). Olysio or Simeprevir is an NS3/4A protease inhibitor drug (PI) for HCV. New protease inhibitors drugs (PIs) belonging to the 2nd generation e.g grazoprevir a macrocyclic compounds, offer a lot of payback over previous generation of protease inhibitor drug , including smaller quantity drug-drug interactions, enhanced dosing profile and with less frequent severity (Keating, 2016). All the mentioned combination therapy drugs are very effective against different HCV genotypes and are affordable.

Materials and Methods

3.1 Ethical Approval

This study was carried out after the approval of Bio-Ethical committee (BEC) of Quaid-i-Azam University Islamabad under an assigned protocol BEC-FBS-QAU2017-10 and by sustaining agreement with the Helsinki Declaration (Association, 2001).

3.2 Study design and Setting

It was a longitudinal study conducted at 3 different locations Quaid-i-Azam University Islamabad, COMSATS University Islamabad with the affiliation of Biotech Diagnostic laboratories, Rawalpindi. The total duration of the study was 34 months commencing from Feb 2016 to December 2018. The HCV positive samples were collected through random sampling from the study areas of different cities of Punjab, KP, Islamabad capital territory and AJK with the help of local collaborators including medical centers, BHUs, RHCs and DHQs. The flow chart below shows the general methodology adopted for the study (Fig 3.1 and 3.2).

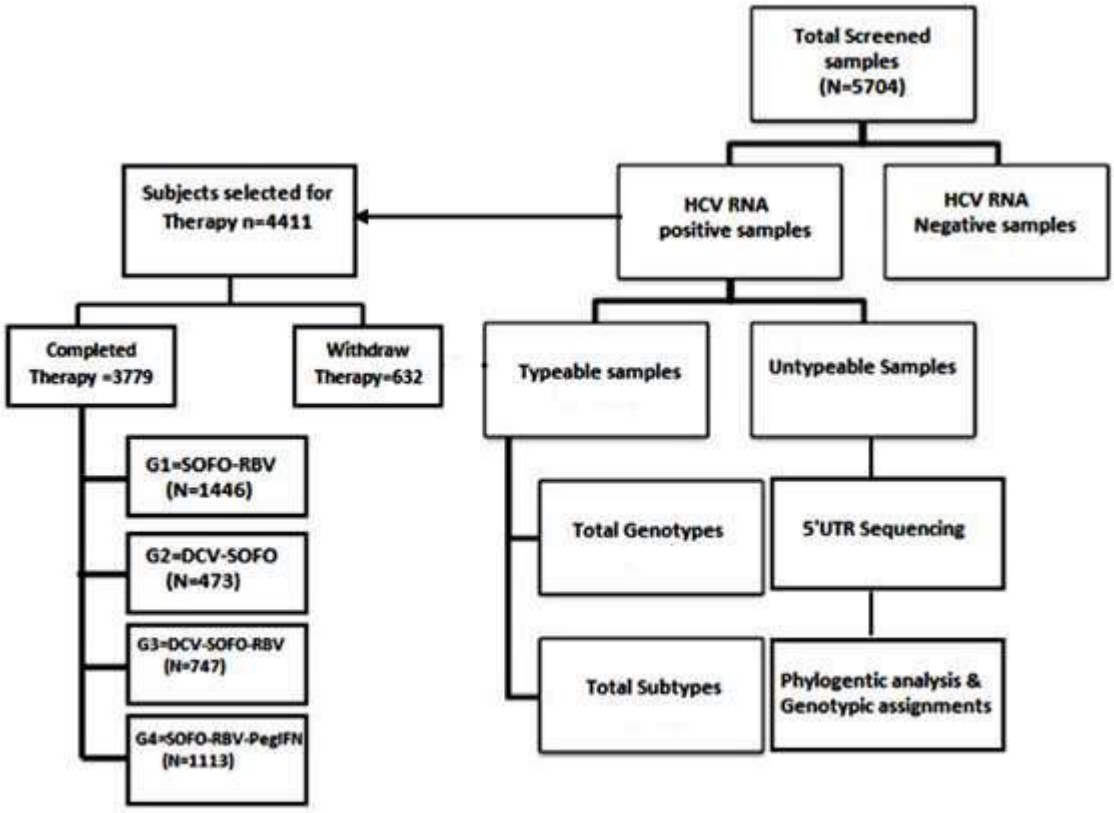


Figure 3.1: Flow chart showing methodology of the study

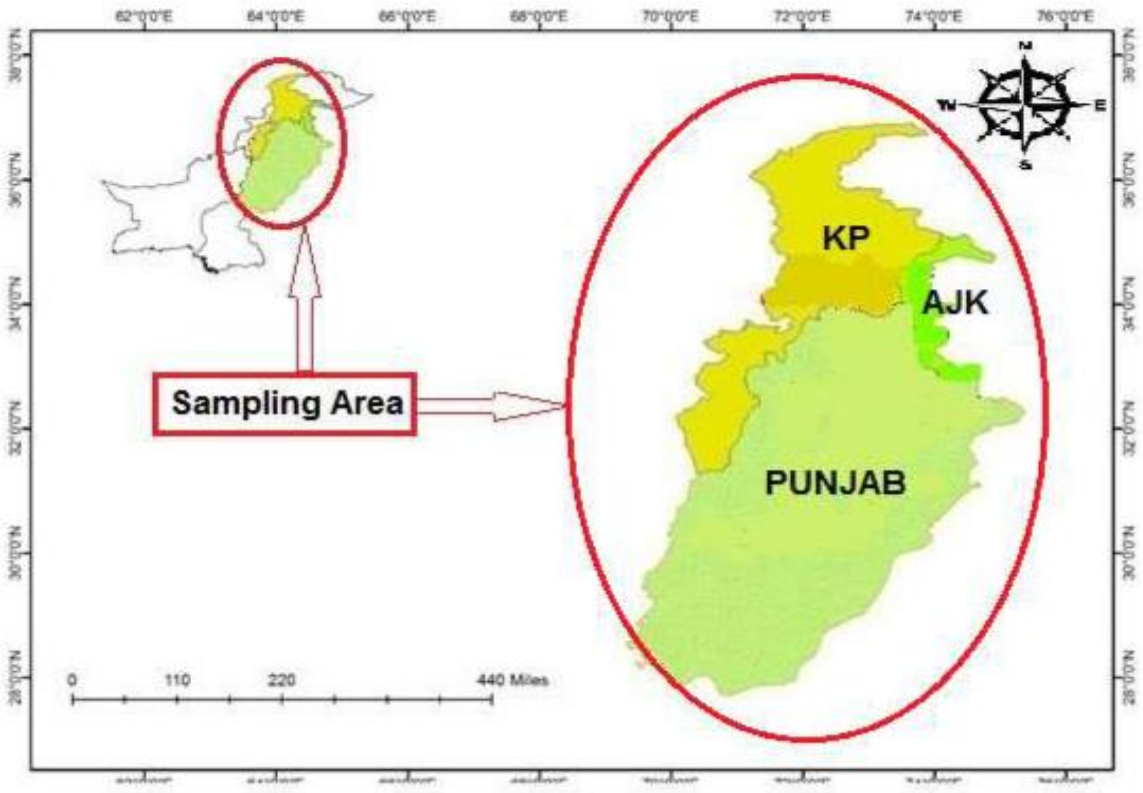


Figure: 3.2 Sampling area's for HCV

3.3 Sampling

3.3.1 Inclusion criteria

Inclusion criteria were intended to screen the irrelevant data information, not adequate to be included into the present investigation. Subjects fulfilling the following criteria were included:

1. Anti HCV positive subjects of different genders, ethnicity and age groups belonging to different provinces of Pakistan.
2. Anti HCV positive subjects of different gender, age groups and ethnic background belonging to AJK and the capital area Islamabad.
3. Only those participants who would willingly participate in the study.
4. Subjects with complete history of the disease.
5. Subjects with elevated LFTs and detectable HCV RNA (for response study).

3.3.2 Exclusion criteria

For the subjects' selection, the following exclusion criterion was used:

1. Subjects with HBV-HCV co-infection.

2. Subjects with other major co-morbidities.
3. Pregnant female subjects and those not willing to participate.
4. Immuno-compromised subjects with lower levels of platelets.
5. Subjects with undetectable HCV RNA.
6. Subjects with normal LFTs and negligible or undetectable viral load (For the treatment groups).

3.3.3 Sample Size

Blood samples of HCV infected subjects were collected from different locations in Pakistan. Slovin's sample size formula was used for the size of sample calculation.

Slovin's Formula: $n = \frac{N}{(1+Ne^2)}$

n = sample size

N= Size of the Population

e= Error margin (to be determined by the researcher)

3.3.4 Data Collection

a. Demographics

Demographic information of the HCV infected subjects including their gender, age, exposure to probable risk factors along with their full contact addresses were recorded. Informed consent was obtained from each subject in written form and detailed information of subjects who were registered for their treatment at respective health care units were noted down on a designed questionnaire. Selection of subjects for HCV viral RNA was done on the basis of anti HCV antibodies detection, complete blood counts (CBC), Liver function test (LFTs) like increased levels of ALT, serum Albumin and Quantitative PCR.

3.3.5 Sampling Techniques

Samples were collected using a probability consecutive sampling technique. This technique of sampling is widely used in qualitative researches and is furthermore acknowledged as a total enumerative sampling in which every subject is selected who meets the criteria of inclusion until the required sample size is achieved.

3.3.6 Samples transportation and handling

Initially positive reported subjects for Anti-HCV on Immuno Chromatographic Techniques (ICT) as well as Enzyme Linked Immunosorbant assay (ELISA) were chosen from different health-care units throughout 24 different locations of Pakistan. A trained staff member was appointed to collect peripheral blood at each site. All specimens were properly collected and transported to Biotech Molecular Diagnostic Laboratories Rawalpindi. 5ml of blood was taken under aseptic conditions in a clot activator gel tube (BD Diagnostics) and serum was separated through centrifugation at 1,000 to 2,000rpm for 10 minutes in centrifuge machine (TDZ4-WSBioridge, China). Subjects were informed about the objectives of study. Oral consent and brief clinical history were also taken and recorded individually.

3.3.7 Samples Processing

All the samples were processed at Biotech Molecular Diagnostic Laboratories Rawalpindi. Serum separated in the form of supernatant liquid portion after centrifugation was transferred immediately to sterilized eppendorf tubes for storage at-80 $^{\circ}$C (Thermo fisher Scientific, USA) until further analysis. For the confirmation of HCV active infection, a nested PCR (Qualitative) was used as mentioned in section 3.6. HCV genotypes were determined through Type-Specific genotyping-based PCR as mentioned in section 3.7.

a. Immuno-chromatography:

Commercially available rapid diagnostic kits were used for initial screening and confirmation of anti HCV antibodies in the concerned samples. Rapid Hepatitis C Virus Test of DIAGNOSTAR© was used for the detection of anti HCV antibodies according to the manufacturer instructions.

3.4. Extraction of HCV RNA

Blood samples for RNA extraction were taken and centrifuged at 8000 rpm for 1min. After centrifugation, the serum was added to a new 1.5 ml microfuge tube. HCV RNA extraction was done through GF-1 (Vivantis, Malaysia) Viral Nucleic Acid extraction kit, with slight modifications. A total of 16µl Proteinase K was first added to each microfuge tube. Afterwards, 7.5µl carrier RNA was added to each tube followed by the addition of 100µl Buffer VL (Lysis buffer). Serum (100µl) was added to labeled microfuge tubes containing VL-carrier RNA buffer

and mixed homogeneously by pulse vortexing for 10-15sec. The tubes were then incubated at 70 ^{o}C for 10min. After incubation, the tubes were centrifuged for 10sec to avoid the drops in the cap of the tubes. Absolute Ethanol (140µl) was added in tubes and vortexed for 10-15sec. The tubes were then subjected to short spin for about 10sec. The lysate was then poured to spin columns and centrifuged at 8000rpm for 1min and the liquid was discarded. 250µl of wash Buffer 1 was added to each microfuge tube and then centrifuged at 8000rpm for 1min. 300 µl of Wash Buffer II was taken in the microfuge tubes and was centrifuged at 8000rpm for 1 min. The previous collection tubes were then thrown out, and spin columns were placed in new collection tubes and were then subjected to drying by centrifugation at 12000 rpm for 2 min. The columns were then placed in freshly labeled 1.5ml microfuge tubes. The lid was opened and tube was placed at room temperature for 3-5 min. In the last step, 50 ul of eluent was added into the adsorption column and placed at room temperature at 2-5 min and centrifuged at 12000 rpm for 1 min. The solution containing the pure RNA was then collected into 1.5 ml centrifuge tube and stored at -20 ºC for further processing.

3.4.1 Primers for qualitative detection and genotyping of HCV

Primers for Qualitative detection and Type-specific PCR were taken from the literature (Ohno et al., 1997, Idrees and Riazuddin, 2008) and modified according to the consensus sequences obtained after alignment of sequences of the Pakistani HCV isolates available in NCBI Genbank. The primers used in the study are given below (Table 3.1).

Table 3.1 Primers for Qualitative and Type-Specific PCR

Primer Name	PCR Rounds	Primers for qualitative detection and sequencing	Bases
MMDS-1/C_1	Round 1	5′ CCCTGTGAGGAACTACTGTCTTCACGC3′	(27mer)
MMDS-2/C_2	Round 1,RT(cDNA)	5′ ACTCGCAAGCACCCTATCAGGCAGTAC3′	(27mer)
MMDS-2A/ C_2	Round 1,RT(cDNA)	5′ ACTCGCAAGCACCCTATCAGGCAGTAC3′	(27mer)
MMDS-2B/ C_2	Round 1,RT(cDNA)	5′ ACTCGCAAGCACCCTATCAGGCAGTAC3′	(27mer)
MMDS-3/ C_3	Round 2	5′ GAAAGCGTCTAGCCATGGCG3′	(20mer)
MMDS-4/ C_4	Round 2	5′ CACAAGGCCTTTCGCGACC3′	(19mer)
		Primers for HCV Genotyping	
G 1	Round 1	5′ GGGAGGTCTCGTAGACCGTGCACCATG 3′	(27mer)
G 2	Round 1,RT(cDNA)	5′GAGMGGKATRTACCCCATGAGRTCGGC 3′	(27mer)

G_3	Round 1	5′AGACCGTGCACCATGAGCAC 3′	(20mer)
G_5	Round 1	5′AACACTAACCGTCGCCCACAA 3′	(21mer)
G_6	Round 1	5′CCTGCCCTCGGGTTGGCTAR 3′	(20mer)
G_7	Round 1	5′CACGTGGCTGGGATCGCTCC 3′	(20mer)
G_8	Round 1	5′GGCCCCAATTAGGACGAGAC 3′	(20mer)
G_9	Round 1	5′CGCTCGGAAGTCTTACGTAC 3′	(20mer)
G_3	Round 2	5′AGACCGTGCACCATGAGCAC 3′	(20mer)
G_{10}	Round 2	5′GGATAGGCTGACCTCTACCT 3′	(20mer)
G_{11}	Round 2	5′GCCCAGGACCGGCCTTCGCT 3′	(20mer)
G_{12}	Round 2	5′CCCGGGAACTTAACGTCCAT 3′	(20mer)
G_{13}	Round 2	5′GAACCTCGGGGGGAGAGCAA 3′	(20mer)
G_{14}	Round 2	5′GGTCATTGGGGCCCCAATGT 3′	(20mer)

3.5 Complementary DNA (cDNA) synthesis

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed for the cDNA synthesis. The genomic RNA was reverse transcribed into cDNA using the protocol: A 20 µl of total HCV RNA was transcribed into cDNA by using MMLV Reverse Transcriptase (Thermo Fisher Scientific, USA). For the formation of cDNA, an enzyme mixture of 9µl volume was prepared for each reaction using reagents mentioned in (Table 3.2).

Table 3.2 Enzyme Mixture preparation

Enzyme Mix	Amount (µl)
RT buffer	4.0
dNTPs (10mM)	2.0
DTT	2.0
RNase inhibitor	0.5
MMLV enzymes	0.5µl

The reagents for cDNA synthesis were initially thawed and then immediately placed on ice after vortexing and centrifugation. A total of 11µl cDNA synthesis Reaction mixture was prepared separately using reagents mentioned in (Table 3.3).

Table 3.3 cDNA synthesis Reaction Mixture

Reaction Mix	Amount (µl)
dH_2O	5.0
Reverse primer C_2	1.0
RNA	5.0
Total volume	**11.0µl**

Reaction mixture was placed in heat block (Wealtec-HB1, USA) for 5 minutes at 85 ^{o}C temperature. A total volume of 20µl RT-PCR reaction was prepared, comprising 9µl enzyme mix and 11µl of reaction mixture at 42 ^{o}C temperature for 48 minutes.

3.6 Qualitative PCR

a. 1st Round PCR

In round first of Qualitative PCR, viral cDNA specific primers for the unstanslated 5′ non coding segments were used (mentioned in Table 3.1). Reagents used for Round 1 PCR are mentioned below in (Table 3.4).

Table 3.4 1st round Qualitative PCR Reagents

Reagents	Amount (µl)
dH_2O	8.2
$MgCl_2$ (25mM)	2.4
Buffer (10X)	2.0
dNTPs (100mM)	1.0
Outer sense primer C_1	1.0
Outer antisense primer C_2	1.0
Taq polymerase	0.4
cDNA	4.0
Total Volume	**20.0 µl**

Thermo cycling conditions for first round of PCR are mentioned below:

Thermal Profile	Temperature	Time
Primary denaturation	94 °C	3 min
Denaturation	94 °C	3 sec
Primer annealing	55 °C	30 sec
Extension	72 °C	30 sec

b. 2nd Round PCR

2 µl of the Round 1 amplified product was used for Round 2 qualitative PCR using same thermo cycling conditions used for Round 1 PCR with exception that a different set of internal primers were used rather than the outer primers (mentioned in Table 3.1) The reagents used for Round 2 PCR are given in (Table 3.5)

Table 3.5 2nd round Qualitative PCR used Reagents

Reagents	Amount (µl)
dH_2O	11.7
$MgCl_2$ (25mM)	2.4
Buffer (10X)	2.0
dNTPs (100mM)	1.0
Internal sense primer C_3	1.0
Internal antisense primer C_4	1.0
Taq polymerase	0.4
1st round product	0.5
Total Volume	**20.0 µl**

3.7 Type Specific PCR

The genotyping of hepatitis C virus was undertaken for all samples using Type-specific PCR as mentioned previously. For the genotyping assay of hepatitis C virus on the basis of 5′ UTR sequences qualitatively positive isolates were chosen.

a. 1st Round of PCR for determination of HCV genotypes

Genotypes of HCV were determined using 4µl of cDNA. The Universal Outer Sense Primer (G_3) as well as Universal Outer Antisense (G_4) primers sequences utilized in round 1st PCR as mentioned in (Table 3.1). For amplification of all the products enzyme *Taq*

polymerase (Thermo scientific, USA) was used. The round 1st PCR was undertaken with 20µl whole volume prepared carefully by mixing reagents listed in (Table 3.4). Thermo cycling conditions for first round of PCR were set as first denaturation was undertaken at a 94°C temperature for 180 sec (30 cycles), subsequent denaturation at 95°C temperature for 30 sec followed by annealing at 57°C temperature for 30 sec and in the end an extension at a of 72°C temperature for 30 sec was given in thermal cycler (T100 BIO-RAD, USA). Last step of extension was undertaken at 72°C temperature for 10 minutes (Table 3.6).

Table 3.6 Regular PCR used reagents

Reagents	Amount (µl)
dH_2O	8.2
Buffer (10X B)	2.0
dNTPs	1.0
Mgcl2	2.4
Forward Primer (G_3)	1.0
Reverse Primer (G_4)	1.0
Taq polymerase	0.4
cDNA	4.0
Total Volume	**20µl**

b. 2nd Round PCR (Type Specific PCR)

In the round 2ndPCR, 1st round amplified product was used in addition to type specific primers mentioned in (Table 3.1). Primers sets were classified into two main groups based on genotype determination and mixes were prepared accordingly. Mix-I was comprised of primers which were specific for (2a, 1b, 3b and 2b) HCV genotypes. Mix-II comprised of primers for (1a, 3a, 4a, 5a and 6a). The reagents used for preparation of both mixes are mentioned in (Table 3.5). Same thermo cycling conditions were used as in round 1st of PCR. First denaturation was under taken at 94°C temperature for 180 sec followed by 30 cycles of denaturation at 95°C temperature for 30 sec, annealing at 57°C temperature for 30 sec and extension at 72°C temperature for 30 sec and last step of final extension was undertaken at 72°C temperature for 10 minutes (Table 3.7).

Table 3.7 Recipe for Type-Specific PCR

Mix I Reagents	Amount (µl)	Mix II Reagents	Amount (µl)
dH_2O	3.2	3.2	3.2
Buffer B	2.0	2.0	2.0
dNTPs	1.0	1.0	1.0
$Mgcl_2$	2.4	2.4	2.4
Primer G_3	1.0	Primer G_3	1.0
Primer G_5	1.0	Primer G_{10}	1.0
Primer G_6	1.0	Primer G_{11}	1.0
Primer G7	1.0	Primer G_{12}	1.0
Primer G_8	1.0	Primer G_{13}	1.0
Primer G_9	1.0	Primer G_{14}	1.0
Taq polymerase	0.4	0.4	0.4
1st Round product	5.0	5.0	5.0
Total Volume	**20µl**	**20µl**	**20µl**

c. Agarose gel electrophoresis

Using 2% Agarose gel, the amplified PCR products were analyzed. Ethidium bromide was used for staining (Thermo Fisher Scientific, USA). 100 bp ladder (Thermo Fisher Scientific, USA) was used to assess the bands size. The amplified DNA fragments were separated at 110V for 40 minutes and visualized under UV transilluminator (Uvitec Limited, Cambridge, UK).

3.8 Determination of Viral load via Real Time PCR

Viral load of the HCV in subjects with undergoing therapy was assessed using Real time PCR Kits and equipment (Qiagen, Germany; equipment: Rotor Gene 3000; Corbett Research, Australia) as per manufacturer's instructions. Viral response against combinational therapies was analyzed by HCV RNA quantification via real time PCR at baseline, 24 and 48 weeks.

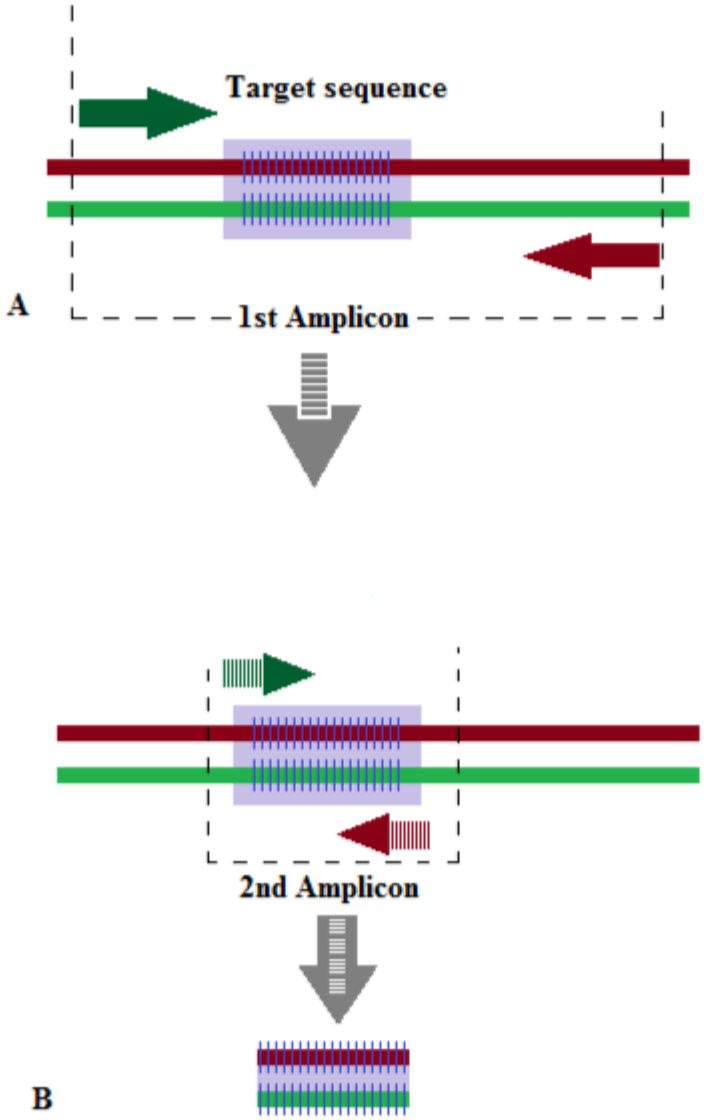


Figure: 3.3. Nested PCR round 1 and 2 (Bio-Rad PCR Principles and Applications)

Figure showing two primers sets used in nested PCR. A) Outer primers are used to amplify the sequences upstream from the second set of primers. B) Amplicons of the first reaction are used as a template for the second reaction by using the second set of primers.

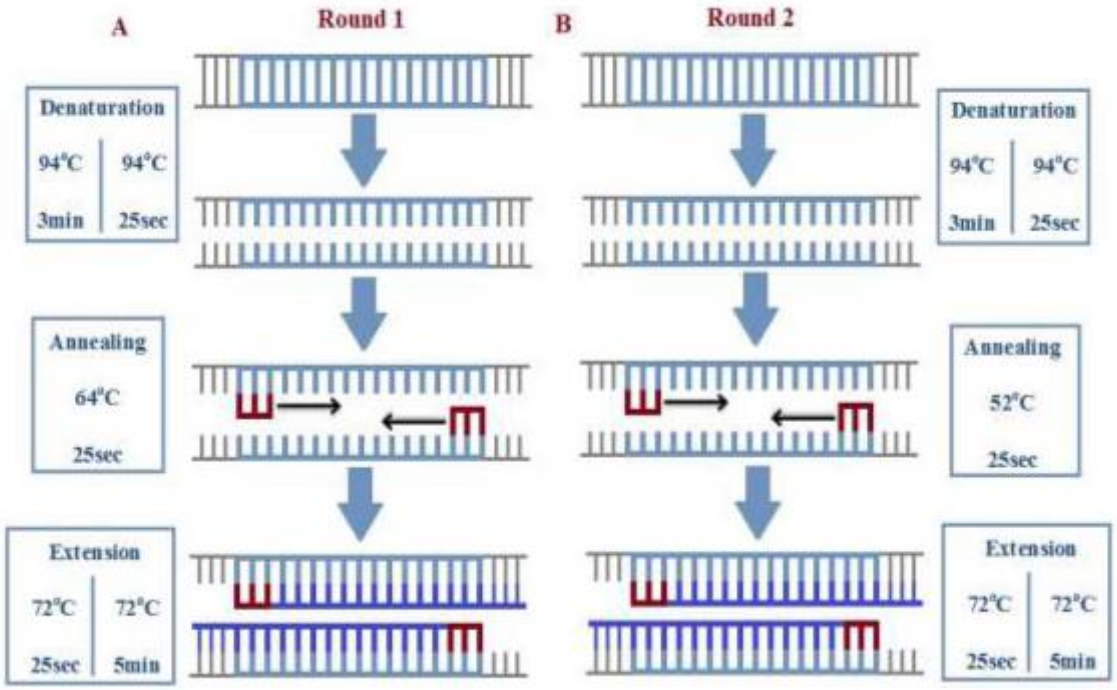


Figure: 3.4 Nested PCR conditions for round 1 and 2(Bio-Rad PCR Principles and Applications) Figure showing the conditions for nested PCR round 1 (A) and 2 (B). The conditions were same for both rounds except for annealing stage in which the temperature was relatively higher in round 1 as compared to the annealing stage of round 2^{nd} .

3.9 Gel Purification of PCR Products

Template preparation for sequencing reaction was carried out via nested RT- PCR of samples positive for HCV. The amplified products were run on 2% agarose gel and a gel extraction kit (QIAGEN, Valencia, California) was used for purification of HCV PCR products using purifying columns as mentioned in kit's protocol with slight modifications as briefly described below:

a. Cut out the desired PCR band (285-bp) using sterilized surgical blade and put in 1.5 ml extraction tube.
b. Spin briefly and estimate the volume of agarose.
c. Add 3 volumes of QG buffer and dissolve agarose for 10 min.
d. Pass the mix into column and spin it at 12000 x g for 1min.
e. Add QG buffer (500 µl) and centrifuge for 1 min.
f. Add PE buffer (750 µl) and centrifuge for 1 min.

g. Spin down for 1 minute again.
h. Place the column in a new extraction tube with the addition of 40 µl of dH_2O.
i. Incubate for 1 min at room temperature.
j. Spin down for 1 min at12000 x g.
k. Add DNA on the column and centrifuge at 12000 rpm for 60 sec.
l. Run DNA (5 µl) on 2% of agarose gel along with Hindi III digested λ DNA marker.
m. Measure the absorbance of quantified andpurified DNA through spectrophotometer (260 nm).

3.10. Sequencing PCR of 5′ UTR Region of Untypable HCV Genotypes

Out of 415 HCV Untypeable genotypes isolates, initially the final purified product of 50 chronically infected Patients with HCV were subjected for 5′UTR Sanger sequencing. For sequencing PCR, sequencing made reaction kit (Applied Biosystems) was used in the Big Dye Terminator cycle and purified DNA was used as templates. Automated sequencer was used for samples analysis (ABI PRISM 3100 genetic analyzer; Applied Biosystems). Consensus sequences were obtained by sequencing products from both strands. Reagents used for preparation of sequencing reaction are mentioned below.

a. Preparation of Sequencing Reaction

For each reaction, reagents mentioned below were poured in separate tubes:

Terminator Ready Reaction Mix	= 8.0 µl
DNA template(2ng/ul)	= 4.0 µl
Primer (5 pM; forward or reverse)	= 1.0 µl
Deionized dH_2O	= 7.0 µl

b. Cycle Sequencing

Reaction tubes were placed in thermal cycler (PE 2700, ABI) and up to 20µl volume was adjusted. The isolates were pre-heated at 96˚C for 1 min and run according to the below cycling parameters: Run 35 cycles at 96 ˚C for 10 seconds, 50 ˚C for 5 seconds and 60 ˚C for 4 minutes.

c. Ethanol Precipitation Method

The ethanol precipitation method was used to purify extension products mentioned below:

a. Spin the contents of reaction tubes.

b. Add 16 µl of dH_2O with 64 µl of 95% ethanol (non-denatured). 60 $\pm$ 3% was the final concentration.
c. Close the tubes and vertex.
d. Leave the tube open for about 15 minutes at room temperature for precipitation of extension products.
e. Spin the tubes at12000 x g for 20 minutes.
f. Carefully aspirate the supernatant of each sample with separate tips and discard them
g. Add 250 µl of 70% ethanol to tubes, mix and spin for 10 minutes.
h. Carefully separate the supernatant.
i. Dehydrate the samples in the dark.

d. Electrophoresis of Samples

Pellet was rehydrated in 15µl formamide and well mixed via pipetting it and then placed in dark for 15 minutes at room temperature. Heat denaturation was given for 5 minutes at 95 ºC in thermal cycler and instantly placed on ice for 5 min. Electrophoresis of sequenced samples was done using ABIPRISM 3100.

3.11 Sequence curetting and &Genbank Submission

5' UTR sequence analysis of HCV isolates from 50 subjects were manually curetted and consensus sequences were derived by alignment of forward and reverse sequences in triplicates. According to the genetic diversity of the isolates, 10 random consensus sequences were identified for phylogenic analysis and submitted to Genbank for accession numbers.

3.12 Phylogenetic Analysis

Molecular Evolutionary Genetics Analysis (MEGA-X) was used to align sequences as per reference sequences. Nucleotide sequences retrieved from sequencing were imported to MEGA-X. For HCV sample sequences with similar nucleotide sequences present in National Center for Biotechnology Information (NCBI), homology analysis was performed through standard nucleotide–nucleotide BLAST (Basic Local Alignment Search Tool) software available at (website http:// www.ncbi.nlm.nih.gov/BLAST). Maximum likelihood (ML) method was used to construct phylogenetic tree while the Kimura 2-parameter method with discrete gamma

distribution was used and the evolutionary distances were computed using MEGA-X software. values was figured out using 1000 bootstrap replicates.

3.13 Online HCV Genotype Prediction

The sequenced samples were confirmed and validated with the help of online prediction tool HCV Genotyper (http://www.bioafrica.net/rega-genotype/html/subtypinghcv.html) by simply submitting the sequences obtained and recording the output.

3.14 Combinations of antivirals dosage and duration

A total of 4411 HCV-positive subjects were enlisted and registration lab number were assigned to subjects according to the site code and subject number. As per the guiding principles of the Asia Pacific Association for the Study of Liver (APASL), all drugs were administered to HCV positive subjects and the follow up was carried out until their treatment ends. Four treatment groups of HCV infected subjects were made according to the treatment regimes they were taking (Table 3.8).

Table 3.8 Treatment groups according to the Type of therapy

Sr.No	Groups	Treatment Combination	Dosage	Duration
1	Group-I	SOFO/RBV	400mg/1000mg	24 weeks
2	Group-II	DCV/SOFO	60mg/400mg	24 weeks
3	Group-III	DCV/SOFO/RBV	60mg/400mg/1000mg	24 weeks
4	Group-IV	SOFO/RBV/PEGIFN	400mg/1000mg/180µg	24 weeks

1. Sofosbuvir (SOFO) (Sovaldi® by Gilead Sciences)
2. Ribavirin (RBV) (Ribazol® by Getz Pharma Pakistan (PVT) Ltd)
3. Daclastavir (DCV)(Daclavia^{TM} by GetzPharma Pakistan (PVT) Ltd)
4. PEG-IFN-α-2a (Ropegra by Roach)

Ribavirin (RBV) was taken two times in a day orally and its dose was determined according to the subjects body weight, e.g. body weight (Subjects <75 kg body weight, 1000mg daily, and in subjects with a body weight ≥75 kg, 1200 mg daily) while Peg-IFN-α-2 was administered subcutaneously once a week with a dose of 180µg in subjects of Group-IV. On

each visit, detailed history along with proper examination with reference to the advancement of the subject disease and conceivable side effects of the treatment were distinctly noted.

3.15 Response rates of combination therapies

Liver function tests (LFTs), Complete blood counts (CBC) were done and PCR were performed before therapy. Monitoring of response to antivirals was assessed by Qualitative of Real-time PCR at different intervals of time including 12, 24 and 48 weeks. Responses were defined as

i. Responders: These include subjects who have received therapy to achieve undetectable limit of HCV RNA during their treatment.

a. Sustained Virologic Response (SVR) as complete absence of HCV RNA after 24 weeks of treatment completion.

b. End of Treatment Response (ETR) means complete absence of HCV RNA after treatment completion at week 24.

ii. Non or Null-Responders **(**NR): The non-responders include subjects who had received therapy, but they failed to achieve an undetectable status of HCV RNA after the end of the treatment at week 24.

iii. Relapsers (R): These include subjects who had undetectable HCV RNA after their treatment ended but the HCV was detectable during the follow up period..

3.16 Statistical analysis

Statistical analysis was done to analyze the data through SPSS version 21 (IBM statistics). The variables for quantitative analysis were mean, standard deviation and ranges while percentages were used as qualitative variables. Various virological and biochemical parameters were assessed, analyzed and correlated with their response to anti-viral therapies. Among the groups, association of variables was studied using Pearson correlation considering p-value <0.05 as statistically significant.

4.1 Results

- **Epidemiology**

a. Determination of Active HCV infection

During the time span of this study, a sum of 5704 serum samples belonging to subjects from 24 different locations of Pakistan were screened for anti HCV using immune-chromato-graphic technique (ICT) which indicated that all the subjects were positive for anti HCV. The positive anti HCV samples were further assessed for active HCV infection by either Qualitative or Real-time PCR which revealed that 5259 (92.1%) subjects had active HCV infection while 445 (7.8%) were found to be PCR negative.

b. Active Infection in different genders

A total of 5704 serum samples collected from different locations were processed for determination of active HCV infection, out of which 3061(53.6%) were females while 2198 (38.5%) were male subjects. Active HCV infection was determined in 5259 (92.1%) samples while 245 (4.2%) female and 200 (3.5%) male, subjects were negative for active infection (Table 4.1). No significant difference was found in the frequency of active infection between male and females subjects (*p* value >0.7).

Table 4.1: Gender wise active HCV infection

Gender	Total Subjects	PCR Negative	PCR Positive (%)
Female	3306(57.9%)	245(4.2%)	3061(53.6%)
Male	2398(42.0%)	200(3.5%)	2198(38.5%)
Total	**5704(100%)**	**445(7.8%)**	**5259(92.1%)**

c. Active Infection in different age groups

All the HCV infected subjects were initially categorized into six age groups. Mean age of subjects enrolled was 44.23 ± 11.71 yrs. In three age groups (26-40, 41-55 and 56-70) the frequency of active infection was n=1906 (36%), n=2300 (43%) and n=885 (13%) which were higher compared to rest of the three age groups (Table 4.2). The frequency of active HCV infection was found comparatively lower in subjects older than 70 years as well as in those younger than 26 years.

Table 4.2: Age-wise active HCV infection

S. No	Ages of Subjects (Years)	Total	PCR Negative	PCR Positive (%)
1	10-25	308	24	284(5.4%)
2	26-40	2066	160	1906(36.2%)
3	41-55	2494	194	2300(43.7%)
4	56-70	742	57	685(13.0%)
5	71-85	84	7	77(1.4%)
6	Above 85	9	2	7(0.1%)
Total	**10-98**	**5704**	**444 (7.7%)**	**5259(100%)**

d. Existing pattern of HCV genotypes in the entire study area

The genotyping analysis showed that out of the 5259 samples with active HCV infection, n=4845 (92.12%) were Typable while n=415 (7.9%) were Untypable by Type specific PCR. Of all the Typable samples, 100 samples belonging to different regions (Table 4.8) were selected for sequencing of their 5′UTRs in order to assess the validity of the type specific assays used in the study with respect to predictability of results. NCBI BLAST and online HCV genotype prediction based on 5′UTR sequences revealed that the Type-Specific PCR results were in agreement with sequenced based characterization.

The most abundant HCV genotype detected across the 24 different cities of Pakistan was HCV 3a (79.9%) followed by a high proportion of the Untypable genotypes (7.9%). Mixed genotypic infections of HCV 3a3b were accounted in (6.7%) subjects while 3b was detected among (3.5 %) of the samples. HCV 2a was detected among (1.7%) samples while the rarely found genotypes were HCV 2b (0.2%), HCV 1a (0.2%) and HCV 4a (0.001) respectively (Fig. 4.1).

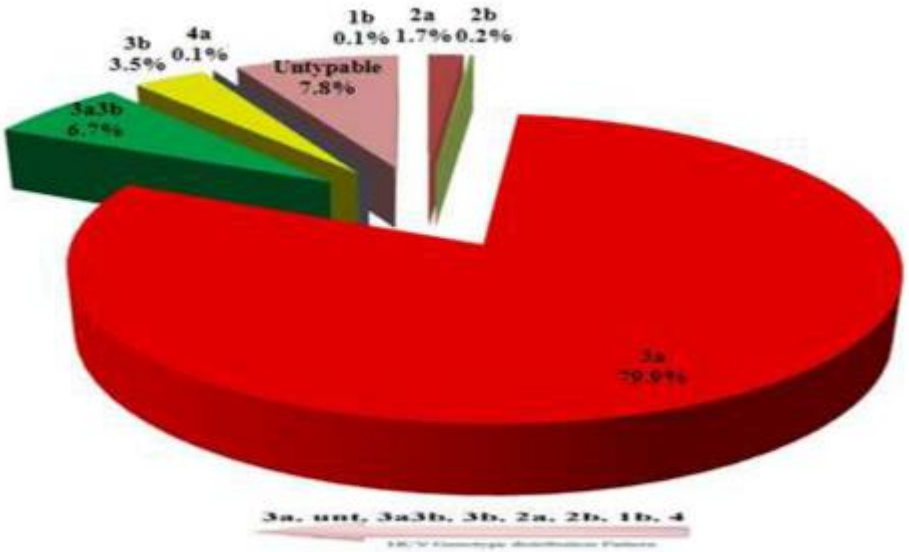


Figure 4.1 : HCV genotypes distribution Pattern in Pakistan (n=5259)

HCV 3a was found highly prevalent in all the cities. In some cities HCV 3a was detected as the sole genotype. Untypable, Mixed genotypes (3a3b) and 3b were mostly found in Rawalpindi, Faisalabad, Gujranwala and Jehlum. Rare HCV genotypes 2a and 2b were mostly prevailing in Rawalpindi, Faisalabad while 4a was only found in Faisalabad (Fig 4.2).

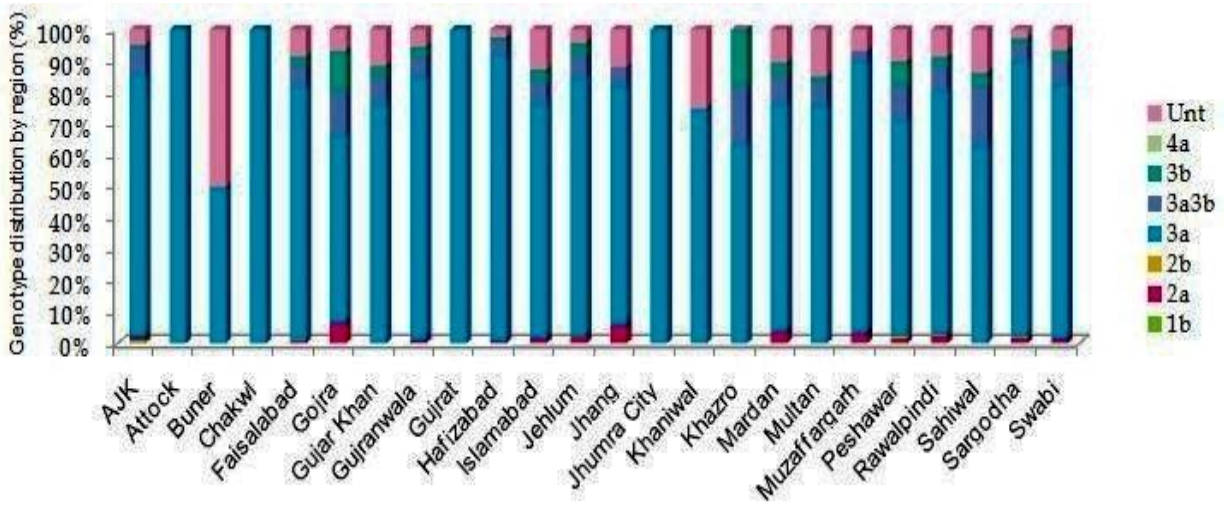


Figure 4.2 : HCV genotypes distribution in 24 different Locations (Cities) of Pakistan

e. Pattern of Gender wise HCV genotypes distribution

HCV genotypes distribution pattern was almost the same in both the genders. However relatively HCV infection (or active HCV infection) was more abundant in female subjects (P<0.01). In male subjects the genotype distribution pattern demonstrated that HCV 3a was abundantly found (33.4%) followed by the Untypable genotypes (3.2%), HCV 3a3b (2.9%), HCV 3b, (1.4%), HCV 2a, (0.8%), HCV 2b, (0.1%) and HCV 1b (0.001%). In female subjects, HCV 3a was slightly more frequent (46.5%) in comparison to male subjects followed by the Untypable genotypes (4.7%), HCV 3a3b (3.9%), HCV 3b (2.0%), HCV 2a (1%), HCV 2b (0.1%) and HCV 1b (0.001%). HCV genotype 4a (0.001%) was found in only one female subject (Table 4.3).

Table 4.3: Gender-wise distribution of HCV genotypes

Genotypes			**1**	**2**		**3**			**4**	**unt**	
Subtypes			**1b**	**2a**	**2b**	**3a**	**3a3b**	**3b**	**4a**	**Untypable**	**Total**
Gender	**Female**	**Count**	1	52	5	2444	204	106	1	249	**3061**
		% age	0.0%	1.0%	0.1%	46.5%	3.9%	2.0%	0.0%	4.7%	**58.2%**
	Male	**Count**	1	40	5	1759	151	76	0	166	**2198**
		% age	0.0%	0.8%	0.1%	33.4%	2.9%	1.4%	0.0%	3.2%	**41.8%**
Total		**Count**	2	92	10	4203	355	182	1	415	**5259**
		% age	**0.0%**	**1.7%**	**0.2%**	**79.9%**	**6.7%**	**3.5%**	**0.0%**	**7.9%**	**100.0%**

f. Age-wise pattern of HCV genotypes distribution

Based on age, six distinct groups of genotyped samples were constituted as described before (Table 4.2). HCV 3a was found abundant in all age groups while Untypable HCV were detected as the second most abundant in majority of the age groups except above 85 years. The two age groups 26-40 yrs and 41-55 yrs with maximum number of actively infected subjects n=4206 (79.9%) revealed the same genotypic distribution pattern as was observed in case of different genders (Fig 4.3) with no considerable variations except 4a which was only reported in a female subject.

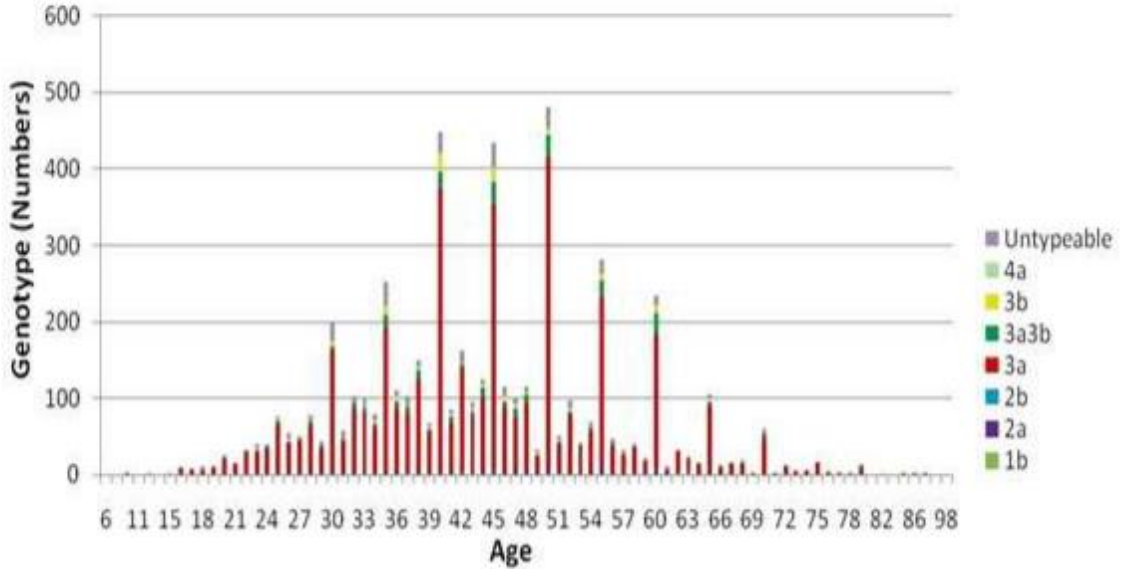


Figure 4.3: Age wise genotype distribution in Pakistan

4.2 Distribution of HCV genotypes in the federal area of Pakistan a. Islamabad

Among 855 HCV-positive subjects enrolled from the capital city and its premises, n=500 (58.4%) were females and n=355 (41.5%) were males. The most prevalent HCV genotype detected among the subjects was HCV 3a n=642 (75.1%) followed by the Untypable n=108 (12.6%), HCV 3a3b n=54 (6.3%), HCV 3b n=35 (4.0%), HCV 2a n=14 (1.6%), HCV 1b n=1, (0.01%) and HCV 2b n=1, (0.01%) respectively (Table 4.4)**.** The genotypes distribution pattern indicated the same trend with HCV 3a as the most abundant followed by the Untypable and other genotypes which were observed in case of the overall pattern detected in the entire area (Table 4.4 and Fig 4.2).

Table 4.4: Distribution of HCV genotypes in Islamabad

S.No	Location		Genotypes								Total
			HCV 1b	HCV 2a	HCV 2b	HCV 3a	HCV 3a3b	HCV 3b	HCV 4a	HCV Unt	
1.	Islamabad	Count	1	14	1	642	54	35	0	108	855
		% age	0.1%	1.6%	0.1%	75.1%	6.3%	4.1%	0.0%	12.6%	100.0%

4.3 Distribution of HCV genotypes in different cities of the Punjab Province a. Rawalpindi

Among the 1200 HCV-positive subjects from Rawalpindi, n=681 (56.7%) were females and n=519 (43.2%) were males. HCV genotype 3a n=933 (77.75%), HCV Untypable n=104 (8.6 %), HCV 3a3b n=91 (7.5%), HCV 3b n = 38 (3.1%), HCV 2a n = 30 (2.5%) and HCV 2b n = 4 (0.3%) (Table 4.5).

b. Gujranwala

A total of 833 samples were received from HCV-positive subjects out of which n=572 (64.8%) were females and n=311 (35.2%) males. In Gujranwala, the genotypes distribution pattern indicated a slight shift with respect to the distribution of HCV 3a3b and the Untypable HCV; however the rest of the pattern reflected the general pattern of distribution as mentioned before in (Fig 4.2). The genotypic distribution pattern indicated that HCV 3a n=743 (84.1%) was the most frequent one followed by mixed infection of 3a3b n=52 (5.8%), Untypable n=47 (5.3%), 3b n = 30 (3.3%) and 2a n = 11 (1.2%) while 2b and 1a/b are absent here (Table 4.5).

c. Faisalabad

Among 675 HCV-positive subjects from Faisalabad, n=346 (54%) were females and n=311 (46%) were males. HCV genotype 3a was found abundantly n=547 (81%), followed by the Untypable n=56 (8.3%), HCV 3a3b n=43 (6.4%), HCV 3b n=20 (3%), HCV 2a n=6 (0.9%), and HCV 2b n=2 (0.1%). Genotype 4a n=1 (0.1%) was also reported in a 47 years old female HCV-positive subject while 1b was absent (Table 4.5).

d. Jhelum

Among 548 HCV- positive subjects from Jehlum city, n=355 (64.7%) were females and n=193 (35.2%) were males. HCV genotype 3a was the most abundant n=453 (82.6%) followed by HCV 3a3b n=37 (6.5%), Untypable n=23 (4.1%), HCV 3b n=22 (4%), HCV 2a n=12 (2.1%) and HCV 2b n=1 (0.2%) while 1b was absent (Table 4.5).

e. Sargodha

Among 228 HCV-positive subjects from Sargodha, n=127 (55.7%) were females and n=101 (44.2%) were males. The distribution pattern of genotypes revealed that HCV 3a n=201 (88.1%) was the most frequent one followed by Untypable n=6 (2.6%), HCV 3a3b n=11 (4.8%), HCV 3b n=5 (2.1%), HCV 2a n=4 (1.75%) and HCV 2b n=1 (0.1%) while 1b was absent (Table 4.5).

f. Hafizabad

Among 126 HCV-positive subjects from Hafizabad city, n=77 (61.1%) were females and n=49 (38.8%) males. The HCV genotype 3a n=115 (91.2%) was most abundant followed by HCV 3a3b n=5 (3.96%), HCV 3b n=2 (1.58%), Untypable n=3 (2.3%) and HCV 2a n=1 (0.1%) while 1b and 4a were absent (Table 4.5).

g. Multan

Among 88 HCV-positive subjects from Multan city, n=50 (56.8%) were females and n=38 (43.1%) were males. HCV genotype 3a n=67

(76.1%) was most abundant one followed by Untypable n=13 (14.8%), HCV 3a3b n=6 (6.8%) and HCV 3b n=2 (2.2%) (Table 4.5).

h. Muzaffargarh

30 samples were collected from this city in which n=18 (60%) were females HCV infected subjects and n=12 (40%) were male subjects. The genotypic distribution pattern was that HCV 3a as the most prevalent one followed by HCV 3a3b and the Untypable while no other types were detected (Table 4.5).

i. Gujar Khan

26 samples were collected from this city in which n=9 (34.6%) were females HCV infected subjects and n=17 (65.4%) were male subjects. The frequency of male subjects was higher than the female subjects. The genotypic distribution pattern was that HCV 3a as the most prevalent one followed by HCV 3a3b and the Untypable while no other types were detected (Table 4.5).

j. Sahiwal

HCV-positive samples collected the from this city were n=22, among in which n=10 (45.4%) were females and n=12 (54.4%) were males. The frequency of male subjects was higher than the female subjects. The genotypic distribution pattern in above mentioned cities was HCV 3a as the most abundant followed by HCV 3a3b and the Untypable while no other types were detected (Table 4.5).

k. Chakwal, Gojra, Gujarat, Jhang, Jhumra, khanewal and Hazro

According to the 2017 census the population of the above mentioned cities were 1.496 million (Chakwal), 0.65 million (Gojra), 0.837 million (Gujrat), 0.332 million (Jhang),2.922 million

(Khanewal) and 0.23 million (Hazro). The most common pattern of HCV genotypes distribution found among these cities was that HCV 3a was the most abundant one followed by the Untypable HCV and then HCV 3a3b (Table 4.5).

Table 4.5: Distribution of HCV genotypes in Punjab

S.No	Location		Genotypes								Total
			HCV 1b	HCV 2a	HCV 2b	HCV 3a	HCV 3a3b	HCV 3b	HCV 4a	HCV Unt	
1.	Attock	Count	0	0	0	2	0	0	0	0	2
		% age	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
2.	Chakwal	Count	0	0	0	11	0	0	0	0	11
		% age	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
3.	Faisalabad	Count	0	6	2	547	43	20	1	56	675
		% age	0.0%	0.9%	0.3%	81.0%	6.4%	3.0%	0.1%	8.3%	100.0%
4.	Gojra	Count	0	1	0	9	2	2	0	1	15
		% age	0.0%	6.7%	0.0%	60.0%	13.3%	13.3%	0.0%	6.7%	100.0%
5.	Gujar Khan	Count	0	0	0	20	2	1	0	3	26
		% age	0.0%	0.0%	0.0%	76.9%	7.7%	3.8%	0.0%	11.5%	100.0%
6.	Gujranwala	Count	0	11	0	743	52	30	0	47	883
		% age	0.0%	1.2%	0.0%	84.1%	5.9%	3.4%	0.0%	5.3%	100.0%
7.	Gujrat	Count	0	0	0	9	0	0	0	0	9
		% age	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
8.	Hafizabad	Count	0	1	0	115	5	2	0	3	126
		% age	0.0%	0.8%	0.0%	91.3%	4.0%	1.6%	0.0%	2.4%	100.0%
9.	Jehlum	Count	0	12	1	453	37	22	0	23	548
		% age	0.0%	2.2%	0.2%	82.7%	6.8%	4.0%	0.0%	4.2%	100.0%
10.	Jhang	Count	0	1	0	13	1	0	0	2	17
		% age	0.0%	5.9%	0.0%	76.5%	5.9%	0.0%	0.0%	11.8%	100.0%
11.	Jhumra	Count	0	0	0	5	0	0	0	0	5
		% age	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%
12.	Khanewal	Count	0	0	0	3	0	0	0	1	4
		% age	0.0%	0.0%	0.0%	75.0%	0.0%	0.0%	0.0%	25.0%	100.0%
13.	Hazro	Count	0	0	0	7	2	2	0	0	11
		% age	0.0%	0.0%	0.0%	63.6%	18.2%	18.2%	0.0%	0.0%	100.0%
14.	Multan	Count	0	0	0	67	6	2	0	13	88
		% age	0.0%	0.0%	0.0%	76.1%	6.8%	2.3%	0.0%	14.8%	100.0%
15.	Muzaffargarh	Count	0	1	0	26	1	0	0	2	30
		% age	0.0%	3.3%	0.0%	86.7%	3.3%	0.0%	0.0%	6.7%	100.0%
16.	Rawalpindi	Count	0	30	4	933	91	38	0	104	1200
		% age	0.0%	2.5%	0.3%	77.8%	7.6%	3.2%	0.0%	8.7%	100.0%
17.	Sahiwal	Count	0	0	0	14	4	1	0	3	22
		% age	0.0%	0.0%	0.0%	63.6%	18.2%	4.5%	0.0%	13.6%	100.0%
18.	Sargodha	Count	0	4	1	201	11	5	0	6	228
		% age	0.0%	1.8%	0.4%	88.2%	4.8%	2.2%	0.0%	2.6%	100.0%

Total	**0**	**67**	**8**	**3178**	**257**	**125**	**1**	**264**	**3900**

4.4 Distribution of HCV genotypes in different cities of the KPK province a. Peshawar

Among the 130 HCV-positive subjects from Peshawar n=68 (52.3%) were females and n=62 (47.7%) males. HCV 3a was detected as the most abundant type n=90 (69.2%) followed by mixed infection with HCV 3a3b n=14 (10.8%), Untypable n=13 (10 %), HCV 3b n=10 (7.6%), HCV 2a n=2 (2.5%) and HCV 1b n=1 (1.2%) while 1b was absent (Table 4.6).

b. Mardan

Among 163 HCV-positive subjects from Mardan area, n=81 (49.9%) were female and n=82 (50.1%) males. The genotypic distribution pattern revealed that HCV 3a n=119 (73%) was the most frequent one followed by Untypable n=17 (10.4%), HCV 3a3b n=14 (8.5%), HCV 3b n=7 (4.2%) and HCV 2a n=6 (3.6%) while 1b, 2b and 4a were absent (Table 4.6).

c. Swabi

Among the 124 HCV-positive subjects from Swabi, n=61 (49.1%) were females and n=63 (50.9%) males. HCV genotype 3a was found as the most abundant n=101 (81.4%) followed by HCV 3a3b n=9 (7.2%), Untypable n=8 (6.4%), HCV 3b n=4 (3.2%) and HCV 2a n=2 (1.6%) while 1b, 2b and 4a were absent (Table 4.6).

d. Buner

Only two samples were obtained from Buner out of which one turned out to be HCV 3a while the other was undetecable (Untypable genotype) (Table 4.6).

Table 4.6: Distribution of HCV genotypes in KP

S.No	Location		Genotypes								Total
			HCV 1b	HCV 2a	HCV 2b	HCV 3a	HCV 3a3b	HCV 3b	HCV 4a	HCV Unt	
1.	Peshawar	Count	0	2	1	90	14	10	0	13	130
		% age	0.0%	1.5%	0.8%	69.2%	10.8%	7.7%	0.0%	10.0%	100.0%
2.	Mardan	Count	0	6	0	119	14	7	0	17	163
		% age	0.0%	3.7%	0.0%	73.0%	8.6%	4.3%	0.0%	10.4%	100.0%
3.	Swabi	Count	0	2	0	101	9	4	0	8	124
		% age	0.0%	1.6%	0.0%	81.5%	7.3%	3.2%	0.0%	6.5%	100.0%
4.	Buner	Count	0	0	0	1	0	0	0	1	2
		% age	0.0%	0.0%	0.0%	50.0%	0.0%	0.0%	0.0%	50.0%	100.0%

4.5 Azad Kashmir

Among the 85 HCV-positive subjects from AJK, n=45 (52.9%) were females and n=40 (47.1%) males. HCV 3a was the most abundant n=71 (83.5%) in AJK followed by HCV 3a3b n=7 (8.2%), Untypable n=4 (4.7 %), HCV 3b n=1 (1.2%), HCV 1b n=1 (1.2%), and HCV 2a n=1 (1.2%) while 2b and 4a were absent (Table 4.7).

Table 4.7: Distribution of HCV genotypes in AJK

S.No	Location		Genotypes								Total
			HCV 1b	HCV 2a	HCV 2b	HCV 3a	HCV 3a3b	HCV 3b	HCV 4a	HCV Unt	
1.	AJK	Count	1	1	0	71	7	1	0	4	85
		% age	1.2%	1.2%	0.0%	83.5%	8.2%	1.2%	0.0%	4.7%	100.0%

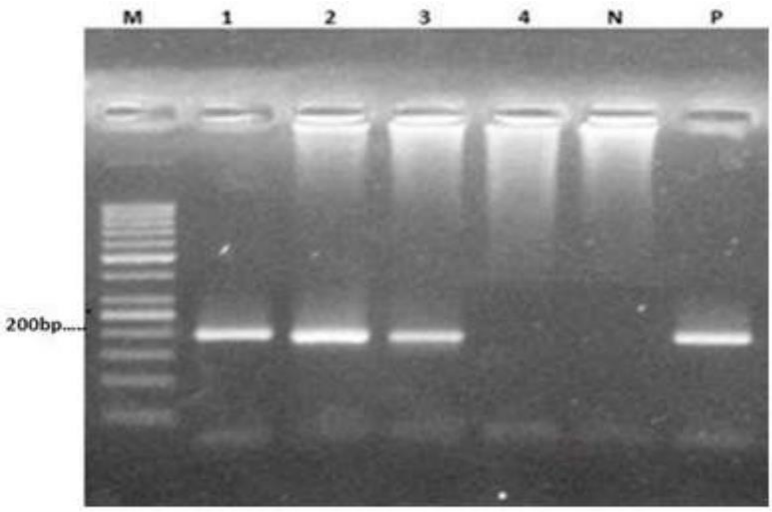


Figure 4.4: Gel picture of HCV PCR amplimer using qualitative PCR. Lane M indicates 100 bps DNA ladder; Lane 1-3: Positive samples; Lane 4: Negative sample; Lane N: Negative control; Lane P: Positive control.

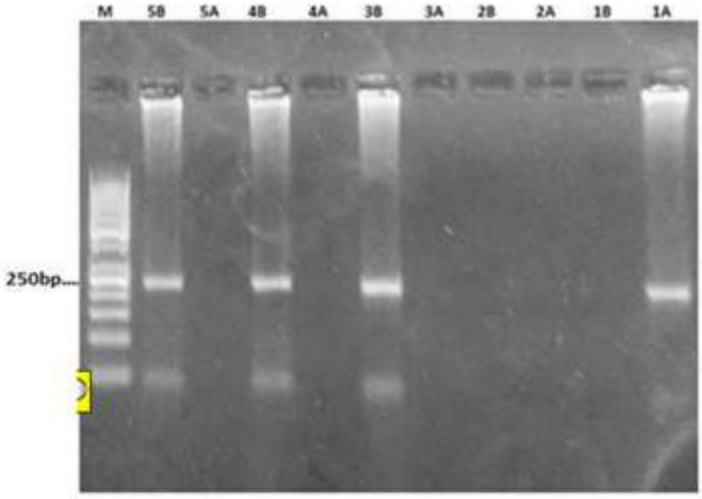


Figure 4.5: Gel picture of HCV PCR amplimer for genotype determination using type-specific PCR. Lane M; 100 bps marker; Lane 5B, 4B and 3B: Genotype 3a: Lane 2A-2B: untypeable genotype; Lane 1A: Genotype 1b

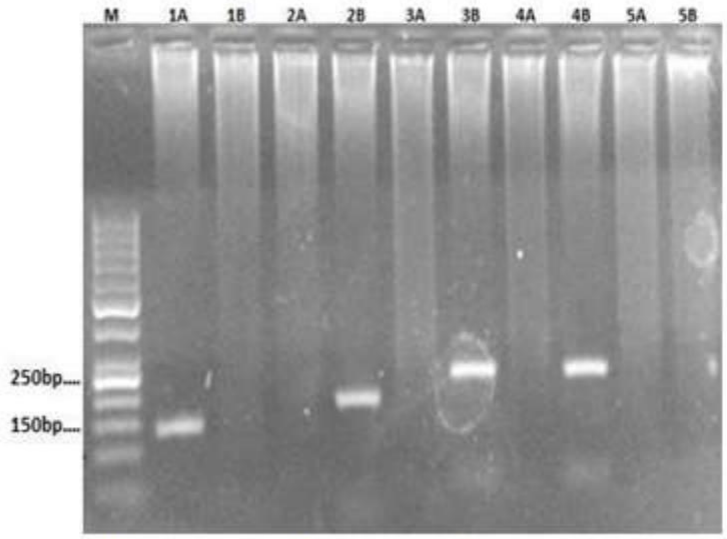


Figure 4.6: Gel picture of HCV PCR amplimer for genotype determination using type-specific PCR. Lane M. 100bps DNA ladder, Lane 1A: Genotype 3b; Lane 2B: Genotype 1a; Lane 3B and 4B: Genotype 3a

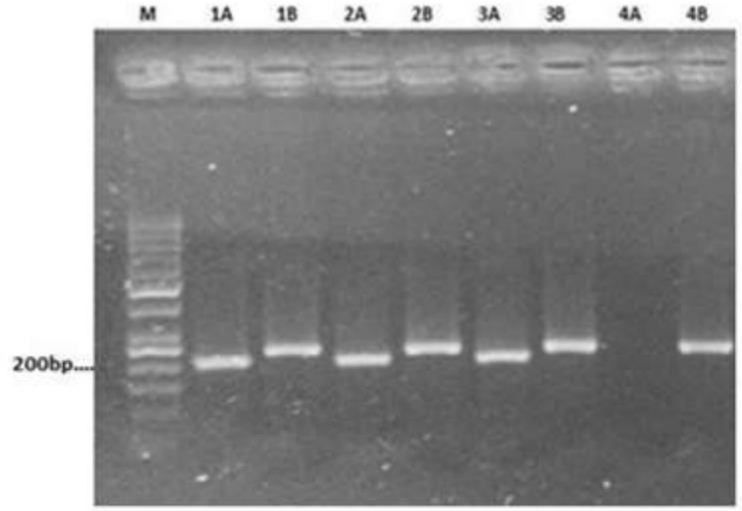


Figure 4.7: Gel picture of HCV PCR amplimer for genotype determination using type-specific PCR. Lane M: 100bps DNA Ladder, Lane 1A and IB: Mixed infection 3a1b; Lane 2A and 2B: Mixed infection 3a1b; Lane 3A and 3B: Mixed infection 3a1b; Lane 4B: genotype 3a

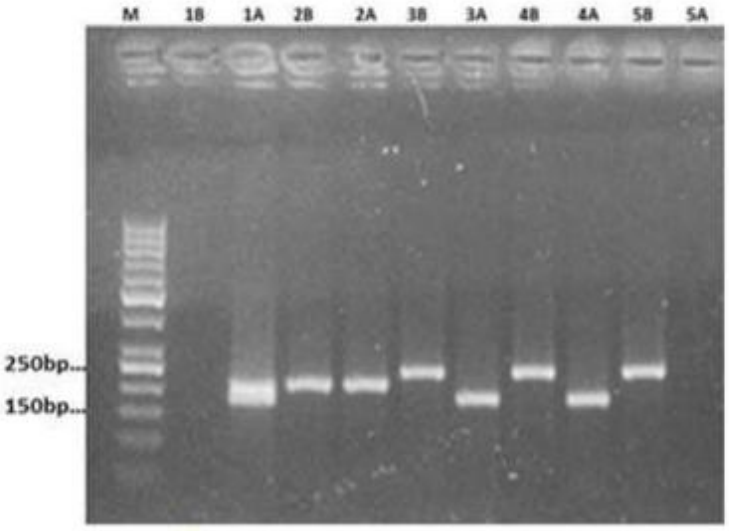


Figure 4.8: Gel picture of HCV PCR amplimer for genotype determination using type- specific PCR. Lane M: 100bp DNA Ladder, Lane 1A: Genotype 1b; Lane 2A and 2B: Mixed infection 1a1b; Lane 3A and 3B: Mixed infection 3a3b; Lane 4A and 4B: Mixed infection 3a3b; Lane 5B: Genotype 3a

Table 4.8: Demographic of Typable Sequenced Subjects (N=100).

S.NO	Area	Males	Females	Total
1	Islamabad	10	12	22
2	Punjab	16	22	38
3	KP	8	18	26
4	AJK	6	8	14
	Total	**40**	**60**	**100**

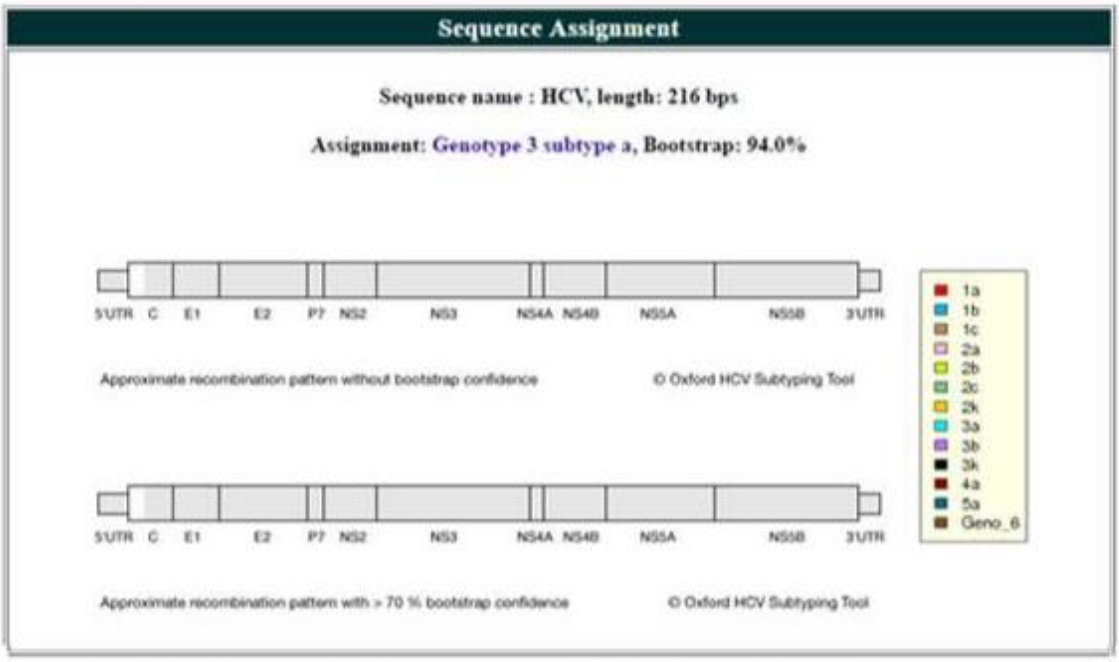


Figure 4.9 : Validation of HCV 3a genotype using online HCV subtyping tool based on HCV 5′UTR. Query sequence belongs to the genotype 3a with 100% bootstrap value.

4.6 Characterization of the Untypable HCV genotypes

One of the objectives of this study was to characterize the Untypable HCV isolates from various geographical areas of Pakistan. During the course of this study, a total of 415 Untypable HCV RNA PCR positive serum samples were detected among the samples collected from different parts of the country. Out of 415 serum samples, 166 (40%) belonged to males and 259 (60%) belonged to females. Fifty 50/415 (12%) randomly selected serum samples were used for sequence analysis of 5′UTR of HCV. The total sequenced samples belonged to each Federal, Punjab, KP, and AJK regions. The demographics of the subjects are demonstrated in (Table 4.9). **Table 4.9:** Demographic of Untypable Sequenced Subjects (N=50).

S.NO	Area	Males	Females	Total
1	Islamabad	5	6	11
2	Punjab	8	11	19
3	KP	4	9	13
4	AJK	3	4	7
	Total	**20**	**30**	**50**

All of the 5' UTRs nucleotide sequences of Untypable isolates were subsequently self-alligned and these Untypable nucleotide sequences showed conserved (*) sequences at position 70G to 166G. Some deletion (-) sequences at position 81, 122 and 123 were observed in all isolates. The alignment revealed a number of sequence variation in the Untypable isolates including PK7 at 69G to 165C, PK8 at 71T to 169A, PK14 at 72A to 165C and PK15 at 71T to 169 A. Alignment of the 5' UTR sequences indicated that there are at least 10 genetically diverse Untypable HCV strains circulating in the study area. The 5'UTR consensus sequences in case of all the 50 isolates were later used for genotype assignment using NCBI and online HCV genotype prediction tool (online software HCV Genotyper) for validation (Fig 4.9). The results indicated that all the 50 Untypable HCV RNA positive samples isolated from both genders were slight variants of HCV 3a at the 5' UTR region (Fig 4.11). The sequences of all studied isolates showed 97 to 100 % sequence conservation to isolates of genotypes 3a existing in the world as well as to previously reported isolates from Pakistan (Fig 4.13). All the genetically diverse 10 HCV Untypable sequences were submitted to Genbank with the accession numbers MK491838, MK491839, MK491840, MK491841, MK732954, MK732955, MK732956, MK732957, MK732958, and MK732959 (Fig 4.10,Table 4.12).

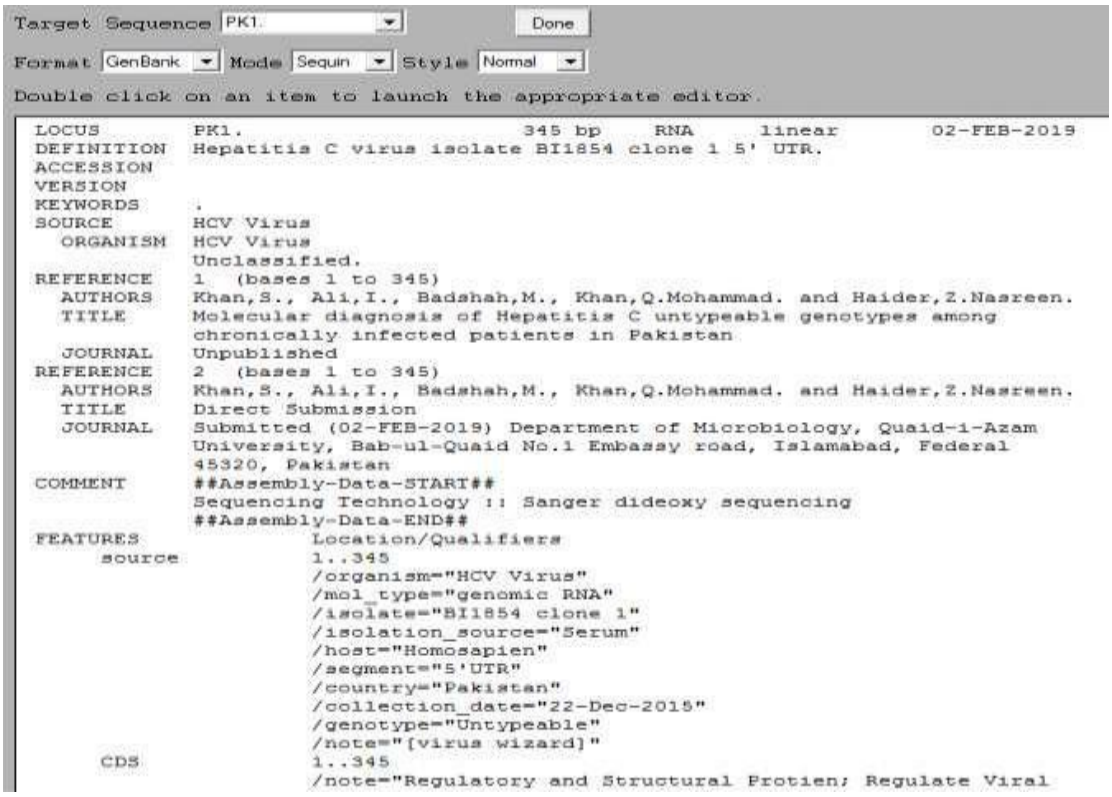


Figure: 4.10 HCV Untypable Isolate PK1.

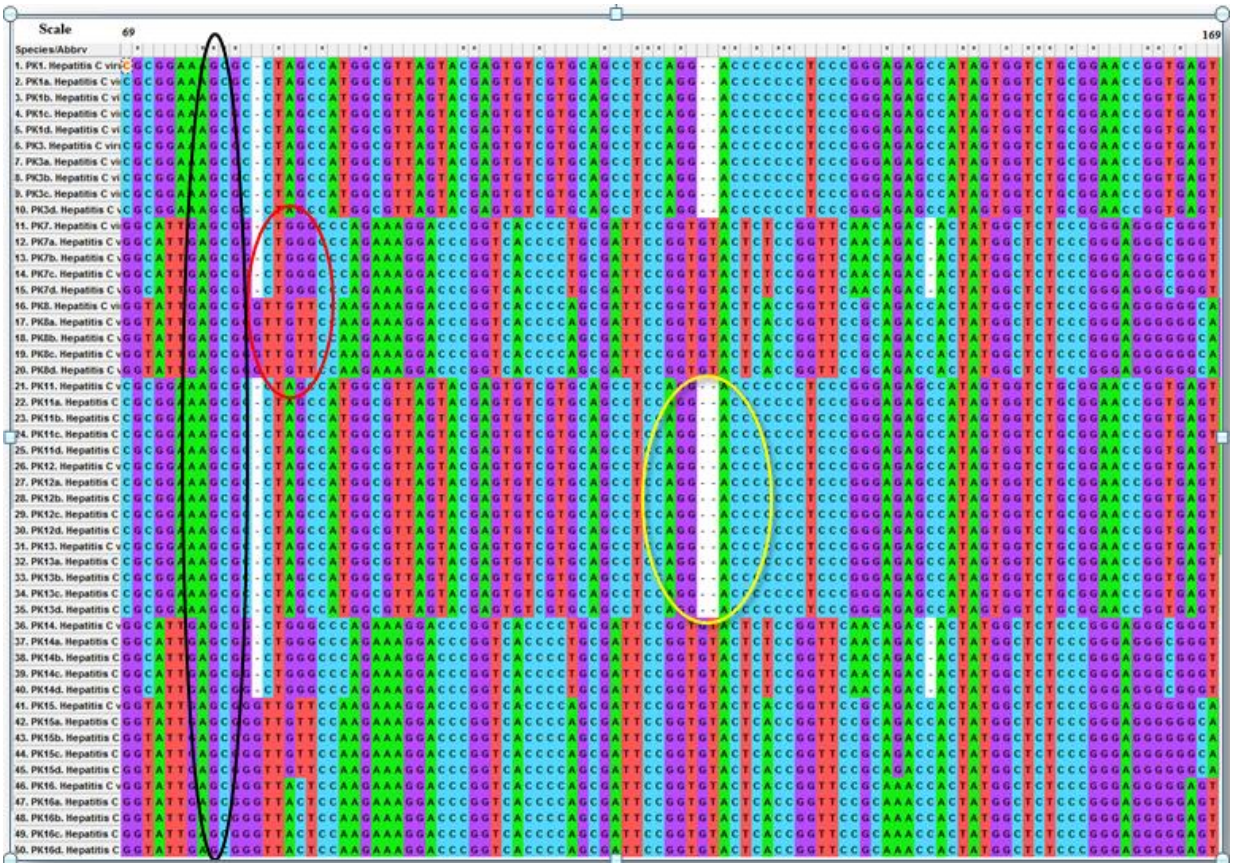


Figure 4.11: 5′ UTR multiple sequence alignment of HCV Untypable isolates

a. Comparative analysis of the Untypable isolates

For comparative analysis of the Untypable isolates, 5′UTR sequences of Pakistan and other regional HCV 3a isolates were downloaded from Genbank (NCBI) and aligned with the 10 genetically diverse Untypable sequences. Alignment results revealed conserved (*) nucleotides at various positions from 159C to 256C and deletion (-) variations at positions 186, 187, 201 and 229 in Untypable and Pakistani isolates. These conserved nucleotides sequences could be important for the identification of HCV variant strains. Transversion variation in Untypable and Pakistani isolates were observed at various positions from 162 to 258 while transversion variation between other countries isolates were at position 194T, 211G and 212T. On the basis of above observation it is analyzed that high variability in the 5′UTR sequences among Untypable isolates and with reference sequences from other countries were noted however similarity between Untypable isolates with Pakistani isolates HCV3a were also observed (Fig 4.12).

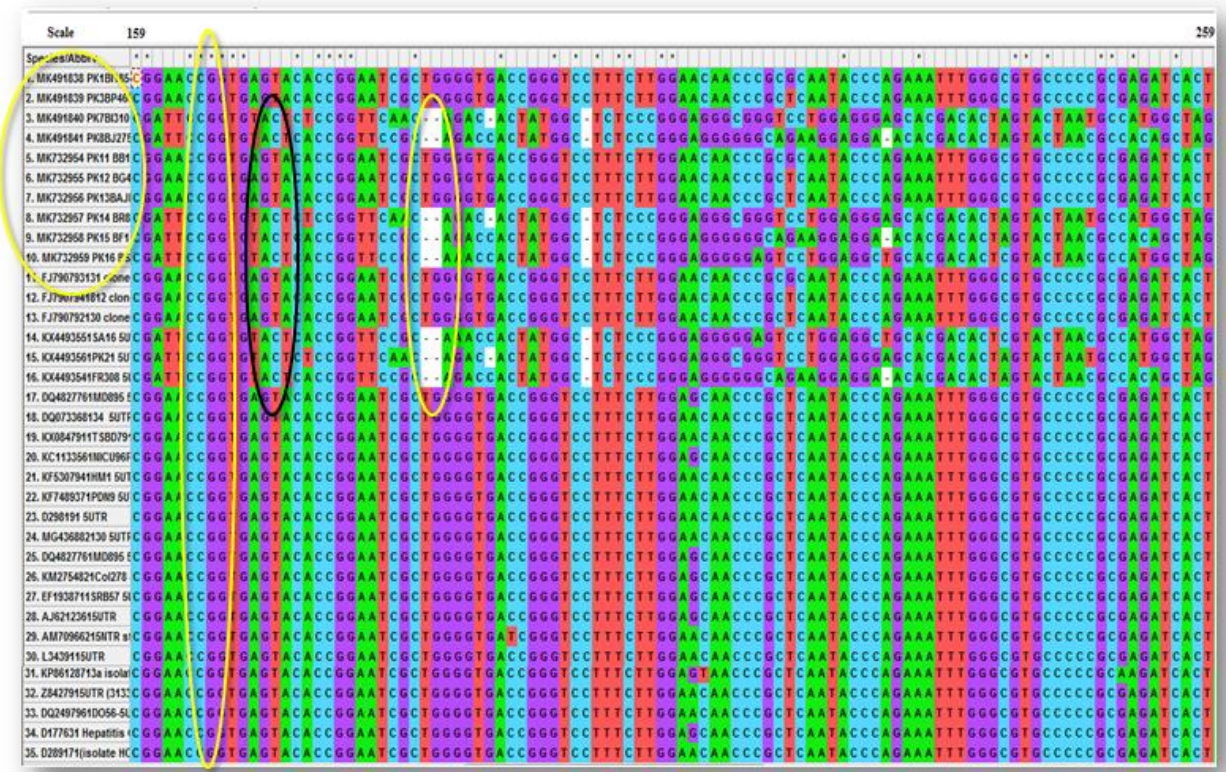


Figure 4.12: Multiple sequence alignment of HCV 5′ UTR sequences of Untypable isolates and reference sequence of HCV genotypes retrieved from NCBI

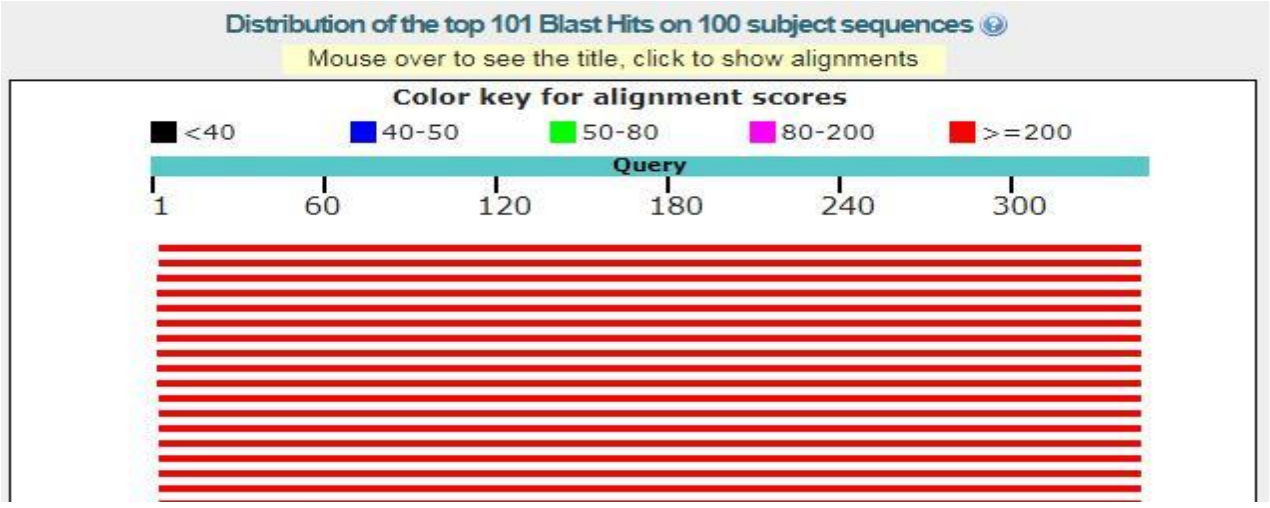


Figure 4.13: Distribution of the top Blast Hits on 100 Subject Sequences

b. Genetic distance Analysis

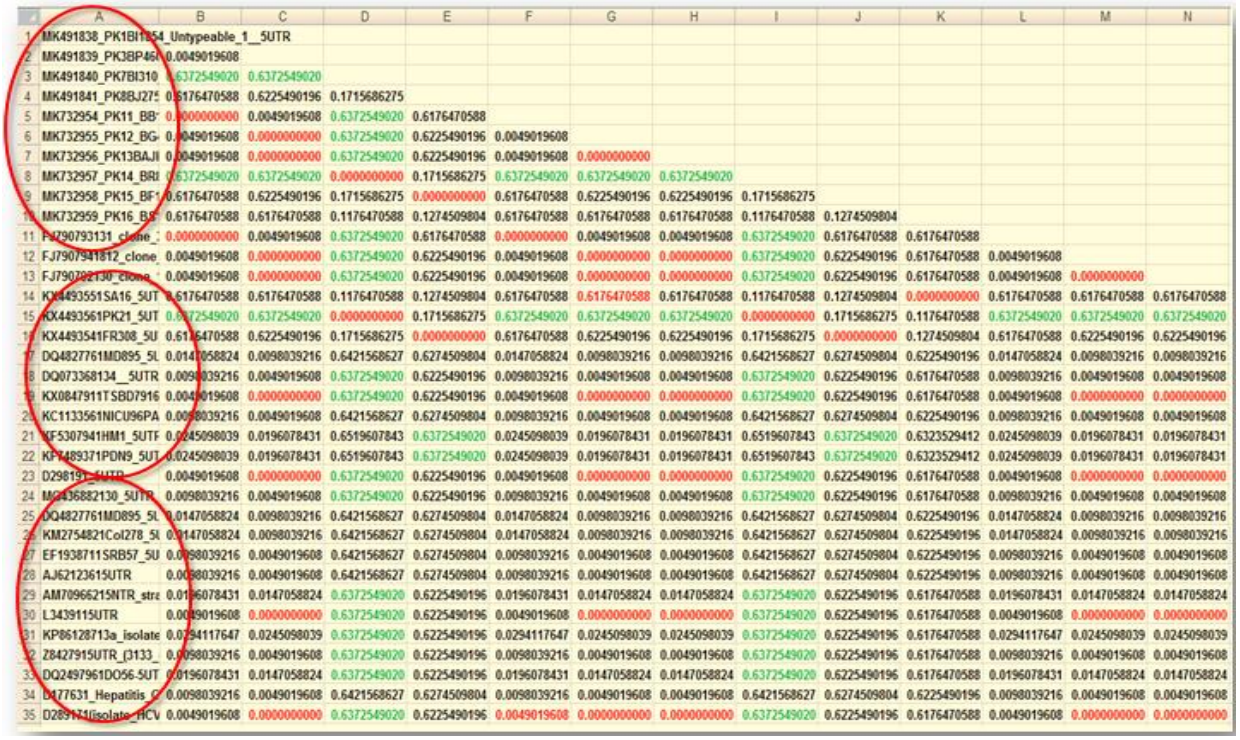
Minimum and maximum genetic distance between Untypable isolates with Pakistani and others countries isolates are described in (Table 4.10)

Table 4.10 Genetic distance Analysis

S.No	Minimum (0.000) genetic distance
1.	Among Untypable isolates PK1,P11 and with Pakistani isolate FJ790793
2.	Among Untypable isolates PK3, PK12, PK13 and with Pakistani isolates FJ790794, FJ790792, KX06491 and D29819 (India), L34391(Canada) and D28917 (Japan)
3.	Among Untypable PK8, PK14 and with Pakistani isolate KX449356
4.	Among Untypable isolates PK11 and PK16
5.	Among Untypable isolates PK12, PK13 and with Pakistani isolates FJ790793, FJ790794, KX084791 and L34291(Canada) and D28917 (Japan)
6.	Untypable PK13 with Pakistani isolates FJ790793, FJ790794, KX084791 and D29819(Japan) L34291(Canada) and D28917(Japan)
7.	Untypable PK14 with Pakistani isolates KX449356
8.	Untypable isolates PK15 with Pakistani isolate KX449354
S.No	**Maximum (0.63) genetic distance**
1.	Among Untypable isolates PK1,PK7,PK14 and PK21
2.	Untypable isolates PK1 with Pakistani isolate KX449356
3.	Among Untypable PK11, PK12, PK13 and with DQ073368 (India), KX0847911 (Brazil), D298191 (Japan), MG436882 (Iran), AM709662 (URUGUAY), L343911 and KP861287 (Canada), Z84279 (UK), DQ249696 (USA), D289171 (Japan).
4.	Untypable PK14 with DQ073368 (India), KX0847911 (Brazil), KF530794 and KF748937 (Malaysia), D298191 (Japan), MG436882 (Iran), AM709662 (URUGUAY), L343911 and KP861287 (Canada), Z84279 (UK), DQ249696 (USA), D289171 (Japan).
5.	Pakistani isolate PK21 with DQ073368 (India), KX0847911 (Brazil), KF530794 and KF748937 (Malaysia), D298191 (Japan), MG436882 (Iran), AM709662 (URUGUAY), L343911 and KP861287 (Canada), Z84279 (UK), DQ249696 (USA), D289171 (Japan).

In the light of above observation it is analyzed that genetic closeness was found among the sequences of HCV Untypable isolates and with Pakistani isolates HCV 3a, however Untypable sequences have variability in comparison to the sequences of isolates from other countries (Table 4.10 and 4.11).

Table 4.11 Pair wise genetic distance between the Isolates



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MK491838_PK1BI1254_Untypeable_1__5UTR													
2	MK491839_PK3BP46	0.0049019608												
3	MK491840_PK7BI310	0.6372549020	0.6372549020											
4	MK491841_PK8BJ275	0.6176470588	0.6225490196	0.1715686275										
5	MK732954_PK11_BB	0.0000000000	0.0049019608	0.6372549020	0.6176470588									
6	MK732955_PK12_BG	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608								
7	MK732956_PK13BAJI	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000							
8	MK732957_PK14_BRI	0.6372549020	0.6372549020	0.0000000000	0.1715686275	0.6372549020	0.6372549020	0.6372549020						
9	MK732958_PK15_BF1	0.6176470588	0.6225490196	0.1715686275	0.0000000000	0.6176470588	0.6225490196	0.6225490196	0.1715686275					
10	MK732959_PK16_BB	0.6176470588	0.6176470588	0.1176470588	0.1274509804	0.6176470588	0.6176470588	0.6176470588	0.1176470588	0.1274509804				
11	FJ790793131_clone_	0.0000000000	0.0049019608	0.6372549020	0.6176470588	0.0000000000	0.0049019608	0.0049019608	0.6372549020	0.6176470588	0.6176470588			
12	FJ790794812_clone_	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000	0.0000000000	0.6372549020	0.6225490196	0.6176470588	0.0049019608		
13	FJ790792130_clone_	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000	0.0000000000	0.6372549020	0.6225490196	0.6176470588	0.0049019608	0.0000000000	
14	KX449351SA16_5UT	0.6176470588	0.6176470588	0.1176470588	0.1274509804	0.6176470588	0.6176470588	0.6176470588	0.1176470588	0.1274509804	0.0000000000	0.6176470588	0.6176470588	0.6176470588
15	KX449356PK21_5UT	0.6372549020	0.6372549020	0.0000000000	0.1715686275	0.6372549020	0.6372549020	0.6372549020	0.0000000000	0.1715686275	0.1176470588	0.6372549020	0.6372549020	0.6372549020
16	KX449354FR308_5U	0.6176470588	0.6225490196	0.1715686275	0.0000000000	0.6176470588	0.6225490196	0.6225490196	0.1715686275	0.0000000000	0.1274509804	0.6176470588	0.6225490196	0.6225490196
17	DQ4827761MD895_5U	0.0147058824	0.0098039216	0.6421568627	0.6274509804	0.0147058824	0.0098039216	0.0098039216	0.6421568627	0.6274509804	0.6225490196	0.0147058824	0.0098039216	0.0098039216
18	DQ073368134__5UTR	0.0098039216	0.0049019608	0.6372549020	0.6225490196	0.0098039216	0.0049019608	0.0049019608	0.6372549020	0.6225490196	0.6176470588	0.0098039216	0.0049019608	0.0049019608
19	KX0847911TSBD7916	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000	0.0000000000	0.6372549020	0.6225490196	0.6176470588	0.0049019608	0.0000000000	0.0000000000
20	KC1133561NICU96PA	0.0098039216	0.0049019608	0.6421568627	0.6274509804	0.0098039216	0.0049019608	0.0049019608	0.6421568627	0.6274509804	0.6225490196	0.0098039216	0.0049019608	0.0049019608
21	KF5307941HM1_5UTF	0.0245098039	0.0196078431	0.6519607843	0.6372549020	0.0245098039	0.0196078431	0.0196078431	0.6519607843	0.6372549020	0.6323529412	0.0245098039	0.0196078431	0.0196078431
22	KF1489371PDN9_5UT	0.0245098039	0.0196078431	0.6519607843	0.6372549020	0.0245098039	0.0196078431	0.0196078431	0.6519607843	0.6372549020	0.6323529412	0.0245098039	0.0196078431	0.0196078431
23	D298191_5UTR_	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000	0.0000000000	0.6372549020	0.6225490196	0.6176470588	0.0049019608	0.0000000000	0.0000000000
24	MG436882130_5UTR	0.0098039216	0.0049019608	0.6372549020	0.6225490196	0.0098039216	0.0049019608	0.0049019608	0.6372549020	0.6225490196	0.6176470588	0.0098039216	0.0049019608	0.0049019608
25	DQ4827761MD895_5U	0.0147058824	0.0098039216	0.6421568627	0.6274509804	0.0147058824	0.0098039216	0.0098039216	0.6421568627	0.6274509804	0.6225490196	0.0147058824	0.0098039216	0.0098039216
26	KM2754821Col278_5U	0.0147058824	0.0098039216	0.6421568627	0.6274509804	0.0147058824	0.0098039216	0.0098039216	0.6421568627	0.6274509804	0.6225490196	0.0147058824	0.0098039216	0.0098039216
27	EF1938711SRB57_5U	0.0098039216	0.0049019608	0.6421568627	0.6274509804	0.0098039216	0.0049019608	0.0049019608	0.6421568627	0.6274509804	0.6225490196	0.0098039216	0.0049019608	0.0049019608
28	AJ62123615UTR	0.0098039216	0.0049019608	0.6421568627	0.6274509804	0.0098039216	0.0049019608	0.0049019608	0.6421568627	0.6274509804	0.6225490196	0.0098039216	0.0049019608	0.0049019608
29	AM70966215NTR_stra	0.0196078431	0.0147058824	0.6372549020	0.6225490196	0.0196078431	0.0147058824	0.0147058824	0.6372549020	0.6225490196	0.6176470588	0.0196078431	0.0147058824	0.0147058824
30	L3439115UTR	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000	0.0000000000	0.6372549020	0.6225490196	0.6176470588	0.0049019608	0.0000000000	0.0000000000
31	KP86128713a_isolate	0.0294117647	0.0245098039	0.6372549020	0.6225490196	0.0294117647	0.0245098039	0.0245098039	0.6372549020	0.6225490196	0.6176470588	0.0294117647	0.0245098039	0.0245098039
32	Z8427915UTR_(3133_	0.0098039216	0.0049019608	0.6372549020	0.6225490196	0.0098039216	0.0049019608	0.0049019608	0.6372549020	0.6225490196	0.6176470588	0.0098039216	0.0049019608	0.0049019608
33	DQ2497961DO56-5UT	0.0196078431	0.0147058824	0.6372549020	0.6225490196	0.0196078431	0.0147058824	0.0147058824	0.6372549020	0.6225490196	0.6176470588	0.0196078431	0.0147058824	0.0147058824
34	D177631_Hepatitis_C	0.0098039216	0.0049019608	0.6421568627	0.6274509804	0.0098039216	0.0049019608	0.0049019608	0.6421568627	0.6274509804	0.6225490196	0.0098039216	0.0049019608	0.0049019608
35	D28917[/isolate_HCV	0.0049019608	0.0000000000	0.6372549020	0.6225490196	0.0049019608	0.0000000000	0.0000000000	0.6372549020	0.6225490196	0.6176470588	0.0049019608	0.0000000000	0.0000000000

c. Phylogenetic analysis

Phylogenetic relationship of the Untypable isolates was carried out in comparison to 25 Pakistani and regional HCV 3a isolates including reference sequences from India, Malaysia, Iran, Japan, UK, USA, Canada, Colombia, Serbia, Brazil and South Africa. (Table 4.13, Fig 4.14). The analysis revealed that the 5′UTR sequences fall in four genetically diverse clades/groups. Clade-I includes all Untypable isolates except HCV PK3. Moreover in clade 1 PK7 clustered close to reference Pakistani isolate KX449356.1; similarly PK14 and PK16 are closely related to Pakistani isolate Kx449355.1; PK8 and PK15 have close phylogenetic relationship to KX449354.1; PK12 and PK13 clustered together with FJ790793.1 (a Pakistani reference sequence HCV 3a). In clade-II and III the Asian, Europian, North and South American countries isolates closely coincided with each other. In clade-IV the sequence of Untypable isolate PK3 is closely related to the reference sequence HCVK3a (Japan) and KX0844791.1 from Brazil with high bootstrap support. The overall results demonstrated that the sequences of Untypable isolates have close phylogenetic relationship with each other and with the Pakistani HCV 3a isolates while different from reference sequences of other countries isolates with some exceptions. It is summarized that most of the Untyapable HCV genotypes encountered in different parts of Pakistan have close evolutionarily relationship with HCV 3a; which is the most

abundantly found HCV genotype in Pakistan. However, there are genetically distinct types e.g PK3, indicating a great deal of diversity among the prevalent Untypable strains (Figure 4.14).

Table 4.12: Genbank submissions of the 5' UTR sequences of the Untypable strains

S.No	Accession No	Isolate/
1	MK491838	**>PK1. Hepatitis C virus isolate BI1854 clone 1 5' UTR** GTCACCTGCCTCTTACGAGGCGACACTCCACCATGGATCACTCCCCTGTGAGGAATTTCTGTCTTCACGCGGAAAGCGCC TAGCCATGGCGTTAGTACGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGG TGAGTACACCGGAATCGCTGGGGTGACCGGGTCCTTTCTTGGAACAACCCGCGCAATACCCAGAAATTTGGGCGTGCCCC CGCGAGATCACTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGG GAGGTCTCGTAGACCGTGCAACGTC
2	MK491839	**>PK3. Hepatitis C virus isolate BP460 clone 3 5' UTR** ACCTGCCTCTTACGAGGCGACACTCCACCATGGATCACTCCCCTGTGAGGAATTTCTGTCTTCACGCGGAAAGCGCCTAG CCATGGCGTTAGTACGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGA GTACACCGGAATCGCTGGGGTGACCGGGTCCTTTCTTGGAACAACCCGCTCAATACCCAGAAATTTGGGCGTGCCCCCGC GAGATCACTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGA GGTCTCGTAGACCGTGCAACGTC
3	MK491840	**>PK7. Hepatitis C virus isolate BI310 Clone 1 5' UTR** ATCCGAAGACCCCTTATCTGGCAGTCTACTCGGCTATTGGTCTCGCAGAGGCACGCCCTATTTCTGGGCATTGAGCGGCT GGGCCCAGAAAGGACCCGGTCACCCCTGCGATTCCGGTGTACTCTCCGGTTCAACAGACACTATGGCTCTCCCGGGAGGG CGGGTCCTGGAGGGAGCACGACACTAGTACTAATGCCATGGCTAGACCCTTTCTGCACTGCCTGATATGGGTGCTTGCGA GACTGCCTGATATGGGTGCTTGCGAGACTGCCTTTTTTTGGTGCTTGCAAGAATCTGAATGTTTGAAATATTTCACAAGTT CTTATTTTCTCCTTGAAACTAGGTC
4	MK491841	**>PK8. Hepatitis C virus isolate BJ2752 Clone 2 5' UTR** ATCCAGAGCCTTTCTCGGGCAGCACTACTCGGCTAGTGATCGCAGCAGGGGCCGCCCCAATTTCTGGGTATTGAGCGGGT TGTTCCAAGAAAGGACCCGGTCACCCCAGCGATTCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCTCCCGGGAG GGGGGCAGAAGGAGGAACACGACACTAGTACTAACGCCACAGCTAGAGGCTTTCTGCCCTGCCTAATATGGATGCTTGC GAGACTGCCTGATCTGGGTGCATGCCAAAATGCCTGATCTTGGGACTTGGCAAAATGGCGAGACATGCTCTGCTACC
5	MK732954	**>PK11. Hepatitis C virus isolate BB1485 clone 2 5' UTR** ACCTGCCTCTTACGAGGCGACACTCCACCATGGATCACTCCCCTGTGAGGAATTTCTGTCTTCACGCGGAAAGCGCCTAG CCATGGCGTTAGTACGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGA GTACACCGGAATCGCTGGGGTGACCGGGTCCTTTCTTGGAACAACCCGCGCAATACCCAGAAATTTGGGCGTGCCCCCGC GAGATCACTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGA GGTCTCGTAGACCGTGCAAC
6	MK732955	**>PK12. Hepatitis C virus isolate BG4327 clone 3 5' UTR** ACCTGCCTCTTACGAGGCGACACTCCACCATGGATCACTCCCCTGTGAGGAATTTCTGTCTTCACGCGGAAAGCGCCTAG CCATGGCGTTAGTACGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGA GTACACCGGAATCGCTGGGGTGACCGGGTCCTTTCTTGGAACAACCCGCTCAATACCCAGAAATTTGGGCGTGCCCCCGC GAGATCACTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGA GGTCTCGTAGACCGTGCAAC

7	MK732956	**>PK13. Hepatitis C virus isolate BAJK4715 clone 1 5' UTR** ACCTGCCTCTTACGAGGCGACACTCCACCATGGATCACTCCCCTGTGAGGAACTTCTGTCTTCACGCGGAAAGCGCCTAG CCATGGCGTTAGTACGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGA GTACACCGGAATCGCTGGGGTGACCGGGTCCTTTCTTGGAACAACCCGCTCAATACCCAGAAATTTGGGCGTGCCCCCGC GAGATCACTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGGA GGTCTCGTAGACCGTGCAAC
8	MK732957	**>PK14. Hepatitis C virus isolate BR86 5' UTR** CGAAGACCCCTTATCTGGCAGTCTACTCGGCTATTGGTCTCGCAGAGGCACGCCCTATTTCTGGGCATTGAGCGGCTGGG CCCAGAAAGGACCCGGTCACCCCTGCGATTCCGGTGTACTCTCCGGTTCAACAGACACTATGGCTCTCCCGGGAGGGCGG GTCCTGGAGGGAGCACGACACTAGTACTAATGCCATGGCTAGACCCTTTCTGCACTGCCTGATATGGGTGCTTGCGAGAC TGCCTGATATGGGTGCTTGCGAGACTGCCTTTTTTTGGTGCTTGCAAGAATCTGAATGTTTGAAATATTTCACAAGTTCTT ATTTTCTCCTTGAAACTAG
9	MK732958	**>PK15. Hepatitis C virus isolate BF1178 5' UTR** CAGAGCCTTTCTCGGGCAGCACTACTCGGCTAGTGATCGCAGCAGGGGCCGCCCCAATTTCTGGGTATTGAGCGGGTTGT TCCAAGAAAGGACCCGGTCACCCCAGCGATTCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCTCCCGGGAGGGG GGCAGAAGGAGGAACACGACACTAGTACTAACGCCACAGCTAGAGGCTTTCTGCCCTGCCTAATATGGATGCTTGCGAG ACTGCCTGATCTGGGTGCATGCCAAAATGCCTGATCTTGGGACTTGGCAAAATGGCGAGACATGCTCTGCT
10	MK732959	**>PK16. Hepatitis C virus isolate BS1466 5' UTR** GTCTTTCGCGACCACACATACTCGGCTAGTGATCTCGCGGGGGCACGCCCAAATTTCTGGGTATTGAGCGGGTTACTCCA AGAAAGGACCCGGTCACCCCAGCGATTCCGGTGTACTCACCGGTTCCGCAAACCACTATGGCTCTCCCGGGAGGGGGAG TCCTGGAGGCTGCACGACACTCGTACTAACGCCATGGCTAGACGCTTTCTGCCCTGCCTGATATGGTTGCTTGCGAGA

Table 4.13: HCV 5′UTR sequences for phylogenetic analysis

S.No	Accession No	Isolate	Country	Year
1.	MK491838	PK1.BI1854 Untypable 1 5' UTR	Pakistan	2017
2.	MK491839	PK3.BP460 Untypable 3 5' UTR	Pakistan	2017
3.	MK491840	PK7.BI310 Untypable 1 5' UTR	Pakistan	2017
4.	MK491841	PK8.BJ2752 Untypable 2 5' UTR	Pakistan	2017
5.	MK732954	PK11.BB1485 Untypable 2 5' UTR	Pakistan	2018
6.	MK732955	PK12.BG4327 Untypable 3 5' UTR	Pakistan	2018
7.	MK732956	PK13.BAJK4715 Untypable 1 5' UTR	Pakistan	2018
8.	MK732957	PK14.BR86 Untypable 5' UTR	Pakistan	2018
9.	MK732958	PK15.BF1178 Untypable 5' UTR	Pakistan	2018
10.	MK732959	PK16.BS1466 Untypable 5' UTR	Pakistan	2018
11.	FJ790793.1	Isolate 31 clone 2 5' UTR	Pakistan	2009
12.	FJ790794.1	Isolate 812 clone 3 5' UTR	Pakistan	2009
13.	KX449355.1	SA16 5' UTR	Pakistan	2016
14.	KX449356.1	PK21 5'UTR	Pakistan	2016
15.	KX449354.1	FR308 5' UTR	Pakistan	2016
16.	DQ482776.1	MD895 5' UTR	India	2006

17.	DQ073368.1	Isolate 34 5' UTR	India	2006
18.	KX084791.1	TSBD7916 5' UTR	Brazil	2016
19.	KC113356.1	NICU96PA 5' UTR	Brazil	2012
20.	KF748937.1	PDN9 5' UTR	Malaysia	2013
21.	KF768418.1	Isolate PDN27 5' UTR	Malaysia	2013
22.	D29819.1	Non coding 5`UTR	Japan	1994
23.	MG436882.1	Isolate 30 5' UTR	Iran	2017
24.	DQ482776.1	MD895 5' UTR	South Africa	2006
25.	KM275482.1	Col_278 5' UTR	Colombia	2014
26.	EF193871.1	SRB57 5' UTR	Serbia	2006
27.	AJ621236.1	5' UTR	Sweden	2004
28.	AM709662.1	5'UTR, strain 18	Uruguay	2007
29.	L34391.1	Non coding 5' UTR	Canada	1995
30.	KP861287.1	3a isolate 15-46/F2PC10 5' UTR	Canada	2013
31.	Z84279.1	Isolate 3133, clone 5 5'UTR	UK	1997
32.	DQ249796.1	DO56-5'UTR-LD-55	USA	2005
33.	D177631	NZL-1_3a	New Zealand	2007
34.	D28917.1	HCVK3a 5' UTR	Japan	1994
35.	MG436882.1	Isolate 30 5' UTR	Iran	2017

(Amina et al., 2016)

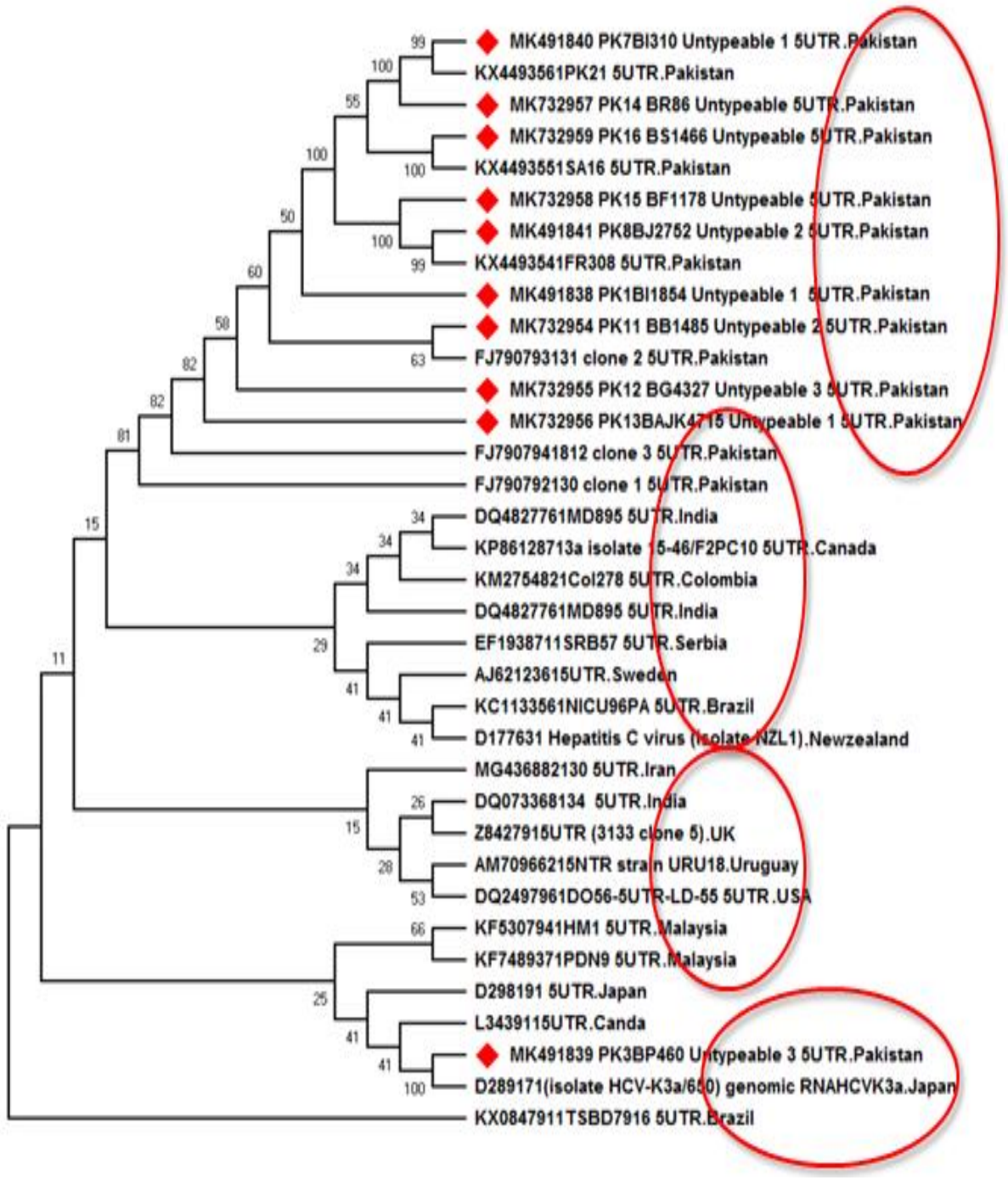


Figure 4.14: Phylogenetic tree of HCV Untypable 5′UTR gene sequences (marked by red box) constructed by Maximum Likelihood algorithm (MEGA-X Software), with Bootstrap values shown on the sub-branches. Tree demonstrates the phylogenetic relationship of 10 genetically diverse Untypable sequences, marked in red with 25 reference sequences from various geographical locations.

4.7 Response rates of Direct Acting Antiviral therapies (DAATs)

A total of 4411 subjects with chronic HCV infection were enrolled for the study out of which n=3779 (85.6%) subjects completed the therapy. Due to a variety of reasons including immune status and other unavoidable circumstances, n=632 (14.3%) HCV-positive subjects discontinued the treatment. Majority of the subjects were in the age of 26-70 years (Table 4.14).

Table 4.14: Demographic of treated subjects (N=3779).

S.No	Ages of Subjects (Years)	Males	Females	Total
1	10-25	93	99	192
2	26-40	587	802	1389
3	41-55	633	997	1630
4	56-70	232	275	507
5	71-85	29	28	57
6	Above 85	1	3	4
Total	**10-98**	**1575**	**2204**	**3779**

a. Baseline characteristics

The baseline characteristics of 3779 HCV infected subjects were recorded at the beginning of the treatment including age, complete blood counts (CBC), viral load and liver function test (LFTs). Initial analysis of the baseline characteristics revealed that viral loads among majority of the CHC subjects were considerably elevated (Mean: $3.41\times10^{6}\pm1.57\times10^{6}$). LFTs levels also indicated a similar elevated trend as compared to the reference values (Table 4.15) while the blood cells profiles were variable with lower number of some leukocytes compared to reference values except the platelets which were above normal. These baselines parameters were indicative of the chronic infection in hepatocytes cells of the subjects by hepatitis C virus. The baseline characteristics of the subjects are given in (Table 4.15).

Table 4.15: Baseline characteristics of four groups (n=3779) in HCV positive subjects

Variables	Minimum	Maximum	Reference range	Mean±SD
Age(years)	11.00	98.00	10-98	44.23±11.71
HCV RNA PCR (IU/ml)	1.55×10^{3}	9.17×10^{7}	$>2\times10^{2}$	3.41×10^{6} ±1.57×10^{6}
Total Bilirubin (mg/dL)	0.30	5.00	Upto 1.0	1.44±0.52
ALT (SGPT) (IU/L)	24.00	233.0	Upto 43	85.10±18.30
ALK-Phos (IU/L)	24.00	695.0	Adults: 65 -360 - Childern: Upto 730	319.51±93.46
Albumin (g/dL)	0.30	6.90	3.2-5.5	5.36±1.02
TLC (/cumm)	4000.0	10200.0	4000-11000	8128.04±1239.56
RBCs (Million/cumm)	4.1	5.8	M: 4.5 - 6.0 : F: 4.0 - 5.5	5.36±0.347
Haemoglobin (gm/dl)	12.3	17.0	M:12.5 - 18: F:11 - 16.5	13.60±1.10
Platlets Count (/cumm	124000.0	425000.0	150000 - 450000	260738.33±65428.84
Neutrophils(%)	46.0	72.0	40-80	59.360±4.0478
Lymphocytes(%)	23.0	39.0	20-40	29.905±3.5294
Monocytes(%)	3.0	9.0	02-10	6.536±1.6000
Eosinophils(%)	2.0	6.0	01-06	4.199± 1.1972

b. Characteristics according to status of subjects

HCV infected subjects were categorized into four groups based on the type of therapy. The base line characteristics were statistically analyzed using Pearson correlation in the case of each treatment group. The results indicated that the mean value of variables (Viral load, LFTs and CBC) were not statistically significant (*p* value >0.05) among the groups. The characteristics of groups are given in (Table 4.16).

Table 4.16: Comparison of different parameters among (1-4) treatment groups

Variables	Group-I (SOFO+RBV)	Group-II (DCV+SOFO)	Group-III (DCV+SOFO+RBV)	Group-IV (SOFO+RBV +PEG-IFN)	P-value
Age(years)	43.717	45.14	43.548	44.978	-
HCV RNA PCR (IU/ml)	285617.253	317102.425	295128.094	451153.8	0.366
Total Bilirubin (mg/dL)	1.44	1.582	1.388	1.424	0.321
ALT (SGPT) (IU/L)	83.903	84.605	81.732	89.141	0.272
ALK-Phos (IU/L)	321.371	301.6	326.332	320.11	0.247
Albumin (g/dL)	5.378	5.439	5.335	5.326	0.359
TLC (/cumm)	8200.888	7855.18	8091.834	8172.442	0.472
RBCs (Million/cumm)	5.372	5.342	5.37	5.358	-
Haemoglobin (gm/dl)	13.588	14.136	13.464	13.483	0.4
Platlets Count (/cumm	260827.732	262644.82	259912.718	260364.991	0.366

Neutrophils(%)	59.389	58.903	59.452	59.454	0.359
Lymphocytes(%)	29.889	29.776	30.019	29.905	0.359
Monocytes(%)	6.505	6.518	6.6	6.54	0.359
Eosinophils(%)	**4.217**	**4.803**	**3.929**	**4.101**	**0.32**

Abbreviations: ALT,=Alanine Aminotransferase, ALP-Phos= Alkaline phosphatase, TLC= Total Leukocyte count, RBCs= Red blood cells, HCV RNA by PCR, Hepatitis C virus ribonucleic acid by polymerase chain reaction

c. Response rates of subjects to different combination therapies

HCV infected subjects were grouped into four categories depending upon the combination of anti-viral therapy received during treatment period. Response rates in different the treatment groups were documented as follows.

c-i. Response rate of SOF-RBV combination therapy (Group-I):

Group-I included 1446 CHC subjects out of which 599 (41.4%) were males while 847 (58.6%) were females. The subjects in group-I were divided into six age groups (Table 4.17b). All the subjects received a combination of SOF and RBV @ 400 mg/day and 1000 mg/day respectively for a period of 24 weeks while the response rate of the therapy was monitored via assessment of viral load and LFTs at different intervals of time particularly at 12 weeks, 24 weeks and 48 weeks.

Majority of the subjects in group-I were infected with HCV 3a, HCV 3b, mixed infection of HCV 3a3b or the Untypable HCV while there were only few cases of infections with other HCV types (Table 4.17a). The ETR and SVR achieved for the combination therapy against HCV 3b infection was the highest (95.8%) as compared to HCV 3a (91.6% & 91.3%); which is the predominant type in Pakistan. Moreover, relapse was observed only in the case of HCV 3a. Interestingly, response of the combination therapy was slightly better in the case of mixed genotypic infections (93.5%) than HCV 3a alone. The combination of SOF-RBV did not prove as much efficient against the Untypable HCV infection with an ETR and SVR of 84.4%, however it was satisfactory against the genotypes found rarely in group-I (Table 4.17a). The overall ETR was (91.7%) and SVR was (91.5%) in the case of infections with all genotypes however 120 (8.2%) subjects turned to be virologically non responders (Table 4.17a). Gender wise response rates in terms of ETR (92.2%) was achieved in female and (89.2%) in male subjects. Similarly SVR was achieved as (92.1%) in female subjects and (88.8%) in male subjects while (7.7%) and (10.7%) non responders and (0.1%) and (0.4%) relapsers were found in female and male subjects respectively (Table 4.21).

The age-group wise response rates revealed that the combination of SOF-RBV was more effective in younger age groups (SVR: 98.2-98.6%) as compared to subjects of >40 yrs (SVR range: 56.2-92.3%) (Table 4.17b). Maximum null response was observed in the elderly (>56 yrs) as compared to the younger age groups with an exception of two subjects of >85 yrs who had achieved 100% SVR.

Table: 4.17a Response of SOF-RBV combination therapy against various HCV genotypes in Group-I

Group-I(SOFO-RBV)							
HCV Genotypes	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapser**
HCV 2a	7	9	16	(93.7%)	(93.7%)	1(6.25%)	0
HCV 3a	471	655	1126	(91.6%)	(91.3%)	94(8.3%)	3(0.2%)
HCV 3a3b	66	88	154	(93.5%)	(93.5%)	10(6.4%)	0
HCV 3b	31	41	72	(95.8%)	(95.8%)	3(4.1%)	0
HCV 4a	0	1	1	(100%)	(100%)	0	0
HCV Untypable	24	53	77	(84.4%)	(84.4%)	12(15.5%)	0
Total	**599**	**847**	**1446**	**(91.7%)**	**(91.5%)**	**120(8.2%)**	**3(0.2%)**

Table: 4.17b Response of SOF-RBV combination therapy in various age groups of Group-I

Group-I(SOFO-RBV)							
Age group(10-98)	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapser**
10-25 Years	42	34	76	(98.6%)	(98.6%)	1(1.3%)	0
26-40 Years	222	305	527	(98.6%)	(98.2%)	7(1.3%)	2(0.3%)
41-55 Years	247	409	656	(92.5%)	(92.3%)	49(7.4%)	1(0.1%)
56-70 Years	80	89	169	(66.8%)	(66.8%)	56(33.1)	0
71-85 Years	7	9	16	(56.2%)	(56.2%)	7(43.7%)	0
Above 85 Years	1	1	2	(100%)	(100%)	0	0
Total	**599**	**847**	**1446**	**(91.7%)**	**(91.5%)**	**120(8.2%)**	**3(0.2%)**

c-ii. Response rate of DCV-SOF combination therapy (Group-II):

Group-II included 473 CHC subjects of both genders out of which 211 (44.6%) were males while 262 (55.4%) were females who were divided into six age groups (Table 4.18b). All the subjects received a combination of DCV and SOF @ 60 mg/day and 400 mg/day respectively for

a period of 24 weeks while the response rate of the therapy was monitored as mentioned for group-I. Majority of the subjects in group-II were infected with HCV 3a, HCV 3a3b mixed infection or the Untypable HCV (Table 4.18a).

The overall ETR rate in case of all the HCV genotypes detected in group-II was (93.2%) while the SVR rate was (92.6%). The overall null-response in case of various HCV genotypes was (6.7%). However, response rate of the combination therapy was variable against different HCV genotypes. The ETR observed in the case of HCV 3a infections was higher (94%) as compared to the other most frequently prevalent types in group-I including the Untypable HCV (91.8%) and mixed infections of HCV 3a3b (88%). A similar trend was observed in the case of SVR as well (Table 4.20a). HCV 2a, 2b and HCV 3b infections were relatively rare in group-II however, the combination therapy worked well in the case of the former as compared to the later (HCV 3b). Relapse was observed only in the case of HCV 3a (Table 4.18a). Gender wise response rates in terms of ETR (92.9%) was achieved in female and (92.3%) in male subjects. Similarly SVR was achieved as (91.7%) in female subjects and (92.3%) in male subjects while (7.0%) and (7.6%) non responders and (1.3%) and (0.0%) relapsers were found in female and male subjects respectively (Table 4.21).

Majority of the subjects in group-II <70 yrs of age. DCV-SOF combination therapy achieved 100% ETR and SVR in the youngest age group (10-25 yrs) followed closely by age group (26-40 yrs) who achieved an ETR and SVR rates of 99.3% with no relapsers (Table 4.20b). However, the combination therapy was not very successful in the elderly (age groups 56-70 & 71-85 yrs) where only 68.7% & 70% of the subjects responded favorably (Table 4.18b).

Table: 4.18a Response of DCV-SOF combination therapy against various genotypes in Group-II

Group-II							
HCV Genotypes	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapser**
HCV 2a	4	2	6	(100%)	(100%)	0	0
HCV 2b	1	1	2	(100%)	(100%)	0	0
HCV 3a	163	204	367	(94.0%)	(93.01%)	22(5.9%)	3(0.8%)
HCV 3a3b	18	24	42	(88.0%)	(88.0%)	5(11.9%)	0
HCV 3b	7	12	19	(89.4%)	(89.4%)	2(10.5%)	0
HCV Untypable	18	19	37	(91.8%)	(91.8%)	3(8.1%)	0
Total	**211**	**262**	**473**	**(93.2%)**	**(92.6%)**	**32(6.7%)**	**3(0.6%)**

Table: 4.18b Response of DCV-SOF combination therapy in various age groups of Group-II

Group-II							
Age group(10-98)	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapser**
10-25 Years	9	16	25	(100%)	(100%)	0	0
26-40 Years	75	89	164	(99.3%)	(99.3%)	1 (0.6%)	0
41-55 Years	84	109	193	(97.4%)	(96.8%)	5 (2.5%)	1(0.5%)
56-70 Years	37	43	80	(70.0%)	(68.7%)	24 (30%)	1(1.2%)
71-85 Years	6	4	10	(80.0%)	(70.0%)	2 (20%)	1(11.1%)
Above 85 Years	0	1	1	(100%)	(100%)	0	0
Total	**211**	**262**	**473**	**(93.2%)**	**(92.6%)**	**32 (6.7%)**	**3(0.6%)**

c-iii. Response rate of triple combination therapy including DCV-SOF-RBV (Group-III):

Group-III included 747 CHC subjects out of which 298 (39.8%) were males while 449 (60.2%) were females. The subjects in group-I were divided into five age groups (Table 4.19b). All the subjects received a triple combination of DCV, SOF and RBV @ 60 mg/day, 400 mg/day and 1000 mg/day respectively for a period of 24 weeks while the response rate of the therapy was monitored via assessment of viral load and LFTs at different intervals of time particularly at 12 weeks, 24 weeks and 48 weeks.

Most of the infected subjects had either HCV 3a genotype or with other types including HCV 3b, mixed infections of 3a3b and the Untypable HCV (Table 4.19a). The overall ETR and SVR rates observed for the triple combination therapy against the HCV types prevalent among the CHC subjects of group-III were 93.9% and 92.8% respectively. The triple combination therapy was particularly more effective against the Untypable HCV as indicated by high ETR and SVR rates of 97.4%. In this study we observed a fairly high response rate of the triple combination (>91%) against the most abundant HCV types in group-III, however, although there were limited subjects infected with HCV 2a, the response rate was relatively poor (SVR: 87.5%). Majority of the null response subjects were infected with HCV 3a and relapse was also observed in the case of the same genotype (Table 4.19a). Gender wise response rates in terms of ETR (93.3%) was achieved in female and (93.9%) in male subjects. Similarly SVR was achieved as (93.0%) in female subjects and (93.9%) in male subjects while (6.6%) and (6.0%) non responders and (0.2%) and (0.0%) relapsers were found in female and male subjects respectively (Table 4.21).

The triple combination therapy was very effective among subjects in the age range of 10-40 yrs with the maximum SVR rate of 98% observed in age group (26-40 yrs), however, the therapy was not very successful among subjects of older age (>40yrs) as maximum null response was recorded in the elderly (Table 4.19b).

Table: 4.19a Response of DCV-SOF-RBV combination therapy against various genotypes in Group-III

Group-III							
HCV Genotypes	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapser**
HCV 2a	2	6	8	(87.5%)	(87.5%)	1(12.5%)	0
HCV 3a	235	360	595	(93.7%)	(93.6%)	37 (6.2%)	1(0.1%)
HCV 3a3b	17	24	41	(92.6%)	(92.6%)	3(7.3%)	0
HCV 3b	9	15	24	(91.6%)	(91.6%)	2(8.3%)	0
HCV Untypable	35	44	79	(97.4%)	(97.4%)	2(2.5%)	0
Total	**298**	**449**	**747**	**(93.9%)**	**(93.8%)**	**45(6.0%)**	**1(0.1%)**

Table: 4.19b Response of DCV-SOF-RBV combination therapy in various age groups of Group-III

Group-III							
Age group(10-98)	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapser**
10-25 Years	16	23	39	(97.4%)	(97.4%)	1(2.5%)	0
26-40 Years	116	186	302	(98.6%)	(98.3%)	4(1.3%)	1(0.3%)
41-55 Years	147	182	301	(93.2%)	(93.2%)	11(3.6%)	0
56-70 Years	19	53	94	(75.5%)	(75.5%)	23(24.4%)	0
71-85 Years	0	5	11	(45.5%)	(45.5%)	6(54.5%)	0
Total	**298**	**449**	**747**	**(93.9%)**	**(93.8%)**	**45(6.0%)**	**1(0.1%)**

c-iv. Response rate of SOF-RBV-PEG-IFN combination therapy (Group-IV):

Group-IV included 1113 CHC subjects out of which 464 (41.6%) were males while 649 (48.4%) were females. The subjects in group-IV were divided into four age groups (Table 4.20b). All the subjects received a triple combination of SOF, RBV and PEG-IFN @ 400 mg/day, 1000 mg/day and 180µg/week respectively for a period of 24 weeks while the response rate of the therapy was monitored via assessment of viral load and LFTs at different intervals of time

particularly at 12 weeks, 24 weeks and 48 weeks.

The most abundant HCV genotype in group-IV was HCV 3a (n=931) followed by the Untypable HCV (n=90), mixed infections of HCV 3a3b (n=47) and HCV 3b (n=41 while there were only 4 subjects infected either with HCV 2a or HCV 1b. The triple combination therapy was most successful against HCV 3b where 100% ETR and SVR rates were observed with no null response or relapses. Similarly, the triple combination was quite effective against the Untypable HCV with an SVR of 94%, however, against HCV 3a and mixed genotypic infections, the therapy was comparatively less effective than the previous double or triple combinations used in other groups (Table 4.20a). A relatively higher percentage of null response was observed in the case of HCV 3a and the number of relapsers in case of the said genotype was also high (Table 4.20a). Gender wise response rates in terms of ETR (93.0%) was achieved in female and (91.9%) in male subjects. Similarly SVR was achieved as (90.3%) in female subjects and (90.0%) in male subjects while (6.9%) and (8.0%) non responders and (2.9%) and (2.0%) relapsers were found in female and male subjects respectively (Table 4.21).

In different age groups, the triple combination proved more effective among subjects of <55 years of age while it was relatively less effective in subjects >55 yrs. Null response and relapses were observed in all age groups with majority of the cases falling in the age group (41-55 yrs) (Table 4.20b).

Table: 4.20a Response of SOF-RBV-PEG IFN against various HCV genotypes in group-IV

Group-IV(SOFO-RBV-PEG INF-α-2a)							
HCV Genotypes	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapse**
HCV 1b	1	1	2	(100%)	(100%)	0	0
HCV 2a	1	1	2	(100%)	(100%)	0	0
HCV 3a	391	540	931	(93.1%)	(90.6%)	64(6.8%)	23(2.5%)
HCV 3a3b	23	24	47	(89.3%)	(87.2%)	5(10.6%)	1(2.1%)
HCV 3b	16	25	41	(100%)	(100%)	0	0
HCV Untypable	32	58	90	(94.4%)	(94.4%)	5(5.5%)	0
Total	**464**	**649**	**1113**	**(93.3%)**	**(91.1%)**	**74(6.6%)**	**24 (2.3%)**

Table: 4.20b Response of SOF-RBV-PEG IFN in various age groups of Group-IV

Group-IV(SOFO-RBV-PEG INF-α-2a)							
Age group(10-98)	**Male**	**Female**	**Total**	**ETR**	**SVR**	**NR**	**Relapse**
10-25 Years	26	24	50	(98.0%)	(92.0%)	1(2%)	3(6.3%)
26-40 Years	180	229	433	(94.6%)	(93.3%)	23(5.3%)	6(1.4%)
41-55 Years	184	308	492	(93.6%)	(91.2%)	31(6.3%)	12(2.5%)
56-70 Years	74	88	138	(86.2%)	(84.0%)	19(13.7%)	3(2.2%)
Total	**464**	**649**	**1113**	**(93.3%)**	**(91.1%)**	**74(6.6%)**	**24(2.3%)**

Table 4.21: Gender wise response against various Therapies

S.No	Therapy	Total Subjects	Gender	ETR	SVR	NR	Relapser
1	**SOFO-RBV**	1446	**Female**	724/785 (92.2%)	723/785 (92.1%)	61/785 (7.7%)	1/724 (0.1%)
			Male	481/539 (89.2%)	479/539 (88.8%)	58/539 (10.7%)	2/481 (0.4%)
2	**DCV-SOFO**	473	**Female**	225/242 (92.9%)	222/242 (91.7%)	17/242 (7.0%)	3/225 (1.3%)
			Male	181/196 (92.3%)	181/196 (92.3%)	15/196 (7.6%)	0/181 (0.0%)
3	**DCV-SOFO-RBV**	747	**Female**	392/420 (93.3%)	391/420 (93.0%)	28/420 (6.6%)	1/392 (0.2%)
			Male	264/281 (93.9%)	264/281 (93.9%)	17/281 (6.0%)	0/264 (0.0%)
4	**DCV-RBV-PEG-IFN**	1113	**Female**	551/592 (93.0%)	535/592 (90.3%)	41/592 (6.9%)	16/551 (2.9%)
			Male	388/422 (91.9%)	380/422 (90.0%)	34/422 (8.0%)	8/388 (2.0%)

d. Comparative analysis of different therapies

All the new DAAs therapies were found very effective while DCV-SOFO-RBV with SVR (93.8%) and DCV-SOFO with SVR (92.6%) were found more efficient as compared to SOFO-RBV (91.5%) and SOFO-RBV-Peg-IFN (91.1%).

- **Genotype wise analysis of different therapies**

  Genotype wise high SVR (93.8%) was achieved by DCV-SOFO-RBV combination therapy against HCV 3a, HCV Untypable, HCV 3a3b and HCV 3b.

The difference between top two therapies was (1.1%) which was not so much significant.

- **Age wise analysis of different therapies**

  Age wise DCV-SOFO combination therapy achieved the high SVR in terms of age range of 26- 70 years in comparison to rest of the three combination therapies.

- **Gender wise analysis of different therapies**

  Gender wise (93.0%) SVR rate in female and (93.0%) in male subjects was achieved against the DCV-SOFO-RBV combination therapy which was the highest one as compared to the rest of three combination therapies.

e.Side effects of the therapies

The side effects were mostly influenza-like syndrome, which occurred in more than 80% subjects. Gastrointestinal, psychiatric, dermatological symptoms and other side effects were mild to moderate. Generally all the subjects in each treated group had adverse events, most of which were mild to moderate in severity. The rate of serious adverse events was less among the subjects who received DCV–SOFO and higher among the subjects who received DCV–SOFO-RBV. The subjects who received SOFO-RBV-PEG-IFN, the occurrence of adverse events was higher than the subjects treated with SOFO-RBV. Subjects received RBV in combination therapy had higher rates of events like fatigue, nausea, insomnia, arthralgia, cough, rash, irritability, dyspnea, and anemia comparatively to the subjects who did not received RBV, same is the case with IFN (Table 4.22).

Table 4.10 Genetic distance Analysis

S.No	Minimum (0.000) genetic distance
9.	Among Untypable isolates PK1,P11 and with Pakistani isolate FJ790793
10.	Among Untypable isolates PK3, PK12, PK13 and with Pakistani isolates FJ790794, FJ790792, KX06491 and D29819 (India), L34391(Canada) and D28917 (Japan)
11.	Among Untypable PK8, PK14 and with Pakistani isolate KX449356
12.	Among Untypable isolates PK11 and PK16
13.	Among Untypable isolates PK12, PK13 and with Pakistani isolates FJ790793, FJ790794, KX084791 and L34291(Canada) and D28917 (Japan)
14.	Untypable PK13 with Pakistani isolates FJ790793, FJ790794, KX084791 and D29819(Japan) L34291(Canada) and D28917(Japan)
15.	Untypable PK14 with Pakistani isolates KX449356
16.	Untypable isolates PK15 with Pakistani isolate KX449354
S.No	**Maximum (0.63) genetic distance**
6.	Among Untypable isolates PK1,PK7,PK14 and PK21
7.	Untypable isolates PK1 with Pakistani isolate KX449356
8.	Among Untypable PK11, PK12, PK13 and with DQ073368 (India), KX0847911 (Brazil), D298191 (Japan), MG436882 (Iran), AM709662 (URUGUAY), L343911 and KP861287 (Canada), Z84279 (UK), DQ249696 (USA), D289171 (Japan).
9.	Untypable PK14 with DQ073368 (India), KX0847911 (Brazil), KF530794 and KF748937 (Malaysia), D298191 (Japan), MG436882 (Iran), AM709662 (URUGUAY), L343911 and KP861287 (Canada), Z84279 (UK), DQ249696 (USA), D289171 (Japan).
10.	Pakistani isolate PK21 with DQ073368 (India), KX0847911 (Brazil), KF530794 and KF748937 (Malaysia), D298191 (Japan), MG436882 (Iran), AM709662 (URUGUAY), L343911 and KP861287 (Canada), Z84279 (UK), DQ249696 (USA), D289171 (Japan).

In the light of above observation it is analyzed that genetic closeness was found among the sequences of HCV Untypable isolates and with Pakistani isolates HCV 3a, however Untypable sequences have variability in comparison to the sequences of isolates from other countries (Table 4.10 and 4.11).

Discussion

Hepatitis C virus (HCV) infection is a major cause of cirrhosis and liver cancer (Kim, 2013). Complications of the end stage liver diseases have been associated with critical mortality and morbidity around the globe (Nagarapu et al., 2013). In Pakistan, over the past two decades, a number of investigators have reported high prevalence of HCV infection from all the four provinces of Pakistan (Kakepoto et al., 1996, Khokhar et al., 2004, Jafri et al., 2006, Ahmad et al., 2007, Idrees and Riazuddin, 2008, Umer and Iqbal, 2016). A more recent study investigating the global prevalence of HCV infection has placed Pakistan at the top second position in terms of the burden of the disease (Waheed et al., 2017, Messina et al., 2015).

Pakistan has a population of more than 200 million (Census report, 2017) with limited resources and a considerable part of the population work as migrant workers in other countries such as the middle east, Europe, USA etc. Moreover, more than 3 million Afghan refugees are also living in Pakistan who are engaged in cross-border trade activities as well. There is considerable evidence (Attaullah et al., 2011) that migrant workers have contributed to the diversity of HCV and the burden of the disease in Pakistan. Phylogenetic analysis of Pakistani HCV isolates based on partial genomic sequences indicate that there is a great deal of genetic diversity among the isolates showing relationship with other regional isolates (Afzal et al., 2014, Stuyver et al., 1993).

Moreover, a number of HCV genotypes abundant in other geographical regions of the world have been reported from different parts of the country which include HCV 1, HCV 2, HCV3, HCV 4, HCV5, HCV6 and their subtypes (Waheed et al., 2009, Attaullah et al., 2011, Khan et al., 2014a, Idrees and Riazuddin, 2008). Due to lack of awareness about disease transmission and numerous risk factors, Pakistan has experienced uncontrolled outbreaks of the infection over the past two decades (Arshad and Ashfaq, 2017, Aleman et al., 2013) thus maximizing the chances for the rise of variants and novel subtypes reported from time to time (Idrees and Riazuddin, 2008). Some studies have reported clusters of Pakistani HCV isolates indicating a region-specific evolutionary trend (Idrees and Riazuddin, 2008, Waheed et al., 2013, Wahid et al., 2018, Umer and Iqbal, 2016). The prevalence of multiple HCV genotypes in the general population with high frequency coupled with limited treatment and diagnostic options in Pakistan has resulted in the failure of control strategies as reflected in the form of increasing burden of HCV infections. Until recently, conventional IFN-therapy alone or in combination with RBV was the only option to treat hepatitis C in Pakistan (Ali et al., 2016a, Umer and Iqbal,

2016) and that too was not available to many due to affordability issues. Due to epidemic situation of hepatitis C, the government started Prime Minister Hepatitis C Control Program in 2005 (Gul et al., 2016) under which free IFN formulations were provided to the patients but that did not achieve the goal of containing the disease due to several issues such as its availability only in some major health care centers not accessible to majority of the patients living in infected areas. HCV genotype is a strong predictor of response to therapy (Waheed et al., 2017) and the dose and duration of antiviral therapy is dependent upon the type of the virus. In Pakistan, genotyping of the virus prior to commencing treatment is not a common practice (Ali et al., 2014) thus complicating assessment of treatment regimens in cases of infections with different genotypes. Although investigators have reported diverse HCV types circulating in different regions of the country, a consensus on the pattern of HCV genotypes distribution has never reached to a conclusion. In fact, some investigators have reported a pattern change in the distribution of HCV genotypes in different parts of the country (Butt et al., 2010, Gul et al., 2016).This lack of consensus on the distribution of HCV genotypes motivated us to figure out the existing pattern of HCV genotypes prevalent in different cities of Pakistan. Moreover, several studies have previously reported HCV types which go undetectable by using Type-specific assays and hence are termed as 'Untypable HCV'(Afzal et al., 2014, Wahid et al., 2018, Zafar et al., 2018). As the Untypable HCV pose challenges to the diagnostics and treatment regimens, it was also of much interest to characterize such species on a representative scale. There have been only a couple of studies describing response rates of various combinations of IFN or PEG-IFN with RBV which have adverse side effects and require a cold chain for optimum results (Abd-Elsalam et al., 2019, Dolatimehr et al., 2017, Moreno et al., 2015). To date, there are limited reports on IFN-free formulations (Ali et al., 2016b, Abbas et al., 2017) which needed further investigations to assess the combinations of the newly approved antiviral therapies (DAAs) against HCV types prevalent among ethnically diverse Pakistan subjects.

The first objective of this study was to investigate the distribution patterns of hepatitis C virus genotypes in different geographical regions of Pakistan. As determination of active HCV infection is necessary prior to genotyping of the HCV isolates so as to avoid errors in the assessment of 'Untypable' HCV strains that is why all the samples were first subjected to determination of active HCV infection. In this study, a total of 5704 anti HCV positive samples

belonging to subjects from 24 different locations of Pakistan were processed for determination of active HCV infection by carrying out RT-PCR based HCV RNA determination which revealed that 5259 (92.1%) subjects had active HCV infection while 444 (7.9%) were found to be PCR negative (Fig 3.1). Previously, several studies have reported active HCV infection from different regions of Pakistan (Ur Rehman et al., 2011, Umer and Iqbal, 2016, Saldanha et al., 2005) reporting active HCV infection of nearly 6% in the common population of Pakistan while even higher (14-28%) prevalence has been claimed in the case of high risk groups which includes the Injection drug users (IDUs) (Ur Rehman et al., 2011, Zein, 2000). The current investigation indicates that the ratio of active HCV infections among anti HCV positive subjects was significantly high (92.1%) however (7.95%) of the subjects with a positive anti HCV were negative for the active infection indicating the self-limiting nature of hepatitis C which has been reported earlier (Saito and Ueno, 2013).

The gender-wise distribution of active infection among 3061(53.6%) female and 2198(38.5%) male subjects respectively had active infection (Table4.1). The frequency of active infection was more in females in comparison to male subjects which is in accordance with the earlier countrywide surveys (Qureshi et al., 2010, Ahmad et al., 2010, Afridi et al., 2013) indicating that active HCV infection is higher in female HCV infected subjects than the male subjects. On the basis of age groups, the results indicated that in the Juvenile and middle age groups (26-40 yrs and 41-55 yrs), the frequency of active infection was 36% and 43% respectively, which was higher compared to the rest of four age groups (Figure 4.3). Our results are in accordance with the earlier studies showing the maximum risk of HCV predominance in middle aged groups (Ahmad et al., 2010, Aslam and Aslam, 2001, Inamullah et al., 2011, Masood et al., 2005). The high predominance of HCV in middle aged groups can be because of more exposure to numerous risk factor factors such as blood transfusions, reuse of syringes etc (Table 4.2).

All the samples with active infection (n=5259) were processed for HCV genotyping using Type-specific PCR and sequencing. The results showed that HCV genotype 3a was the most prevalent one with a frequency distribution of n=4203 (79.9%) followed by a considerable prevalence of the Untypable HCV (7.9%), mixed infection of HCV 3a3b (6.7%), HCV 3b (3.5%), HCV 2a (1.7%), and sporadic cases of the rarely found HCV 2b, HCV 1b and HCV 4 (Fig 4.1). Various investigations have recognized HCV genotype 3a as the most common hepatitis C virus in Pakistan cite (Hamid et al., 2004, Waheed et al., 2009, Ali et al., 2014, Idrees

et al., 2009, Al Kanaani et al., 2018) with some exceptions claiming HCV 1a as the most abundant in the Baluchistan province of Pakistan (Idrees and Riazuddin, 2008), however the later claim has not been validated by other studies from the same province (Umer and Iqbal, 2016, Attaullah et al., 2011, Afridi et al., 2009). Similarly a couple of studies in 2010 and 2011 contradicting the current study have demonstrated an ascent in the frequency of genotype 2a, especially in the north-western region of Khyber Pakhtunkhwa (KP) (Ali et al., 2011a, Khan et al., 2014a) however majority of the studies (Gul et al., 2016, Umar et al., 2010, Umer and Iqbal, 2016) including this study is in contradiction to these reports and have reported that HCV 3a was the most frequent genotype prevalent in different parts of KP as well as in other provinces of Pakistan (Gul et al., 2016, Idrees and Riazuddin, 2008).

An interesting finding of our observation is the vast number of Untypable HCV genotypes n=415, (7.9%) that produced no genotype-specific PCR fragments in our genotyping assay (Fig 4.1). All the Untypable HCV genotypes had adequate viral titer showing that the Untypability was not because of low HCV RNA levels and may be due to variations in their genomes. As RNA viruses mutate frequently (Nazir et al., 2017, Afzal et al., 2014), the rise of variants or new subtypes in the form of Untypable genotypes going undetectable by the current techniques is unavoidable. Previously, a number of studies have reported Untypable HCV genotypes in the range of (4% to 6%) from various parts of the country including parts of our study area (Khan et al., 2017, Zafar et al., 2018, Afzal et al., 2014). The frequency of the Untypable HCV in our study is relatively higher than reported earlier, which is an indication of the fact that uncontrolled outbreaks of HCV in various parts of the country have been contributing towards the rise of variants and need through characterization for designing futuristic diagnostic and therapeutic strategies. There is a critical need to overhaul genotyping techniques by utilizing latest HCV sequence information, so as to build up a consensus reference genotyping strategy so as to avoid errors due to the use of different assays (Khan et al., 2014a, Attaullah et al., 2011).

Due to increasing burden of HCV infections in Pakistan, it seems plausible that mixed genotypic infection will also rise as HCV genotypes do not provide cross protection (Umer and Iqbal, 2016). In the present research study, mixed genotypic infections of HCV 3a3b were detected among 6.7% subjects (Fig 4.1). The number of HCV infected subjects experiencing mixed infections is closer to that described by Idrees and Riazuddin (2008) who had reported

4.8% predominance of mixed HCV genotype infections in Pakistan (Idrees and Riazuddin, 2008, Afzal et al., 2014). Several recent studies have also described mixed HCV infections in Pakistani population where different combinations such as HCV 3a3b, 3a1a, 3a2a etc have been reported (Idrees and Riazuddin, 2008, Umer and Iqbal, 2016, Butt et al., 2011). HCV 3a3b is common among most of the mixed genotypic infections indicating its high frequency distribution in Pakistan. Retrospective studies have described a much higher number of HCV 2a (3-4%) from different parts of Pakistan (Gul et al., 2016, Attaullah et al., 2011).

In this study, HCV genotype 2a was detected among 1.7% subjects (Fig 4.1). The lower frequency of HCV 2a in our study indicates that the dynamics of HCV 2a infection have also been affected over the past couple of years. Another important finding in our study was the detection of genotype 4a in Faisalabad (Table 4.5). HCV genotype 4a has been reported to be very rare in Pakistan. In 2010, Ahmad *et al* in Lahore, (Punjab) and in 2018 Braira *et al* in Mardan,(KP), reported HCV genotype 4a among HCV infected subjects in Pakistan (Khan et al., 2011b, Butt et al., 2011). The occurrence of genotype 4a has been reported in the neighboring countries like Iran, Iraq and UAE(Ur Rehman et al., 2011). In Pakistan, a huge number of people are working as migrant workers in the middle East and like-wise in our study wherein it was detected in a subject with a history of working in the middle east, it is plausible that the subject would have been infected abroad and that contracting HCV infection abroad would have affected the diversity of HCV in Pakistan as well (Wahid et al., 2018).

The second objective of the study was to sequence the 5′UTR region of the Untypable HCV genotypes. HCV 5′ UTR is conserved part of HCV genome (Beales et al., 2001). This high level of conservation inside the 5′ UTR has made it ideal part for recognition of sequence oriented genotypes of hepatitis C virus. Various investigators have used 5' UTR for the diagnosis of HCV genotypes (Anjum et al., 2013, Germer et al., 1999, Shier et al., 2014). In current study using the NCBI BLAST tool (ncbi.nlm.nih.gov) and the online HCV genotyping tool, analysis of the 50 sequences of the Untypable HCV 5'UTR indicated that all the randomly selected Untypable samples (n=50/50, 100%) were genetically closer to HCV 3a. The finding of Untypable genotypes genetically closer to type 3a in Pakistan is not surprising because genotype 3a is the most abundant in Pakistan (Afzal et al., 2014, Al Kanaani et al., 2018, Zafar et al., 2018) and uncontrolled outbreaks of HCV infections would have added to the rise of variants,

however, the possibility of identifying more variants cannot be ruled out in the present situation. Self-alignment of the 50 5′UTR sequences identified 10 genetically diverse isolates which were subsequently used for alignment using 5′UTR sequences from other parts of the world including neighboring countries (Table 4.13). Most frequent variations observed in the HCV variants detected in this study in comparison to prototype sequences included nucleotide conservation(*), deletions (-) and transversion, no other sequence variation was found in our study. Comparative analysis of all sequences showed some nucleotide substitutions within 5'UTR as mentioned in (Fig 4.12). Some of these sequence variations have previously been reported by (Yasmeen et al., 2009, di Filippo et al., 2012) in the 5'UTR of HCV from multi-transfused subjects in Colombia. The overall nucleotides similarity among different Pakistani isolates was 97.50% $\pm$ 0.50%. This nucleotides similarity among different isolates is approximately the same as described by Kavita and colleagues (2003) in India that was 94.55%. For type 3 sequences, the PNI was 97.97% $\pm$ 0.50% that was 1-2% less than that reported from India (98.75%) which is indicative of a similar evolutionary pattern in the neighboring countries. Minimum genetic closeness (0.00) was observed among HCV Untypable isolates and with Pakistani isolates HCV 3a while maximum genetic distance (0.63) was analyzed among Untypable HCV isolates and other countries isolates (Table 4.11) which showed that majority of the Untypable HCV isolates were closer among each other and to Pakistani HCV 3a isolates, however they were distant from the isolates of other regions with some exceptions. In the case of HCV 3a isolates, similar results have been documented earlier (Yasmeen et al., 2009).

Results of the phylogenetic analysis demonstrated that the most of the Pakistani Untypable HCV clustered in clade-I with maximum bootstrap value (1000) while HCV PK3 fell in clade-IV (Fig 4.13, 4.14). Most of the Untypable strains characterized in this study were closer to previously reported HCV 3a sequences from Pakistan in clad-I (Afzal et al., 2014, Umer and Iqbal, 2016) however, slight divergence from the previously reported sequences indicate that genotype HCV 3a in Pakistani population is evolving into different variants. PK 3 did not cluster with any of the Pakistani isolates, it rather clustered with HCV 3a strains reported from Japan, and Brazil. It is an indication of the fact that genetically distinct Untypable HCV strains are evolving in different areas of Pakistan which may have been transmitted by migrants (Afzal et al., 2014, Khan et al., 2017, Zafar et al., 2018). Clustering together of all Pakistani HCV 3a isolates in a single group indicates a distinct evolutionary trend. Similar results of a

phylogenetically distinct HCV 3a have earlier been documented in Pakistan using the whole genome phylogeny using the first whole genome HCV 3a (Rehman et al., 2011).

The third objective of the study was to evaluate the response rates of different antiviral therapies among chronically infected Patients with HCV in Pakistan. The principle motivation behind antiviral treatment in hepatitis C is either to root out the infection from the subject's body or to decrease the chances of infection progression to advanced stages like cirrhosis and hepatocellular carcinoma (Ali et al., 2011b). At present, the best starting therapy for treatment of subjects infected with chronic HCV infection is the new direct acting antivirals (DAAs) combination therapies (Waheed et al., 2017, Siddique et al., 2017). These combination regimens are reported to achieve SVR24 among 97% or 99% of treatment subjects. Among different factors that predict a response to these combination therapies include age, gender, viral load and HCV genotype etc (Iqbal et al., 2017, Ali et al., 2016a).

A total of 4411 subjects with chronic HCV infection were enrolled for this study out of which n=3779 (85.6%) including n=2204 (58.3%) females and n=1575 (41.7%) male subjects completed the therapy. The subjects were divided into four treatment groups each receiving a different combination therapy. A combination of SOF-RBV was used in group-I. The ETR and SVR24 achieved for the combination therapy against HCV 3b infection (95.8%) and mixed genotypic infections (93.5%) were higher as compared to HCV 3a (91.6% & 91.3%); which is the predominant type in Pakistan. Moreover, relapse was observed only in the case of HCV 3a. The combination therapy was not efficient against the Untypable HCV infection with an ETR and SVR24 of 84.4%, however it was satisfactory against the genotypes found rarely in group-I. The overall ETR was (91.7%) and SVR24 was (91.5%) in the case of infections with different genotypes detected in the study group, however 120 (8.2%) subjects showed null response (Table 4.17a).

Few clinical trials available in literature for genotype 3, includes FISSION, FUSION, POSITRON, ALLY-3 and BOSON studies suggesting good acceptance of SOFO combination therapy with RBV (Ahmad et al., 2018). A multi-centre RESiP trial from Pakistan including more than 5000 subjects with (94%) HCV genotype 3 subjects demonstrated SVR12 of 97% in non cirrhotic and 89% cirrhotic treatment naïve subjects respectively (Akhter et al., 2017) which is comparable with the current study. However, reports on relapse indicate the need for more rigorous and advanced research in the field in order to cope with challenges posed by the emergence of variants and it

needs serious consideration of the scientific community (Wahid et al., 2017). In case of Untypable HCV genotype we did not find any data for comparison to measure the SVR24 rate in respect to SOFO-RBV combination therapy, however the lower response rate is indicative of the fact that these types might have accumulated mutations thus making them respond differently to the combination therapy. The age-group wise response rates revealed that the combination of SOF-RBV was more effective in younger age groups (SVR24 range: 98.2-98.6%) as compared to subjects of >40 yrs (SVR24 range: 56.2-92.3%). Maximum null response was observed in the elderly (>56 yrs) as compared to the younger age groups (Table 4.17b). In agreement to the current study, it was reported in different studies that the age <40 years showed higher SVR24 rates compared with age >40 years (Aziz et al., 2011, Iqbal et al., 2017). It showed that subjects with younger group with hep C are suitable candidates to treat.

In terms of gender wise response, high SVR24 rate (92.1%) was achieved in female subjects in comparison to male subjects (88.8%) mostly in age range of 26-70 (Table 4.21). In consent to this study, Belci et al. demonstrated in their study that remarkably higher SVR24 in female subjects was achieved as compared to male subjects (Belci et al., 2016) which may be due to more active Interferon signaling in the presence of estrogen (Pol et al., 2016) in the case of the combination used. Martin et al. demonstrated that DAAs aside from their direct action of HCV also boost up the immunity to fight against the virus, the mechanism are very parallel to that of IFNs (Pol et al., 2016). In current study SOFO-RBV combination therapy has revealed a better safety profile in HCV infected subjects of Pakistani population.

No serious side effects have been reported however fatigue and generalized weakness were the most common severe effects. Other side effects were headache, myalgia, fever, dry cough, oral ulcers, rash and pruritis while anemia was observed in rare subjects (Table 4.22). In consent to the current study, same side effects were reported by Akhter et al in their studies (Akhter et al., 2017). Conclusively the results indicated that SOFO with RBV is a safe and cost effective treatment modality for HCV infected subjects in Pakistan (Jamil et al., 2018).

Group-II subjects received a combination of DCV-SOFO for a period of 24 weeks. The overall ETR rate in case of all the HCV genotypes detected in group-II was 93.2% while the SVR24 rate was 92.6%. However, response rate of the combination therapy was variable against different HCV genotypes. The ETR observed in the case of HCV 3a infections was higher (94%) as compared to the Untypable HCV (91.8%) and mixed infections of HCV 3a3b (88%).

A similar trend was observed in the case of SVR24 as well. HCV 2a, 2b and HCV 3b infections were relatively rare in group-II however, the combination therapy worked well in the case of the former as compared to the later (HCV 3b). Relapse was observed only in the case of HCV 3a (Table 4.18a). Some recent studies have come up with similar results where SVR24 of 90.8% or more (upto 94%) was achieved in the case of DCV-SOFO combination therapy administered for 12 or 24 weeks (Belperio et al., 2019, Butt and Shah, 2019, Ahmed et al., 2018, Pol et al., 2016). A common observation in the above mentioned studies was a better response of the combination among the treatment naive subjects as compared to the treatment experienced subjects. Although there were no treatment-experienced subjects in group-II of this study, the response rate of the combination therapy among the treatment naive subjects was slightly better against HCV 3a infections in Pakistani population. Moreover we did not find any data for comparison regarding the response rates of mixed and Untypable HCV genotypes in respect to DCV-SOFO combination therapy. However like group-I, a comparatively lower response rate revealed that these types might have accumulated mutations thus making them respond differently to the combination therapy.

Majority of the subjects in group-II were <70 years of age. DCV-SOF combination therapy achieved 100% ETR and SVR24 in the youngest age group (10-25 yrs) followed closely by age group (26-40 yrs) who achieved an ETR and SVR24 rates of 99.3% with no relapses. However, the combination therapy was not very successful in the elderly (age groups 56-70 & 71-85 yrs) where only 68.7% & 70% of the subjects responded favorably (Table 4.18b). So far no data is available for comparison in terms of the above mentioned age groups for the DCV-SOFO combination therapy. SVR24 was achieved among 91.7% female subjects and 92.3% male subjects. In contradiction to the current study Bhattacharya et al in 2019 demonstrated that female subjects showed high SVR as compared to male subjects when treated with DCV-SOFO combination (Butt and Shah, 2019) however, the difference in the SVR24 in the case of genders was not significant in this study.

Side effects of the combination therapy as observed among the subjects included headache, fatigue, nausea, diarrhea, anemia, rash, insomnia, dizziness and drowsiness (Table 4.22) which have been reported by other investigators as well (Pellicelli et al., 2014). DCV-SOFO was not related with any new or serious adverse events, however, it has been reported earlier that the subjects who received DCV-SOFO-RBV had more side effects listed in (Table 4.22) that are predictable as the known side effects of RBV (Leroy et al., 2016).

In Group-III, a triple combination therapy of DCV-SOFO-RBV was administered for 24 weeks. The overall ETR and SVR24 rates observed for the triple combination therapy against various HCV types prevalent in group-III were 93.9% and 92.8% respectively. The triple combination therapy was particularly more effective against the Untypable HCV (SVR24: 97.4%) and all abundant HCV types detected in the subjects (>91%), however, it did not respond comparatively well against HCV 2a (SVR24: 87.5%). Majority of the null response subjects were infected with HCV 3a and relapse was also observed in the case of the same genotype (Table 4.19a). In consent to the current study, SVR24 rate of 89% was observed for the triple combination in subjects infected with HCV genotype 3a and HCV mixed genotype 3a3b (Pol et al., 2016). No information was found for comparison of response rate in the case of the Untypable HCV infection.

The triple combination therapy was very effective among subjects in the age range of 10-40 yrs with the maximum SVR24 rate of 98% observed in age group (26-40 yrs), however, the therapy was not very successful among subjects of older age (>40yrs) (Table 4.19b). In case of age groups, again no data was available for comparison regarding the response rate of DCV-SOFO-RBV combination therapy. In consent to our study, a trial study (ALLY-31) reported that the addition of RBV to DCV-SOFO enhanced SVR12 response in cirrhotic subjects in comparison to that of DCV-SOFO without RBV in ALLY-3, but did not remarkably change the tolerability and safety profile. There was no increase in overall grade 3-4 laboratory abnormalities in ALLY-31 (3 events in 50 subjects) compared with ALLY-3 (8 events in 152 subjects), and the addition of RBV to DCV-SOFO resulted in only a single case of grade 3 treatment-related anemia in ALLY-31 against HCV 3a (Leroy et al., 2016). Gender-wise response indicated that SVR24 in female subjects (93.0%) was slightly lower in comparison to males (93.9%) but statistically insignificant (Table 4.21).

In contradiction to the current study, But et al in 2019 demonstrated that female subjects were more responsive as compared to male subjects when treated with DCV-SOFO-RBV (Hézode et al., 2015) which is a general observation reported by several investigators in the case of IFN-based regimes which particularly achieved better SVR among the female subjects (Abd-Elsalam et al., 2019, Aziz et al., 2011, Ali et al., 2016a). However, the gender-based significant differences in response rates have not been reported in cases of DAAs combinations by other investigators (Abbas et al., 2017, Jamil et al., 2018). Side effects like fatigue, headache, and nausea were the most common in the current group(Table 4.22) which have been reported earlier (Pol et al., 2016).

In summary, the DCV-SOFO-RBV combination is associated with a high SVR24 rate in subjects infected with various HCV genotypes.

In 2013, SOFO was introduced as a pan genotypic antiviral and was used in combination therapy with PEG-IFN and RBV (Bhatia et al., 2014).The group-IV received triple combination therapy consisting of SOF-RBV-PEG IFN which was most successful against HCV 3b and the Untypable HCV with ETR and SVR24 rates of 100% and 94% respectively, however, against HCV 3a and mixed genotypic infections, the therapy was comparatively less effective than other double or triple combinations therapies (Table 4.20a). A relatively higher percentage of null response was observed in the case of HCV 3a and the number of relapses in case of the said genotype was also high (Table 4.20a). Other investigators have reported that the SOFO-RBV-PEG IFN therapy was far better than the double combination of PEG-IFN and RBV therapy with higher efficacy, shorter treatment duration, and fewer side effects (Bhatia et al., 2014). For correlation in BOSON study, the addition of PEG-IFN-a to SOFO-RBV for 12 weeks achieved better SVR12 in genotype 3-infected subjects with cirrhosis (88%) relative to SOFO-RBV alone for 16 weeks (51%) or 24 weeks (79%) (Foster et al., 2015). It has been reported that 95% of HCV-infected subjects at week 4 treated with combination therapy of PEG-IFN-RBV-SOFO results in complete eradication of hepatitis C (Lawitz et al., 2014, Kowdley et al., 2013, Lawitz et al., 2013). In consent to current group study 96-98% of SVR24 was achieved by HCV genotypes 3 and its subtypes in respect to SOFO-PEG IFN-a-RBV combination therapy (Lawitz et al., 2013). No data is available for comparison regarding the response of Untypable HCV genotypes in respect to PEG-IFN-a-SOFO-RBV combination therapy.

In different age groups, the triple combination proved more effective among subjects of <55 years of age while it was relatively less effective in subjects >55 yrs. Null response and relapses were observed in all age groups with majority of the cases falling in the age group (41-55 yrs) (Table 4.20b). In respect to age range we did not find any data for correlation regarding the triple combination therapy of SOFO- PEG IFN-a-RBV. Gender wise response rates in terms of ETR was 93.0% in female and 91.9% in male subjects. Similarly SVR24 was achieved as 90.3% in female subjects and 90.0% in male subjects (Table 4.21). Similar results were reported in a study carried out in Egypt in which female subjects were more responsive to SOFO-PEGIFN-a-RBV combination therapy as compared to male subjects (Abd-Elsalam et al., 2019). Consistent with the established safety profile of PEG-IFN and RBV treatment, the addition of PEG-IFN-a led to a higher incidence of

complications and side effects. The overall percentage of side effects was higher in the subjects treated with triple therapy regimen as compared to those treated with double therapy regimen (SOFO-RBV) (Table 4.22). These listed side effects were reported in earlier studies in subjects treated with RBV and PEG-IFN alfa-2a including severe neuropsychiatric side effects (Akiyama et al., 2013, Moreno et al., 2015). In conclusion, PEG-IFN, RBV plus SOFO regimen is a highly effective therapy for treatment of HCV genotype 3 along with its subtypes in Pakistan as reported by other studies as well (Butt et al., 2019).

❖ Conclusion

This study concludes that there is a uniform pattern of HCV genotypes distribution across the study area with HCV 3a, HCV 3b and the Untypable HCV as the most dominant genotypes followed by HCV 2a while other genotypes are rarely found in some areas. Furthermore, genetically diverse types of the Untypable HCV exist in different parts of the country. Although partial genetic information reveal that the Untypable HCV may be slight variants of HCV 3a, yet whole genome sequencing on a representative scale could help reveal their entire genetic divergence and their true identity as variant or novel types. Although the response rate of all combination therapies was quite satisfactory in subjects <41 years among subjects infected with various HCV types, DCV-SOF and DCV-SOF-RBV particularly achieved high SVR24s in the case of HCV 3a infections while SOF-RBV and SOF-RBV-PEG IFN combinations were more effective against HCV 3b. In the case of the Untypable HCV, DCV-SOF-RBV combination was comparatively more efficient although DCV-SOF and SOF-RBV-PEG IFN also achieved high SVR24. SOF-RBV, DCV-SOF and the triple combinations used in group-IV worked very efficiently against HCV 4a, HCV 2a and 2b, and HCV 1b respectively.

Future Prospects

1. The characterization of whole genome of the prevailing HCV types in Pakistan should be conducted to understand the evolution of HCV in specific ethnic backgrounds and to have a broader background for finding association of viral factors with response to different therapies.
2. Assays based on Type-specific PCR used for HCV genotyping should be updated after 5-10 years in the light of newer sequencing data for designing relatively trustworthy genotyping systems and assessment of viral epidemiological.
3. DAAs efficacy should be evaluated against the evolving HCV genotypes and subtypes prevalent in Pakistan.
4. Host factors (such as IL28B genotype) should be assessed so as to clearly evaluate the anti-viral resistance in our population.
5. In the light of viral and host genomics, extensive bioinformatics studies should be carried out to find out useful information about host susceptibility or resistance and design of newer strategies for containing the virus.
6. A therapeutic vaccine-based strategy or other vaccine design strategies should be explored against the virus.

Publications from this research work:

1. Shahroz Khan[1*],Ijaz Ali[2] , Malik Badshah[1] , Qaiser Mohammad Khan[3] , Zahida Nasreen Haider[3] , Sajid Ali[4] , Imtiaz Ali Khan[5] and Anwar Ullah[2] "Molecular Epidemiology of Hepatitis C Virus Genotypes Among Chronically Infected Patients in Pakistan" Jundishapur J Microbiol. 2019 March; 12(3):e86428. doi: 10.5812/jjm.86428.

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Submitted research Paper:

2. Sofosbuvir and ribavirin combination therapy without interferon for treatment of hepatitis C virus genotype 3a in Pakistan: comparison between treatment naïve and treatment experienced patients (Jundishapur Journal of Microbiology)
3. Response rates of new direct-acting antiviral therapies Daclatasvir, Sofosbuvir in combination with Ribavirin and Pegylated Interferon among chronically infected Patients with HCV in Pakistan (Hepatitis Monthly)
4. Molecular diagnosis of Hepatitis C Untypeable genotypes among chronically infected patients in Pakistan (Plos One)

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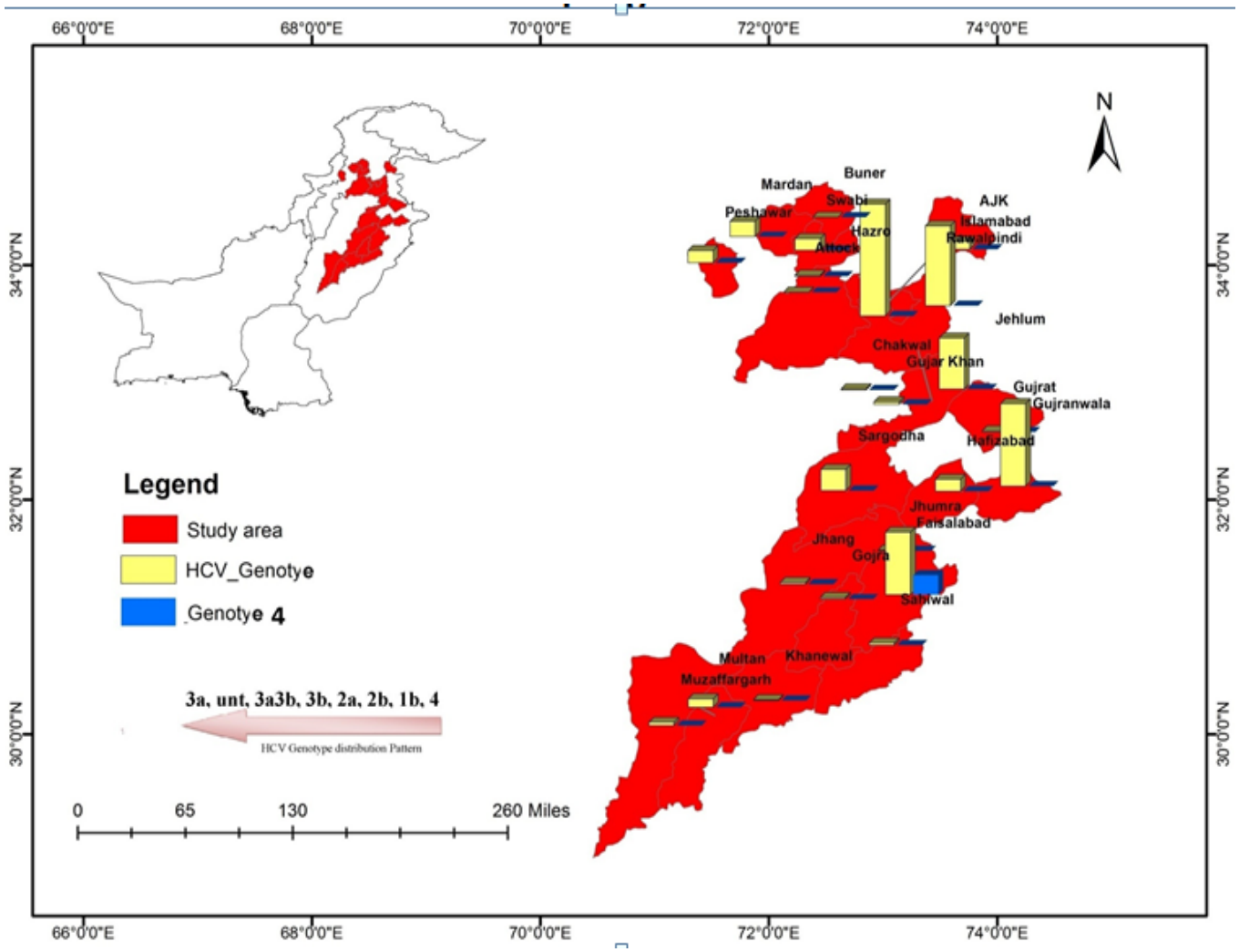

Sampling area (Fedral, Punjab, KPK and AJK)